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Functional Analysis of Sequences Adjacent to *dapE* of *Corynebacterium glutamicum* Reveals the Presence of *aroP*, Which Encodes the Aromatic Amino Acid Transporter

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An initially nonclonable DNA locus close to a gene of L-lysine biosynthesis in *Corynebacterium glutamicum* was analyzed in detail. Its stepwise cloning and its functional identification by monitoring the amino acid uptakes of defined mutants, together with mechanistic studies, identified the corresponding structure as *aroP*, the general aromatic amino acid uptake system.

The gram-positive bacterium *Corynebacterium glutamicum* is used for the large-scale production of amino acids. We have investigated the cellular biology of this organism, including its physiological and genetic characterizations (3, 4) as well as aspects of transport (9, 10, 14). A peculiarity of *C. glutamicum* is its split pathway of L-lysine synthesis (12). We succeeded in cloning *dapE* of the succinylase branch of this pathway but experienced problems in cloning this locus on a larger fragment (13). On the bases of the idea that a transporter could be present close to the L-lysine biosynthesis gene and our interest in L-lysine export (14), we decided to investigate the region adjacent to *dapE* in more detail. The results presented here include the functional identification of a transporter which has not been described previously and which is furthermore the first functionally characterized gram-positive transporter within cluster 1 of the amino acid-polyamine-choline family of facilitators (11).

The starting point of the analysis was the structurally intact *Bam*HI 3.4-kb fragment of *C. glutamicum* (13) with the known sequence of the *dapE* and the ORF2′ encompassing part (Fig. 1, top). Sequencing of the other part of the fragment revealed the presence of one complete open reading frame (ORF3) as well as a truncated one (ORF5n), both located upstream of *dapE* on the opposite strand. While no homologies with data bank entries for the deduced ORF3 gene product were apparent, that of the truncated ORF5n exhibited high identities (see below) with members of a family of transporters specific for amino acids, polyamines, and choline (11). This prompted us to isolate the carboxy-terminal part of ORF5, too. For this purpose, the 276-bp *Bam*HI-*Bst*I fragment from the end of the *Bam*HI 3.4-kb fragment was integrated into the genome of *C. glutamicum* via pEM1 (12), to eventually isolate the ORF5c 3.9-kb fragment (ORFc3.9) by plasmid rescue. The ORF5c1.4 part, chosen to overlap the known structure, was again sequenced and was melted with ORF5n. The deduced ORF5 gene product (size, 463 amino acids; molecular mass, 49,268 Da) is a hydrophobic polypeptide presumably spanning the membrane 12 times (Fig. 2). Its identities with members of the amino acid-polyamine-choline family of transporters are 40.5% with GabP (translocating γ-aminobutyrate) and 32.5 to 33.6% with LysP (lysine), PheP (phenylalanine), and AroP (aromatic amino acids) of *Escherichia coli* (1, 5).

To identify the function of ORF5, the fusion of the fragment containing ORF5c1.4 plus ORF5n1.6 was attempted but was not possible in the pUC vectors but was obtained with vector pJC1 to give pJCorf5. Vector pJC1 carries the pACYC replicon and has moderate copy number in *E. coli*. Plasmid pJCorf5 was introduced into the wild-type strain to yield *C. glutamicum* pJCorf5, which was expected to oversynthesize the ORF5 gene

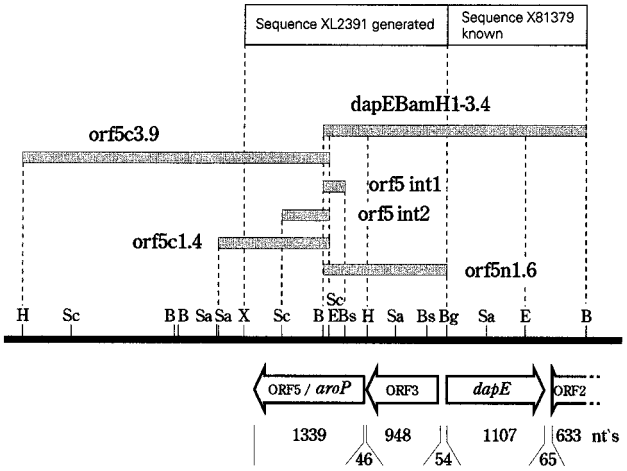

FIG. 1. Overview of the *dapE aroP* locus of *C. glutamicum*. The original clone for studying the structure of ORF5 is shown at the top of the figure (13) and sizes of fragments (in nucleotides [nt's] are shown at the bottom. The original clone was completely sequenced, and the information was used to clone a chromosomal 3.9-kb *Hin*dIII-*Eco*RI fragment (orf5c3.9). Functional studies with reconstituted and plasmid-encoded ORF5 identified this ORF as *aroP* of *C. glutamicum*, encoding the general aromatic amino acid uptake system of this gram-positive bacterium. Selected restriction sites within the chromosome, including those relevant for the construction of plasmids and strains for structural and functional identification, are given. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EI; E, *Eco*RV; H, *Hin*dIII; Sa, *Sal*I; Sc, *Sac*I; X, *Xho*I.

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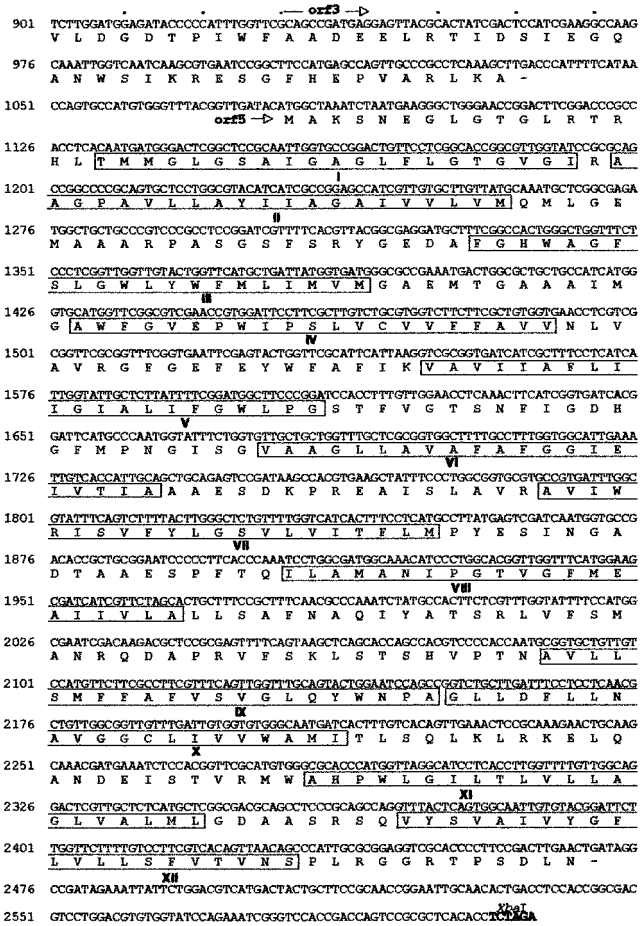

FIG. 2. Sequence of *aroP* of *C. glutamicum*. The 3′ end of ORF3 and the deduced amino acid sequence of the general aromatic amino acid permease are shown. The putative membrane-spanning helices are also indicated. The GenBank accession number of the sequence is XL2391.

product. In addition, the derivative *C. glutamicum*::pEMorf5int2, in which vector sequences disrupt the middle of the ORF, was constructed (Fig. 1). As a consequence, together with the wild-type strain three strains which were expected to differ greatly in the presumed amino acid transport were available. However, no difference in L-lysine export (14) was found. The similarity between the DNA sequences suggested that γ-aminobutyrate might be transported, but no differences in the rates of uptake of this compound by the strains were observed. Therefore, we directly assayed growing cultures by high-performance liquid chromatography analysis for consumption of all proteinogenic amino acids. To do this, minimal medium CGXII (7) in which ammonium and urea were replaced by 1 mM each amino acid was used. After overnight growth, amino acids were consumed either completely (Leu, Thr, Ala, Ile), by about 50% (Val, His, Phe, Trp, Tyr), or by only 20% (Gly, Arg, Lys). The main difference found with respect to the three different *C. glutamicum* strains used was in the consumptions of L-tryptophan, L-tyrosine, L-phenylalanine, and L-histidine. After inoculation, the aromatic amino acids were directly taken up, with rates of about 0.5 nmol/min/mg of dry weight for *C. glutamicum* pJCorf5 and about 0.06 nmol/min/mg of dry weight for the strain with ORF5 interrupted. This result, together with the sequence, identifies ORF5 as *aroP* of *C. glutamicum*. On the basis of these results and on the sequence similarity, ORF5 was thus identified as the general aromatic amino acid uptake system of *C. glutamicum* and was consequently designated *aroP*, by analogy to the corresponding *E. coli* system (1, 5).

TABLE 1. Uptake rates of three ^{14}C-labeled aromatic amino acids by *C. glutamicum*

C. glutamicum strain	Mean uptake ± SD (nmol/min/mg dry weight) [no. of assays]) of:		
	L-Tyrosine	L-Phenylalanine	L-Tryptophan
pJCorf5/aroP	2.88 ± 0.14 (6)	0.74 ± 0.13 (3)	0.66 ± 0.19 (3)
Wild type	0.64	0.40 ± 0.01 (2)	0.21 ± 0.12 (2)
::orf5int2 (*aroP*)	0.30 ± 0.07 (2)	0.24 ± 0.03 (3)	≤0.1 (2)

In order to further functionally characterize the *aroP* gene product, we determined the uptake activities of ^{14}C-labeled substrates for the three *C. glutamicum* strains by the rapid filtration technique. For this purpose, cells were pregrown overnight in brain heart infusion, washed with CGXII medium lacking carbon and nitrogen sources but containing 0.9% NaCl, and suspended in the same medium. After 2.5 h of cultivation, the cells were harvested, again washed as before, and resuspended in the same buffer at a cell density of 0.4 to 2.0 mg (dry weight) per ml. Cells were preincubated for 3 h, and uptake was initiated by the addition of 1 to 200 μM ^{14}C-labeled amino acids. Portions were withdrawn at 15- to 30-s intervals, filtered on nitrocellulose filters (pore size, 0.45 μm), and washed two times with cold 0.1 M LiCl prior to liquid scintillation counting. The uptake activity was clearly dependent on the expression of *aroP* (Table 1). In comparison with L-phenylalanine and L-tryptophan, the most pronounced effect was observed with L-tyrosine, presumably because the membrane of *C. glutamicum* has a very low passive permeability for the latter amino acid (unpublished results). Competition studies confirmed these results, since the addition of 200 μM L-tryptophan or L-phenylalanine reduced the uptake of 20 μM labeled L-tyrosine to less than 14%. We furthermore determined a K_m of 3.0 ± 0.3 μM for the uptake of L-tyrosine by *C. glutamicum* pJCaroP (Fig. 3). The residual uptake with the insertion mutant could indicate an additional uptake system. As is known

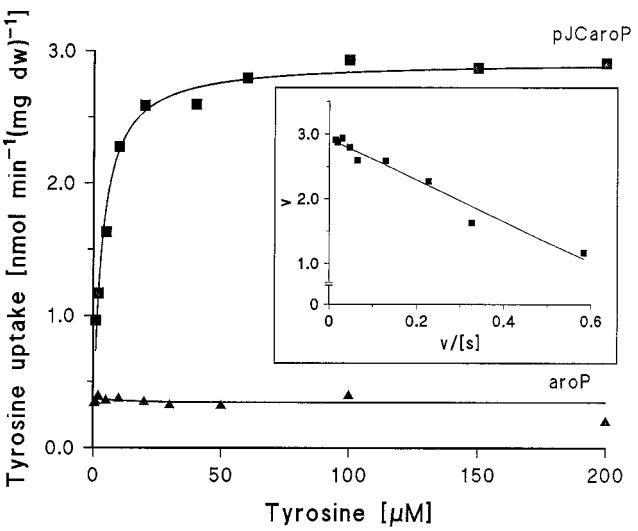

FIG. 3. Initial reaction velocities for tyrosine uptake by *C. glutamicum* pJCaroP and the insertion mutant. Conversion of the direct data into an Eadie-Hofstee diagram is shown in the inset.

from the *E. coli* system, the *C. glutamicum* general aromatic amino acid uptake system functions according to a secondary mechanism. This was indicated by the fact that the addition of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone instantly stopped the L-tyrosine uptake under conditions under which the internal ATP level was confirmed not to be decreased significantly (8).

With all of our results taken together, our analysis structurally and functionally identified the gene for the uptake of the aromatic amino acids of *C. glutamicum*. Recently, in an attempt to assay for altered productivity of aromatic amino acids, a DNA fragment of *C. glutamicum* was isolated, which, when plasmid encoded, resulted in a fewfold increase of the aromatic amino acid uptake (6) and which is therefore also likely to contain the general aromatic amino acid permease. The *C. glutamicum aroP*, as characterized in this paper, is the first gram-positive member within cluster 1 of the amino acid-polyamine-choline family of secondary transporters. It is interesting that *E. coli* has five different aromatic uptake systems which were originally isolated on the bases of their functions but that there is in addition a sixth candidate, for which only the sequence is known (2). Our analysis of mutants indicates that, in addition to *aroP*, more than one system could also be present in *C. glutamicum.*

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