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Structure of the *gluABCD* Cluster Encoding the Glutamate Uptake System of *Corynebacterium glutamicum*

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To assess the mechanism and function of the glutamate uptake system of gram-positive *Corynebacterium glutamicum*, a mutant deficient in glutamate uptake was isolated and was then used to isolate a DNA fragment restoring this deficiency. In a low-copy-number vector, this fragment resulted in an increased glutamate uptake rate of 4.9 nmol/min/mg (wild type, 1.5 nmol/min/mg). In addition, carbon source-dependent regulation of the glutamate uptake system was determined with the fragment, showing that the entire structures required for expression and control reside on the fragment isolated. Sequencing of 3,977 bp revealed the presence of a four-gene cluster (*gluABCD*) with deduced polypeptide sequences characteristic of a nucleotide-binding protein (GluA), a periplasmic binding protein (GluB), and integral membrane proteins (GluC and GluD), identifying the glutamate transporter as a binding protein-dependent system (ABC transporter). This identification was confirmed by the kinetic characteristics obtained for cells grown in the presence of globomycin, which exhibited an increased K_m of 1,400 μM (without globomycin, the K_m was 1.5 μM) but a nearly unaltered maximum velocity. By applying gene-directed mutagenesis, a strain with the entire cluster deleted was constructed. With this mutant, the glutamate uptake rate was reduced from 1.4 to less than 0.1 nmol/min/mg, which is proof that this system is the only relevant one for glutamate uptake. With this strain, the glutamate excretion rate was unaffected (18 nmol/min/mg), showing that no component of *gluABCD* is involved in export but rather that a specific machinery functions for the latter purpose.

Gram-positive *Corynebacterium glutamicum* is in use for the large-scale production of amino acids, in particular for glutamate. However, compared with what is known about many other bacteria, molecular details on the amino acid transport systems of this bacterium are severely restricted. So far, only the structure of the lysine uptake carrier, *lysI*, is known (33). Functional studies with intact cells on the kinetics and energetics of several amino acid carriers of *C. glutamicum*, including that of the glutamate uptake system, have been carried out (for a review, see reference 20). This uptake system (i) exhibits high affinity, (ii) results in extremely high internal glutamate accumulation, (iii) is irreversible, and (iv) has activity directly correlated to the cytosolic ATP content of the cell (21). These results were interpreted in terms of a primary, binding protein-dependent, ATP-driven uptake system for glutamate. Interestingly, the total catalytic activity of the system is induced when cells are grown on glutamate but it is subject to catabolite repression by glucose (22).

Binding protein-dependent systems belong to the large family of ABC transporters (for a review, see reference 11). These multicomponent systems typically consist of two membrane-inserted subunits (domains), one component inside the cytoplasm which carries the ATP-binding site, and finally the binding protein located outside the cytoplasm. The systems are well characterized in gram-negative bacteria, with particularly well-studied members being the uptake systems for histidine (2), oligopeptides (13), and maltose. Only a few systems have been characterized for gram-positive bacteria, e.g., those for peptide uptake in *Bacillus subtilis* (25, 28), *Streptococcus pneumoniae* (1), and *Lactococcus lactis* (37); the glutamine uptake system of *Bacillus stearothermophilus* (41); and the iron-hydroxamate uptake system of *B. subtilis* (31). According to the architecture of the translocator, the respective operons (if characterized at all) consist of at least four genes. But frequently genes not directly linked to uptake are localized within the clusters, like *dciAA* within the *B. subtilis dci* cluster which is correlated with sporulation (25) or *amiB* within the *S. pneumoniae ami* locus which is assumed to modulate arsenate export activity (1).

Our interest in studying the glutamate uptake of gram-positive *C. glutamicum* is in correlating the established functional description with molecular data. Moreover, such characterization is intriguing in view of the impressive activity of glutamate excretion feasible with *C. glutamicum*, the functional and molecular basis of which is still undefined.

MATERIALS AND METHODS

Bacterial cultures. The wild type of *C. glutamicum* ATCC 13032, as well as its glutamate-uptake-deficient mutant M10 derived by UV mutagenesis, was used. M10 had been made restriction deficient by the method of Liebl et al. (24). Strains were grown at 30°C on rich medium CGIII or minimal medium CGIX (4) containing tetracycline (15 μg/ml) or kanamycin (50 μg/ml) when appropriate. For isolation of uptake-deficient strains, the minimal medium contained either sodium glutamate or sodium glutamine (10 g/liter, each) as the carbon source.

Plasmid constructions. In order to construct versatile low-copy-number shuttle vectors with the *cos* site, the basic constructions required were made in pUC19 (38). The steps taken were as follows. First, a 0.5-kb *Pst*I fragment of pBTI-1 providing the *cos* site (Boehringer, Mannheim, Germany) was introduced into the *Pst*I site of pUC19. Second, the *C. glutamicum* replicon of the low-copy-number vector pGA2 (34a) was excised as a 3.4-kb *Hin*dIII-*Sal*I fragment before being made blunt and introduced into the *Sal*I site of pUC19-cos. Third, the *tet*α1 gene was isolated as a 1.7-kb *Eco*RI-*Acc*I fragment from pHY163PLK (16) before being made blunt and introduced into the *Sma*I site of pUC19-cos-oriCg. Fourth, after a partial *Hin*dIII digestion, the fragment carrying *cos-ori*$_{Cg}$-*tet* was isolated and ligated with a 3.3-kb fragment of pOU71 (23), providing the *Escherichia coli* replicon with temperature-sensitive control of replication. The final plasmid, pKW0, has unique restriction sites for *Eco*RI, *Kpn*I, and *Bam*HI. For construction of pKW3 (Fig. 1) carrying the kanamycin resistance gene replacing *tet*α1, the kanamycin resistance gene obtained as a *Bam*HI cassette from pUC4K (38) was introduced into the *Bam*HI site of pKW0. The tetracycline resistance

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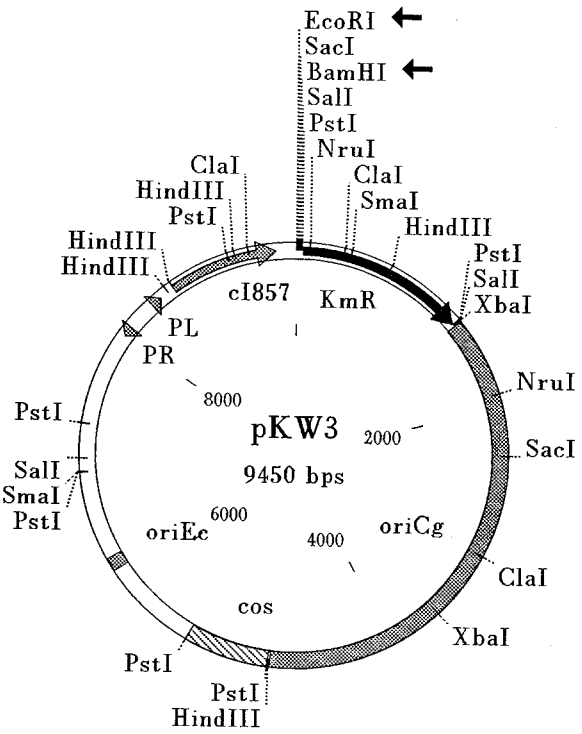

FIG. 1. Vector pKW3 with low copy number in *C. glutamicum* and low but inducible copy number in *E. coli*. The vector is based on pOU71 (open segment) (23) and pGA2 (shaded segment) (34a). The *cos* site as well as the location of the origins of replication of *C. glutamicum* (oriCg) and *E. coli* (oriEc) and the lambda phage promoters (PR and PL) driving replication in *E. coli* under control of the temperature-sensitive repressor cI857 are marked. Two singular restriction sites are marked.

gene was deleted by partial *Bam*HI restriction followed by *Kpn*I restriction, and one of the two *Bam*HI sites was deleted by partial digestion and was made blunt with Klenow enzyme. The copy numbers of pKW0 and pKW3 were estimated to be less than 5 copies in *C. glutamicum* and less than 5 copies in *E. coli* when grown at 30°C, but when the temperature is shifted to 42°C copy numbers in *E. coli* are higher than 500.

Selected subclones of the cluster were made as follows: pKW12 carries a *Sau*3A fragment of 19 kb in the *Bam*HI site of pKW0, pHSGglu carries a 3.7-kb *Eco*RI-*Sal*I fragment of pKW12 in pHSG576 (35), pHSGglu$_{inv}$ carries the same fragment in the opposite orientation, and pKW12Eco was made by *Eco*RI digestion of pKW12 and religation of the largest fragment isolated from gels.

Ligations and transformations. Chromosomal DNA was partially *Sau*3A restricted, size fractionated by gel electrophoresis, and purified with EluTip columns (Schleicher and Schüll, Dassel, Germany). Plasmid pKW3 was linearized with *Bam*HI before it was dephosphorylated and 500 ng of it was precipitated with 2-μg fragments by the use of LiCl and ethanol. The precipitate was washed with 70% ethanol and dissolved in water, and ligation was carried out overnight. The ligation products were then heat inactivated (10 min, 70°C), purified by phenol-chloroform extraction, precipitated with 1.45 M ammonium acetate and three volumes of ethanol, and after a final washing step dissolved in 10 μl of water.

To make *C. glutamicum* competent, strains were grown in CGIII in the presence of 0.4% isonicotinic acid hydrazide, 2.5% glycine, and 0.1% Tween 80. Cells were washed with ice-cold water and 0.7 mM phosphate buffer (pH 7.5) containing 0.5 M sorbitol. Transformation was carried out by electroporation with 1 to 3 μl of ligation mixture (~0.4 μg of DNA). Cells were immediately transferred to BHIS (24), shaken for 1 h at 30°C, and then plated on Luria broth containing 37 g of brain heart infusion (Difco) per liter, 0.5 M sorbitol (final pH of 7.5), and 10 μg of kanamycin or 2.5 μg of tetracycline per ml.

Glutamate uptake and excretion. For uptake measurements, *C. glutamicum* was grown on the supplemented minimal medium of Krämer et al. (22) with either 40 g of glucose per liter or 10 g of glutamate per liter as the carbon source up to the exponential phase. Cells were washed with buffer (50 mM MES [morpholineethanesulfonic acid]-Tris, 50 mM NaCl, 10 mM KCl [pH 8]) and were resuspended to a concentration of 1 mg (dry weight) per ml. A 2-ml cell suspension was stirred to provide oxygen, and the assay was started by the addition of ^{14}C-labelled glutamate (final concentration, 100 μM). Within 3 min, six aliquots were taken. They were processed as described previously (22) to obtain the kinetic data.

For the experiments with globomycin, cells were pregrown as before but were then inoculated into small flasks with 10 ml of the identical medium with 50 μg of globomycin per ml. The globomycin was dissolved in ethanol which was also added to the control culture. Cultures were grown overnight and then were used for uptake determinations as before, but with various glutamate concentrations.

For the determination of glutamate excretion rates, cells were grown on minimal medium CGXII (17) with the concentration of biotin reduced to 10 μg/liter. The cells were harvested in the exponential phase and inoculated into fresh CGXII supplemented with only 1 μg of biotin per liter. After about 15 h, growth was arrested because of the limited biotin supply and glutamate secretion started. At several points, the glutamate secreted was quantified enzymatically and the resulting curve was used to calculate the glutamate excretion rate. All rates are given in nanomoles of substrate translocated per minute per milligram (dry weight).

Nucleotide sequence accession number. The entire DNA sequence for the *gluABCD* cluster has been deposited in GenBank under accession number X81191.

RESULTS

Isolation of a glutamate uptake mutant. The glutamate uptake system of *C. glutamicum* is clearly different from the glutamine uptake system of this organism (14). Moreover, growth with glutamate as a carbon source is directly dependent on the capacity of the glutamate transport system (21). Therefore, mutagenized cells of the wild type of *C. glutamicum* were replica plated on minimal medium with either glutamate or glutamine as the carbon source. Several clones obtained were retested, and finally one mutant with reduced growth on glutamate but normal growth on glutamine was selected. In transport assays, this mutant exhibited a reduced glutamate uptake activity of 0.53 nmol/min/mg (dry weight) (wild type, 1.83 nmol/min/mg [dry weight]). However, the mutant was not suitable for homologous complementation because of its low transformation efficiency. Therefore, a restriction-deficient derivative, designated M10, which exhibited high transformation efficiency was made. This mutant was identical to the parent strain with respect to the impaired glutamate uptake (data not shown).

Isolation of a complementing fragment. Since we expected to clone a membrane protein with possibly deleterious effects when oversynthesized, new vectors with low copy numbers had to be constructed in *C. glutamicum* (see Materials and Methods). Plasmid pKW3 (Fig. 1) bearing the Kan^r gene of Tn*5* was actually used in the experiments. It was *Bam*HI restricted and was ligated with *Sau*3A-derived DNA fragments of *C. glutamicum* ATCC 13032. By an optimized electroporation protocol, strain M10 was transformed to Kan^r. Two clones with growth characteristics on glutamate comparable to wild-type characteristics were identified. Both clones contained a plasmid with an insert of about 19 kb and of a restriction pattern very similar to each other. The plasmid eventually chosen was designated pKW12. A specific glutamate uptake rate of 4.92 (nmol/min/mg [dry weight]) was determined for the recombinant strain M10 carrying this plasmid, whereas that of the wild type was 1.58. This is thus functional proof that pKW12 contains genes involved in glutamate uptake.

Subcloning and sequence analysis. To reduce the size of the complementing fragment, we made use of the biochemical indications that an ATP-binding protein was expected to be part of the glutamate uptake system of *C. glutamicum* (21). ATP-binding proteins have a well conserved nucleotide-binding site (40). Accordingly, a probe based on the respective sequences known for the gram-positive transporters and on the codon bias for moderately expressed genes of *C. glutamicum* was synthesized (6). Upon hybridization with restricted pKW12 (data not shown), the two smallest positively reacting fragments were a 4.5-kb *Eco*RI fragment and a 3.7-kb *Eco*RI-

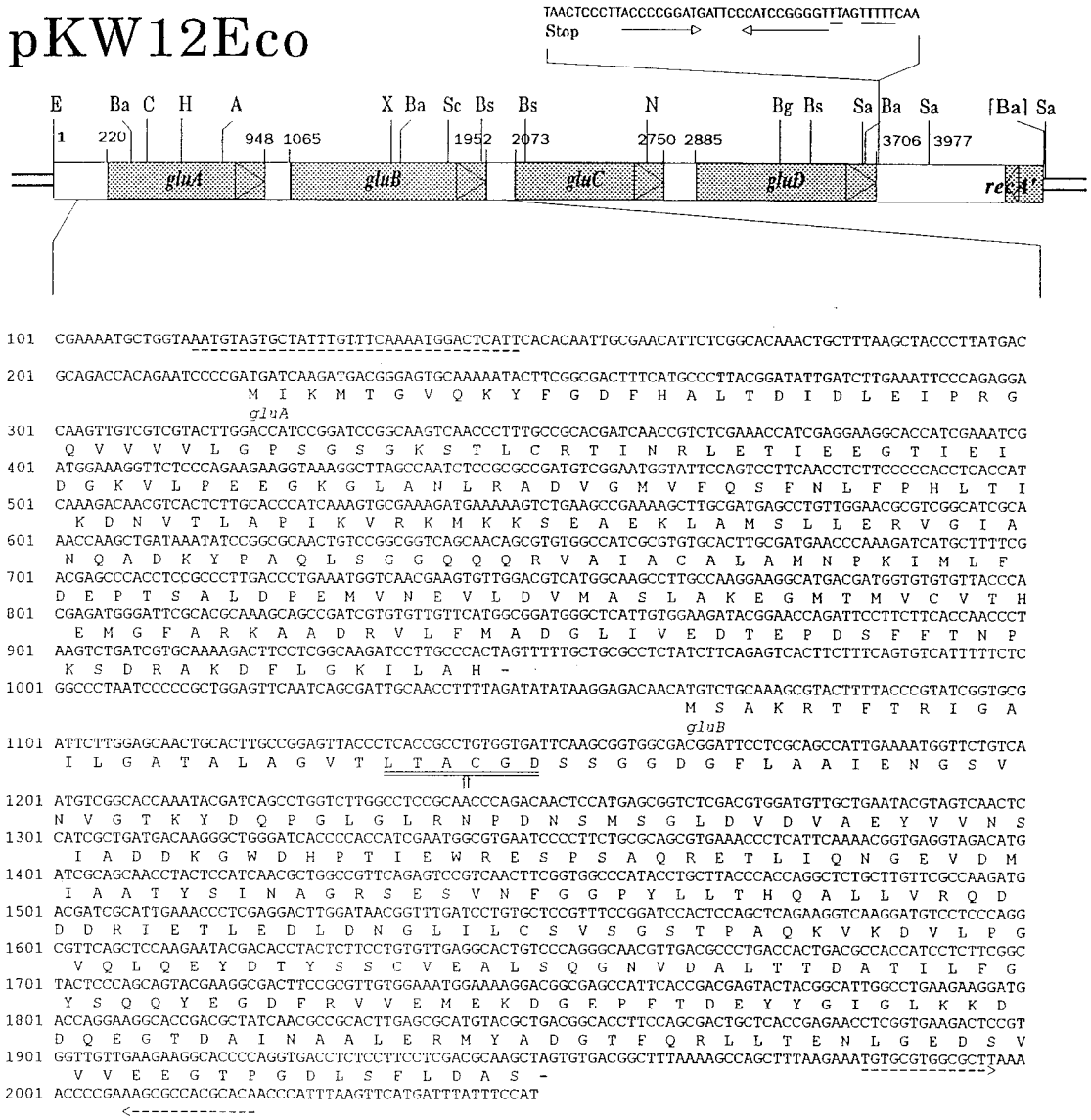

FIG. 2. Structure of the *gluABCD* cluster of *C. glutamicum*. The general organization is shown at the top of the figure, with the prominent restriction sites and the lively terminator of the cluster. Part of the total sequence of 3,977 bp is shown at the bottom, along with the deduced amino acid sequence of GluA and GluB. The arrows indicate the inverted repeats, the broken line at the beginning of the sequence (nucleotides 115 to 148) marks a site similar to a CRP recognition site of *E. coli*, and the double line with the arrow marks the consensus cleavage site of prolipoproteins. The prominent restriction sites in the cluster are *Aat*II (A), *Bam*HI (Ba), *Bgl*II (Bg), *Bst*EII (Bs), *Cla*I (C), *Eco*RI (E), *Hin*dIII (H), *Nru*I (N), *Sal*I (Sa), and *Sca*I (Sc).

*Sal*I fragment. The *Eco*RI-*Sal*I fragment was subcloned into low-copy-number vector pHSG576 (35) to yield pHSGglu, since attempts to clone the fragment with pUC failed. Initial sequence analysis of subclones of the pHSG576 derivatives revealed that one open reading frame was incomplete. Therefore, upon combination of hybridization and sequence data further fragments of pKW12 were cloned to finally yield a sequence of 3,977 bp which is present in pKW12Eco (Fig. 2).

The region from 100 to 2,050 bp is shown in Fig. 2, with the end of the entire sequence included. Four complete potential coding regions were identified. They were designated *gluA*, *gluB*, *gluC*, and *gluD*, because of their functional and structural characteristics (as described below). All of the regions are transcribed in the same direction and start with an ATG codon. No obvious promoter structures or ribosome binding sites could be detected, whereas three inverted repeats are present in the sequence. The inverted repeat at the end of *gluD* (Fig. 2) has a high potential for forming a stem-loop structure rich in G/C ($\Delta G = -26.6$ kcal/mol [ca. -111 kJ/mol]) and is followed by a stretch of T residues. It therefore has characteristics of a rho-independent terminator. A second inverted repeat is present between *gluB* and *gluC* ($\Delta G = -27.4$ kcal/mol [ca. -115 kJ/mol]).

In addition to the sequence of the *glu* cluster, sequence data for fragments extending to the left and right sides of the 3,977 bp fragment were also obtained. The 3′ end of *recA* of *C. glutamicum* (2a) was identified 0.63 kb downstream of *gluD*. This sequence analysis excludes the presence of genes involved in transport directly downstream of *gluD*. Similarly, we have no indication that a gene is present within at least 0.40 kb upstream of *gluA*.

TABLE 1. Glutamate uptake rates with recombinant clones of the wild type and induction by growth on glutamate[a]

Plasmid with *C. glutamicum*	Carbon source	Transport rate (nmol/min/mg [dry wt])
pKW3	Glucose	0.43
pKW12	Glucose	4.61
pKW12Eco	Glucose	2.11
pKW3	Glutamate	5.00
pKW12	Glutamate	17.10
pKW12Eco	Glutamate	14.90

[a] pKW3 is the control plasmid without insert, and pKW12Eco carries the smallest fragment with *gluABCD* (Fig. 2).

pKW12Eco encodes the Glu-binding system. Plasmid pKW12Eco, containing the sequenced fragment (Fig. 2), was introduced into the wild type of *C. glutamicum*. After being grown on glutamate, this recombinant strain (as well as that carrying the original plasmid pKW12) yielded a specific uptake rate about threefold greater than that of the control (Table 1). This result is proof that the sequenced fragment carries the essential elements of the uptake system. The degree of overexpression is in accord with our estimates of fewer than five copies for the vector we had constructed (pKW3). Since in the wild type the synthesis of the glutamate uptake system is regulated (21), we also assayed whether a responsible structure is present in pKW12Eco. As can be seen from Table 1, the markedly decreased uptake rate obtained after strains had been grown on glucose (compared with uptake rates on glutamate) verifies that this is the case.

In sequence comparisons, the deduced amino acid sequence of the *gluA* gene product (241 amino acids [aa], 26.5 kDa) exhibited strong similarity to the ATP-binding proteins characteristic of the ABC transporters and in particular to those translocating amino acids and opines. The strongest similarities are exhibited by the proteins of the glutamine transport systems. Thus GlnH2 of *B. stearothermophilus* (41) shares 62% identical (77% similar) amino acid residues, and that of *E. coli* (26) shares 58% identical (74% similar) amino acid residues. An almost perfect fit exists with respect to the nucleotide binding site motifs A and B, including that of HisP of *Salmonella typhimurium* (12), with additional long stretches of extensive homologies extending beyond the ATP-binding region. GluB (295 aa, 31.7 kDa) exhibits similarities to binding proteins. The similarity to the corresponding component of the glutamine transport system of *B. stearothermophilus* (32% identical [51% similar] over a stretch of 203 aa) is again greatest, with the exception of an additional C-terminal part of 44 aa residues present in GluB of *C. glutamicum*. At the N-terminal end, use of SIGNALSEQ identifies 26 aa residues as a potential signal sequence, indicating translocation of this protein through the cytoplasmic membrane. The great similarities of this leader sequence to that of MalX of *S. pneumoniae* (9) and also to the consensus sequence of prolipoproteins of gram-negative bacteria (42) are remarkable. GluC (225 aa, 24.3 kDa) and GluD (273 aa, 29.8 kDa) have significant similarities to the membrane proteins of ABC transporters. Both proteins contain the sequence $EAA(X_3)G(X_9)I(X)LP$ (with the exception that in GluC leucine is replaced by phenylalanine). This configuration fits exactly to the consensus sequence of the integral membrane proteins of ABC transporters in gram-negative bacteria (29). GluC and GluD are very hydrophobic, and the use of the HELIXMEM analysis of Eisenberg et al. (7) identifies five regions of GluC and also five of GluD with a high potential for representing membrane-spanning helices.

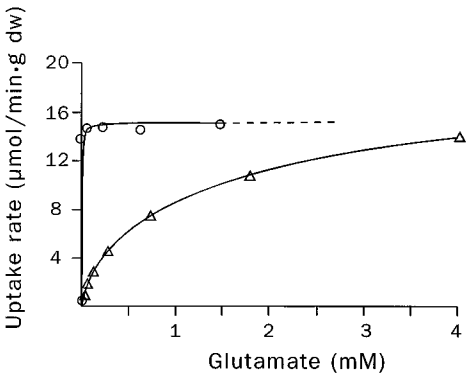

FIG. 3. Effects of globomycin on glutamate uptake rates of *C. glutamicum* after incubation with (Δ) and without (○) 50 μM globomycin. dw, dry weight.

Inhibition of glutamate uptake. From the sequence data, we conclude that processing and modification of the *gluB* gene product occur. In order to investigate this assumption further, we used globomycin, which inhibits the processing of prolipoproteins in *E. coli* (15). When cells of *C. glutamicum*, pregrown with glucose, were shifted to a medium with glutamate as the carbon source, the glutamate uptake system was induced and the typically high uptake rate of 12 to 16 nmol/min/mg (dry weight) was determined. When 50 μg of globomycin per ml was added to the glutamate medium, a very low uptake rate of 0.8 nmol/min/mg (dry weight) was measured in the standard assay. Surprisingly, in spite of the negligibly low glutamate uptake rate, the globomycin-treated *C. glutamicum* cells grew at an only slightly reduced growth rate. This discrepancy was resolved by studying the basic kinetic parameters of glutamate uptake (Fig. 3). The high affinity of the glutamate uptake system, typical of binding protein-dependent systems, is not resolved by the data in the figure. Globomycin-treated cells, on the other hand, showed a completely different pattern. Although V_{max} was more or less unchanged, the affinity of the transport system was strongly reduced. Calculation of the kinetic data for globomycin-treated cells by direct fitting yielded a V_{max} of 15.4 nmol/min/mg (dry weight) and a K_m of 1.52 mM. A small proportion of the overall activity (about 2 nmol/min/mg [dry weight]) still exhibited a high substrate affinity.

Construction of a deletion mutant. To unequivocally assess the role of *gluABCD* in the glutamate transport capability of *C. glutamicum*, we used the procedure established only recently for directed chromosomal mutations in *C. glutamicum* (30). In a process starting with pKW12, in several steps (Fig. 4) plasmid pKK14, deleted of almost the entire *gluABCD* cluster and carrying in addition a mobilizable function and the *sacB* gene, was constructed. This plasmid was transferred by conjugation to the wild type of *C. glutamicum*, yielding four Kan^r Suc^s clones which were verified to be plasmid free. Two of them were identified on minimal medium as Glu^-, whereas the other two were still Glu^+, probably indicating that in these strains recombination occurred with the *gluA* part of pKK14, leaving one copy of the cluster intact. Strain KK40 was grown overnight on Luria broth (plus 0.2% glucose) and was plated on the same medium containing 10% sucrose. Of 100 sucrose-resistant clones, 19 were Kan^s and all were still Glu^-. Chromosomal DNA was isolated from four of these clones and analyzed by Southern hybridization using a 1.2-kb *Bam*HI fragment of the cluster as a probe (data not shown). Two clones did not hybridize with the probe, confirming the deletion of the cluster. One of them was designated *C. glutamicum* Δ*glu*.

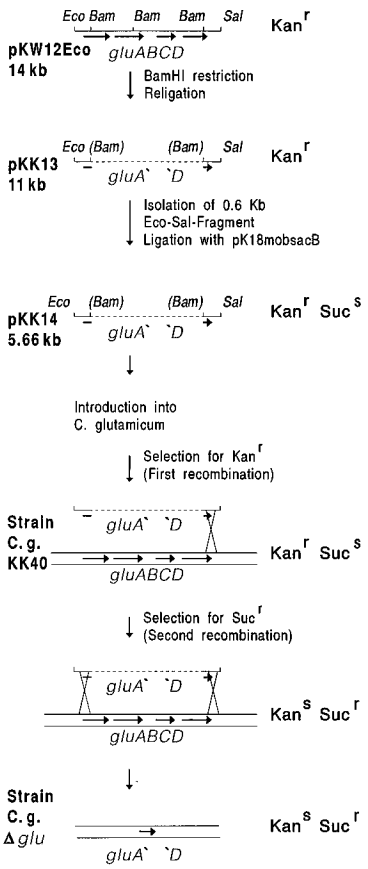

FIG. 4. Construction scheme for deletion of the *gluABCD* cluster in the chromosome of *C. glutamicum*. In the first step, a fragment carrying the 3′ end of the cluster with 109 nucleotides of the beginning of *gluA* and the 5′ end of the cluster, including 42 nucleotides of the end of *gluD*, was generated. This fragment was inserted in a mobilizable nonreplicative plasmid carrying the *sacB* gene to yield pKK14. This plasmid was introduced into *C. glutamicum* (C.g.) ATCC 13032, and the strain with the cluster deleted was generated in two successive selection procedures, as described by Schäfer et al. (30). Eco, *Eco*RI; Bam, *Bam*HI; Sal, *Sal*I.

Glutamate uptake and excretion with the deletion mutant. The wild type of *C. glutamicum* and its isogenic deletion derivative were grown with glucose, and the uptake rates were determined in the standard assay. Strain *C. glutamicum* Δ*glu* had almost no activity compared with the wild type (import rates were 0.06 and 1.26 nmol/min/mg [dry weight] for the mutant and the wild type, respectively), thus showing that no further system contributes to glutamate import. To determine excretion rates for glutamate, we used the unique property of *C. glutamicum*, i.e., the excretion of glutamate triggered by biotin limitation (34). The efflux rates of *C. glutamicum* and *C. glutamicum* Δ*glu* (22 ± 4 and 18 ± 5 [mean ± standard error of the mean] nmol/min/mg [dry weight], respectively) thus initiated were indistinguishable. This result is proof that the great potential of *C. glutamicum* to excrete glutamate is not due to a component of the *gluABCD* cluster but rather that specific machineries are functioning for glutamate import and export.

DISCUSSION

The structural and functional analysis of *gluABCD* clearly identifies the glutamate uptake system of *C. glutamicum* as a binding protein-dependent system (ABC transporter), with all structures for transport and regulation present on the analyzed fragment. The presence of a terminator structure downstream of *gluD* suggests that the four genes are organized as an operon. The great distances between the genes (ranging from 120 to 138 bp) are remarkable, since in most gram-positive species the intergenic regions of ABC transporters are often less than 20 nucleotides apart or the genes even overlap. Only in the downstream region of binding protein-encoding genes, as exemplified by the *opp* cluster of *B. subtilis* (28) or that of the gram-negative *S. typhimurium* (13), the intergenic region extends up to 114 bp. In this region also, an additional stem-loop structure which is presumed to stabilize upstream mRNA is present. Similarly, the stem-loop structure following *gluB* of *C. glutamicum* (Fig. 2) is likely to result in an increased steady-state level of the upstream message, as has been proven for such structures within the *ilvBNC* or *gap* operon of *C. glutamicum* (17, 32). The ultimate result would be an increased ratio of binding protein relative to the membrane complex. Compared with the genetic organization of other binding protein-dependent systems, the location of *gluB* of *C. glutamicum* within the middle of the cluster is unusual. The presence of *gluA* in front of *gluB* might also ensure formation of the proper stoichiometric ratios of the domains of the translocator complex, generally constructed of two ATP-binding proteins (*gluA* in *C. glutamicum*), two transmembrane proteins (*gluC* and *gluD*), and a binding protein (*gluB*) (11).

The derived sequence of the *C. glutamicum* cluster contains the part relevant for carbon source-dependent regulation (Table 1). In fact, on inspection of the only 220 bp which have to be taken into consideration in front of *gluA*, a likely target site at nucleotides 115 to 148 was identified (marked in Fig. 2). This site has weak similarities to the cyclic AMP (cAMP) receptor protein (CRP) binding site from the *lac* operon of *E. coli*. Nothing is known currently about carbon source-dependent regulation in *C. glutamicum*. However, the recent identification of an adenylate kinase gene of this organism (19) confirms the assumption that carbon source-dependent regulation might share elements with the cAMP-CRP system of *E. coli.*

Several lines of evidence for the structure of GluB of *C. glutamicum* are in accord with the view that in gram-positive bacteria the binding proteins are lipoproteins. First of all, a signal sequence with a basic region at the N terminus carrying three positive charges followed by a stretch of hydrophobic and apolar residues is present. Such a structure is characteristic of signal sequences (10). Secondly, the hydrophobic core is followed by the short sequence LTACGD (Fig. 2). This sequence is in exceptional agreement with the consensus sequence of prolipoproteins, in which it is the target of signal peptidase II, within a series of reactions resulting in lipid modification of the N-terminal cysteine. Thirdly, since globomycin inhibits the processing of lipoproteins (15), our functional analysis with this inhibitor also suggests the formation of a glycerol-cysteine-lipid residue of GluB of *C. glutamicum* which could be responsible for anchoring the binding protein in the membrane. Interestingly, in gram-negative species the presence or absence of a negatively charged residue following the cleavage site determines the location of the lipoprotein in the inner or outer membrane (42). Obviously, such a signal is not required in gram-positive species. Our finding of Asp at this site contrasts with the absence of such a charged residue from all cleavage sites of gram-positive bacteria.

The kinetic analysis with globomycin-grown cells (Fig. 3) led us further to conclude that the binding protein of *C. glutamicum* needs to be tethered correctly to the membrane for proper functioning. Upon inhibition, the capacity of the system (V_{max}) turned out to be more or less unchanged, whereas its

affinity (K_m) was dramatically decreased. We assume that the former property is more directly related to the membrane-bound subunits (GluC and GluD), whereas the latter depends on a functionally active binding protein (GluB). These results obviously resemble those obtained with mutants whose maltose uptake systems were binding protein deficient (36), in which suppressor mutations located in the membrane components still enabled translocation of substrate with a strongly reduced affinity. It is therefore likely that in *C. glutamicum* the complex GluACD in the presence of unprocessed binding protein can still confer the basic functions of substrate translocation, although the binding step in the reaction cycle is dramatically changed.

For the two hydrophobic membrane proteins of the glutamate uptake system of *C. glutamicum*, the sequence analysis predicts five membrane-spanning segments, with a charge distribution according to the "positive-inside" rule of von Heijne (39). There is experimental proof of six membrane-spanning segments for OppB, OppC (27), and MalG (5), but there is also evidence of five segments as is the case for HisQ and HisM (18). The sequence predictions for the final two helices occupying about 100 aa of the C terminus are rather reliable (11, 29). These two helices are preceded by the consensus motif $EAA(X_3)G(X_9)IXLP$, which is very well conserved within GluC and GluD of *C. glutamicum* (data not shown). A remarkable feature of GluD of *C. glutamicum* is the extraordinary accumulation of 10 positively and 9 negatively charged aa residues within the final stretch of 51 aa following the last helix.

There is increasing evidence that *C. glutamicum* possesses specific excretion carriers to extrude excess amino acids under particular metabolic conditions (20). Such types of transport systems have been shown to be necessary under conditions of growth on peptides (8), since those amino acids which are constituents of the peptides, but which are not metabolized, otherwise accumulate at high levels within the cell. Evidence of carrier-mediated, energy-dependent excretion of glutamate by *C. glutamicum* has been presented previously (14). On the other hand, inversion of the glutamate uptake system has also been held responsible for glutamate export (3), which could be triggered by specific membrane alterations required for the excretion of glutamate by *C. glutamicum*. Since we determined that the glutamate excretion of the deletion strain was unaltered, we have genetically differentiated between uptake and excretion and can definitely rule out the involvement of any of the four components of the binding protein-dependent system of *C. glutamicum* in excretion. Moreover, our experiments with the deletion strain clearly show that the *gluABCD* cluster codes for the only transport system for glutamate uptake in *C. glutamicum.*

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