Methionine Uptake in *Corynebacterium glutamicum* by MetQNI and by MetPS, a Novel Methionine and Alanine Importer of the NSS Neurotransmitter Transporter Family†

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ABSTRACT: The soil bacterium *Corynebacterium glutamicum* is a model organism in amino acid biotechnology. Here we present the identification of two different L-methionine uptake systems including the first characterization of a bacterial secondary methionine carrier. The primary carrier MetQNI is a high affinity ABC-type transporter specific for L-methionine. Its expression is under the control of the transcription factor McbR, the global regulator of sulfur metabolism in *C. glutamicum*. Besides MetQNI, a novel secondary methionine uptake system of the NSS (neurotransmitter:sodium symporter) family was identified and named MetP. The MetP system is characterized by a lower affinity for methionine and uses Na^+ ions for energetic coupling. It is also the main alanine transporter in *C. glutamicum* and is expressed constitutively. These observations are consistent with models of methionine, alanine, and leucine bound to MetP, derived from the X-ray crystal structure of the LeuT transporter from *Aquifex aeolicus*. Complementation studies show that MetP consists of two components, a large subunit with 12 predicted transmembrane segments and, surprisingly, an additional subunit with one predicted transmembrane segment only. Thus, this new member of the NSS transporter family adds a novel feature to this class of carriers, namely, the functional dependence on an additional small subunit.

Corynebacterium glutamicum is a model organism in biotechnology and is well-known for its capacity to effectively produce amino acids and to actively export these solutes from the cytoplasm into the medium (*1*−*3*). Extended classifications of transport systems including those of amino acid uptake and excretion are available (*4*), and numerous amino acid uptake systems have been identified at the functional level. However, there is still a large number of amino acid transport systems not yet identified even in model organisms such as *E. coli*, *B. subtilis*, or *C. glutamicum*.

The sulfur-containing amino acid methionine plays a pivotal role in cellular metabolism because it is the universal initiator of the translation process. Additionally, its derivate *S*-adenosylmethionine (SAM[1]) is the major methyl donor and thereby essential for biosynthesis of phospholipids, proteins, DNA, and RNA (*5*).

Methionine uptake in the Gram-negative strains *E. coli* and *Salmonella typhimurium* was one of the first bacterial amino acid transport systems to be studied in detail in fundamental contributions by R. Kadner (*6*−*9*) and P. D. Ayling (*10*−*12*). The simultaneous presence of two and in the case of *S. typhimurium* three uptake systems with different affinity as well as their specificity and regulation on the level of transcription was studied. By biochemical investigations, the high-affinity MetD and the low-affinity MetP transporter, which have a K_m of 0.1 μM and 40 μM, respectively, were characterized in *E. coli* (*6, 7*). Additionally, the methionine uptake by MetD was found to be ATP-dependent. Whereas MetP was shown to accept only L-methionine, the MetD system is able to transport the D-isoform as well (*8, 9*). *S. typhimurium* possesses three L-methionine uptake systems: one with high affinity (K_m = 0.3 μM) and two with lower affinity (K_m of 24 μM and 1.8 mM, respectively) (*10*). The molecular nature of these transporters, however, remained elusive until 2002, when the primary active MetD (MetNIQ) system was described in *E. coli* (*13, 14*). In these studies, regulation of the MetD locus by MetJ, the central regulator of the Met regulon in *E. coli* was investi-

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[1] Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; cdm, cellular dry matter; CMMC, Centre for Molecular Medicine, University of Cologne; CSPD, disodium 3-(4-methoxyspiro(1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.13,7]decan)-4-yl)phenyl phosphate; HMAP, hybrid multidimensional alignment profile; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, 2-amino-2-(hydroxymethyl)-propane-1,3-diol; PLOP, Protein Local Optimization Program; SAM, *S*-adenosylmethionine.

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Table 1: Strains and Plasmids Used in This Work

strain or plasmid	relevant characteristics[a]	reference or source[b]
	Strains	
E. coli		
DH5αmcr	*endA1 supE44 recA1 gyr96 relA1 deoR U169 ϕ*80dlacZΔM15 *mcrA* Δ(*mrr-hsdRMS-mcrBC*)	Grant et al. 1990
C. glutamicum		
ATCC 13032	wild type	ATCC
Δ*mcbR*	wild type carrying a 0.8 kb *Hin*dIII deletion in the *mcbR* locus	Rey et al. 2003
Δ*metNI*	wild type deleted of a 1.8 kb fragment of *metN-metI*	Trötschel et al. 2005
Δ*cgl1029*	wild type deleted of a 0.2 kb fragment of *cgl1029*	this work
Δ*cgl1029/30*	wild type deleted of a 1.8 kb fragment of *cgl1030−1029*	this work
Δ*metNI* Δ*cgl1029/30*	13032Δ*metN-metI* deleted of a 1.8 kb fragment of *cgl1030−1029*	this work
metN::pK18mob	13032 with a pK18mob insertion in *metN*	this work
metI::pK18mob	13032 with a pK18mob insertion in *metI*	this work
Δ*metNI cgl1030*::pDrive	13032Δ*metN-metI* with a pDrive insertion in *cgl1030*	this work
	Plasmids	
pZ8−1	Expression vector, p*tac*, Km^r, *ori* pUC, *ori C. glutamicum*	Dusch et al. 1999
pZ8−1*cgl1029*	pZ8−1 carrying *cgl1029*	this work
pZ8−1*cgl1030*	pZ8−1 carrying *cgl1030*	this work
pZ8−1*cgl1029/30*	pZ8−1 carrying *cgl1030−1029*	this work
pK18mob	Integration vector, Km^r *ori*V_{Ec}*oriT*	Schäfer et al. 1994
pK18mob*metN′*	pK18mob with an internal 0.9kb fragment of *metN*	this work
pK18mob*metI′*	pK18mob with an internal 0.6kb fragment of *metI*	this work
pDrive	Km^R, Ap^R, *p lpp/lac′*, f1 *ori*	Qiagen AG (Hilden, Germany)
pDrive*cgl1030*	pDrive with an internal 1.5kb fragment of *cgl1030*	this work
pK18mobsacB	Integration vector, Km^R *ori*V_{Ec}*oriT sacB*	Schäfer et al. 1994
pK18mobsacBΔ*metNI*	pK18mobsacB with a 2kb fragment carrying the flanking regions of *metN* and *metI*	Trötschel et al. 2005
pK18mobsacBΔ*cgl1029*	pK18mobsacB with a 2kb fragment carrying the flanking regions *cgl1029*	this work
pK18mobsacBΔ*cgl1029/1030*	pK18mobsacB with a 2kb fragment carrying the flanking regions of *cgl1030* and *cgl1029*	this work

[a] Km^r, kanamycin resistant; Ap^r, ampicillin resistant. [b] ATCC, American type Culture Collection.

gated, showing that the promoter of the MetNIQ cluster is in fact repressed by MetJ. Furthermore, the structure of MetNI was solved, and an allosteric regulation of the ATPase subunit MetN by methionine was proposed (*15*). Subsequently, the related ABC-type methionine uptake systems AtmBDE and MetNPQ were identified in the Gram-positive bacteria *Streptococcus mutans* and *Bacillus subtilis*, respectively (*16, 17*). However, in spite of extensive screenings, the gene(s) encoding the secondary methionine carrier MetP were not identified in *E. coli*, *S. typhimurium*, and *Brevibacterium linens* (*18*). The only known bacterial secondary transporter for methionine is BcaP, which is a branched-chain amino acid permease of *Lactococcus lactis* accepting isoleucine, leucine, valine, and methionine as substrate (*19*). An interrelation of the uptake of methionine and other amino acids was frequently described because methionine transport was inhibited by the addition of leucine, valine, alanine, and in the case of *B. linens* by cysteine (*18*).

Methionine uptake has not yet been studied in *C. glutamicum*, although we recently described the active export of methionine in this organism catalyzed by the BrnFE system (*20*). In the present article, we report the molecular identification and biochemical characterization of an ABC-type carrier of the MUT family and a novel secondary methionine and alanine uptake system in bacteria.

In view of the situation that a significant number of amino acid transport systems have not been identified at the molecular level, there is still a fair chance of discovering transport systems of a new type, both in terms of functional and structural properties. This turned out to be true in the case of the MetP methionine uptake system in *C. glutamicum*. Recent additions to the spectrum of structural families of amino acid transporter systems were mainly due to newly described amino acid exporters (*1, 21−23*). Among these, secondary uptake and efflux systems composed of multiple subunits have also been found, e.g., export systems of the BrnFE type (*20, 24*) or of the ThrE type (*23, 25*). Here, we report the identification of the amino acid uptake system MetPS that is composed of two very different subunits: a large subunit with 12 predicted transmembrane segments and a small, accessory subunit with only 60 amino acids and one single predicted transmembrane segment. Interestingly, the large subunit of this system turned out to be a member of the neurotransmitter:sodium symporter family (NSS). These are ubiquitous mammalian sodium and chloride-coupled transporters, which catalyze the uptake of neurotransmitters, such as glycine and dopamine, from the synaptic cleft or are involved in amino-acid uptake in the gut (*26*). A number of bacterial NSS homologues are known, namely, TnaT (*27*), Tyt1 (*28*), and LeuT from *Aquifex aeolicus*, the 3D structure of which has been solved recently (*29*). In particular, LeuT serves as a model for studying structure−function relationships and regulatory properties of this important transporter family (*30*). On the basis of its novel structural properties, MetPS thus adds a new feature to this class of transporters, namely, the requirement of an additional small subunit.

MATERIALS AND METHODS

Bacterial Strains and Growth. The strains and plasmids used are listed in Table 1. *C. glutamicum* cells were grown either in brain heart infusion (BHI) medium (Becton-

Dickenson, Heidelberg, Germany) or in minimal medium MMI (*31*) at 30 °C. For all experiments, cells were precultured in 5 mL of BHI medium for approximately 8 h and subsequently used for inoculation of 20 mL of medium. After approximately 20 h, the culture was used to inoculate fresh MMI medium to an A_{600} of 1−2, and the experiments were started after 2 h of incubation at A_{600} of 3−5.

WT strain served *C. glutamicum* ATCC 13032. All strains were cultivated in flasks shaking at 130 rpm, and, when appropriate, the medium was supplemented with kanamycine (15 or 25 μg mL^{-1}). *E. coli* DH5α*mcr* cells were grown in Luria−Bertani (LB) medium at 37 °C and used for molecular cloning procedures.

Construction of Mutant Strains. For construction of insertion mutants, an approximately 500 bp fragment of the gene of interest was amplified by PCR (Mastermix, Qiagen, Hilden, Germany) and cloned into the pDRIVE vector system (Qiagen, Hilden, Germany) or the vector pK18mob (*32*). Subsequently, *C. glutamicum* was transformed by electroporation (*33*), and clones were selected on kanamycine-containing agar plates as well as proven by PCR analyses confirming the chromosomal integration of the plasmid sequence into the according genomic locus. The genes *metN*, *metI*, and *Cgl1030*, were inactivated using the corresponding plasmids indicated in Table 1. All genes inactivated for this study are listed in Table S1 in the Supporting Information, including primer sequences for amplification of internal gene fragments as well as for the control of insertion inactivation of the particular locus.

Deletion of genes in *C. glutamicum* was carried out as described (*32*). Briefly, fragments of approximately 1000 bp directly flanking the open reading frame of interest were amplified by PCR using primers mediating the introduction of restriction sites to the 5′ as well as 3′ end of the PCR products. For the deletion of *Cgl1029/30*, the primers Del1030F1SphI 5′-GCGC**GCATGC**GAATGTGAGCACCAACAC-3′, Del-1030F1*Hin*dIII 5′-GCGC**AAGCTT**GGGAACTACTGCAA-TCATC-3′, Del1030F2*Hin*dIII 5′-GCGC**AAGCTT**GAA-CAGTCCTGTGTGTGGT-3′, and Del1030F2XbaI 5′-GCGC**TCTAGA**CGAAGGT-TTTCGCGGATG-3′, and for the deletion of *Cgl1029*, the primers Cgl1029-*Hin*dIII 5′-GCGC**AAGCTT**GTAGACCGATGCCAATGA-3′, Cgl1029-SphI-as 5′-GCGC**GCATGC**AGATCTCTGTAGGAAACAC-3′,Cgl1029-SphI-sense5′-GCGC**GCATGC**GTTCCCATGATTT-AGGAC-3′, and Cgl1029-XbaI 5′-GCGC**TCTAGA**CACTG-TCAGTGGGATTCG-3′ were used (restriction sites in bold). After digestion of the fragments by the corresponding restriction enzymes, the DNA fragments were purified and cloned into the shuttle vector pK18mobsacB (*32*). After confirmation of fragments by sequencing (CMMC, University of Cologne), the vectors indicated in Table 1 were used for the transformation of *C. glutamicum*. After a first homologous recombination, the plasmid was integrated into the genome directly adjacent to the gene of interest, mediating kanamycine resistance and sucrose intolerance to recombinant clones. A second recombination event led to the deletion of the gene or to restoration of the WT genotype, which can be monitored by the sucrose tolerance of selected clones. Finally deletions were confirmed by PCR indicating the complete loss of the corresponding gene or genomic region. The *metN-metI*, *Cgl1029*, and *Cgl1029/30* deletion mutant strains were generated by using the plasmids listed in Table 1. Deletion of *metNI* was described previously (*20*). For expression of genes in *C. glutamicum* for complementation, the gene or genomic region of interest was amplified by PCR, and the product was cloned into the shuttle vector pZ8−1 (*34*) mediating kanamycine resistance. After sequence confirmation, *C. glutamicum* was transformed, and positive clones were proven by PCR. In Table 1, all constructed plasmids and the corresponding mutants are listed.

RNA Techniques and RT-PCR. For RNA preparation, *C. glutamicum* cells were harvested from 10 mL of culture (A_{600} = 5) by centrifugation. After disruption of cells by glass beads, RNA was isolated using the NucleoSpin RNAII Kit according to the supplier protocol (Macherey-Nagel, Düren, Germany). Total RNA (5 μg) was blotted onto nylon membranes (BioBond Sigma, Taufkirchen, Germany) using a Minifold I Dot Blotter (Schleicher & Schuell, Dassel, Germany), and hybridization of digoxigenin-labeled RNA probes was performed (*35*) and detected using a luminescent image analyzer LAS1000 (Fuji, Raytest, Straubenhardt, Germany) using alkaline phosphatase-conjugated antidigoxigenine Fab fragments and CSPD as light-emitting substrate as recommended by the supplier (Roche Diagnostics, Mannheim, Germany). In order to obtain antisense RNA probes, a DNA fragment of 544 bp, representing the *metN* gene, was amplified by PCR using the primers metNprobe5 5′-CTCAACGGCACCGACATC-3′ and metNprobe3 5′-GCGCGCTAATACGACTCACTATAGGGGCGCGGTGGCC-ACGAAC-3′ containing the T7-promoter (Table S2, Supporting Information). Subsequently, probes were produced by *in vitro* transcription using T7-polymerase (Roche, Mannheim, Germany).

For RT-PCR experiments, the RNA was additionally treated by Turbo DNase (Ambion, Austin, USA) and after purification analyzed by using One Step RT-PCR as recommended by the supplier (Qiagen, Hilden, Germany). The primer P1 for reverse transcription was 5′-CGTTGCAG-GTCGTTCAGA-3′, and the additional primer for the subsequent PCR was Cgl1030_5 5′-CCGCTGTTGTTTCTCGAT-3′.

Amino Acid Uptake Assay. At an A_{600} of 5, *C. glutamicum* cells were harvested by centrifugation, washed twice with MES/Tris buffer (50 mM, pH 8.0, 10 mM NaCl, 10 mM KCl, and 10 mM glucose), and kept on ice. Uptake rates for methionine or alanine were determined using a filtration assay as described (*36*) and different concentrations of ^{14}C-labeled L-methionine or L-alanine (Hartmann Analytik, Braunschweig, Germany). Where indicated, the protonophore CCCP was added, or MES/Tris buffer with different sodium concentrations was used. In all assays, the initial uptake rates were determined in the first 3 min by sampling every 30 s and used for further calculations. Kinetic parameters as well as standard errors were derived from nonlinear regressions according the Michaelis−Menten equation by using the Sigma Plot software package providing automated performance of several iterations for the calculation of corresponding parameters.

Homology Modeling of MetP. The homology model of MetP was constructed as described (*37*), using the structure of LeuT as a template, following the HMAP alignment (*38*) shown in Figure S1 of the Supporting Information. The initial model was constructed using Nest (*39*). Two sodium ions and one amino acid substrate were modeled on the basis of the equivalent molecules in the LeuT structure. For the amino acid substrates, either the entire leucine molecule was copied,

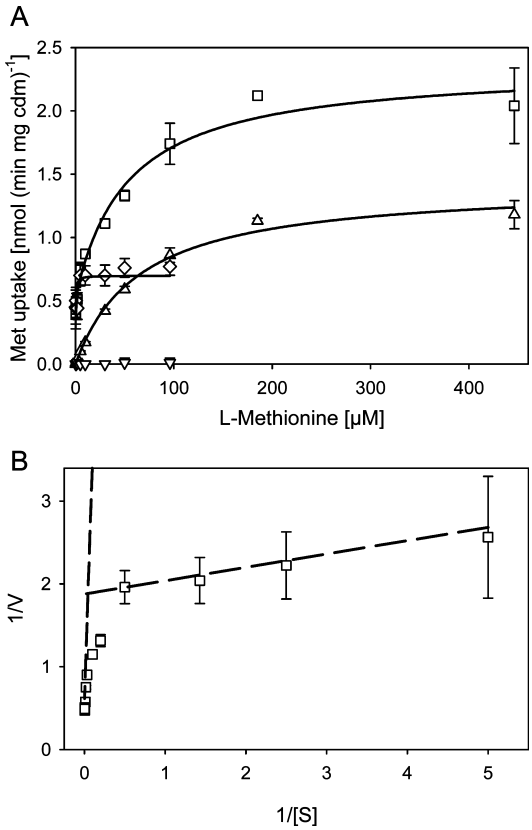

FIGURE 1: Methionine uptake in *C. glutamicum*. Uptake of ^{14}C labeled L-methionine dependent on the external methionine concentration in *C. glutamicum* wild type (□), *metNI* deletion strain (△), *metPS* deletion strain (◇) as well as the *metNI* and *metPS* deletion strain (▽). Data were plotted according to Michaelis and Menten (A) or Lineweaver and Burk (B). All values have been determined at least in triplicate, and standard deviations are indicated. The lines in panel A are the result of direct fits according to Michaelis−Menten equations.

or in the case of methionine and alanine, only the backbone atoms were copied. In all cases, the substrate-bound model was refined by energy minimization of small clashes, followed by side chain optimization for the protein and substrate, using PLOP (*40, 41*).

RESULTS

In order to characterize methionine uptake in *C. glutamicum* in terms of its kinetic properties, we used ^{14}C-labeled L-methionine at different concentrations (Figure 1). Plotting the data according to the Michaelis−Menten equation revealed a saturation kinetic (Figure 1A), but by fitting of the data for calculation of kinetic parameters, we obtained values with high standard errors. After plotting the data according to Lineweaver and Burk, a biphasic dependence of L-methionine uptake on substrate concentration was observed, and the data for high and low substrate concentrations could be fitted separately (Figure 1B). This indicates the presence of at least two transport systems with different transport affinities. By assuming two uptake systems, we fit the data of Figure 1A according to a composite Michaelis−Menten equation by nonlinear regression and obtained the following kinetic constants: high affinity system, $K_M \sim 0.1$ μM, $V_{max} = 0.53$ (±0.09) nmol (min mg cdm)$^{-1}$; low affinity system, $K_M = 53$ (±16) μM, $V_{max} = 1.82$ (±0.13) nmol (min mg cdm)$^{-1}$. These data fit the model better than those obtained by assuming only one uptake system; however, identification of the particular carrier was required in order to verify the kinetic parameters.

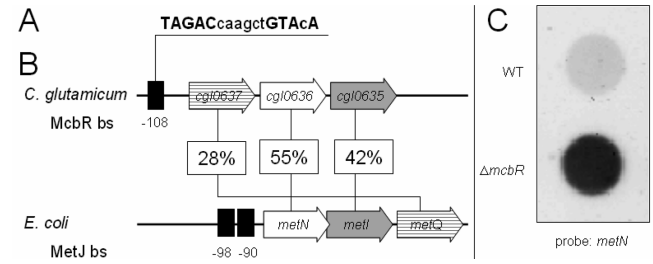

FIGURE 2: Genomic locus of the genes encoding the primary methionine uptake system MetQNI in *C. glutamicum* and MetNIQ in *E. coli*. In (B), the locus tags, the extent of similarity of the corresponding protein sequences, and the distance of the binding sites (bs in black boxes) from the last nucleotide of the McbR and the MetJ binding motif to the first nucleotide of the start codon are indicated. In (A), the sequence of the McbR binding site is given (palindromic sequence in bold). In a Dot blot analysis (C), total mRNA of the wild type (WT) and of a mutant strain lacking the *mcbR* gene was analyzed by a probe representing the *metN* gene.

Molecular Identification of the MetQNI (MetD) System. Since the high affinity D-methionine uptake system MetNIQ, previously called MetD and belonging to the class of ABC-type transporters, was recently published for *E. coli* (*13, 14*), we screened the *C. glutamicum* genome for the presence of a similar gene cluster. By this approach, a cluster of three genes was identified, the genomic structure of which, in comparison to the similar locus in *E. coli*, is shown in Figure 2 including the degree of amino acid similarity of the three corresponding subunits. When we deleted both the *metI* and *metN* genes in *C. glutamicum*, the kinetic analysis of the recombinant strain *C. glutamicum* Δ*metNI* showed a specific loss of the high affinity kinetic component (Figure 1A) and a reduced L-methionine uptake rate at high substrate concentrations. In the following, this uptake system is named MetQNI, according to the gene order in the operon and the corresponding denomination of subunits in *E. coli* (*13*). Thereby MetQ represents the periplasmic substrate binding protein, MetN the cytosolic ATPase component, and MetI the membrane bound subunit.

Functional Characterization of the MetP Methionine Uptake System. For a basic functional characterization of the methionine uptake system MetP, we analyzed its type of energy coupling and its dependence on the presence of cosubstrates (Figure 3). Addition of the uncoupler CCCP (50 μM) led to a significant decrease in methionine uptake under conditions in which the internal ATP concentration was not significantly decreased due to the high glycolytic activity in *C. glutamicum* (data not shown). This was true both for the wild type, in which both systems are active, as well as for the Δ*metNI* disruption strain. Moreover, L-methionine uptake catalyzed by MetP proved to be strictly dependent on the presence of Na^+ (Figure 3B). The derived transport affinity (apparent K_M) for Na^+ in the presence of 30 μM methionine was 0.34 mM (±0.06). Taken together, these results indicate that the putative MetP uptake system most likely functions as a secondary, Na^+-coupled transport system.

Molecular Identification of the MetP Methionine Uptake System. By a systematic approach, we selected all genes encoding putative Na^+-coupled, secondary carrier systems

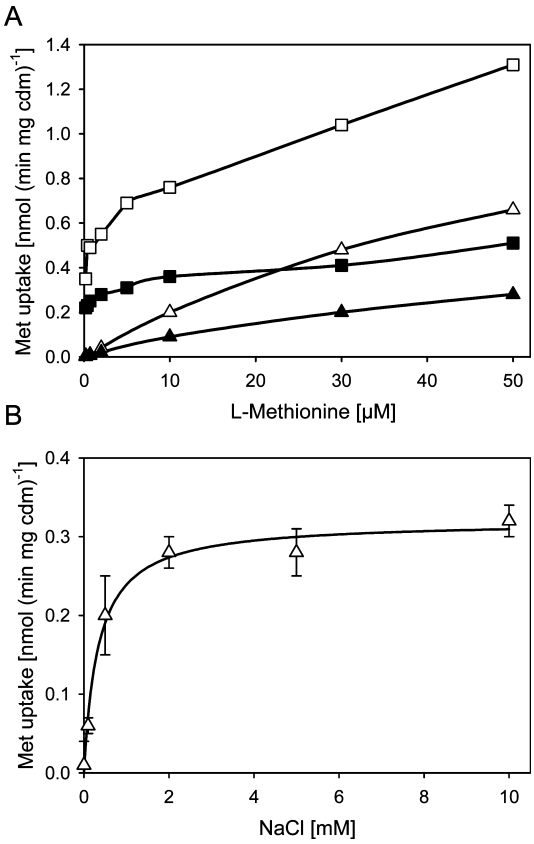

FIGURE 3: Characterization of methionine uptake by MetP. (A) Uptake of ^{14}C labeled L-methionine dependent on the external methionine concentration for wild type *C. glutamicum* without further addition (□), upon the addition of 50 μM of the uncoupler CCCP (■), and for the *metNI* disruption strain in the absence (Δ) and in the presence of 50 μM CCCP [▲]. (B) Methionine uptake in the *metNI* deletion strain dependent on externally added sodium.

in the genome of *C. glutamicum* as well as candidates for methionine transporters, which led to a list of 18 genes shown in Table S1 (Supporting Information). Among these were three genes similar to the described *S*-methylmethionine uptake system *ykfD* of *E. coli*, namely, *cgl1155*, *cgl0470*, and *cgl1107* (*42*). Using the *metNI* disruption strain *C. glutamicum* Δ*metNI*, we inactivated the putative target genes one by one. The obtained recombinant strains were assessed for activity of L-methionine uptake, and all but one strain tested showed amino acid uptake in the range of ± 20% of the *metNI* disruption strain. The exception was the strain in which gene *cgl1030* was interrupted, where L-methionine uptake in the recombinant strain was negligible. This result indicates that (i) the gene product of *cgl1030* represents the medium affinity L-methionine uptake system MetP and (ii) that there is no further L-methionine uptake system present in *C. glutamicum* under our experimental conditions besides MetQNI and MetP.

Subsequent construction of a *metP* deletion strain resulted in the inactivation of *cgl1030* as well as *cgl1029*, which is a small adjacent ORF downstream. Accordingly, in the WT background strain, *C. glutamicum* Δ*cgl1029/30* was generated, and in the Δ*metNI* background strain, *C. glutamicum* Δ*metNI*Δ*cgl1029/30* was generated. Both mutations had the expected impact on methionine transport activity (Figure 1A). Whereas the high affinity system, associated with MetQNI, was still present in the strain *C. glutamicum* Δ*cgl1029/30,* a complete loss of L-methionine uptake was observed in the double deletion strain.

Functional Characterization of the Primary Active MetQNI System. Once the *C. glutamicum*Δ*cgl1029/30* strain was available in which the MetP system is absent, a functional characterization of the remaining primary active MetQNI system was possible. The obtained kinetic constants (K_M = 0.2 (±0.09) μM; V_{max} = 0.7 (±0.05) nmol (min mg cdm)$^{-1}$) of the separately measured MetQNI L-methionine uptake system match the data for the high affinity system derived from the initial calculation using the data for methionine uptake by WT cells (see above and Figure 1A). We also determined the substrate specificity of the MetQNI system in detail. In the uptake assay of labeled L-methionine, we added other amino acids in unlabeled form and in 10-fold excess. We did not observe competition by any of the 20 L-amino acids tested, and surprisingly, this held true also for D-methionine, which is the preferred substrate of the homologous *E. coli* system (*13, 14*).

We further studied the regulation of the *metQNI* gene cluster on the level of expression. While in *E. coli* the transcriptional regulator MetJ mediates the control of methionine biosynthesis and binds to the two operator sites found in the promoter region of the *metNIQ* operon (Figure 2), in *C. glutamicum* the regulator McbR was identified as a regulator of genes involved in sulfur metabolism (*43*−*45*). In the promoter region of the *metQ* gene *cgl0637*, a McbR binding site was found that matched the proposed consensus sequence 5′-TAGAC-N_6-GTCTA-3′ (see Figure 2). In order to verify the regulation of *metQNI* expression by the repressor McbR, we compared the expression of *metN* in WT and in the Δ*mcbR* mutant. Dot blot analysis revealed that in the McbR lacking mutant, the *metN* gene is strongly expressed in contrast to the WT (Figure 2C). This is in agreement with bioinformatics predictions as well as results obtained by DNA microarray experiments using the *mcbR* mutant of *C. glutamicum* performed by Rey and co-workers (*43*). Consequently, the primary L-methionine uptake system MetQNI in *C. glutamicum* is in fact under the control of the transcription factor McbR.

Functional Characterization of the Secondary Transport System MetP. Having proven that MetQNI and MetP are the only two functionally active L-methionine uptake systems in *C. glutamicum* under our experimental conditions, we were able to study the functional properties of MetP in the Δ*metNI* deletion strain. Using a direct fit to the concentration dependence of methionine uptake shown in Figure 1A, a K_M value of 65.6 (±6.7) μM and a V_{max} value of 1.42 (±0.05) nmol (min mg cdm)$^{-1}$ were derived, close to the values obtained in the wild type strain by calculation (see above).

In order to investigate the substrate specificity of MetP, we tested all 20 and selected L-amino acids and in addition D-methionine, for competition of MetP mediated uptake of L-methionine (Table 2). We found that a number of amino acids inhibited methionine uptake to varying extents when present in 10-fold excess. A strong inhibition was caused by branched chain amino acids, by cysteine, and, somewhat less effectively, alanine, indicating that these amino acids are putative substrates for MetP. The positive control experiment, i.e., testing inhibition of the uptake of these amino acids in labeled form by addition of excess methionine, was not possible in the case of cysteine because of the

Table 2: Screening for Additional Substrates of the Transporter MetP by Measuring of Methionine Uptake of the Mutant Lacking the Primary Uptake System MetQNI in the Absence (None) and Presence of a Combination of All Amino Acids (All) as Well as Selected Amino Acids in a 10-Fold Excess[a]

amino acid addition	uptake rate [nmol (min mg cdm)$^{-1}$]
none	0.65
all	n.d.
L-Ala	0.14
L-Val	n.d.
L-Leu	n.d.
L-Ile	n.d.
L-Cys	n.d.
L-Ser	0.62
L-Thr	0.34
D-Met	0.46
L-Pro	0.61
Gly	0.62

[a] The L-methionine concentration was 50 μM, and n.d. means not detectable. The results were obtained by two independent experiments.

difficulty in obtaining correctly labeled and pure cysteine. Moreover, additional cysteine and/or cystine uptake systems are present in *C. glutamicum* (data not shown), which makes it impossible to address the consequences of the deletion of the *metP* gene for cystine or cysteine transport on the physiological level. In the case of branched chain amino acids, the highly active BrnQ system was found to represent the only importer for branched chain amino acids in *C. glutamicum*, and in a *brnQ* knock out mutant, no residual uptake activity for leucine was found (*46*). Therefore, we concluded that leucine inhibits the transport activity of MetP, although it is not transported. For analysis of alanine as a putative substrate of MetP, we first characterized the uptake of labeled alanine in the Δ*metNI* and in the Δ*metNI*Δ*cgl1029/30* deletion strain (Figure 4). The obtained results show that (i) MetP in fact accepts alanine as a transport substrate and that (ii) MetP is the major alanine uptake system in *C. glutamicum* since the residual activity in the deletion strain *C. glutamicum* Δ*metNI*Δ*cgl1029/30* amounts to only about one-third of the alanine uptake in the Δ*metNI* strain. The kinetic values derived from the difference between the two experimental results led to a K_M of 69 (±11) μM and a V_{max} of 3.3 (±0.2) nmol (min mg cdm)$^{-1}$ for alanine uptake (Figure 4A). Figure 4B shows competition of methionine uptake by alanine and vice versa. Thereby we obtained a half-maximal inhibition of methionine uptake at an alanine concentration of 160 (±20) μM and a half-maximal inhibition of alanine uptake at a methionine concentration of 139 (±15) μM indicating that the two amino acids are equally good substrates for MetP. Interestingly, MetP was shown to accept the structurally diverse amino acids methionine and alanine but did not accept β-alanine as a substrate (results not shown).

Finally, similar to the experiments described for the MetQNI system, we investigated whether MetP is under the control of the McbR regulatory network. This was not the case (results not shown). Furthermore, we tested whether expression of the *metP* gene is influenced by the presence of various amino acids, including L- and D-methionine; however, no influence on the level of expression was observed, and a constitutive mRNA level was found (results not shown).

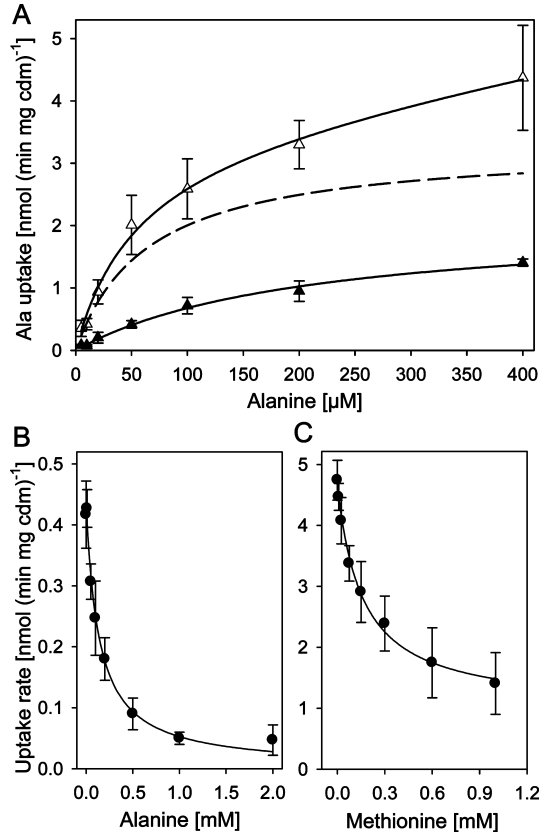

FIGURE 4: Alanine uptake in *C. glutamicum*. (A) Uptake of ^{14}C labeled L-alanine dependent on the external alanine concentration in the *metNI* (Δ) and in the *metNIcgl1029/30* deletion strain (▲) as well as the calculated uptake rate for the MetP system (dashed line). Competition of ^{14}C labeled L-methionine uptake by alanine (B) and ^{14}C labeled L-alanine uptake by methionine (C). The residual uptake rate of the originally added, labeled amino acid methionine and alanine, respectively, is shown upon the addition of increasing concentrations of the competitor alanine (B) and methionine (C), respectively.

Structural Modeling of MetP Bound to Its Substrates. Further support for the role of the *cgl1030* gene product in amino-acid uptake was obtained by modeling of its substrates bound to a homology model of MetP. MetP has a high degree of similarity (25−28% identity and 44−51% similarity, depending on the sequence length used for normalization) to the NSS (neurotransmitter:sodium symporter family) transporter LeuT from *Aquifex aeolicus* (Figure 5 and Figure S1 (Supporting Information)), the first bacterial transporter of the NSS family whose 3D structure was solved by X-ray crystallography (*29*). MetP has longer C- and N-terminal regions than LeuT, but the loops are of similar lengths, and the sequence identity is particularly high in the regions known from LeuT to bind leucine and the cosubstrate sodium (Figure S1, Supporting Information). On the basis of the known 3D structure of LeuT, we generated a homology model of MetP (Figure 5), which is likely to be accurate within 1−2 Å for the Cα atoms (*37*). We found it was possible to model the binding of substrates within the binding pocket of MetP (Figure 5 B−D). As in the LeuT structure, the carboxyl and amino group of each substrate were placed in close proximity to the two sodium ions. The conserved interactions are from Gly53, Gly55, and Tyr131 to the substrate carboxylic group and from Ala51, Ser264, and Phe261 the substrate amino group (Figure 5). The residues contacting the side chains of the bound amino acids in MetP

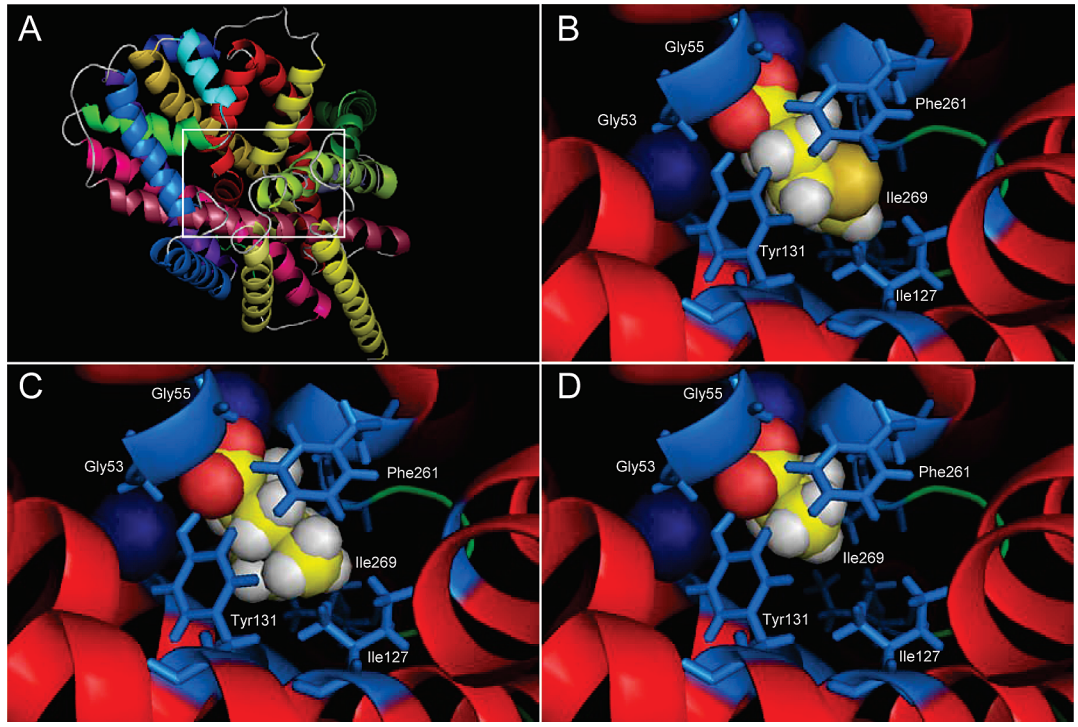

FIGURE 5: Structural model of MetP and substrate binding. The protein was homology modeled using the structure of LeuT (pdb id: 2a65 (*19*)) as a template, according to a sequence alignment constructed using HMAP (see Figure S1 in Supporting Information). (A) Individual helices are shown in different colors as viewed from the extracytoplasmic site. The white box indicates the substrate binding pocket. The methionine (B), leucine (C), and alanine (D) substrates and sodium ions are shown as spheres, and the residues involved in the formation of the substrate binding pocket are shown as sticks. Selected residues are labeled according to the description in the text.

are Ile127 and Ile269. These residues correspond to Val and Ala in LeuT, respectively, reflecting their different substrate specificity profiles.

Subunit Composition of the MetP System. When testing for complementation, we found that in recombinant *C. glutamicum* strains in which the gene *cgl1030* was deleted together with parts of the closely adjacent, small gene *cgl1029* (*C. glutamicum* Δ*cgl1029/30*) (Figure 6) complementation could not occur by a plasmid encoding the *cgl1030* gene only (Figures 6 and 7). However, complementation with a plasmid, in which both genes *cgl1030* and *cgl1029* were present, in fact restored L-methionine uptake. The differences in the observed methionine uptake rates between the mutant strain Δ*metNI* and Δ*metNI*Δ*cgl1029/30* harboring the plasmid pZ8−1*cgl1029/30* are most probably caused by a low ectopic expression in the latter strain. As a control, we also tested complementation of the double deletion strain by a plasmid harboring the *cgl1029* gene only, which was not successful. Finally, the gene *cgl1029* downstream of gene *cgl1030* was deleted separately. The resulting strain *C. glutamicum* Δ*cgl1029* had the same phenotype in terms of methionine uptake as observed for strain *C. glutamicum* Δ*cgl1029/30* (data not shown). In view of this result, we investigated whether the two genes are transcriptionally coupled. For this purpose, RNA of *C. glutamicum* wild type was isolated and analyzed by RT-PCR (Figure 6). After performing the reverse transcription by using a primer that binds downstream of the gene *cgl1029*, the resulting DNA was amplified by PCR using this primer in combination with a primer binding to the upstream region of *cgl1030*. The results of these experiments demonstrate that the transcripts of the two genes *cgl1030* and *cgl1029* in fact form a polycistronic mRNA. Consequently, we propose the name *metS* for the gene *cgl1029* downstream of the *metP* gene.

Taken together, these results strongly indicate that both genes *cgl1030* and *cgl1029* together are necessary for the functional L-methionine uptake system MetPS in *C. glutamicum*. The two gene products *Cgl1030* and *Cgl1029* are predicted to be composed of 579 and 60 amino acids, respectively, and contain 12 and 1 transmembrane segments, respectively, according to predictions by SOSUI (*47*). The *cgl1029* gene product MetS does not show sequence similarity to any protein with known function. However, by inspecting bacterial genomes for the co-occurrence of genes encoding proteins with sequence similarity to *metP* and *metS* from *C. glutamicum*, a number of species, both closely related as well as unrelated to *C. glutamicum*, an identical organization of genes similar to *metP* and *metS* was found by bidirectional blast searches (Figure 6). The neighborhood of this particular gene cluster, however, does not seem to be strongly similar in different bacterial species, except for the frequent occurrence of a gene encoding a predicted GTPase (*cgl1032*).

DISCUSSION

Originally, this work was driven by the biotechnological significance of methionine uptake in *C. glutamicum*. With respect to transmembrane fluxes, amino acid production generally depends on at least three transport events, i.e., (i) uptake of substrate, usually glucose, (ii) export of product, which, in the case of methionine was recently elucidated (*20*), and (iii) reimport of product (*48*). The latter of these events is, in terms of amino acid production, an unwanted side effect of the presence of amino acid uptake systems for physi-

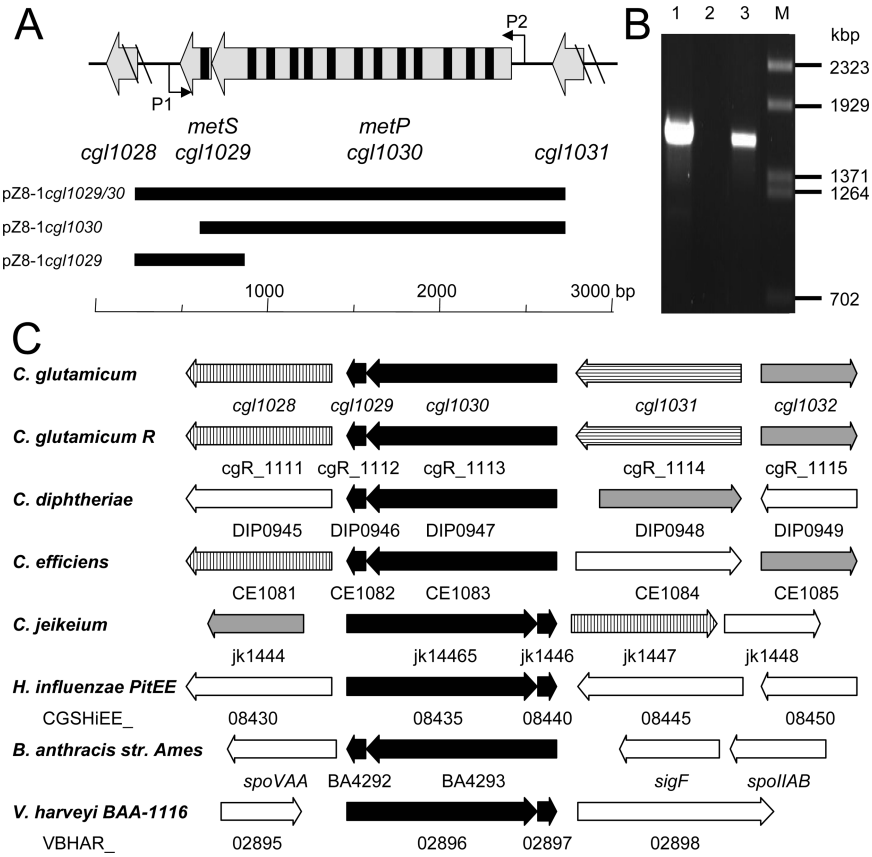

FIGURE 6: Genomic locus of the genes encoding the methionine uptake system MetPS in *C. glutamicum* (A) including proposed gene names and locus tags, the position of predicted transmembrane helices encoding regions (black boxes) as well as the size of the genomic fragments pZ8−1*cgl1029/30*, pZ8−1*cgl1030*, and pZ8−1*cgl1029* (black bars) used for complementation of the strain *C. glutamicum* Δ*metNI*Δ*cgl1029/30*. (B) Cotranscription of the genes *cgl1030* and *cgl1029* was shown by RT-PCR using the primer P1 for first strand synthesis by reverse transcription and primer P2 for the subsequent PCR, as indicated in part (A). Experiments were performed with total RNA of wild type with or without RT steps (lane 1 and 2, respectively) and with genomic DNA of the wild type (lane 3). (C) A search for proteins homologous to MetP and MetS by bidirectional blast search revealed comparable genomic loci in *C. glutamicum* strain R, *Corynebacterium diphtheriae*, *Corynebacterium efficiens*, *Corynebacterium jeikeium*, *Haemophilus influenzae PitEE*, *Bacillus anthracis*, and *Vibrio harveyi BAA-1116*. Encoded genes homologous to MetP and MetS (black), a predicted GTPase (gray), and proteins of unknown function (horizontally and vertically streaked) and proteins of other functions (white) are shown and indicated by their locus tags.

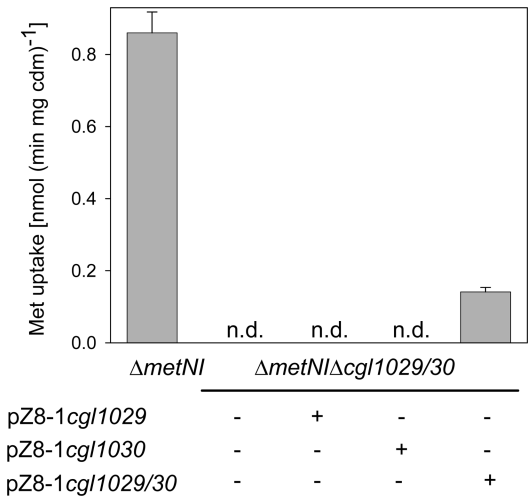

FIGURE 7: Complementation of *metPS* deletion in *C. glutamicum*. Methionine uptake rates are shown for the MetQNI lacking *C. glutamicum* strain as well as for the strain lacking both MetQNI and MetPS at a substrate concentration of 100 μM methionine. The latter strain was complemented with the genes *cgl1029* (*metS*), *cgl1030* (*metP*), or a combination of both. The size of the complementing gene fragments in the respective plasmids is indicated in Figure 6, and n.d. means not detectable (below the detection limit of 0.05 nmol (min mg cdm)$^{-1}$).

ological reasons. In fact, it has been shown that during methionine production by *C. glutamicum*, the product methionine is taken up again by the production strain when the substrate glucose is consumed (*49*). This certainly also occurs unnoticed during the production process because of the dominating methionine export flux. Consequently, knowledge and possibly elimination of relevant uptake systems is a common strategy in improving amino acid production efficiency (*1, 48, 50*).

For valuable substrates, bacteria frequently harbor a minimum of two different uptake systems: a medium affinity system with moderate specificity, which is constitutively expressed, and a specific transport system with high affinity, which is regulated according to the availability of the substrate. This is exactly the scenario we have found for methionine uptake in *C. glutamicum*, and a similar situation was described many years ago by biochemical analysis for *E. coli* (*6*). Primary active methionine uptake systems of the ABC-carrier type, originally named MetD (*7*), have recently been identified for *E. coli* (*13, 14*) as well as for *Bacillus subtilis* (*16*). The secondary system in *E. coli*, originally designated MetP (*7*), however, remained elusive and is still not known because no homologue of MetPS of *C. glutamicum* is present in *E. coli*.

By using a direct approach based on both genomic data of *C. glutamicum* and on biochemical analysis of methionine uptake, we were able to identify two methionine uptake systems in this Gram-positive soil bacterium, one being highly similar to the known, primary active MetNIQ system of *E. coli* and a novel one with a secondary mechanism, showing structural similarity to carriers from the neurotransmitter:sodium symporter family. The latter transporter MetPS is in fact a methionine and alanine transporter. Knowledge of bacterial carrier systems for methionine and alanine is extremely limited so far. To our knowledge, there is only one report of methionine being transported by the branched chain amino acid transporter BcaP from *Lactococcus lactis* (*19*) and one reference that alanine is a substrate of MctP from *Rhizobium leguminosarum* (*51*). Alanine transport by the AspT system (aspartate:alanine antiporter), by contrast, has no physiological significance for alanine import and instead was proposed as an electrogenic system used to generate an electrochemical potential in bacteria under particular conditions (*52*).

On the basis of the available MetP deletion strain, the remaining ABC-type MetQNI (MetD) system in *C. glutamicum* was characterized. It turned out to be rather similar to the *E. coli* MetNIQ system (*13, 14*), being characterized by high affinity, strict specificity, and regulation by a methionine specific repressor. In the promoter region of the *C. glutamicum metQNI* cluster, we identified a McbR binding site. McbR is a TetR-type of transcription regulator that uses *S*-adenosylhomocysteine as a corepressor (*43, 44*). Methionine did not affect McbR DNA binding and therefore is not an effector of McbR (*44*). This is in agreement with the observation that *C. glutamicum* can use methionine only as very poor sole sulfur or sole nitrogen source so that the availability of methionine itself is most likely not a signal for *metQNI* expression directly. In the case of *E. coli*, a similar mechanism was found, in which the transcription factor MetJ is used, which binds S-adenosylmethionine as an effector (*53, 54*). In comparison, in *Streptococcus mutans* the MetR protein was found to activate the transcription of the AmtBDE ABC-type transporter by binding to a palindromic sequence located in the promoter region. The AmtBDE system was found to bind D- and L-methionine as well as selenomethionine and homocystein. The latter was proposed to act as a coeffector of the MetR regulator (*17*). In *B. subtilis*, however, no transcription factor is needed at all, but *S*-adenosylmethionine binds directly to the mRNA of particular genes, thereby increasing the efficiency of expression (*55*). There is, however, one striking difference between *C. glutamicum* and *E. coli*, in that the *E. coli* MetNIQ system prefers D-methionine to L-methionine (*13, 14*), whereas the MetQNI system from *C. glutamicum* exclusively accepts L-methionine.

Identification of MetPS in the MetQNI deletion background was possible by a systematic inactivation of putative candidate genes one by one, after the biochemical confirmation of a single remaining secondary methionine uptake system. After the concomitant knock out of the MetP encoding gene, no further methionine uptake activity was found (Figure 1A) indicating the presence of only two methionine uptake systems in *C. glutamicum* under our experimental conditions. In addition, we concluded that inhibition of MetPS by CCCP (Figure 3) was incomplete. In comparison to MetP of *E. coli*, which is specific for L-methionine and is induced on the level of transcription by the availability of its substrate methionine (*8*), MetPS of *C. glutamicum* is constitutively synthesized and accepts methionine and alanine as substrate. We propose that the inhibition of methionine transport by alanine and vice versa is most likely a competitive inhibition. The inhibition of MetPS dependent methionine transport by cysteine is in agreement with results obtained for methionine transport in *Brevibacterium linens* CNRZ 918. In this strain, a secondary methionine carrier was described biochemically, which was sodium dependent and inhibited by cysteine addition like the MetPS system in *C. glutamicum* (*18*). A further analysis of the substrate specificity of MetPS with respect to the impact on physiology was hindered by the presence of other transport systems for particular substrates such as cysteine, cystine and branched chain amino acids as well as the fact that *C. glutamicum* can utilize D-methionine or methionine sulfoxide only very poorly as the sole sulfur source. The latter one might represent a substrate of the MetQNI system as shown for the MetNPQ carrier in *B. subtilis* (*16*); however, metabolic utilization of methionine sulfoxid seems to be inefficient in *C. glutamicum*.

By sequence comparison, it was revealed that MetP belongs to the neurotransmitter:sodium symporter family (NSS), and thus is related to LeuT from *A. aeolicus*. The two proteins are characterized by a high similarity throughout the whole sequence, and structural modeling of substrate binding to MetP on the basis of the known 3D structure of LeuT is supportive of a common binding mode. Whereas methionine was not tested as a putative substrate of LeuT, alanine was proven to represent the preferred substrate (*29, 30*). While the affinity of LeuT for leucine is also very high (K_D ~20 nM), binding and transport kinetics for leucine, as well as molecular dynamics simulation studies imply that the LeuT transporter is trapped in an occluded state by this particular amino acid (*30, 56*). We suggest from our experiments and modeling that MetP of *C. glutamicum* can indeed bind methionine, alanine, and leucine (Figures 4 and 5) but that the latter amino acid may lead to the formation of an occluded state, as proposed for LeuT. This hypothesis is in agreement with the transport of methionine and alanine by MetPS and the inhibition of methionine uptake by leucine (Table 2). Leucine itself is not or is only very slowly transported by MetPS because the BrnQ transporter was found to represent the only uptake system for branched chain amino acids in *C. glutamicum* (*46*).

The conservation of residues in MetP and LeuT involved in binding of the carboxy and amino group of amino acid substrates was demonstrated by modeling of all three amino acids into the substrate binding pocket. We propose that the recognition of the functional groups at the α-carbon of the substrate amino acids is essential for binding because β-alanine, which lacks the amino group in the α-position, is not a substrate of MetPS (data not shown). The coordination of the substrate side chains seems to be less well defined, as underlined by the observed amino acid differences in MetP in comparison

to LeuT. A complete understanding of the selectivity for different amino-acid side chains will require further structural knowledge of, e.g., other states of the protein during transport.

On the basis of the complementation analysis, MetPS turned out to be a novel type of bacterial transport system in that, besides the major subunit represented by the gene product of *cgl1030* (*metP*), which comprises a membrane protein with 12 transmembrane segments, a second, smaller protein, encoded by the gene *cgl1029* (*metS*), with only one putative transmembrane segment is essential for functional activity. Dot blot analyses of WT and the *cgl1029* lacking strain revealed comparable mRNA levels for *cgl1030* indicating that at least transcription of this gene can occur in a *cgl1029* independent manner (data not shown). Amino acid transport systems in bacteria composed of two subunits are not unknown, as exemplified by the recently described methionine and branched chain amino acid exporter BrnFE from *C. glutamicum* (*20, 24*), as well as by transport systems of the ThrE type (*23*). In the latter two transport systems, the expression of both subunit encoding genes is essential for active transport, as shown here for *metP* and *metS* with respect to methionine transport. Moreover, by bidirectional blast search, we identified a number of structurally similar gene loci composed of two genes encoding proteins similar to MetP and MetS from *C. glutamicum*. Furthermore, ActP, the proposed acetate permease of *E. coli*, is cotranscribed with a small gene *yjcH* encoding a small putative membrane protein (*57*). However, no function could be assigned to the gene product of *yjcH*. ActP itself, however, is not structurally similar to MetP.

Transport systems composed of two subunits with such an extremely different number of transmembrane domains are so far only known in eukaryotes, namely, the heterodimeric amino acid transporter (HAT) systems (*58, 59*). In spite of the functional similarity however, the two components of the HAT systems are rather different. Although the so-called light chains are in fact predicted to contain 12 transmembrane segments, as for the MetP subunit Cgl1030, the heavy chain subunits of HATs, containing only a small number of (most probably only one) transmembrane segments, also carry huge extramembraneous, heavily glycosylated hydrophilic domains and are linked to the light chain by a disulfide bridge. Moreover, the light chain subunits of HAT do not display significant sequence similarities to Cgl1030 and have amino acid transport activity on their own, which is obviously not the case for MetP.

Although the relevance of the small, accessory subunit MetS for a functionally active MetPS system has been unequivocally shown here, it is not clear whether this small protein is in fact an integral part of the functional transport system. An alternative explanation could be the function of MetS as a specific molecular chaperone, which, for example, is discussed in the case of the KdpF subunit of the KdpABCF ATPase in *E. coli* (*60, 61*). In the future, the functional participation of MetS in the transport process will be addressed by further studies involving the reconstitution in proteoliposomes in a functionally active state and/or structural studies on purified proteins.

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SUPPORTING INFORMATION AVAILABLE

A sequence alignment between LeuT and MetP used to construct the model as well as a comparison of the predicted secondary structure of MetP and that of LeuT including the positions of highly conserved residues involved in coordination of sodium ions and substrate binding, and a list of all genes inactivated for identification of MetP and the corresponding primer sequences. This material is available free of charge via the Internet at http://pubs.acs.org.

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