

Molecular Identification of the Urea Uptake System and Transcriptional Analysis of Urea Transporter- and Urease-Encoding Genes in *Corynebacterium glutamicum*

Gabriele Beckers, Anne K. Bendt,† Reinhard Krämer, and Andreas Burkovski*

Institut für Biochemie der Universität zu Köln, Cologne, Germany

Received 9 July 2004/Accepted 16 August 2004

The molecular identification of the *Corynebacterium glutamicum* urea uptake system is described. This ABC-type transporter is encoded by the *urtABCDE* operon, which is transcribed in response to nitrogen limitation. Expression of the *urt* genes is regulated by the global nitrogen regulator AmtR, and an *amtR* deletion strain showed constitutive expression of the *urtABCDE* genes. The AmtR repressor protein also controls transcription of the urease-encoding *ureABCEFGD* genes in *C. glutamicum*. The *ure* gene cluster forms an operon which is mainly transcribed in response to nitrogen starvation. To confirm the increased synthesis of urease subunits under nitrogen limitation, proteome analyses of cytoplasmic protein extracts from cells grown under nitrogen surplus and nitrogen limitation were carried out, and five of the seven urease subunits were identified.

Urea is a readily available nitrogen source, since it is excreted by a variety of organisms into the environment. In bacteria able to metabolize this solute, urea is hydrolyzed by the cytoplasmic urease enzyme complex, leading to one CO₂ and two ammonium molecules (for review, see reference 20). Although urea is small and uncharged and can therefore easily pass the bacterial membrane, many prokaryotes, including *Corynebacterium glutamicum*, synthesize energy-dependent transport systems for its uptake. When present in high concentrations, sufficient urea crosses the *C. glutamicum* cytoplasmic membrane by passive diffusion, and only under conditions of nitrogen starvation is an energy-dependent urea uptake system synthesized (27). With a K_m of 8 μ M for urea, the affinity of this uptake system is much higher than the affinity of urease for its substrate (K_m of approximately 55 mM). The maximum urea uptake rate depends on the level of expression and is relatively low at 2.0 to 3.5 nmol mg (dry weight)⁻¹ min⁻¹ (27). Genes coding for the *C. glutamicum* urea uptake system have not been previously identified.

The genes encoding the urease enzyme complex were isolated and sequenced (21, 24). While *ureA*, *ureB*, and *ureC* encode the urease structural subunits, the *ureE*, *ureF*, *ureG*, and *ureD* genes code for accessory proteins. As shown by mutant analyses, at least the *ureC* and the *ureD* products are essential for a functional urease (21). Depending on the growth conditions, urease activity observed in the wild type varies between 0.9 and 2.2 U mg of protein⁻¹ for cells grown in ammonium-rich minimal medium (21, 24), between 1.0 and

1.6 U mg of protein⁻¹ when glutamine is used as nitrogen source (24), and between 2.0 and 6.1 U mg of protein⁻¹ when different concentrations of urea were added to the medium as sole nitrogen source (21, 24). These medium-dependent changes in urease activity indicate that the enzyme is moderately regulated, either on the level of activity or on the level of gene expression.

Here, we describe the identification of genes encoding the *C. glutamicum* urea uptake system and their transcriptional organization and regulation, as well as the organization and control of the genes coding for the urease enzyme complex.

MATERIALS AND METHODS

Bacterial strains and growth. *C. glutamicum* and *Escherichia coli* strains and plasmids used in this study are listed in Table 1. *E. coli* cells were routinely grown in Luria-Bertani medium (25) at 37°C, and *C. glutamicum* strains were grown in brain heart infusion (Difco, Sparks, Md.) at 30°C. To study the effects of nitrogen starvation under comparable conditions, a standard inoculation scheme was applied. A fresh *C. glutamicum* culture grown in brain heart infusion medium was used to inoculate minimal medium [per liter, 42 g of morpholinepropanesulfonic acid, 20 g of (NH₄)₂SO₄, 5 g of urea, 0.5 g of K₂HPO₄ · 3H₂O, 0.5 g of KH₂PO₄, 0.25 g of MgSO₄ · 7H₂O, 0.01 g of CaCl₂, 50 g of glucose, 0.2 mg of biotin, 10 mg of FeSO₄, 10 mg of MnSO₄, 1 mg of ZnSO₄, 0.2 mg of CuSO₄, 0.02 mg of NiCl₂ · 6H₂O, 0.09 mg of H₃BO₃, 0.06 mg of CoCl₂ · 6H₂O, 0.009 mg of NaMoO₄ · 2H₂O; pH adjusted to pH 7.0 using NaOH (16)] for overnight growth. This culture, with an overnight optical density at 600 nm (OD₆₀₀) of approximately 25 to 30, was used to inoculate fresh minimal medium to an OD₆₀₀ of approximately 1, and cells were grown until the exponential growth phase was reached (OD₆₀₀ of approximately 4 to 5). To induce nitrogen starvation, cells were harvested by centrifugation and the pellet was suspended in and transferred to prewarmed minimal medium without a nitrogen source. Media were supplemented with antibiotics (50 μ g of carbenicillin/ml, 25 μ g of chloramphenicol/ml, 25 μ g of kanamycin/ml [final concentrations]) if appropriate.

Determination of urea and amino acid uptake. Since urea is immediately decomposed within the cell by the action of urease and, as a consequence, it is not possible to follow its accumulation within the cell, the decrease of the urea concentration in the medium was determined as described previously (21, 27). For this purpose, log-phase cells grown in minimal medium were harvested, washed, and suspended in nitrogen-free minimal medium to an OD₆₀₀ of 0.5. After 3 h of nitrogen starvation at 30°C, [¹⁴C]urea (specific activity, 55 mCi

* Corresponding author. Mailing address: Institut für Biochemie der Universität zu Köln, Zùlpicher-Str. 47, D-50674 Köln, Germany. Phone: 49 221 470 6472. Fax: 49 221 470 5091. E-mail: a.burkovski@uni-koeln.de.

† Present address: Department of Biochemistry, National University of Singapore, Singapore 117597.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description ^a	Reference or source
<i>C. glutamicum</i> strains		
ATCC 13032	Type strain	1
MJ6-18	ATCC 13032 Δ <i>amtR</i>	14
RES167	Restriction-deficient mutant of type strain ATCC 13032; Δ (<i>cgIIM-cgIIR-cgIIR</i>)	28
ATCC::pDRIVE-urtC	<i>urtC</i> insertion in wild-type strain	This study
RES167::pDRIVE-urtA	<i>urtA</i> insertion in restriction-deficient strain RES167	This study
RES167::pDRIVE-urtE	<i>urtE</i> insertion in restriction-deficient strain RES167	This study
RES167::pDRIVE-ureR	<i>ureR</i> insertion in restriction-deficient strain RES167	This study
<i>E. coli</i> strain		
DH5 α <i>mcr</i>	<i>supE44 hsdR17 recA1 endA1 gyrA96 thi1 relA mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>)	7
Plasmids		
pDRIVE	Ap ^r Km ^r ; A-T cloning vector	Qiagen
pDRIVE-urtA	pDRIVE carrying an internal 1.1-kb <i>urtA</i> fragment	This study
pDRIVE-urtC	pDRIVE carrying an internal 0.8-kb <i>urtC</i> fragment	This study
pDRIVE-urtE	pDRIVE carrying an internal 0.6-kb <i>urtE</i> fragment	This study
pDRIVE-ureR	pDRIVE carrying an internal 0.5-kb <i>ureR</i> fragment	This study
pGEM-3z	<i>E. coli</i> plasmid for in vitro transcription, Ap ^r	Promega
pGEM-urtA	0.5-kb internal <i>urtA</i> fragment in pGEM-3z	This study
pGEM-ureA	0.3-kb internal <i>ureA</i> fragment in pGEM3z	This study
pUC11-1.8	AmtR delivery plasmid for gel retardation experiments	14
pUC18	<i>plac</i> , Ap ^r	32

^a Abbreviations: Ap, ampicillin; Km, kanamycin.

mmol⁻¹) was added (4.0 μ M final concentration). Samples were taken at intervals and directly added to 2 ml of scintillation cocktail (Roth, Karlsruhe, Germany). The [¹⁴C]urea concentration in the medium was determined using a Beckman LS6500 multipurpose scintillation counter (Beckman, Munich, Germany), and uptake rates were calculated from the decrease in the label.

For measurement of amino acid uptake rates, cells were incubated as described for the urea transport measurements. The uptake determination was started by the addition of ¹⁴C-labeled L-amino acids (specific activity, 254 mCi mmol⁻¹; 1.5 μ M final concentration). In this case, radioactivity transported into the cell was measured directly by a filtration assay as described earlier (18).

Determination of urease activity. Urease activity was measured by the indophenol method (13). Cells were harvested and washed once with 50 mM potassium phosphate buffer at pH 7.0, resuspended to an OD₆₀₀ of 200, and disintegrated by ultrasonic treatment with a Branson sonifier 250 at an output control of 2.5 and a duty cycle of 25% for 4 min. After centrifugation at 4°C for 30 min at 18,000 \times g, urease activity was determined immediately in the cell-free supernatant by measuring the amount of ammonia produced from urea in 15 min at 30°C. A calibration curve was obtained from appropriate dilutions of a freshly prepared NH₄Cl solution. One unit of enzyme activity was defined as 1 micromole of ammonia released per minute.

Measurement of the intracellular ATP pool. Intracellular ATP concentrations were determined as described previously by using a modified firefly luciferase assay (17).

General molecular biology techniques. Standard techniques for plasmid isolation, transformation, and cloning were used (2, 25).

Construction of mutant strains. For gene disruption mutagenesis, internal gene fragments of the genes *ureR*, *urtA*, *urtC*, and *urtE* were amplified by PCR and ligated to pDRIVE vector DNA, resulting in the plasmids pDRIVE-ureR, pDRIVE-urtA, pDRIVE-urtC, and pDRIVE-urtE. Plasmids pDRIVE-ureR, pDRIVE-urtA, and pDRIVE-urtE were used to create gene disruption mutants with the restriction-deficient *C. glutamicum* strain RES167 as parental strain; for plasmid pDRIVE-urtC, the type strain ATCC 13032 was used as parental strain. For the construction of the different gene disruption plasmids, internal DNA fragments of the corresponding genes were amplified via PCR and ligated to DNA of the pDRIVE AT cloning vector as recommended by the supplier (QIAGEN, Hilden, Germany). The following primer combinations were used for PCR: 5'-AGCAACACCGCAGCTTCC-3' and 5'-GCAGCGGTGGTCTTGGC-3' (*urtA*); 5'-TGCGGTATTGCTGTGCGC-3' and 5'-CCACCAGCCCAGCACC-3' (*urtC*); 5'-ATTTGTGTGCAGTTATGGC-3' and 5'-GCGCCTTGTCCCATTCC-3' (*urtE*); 5'-TTACGAACCTTGGATGCGCG-3' and 5'-CGACAGGCCACTCCCATT-3' (*ureR*). Mutant strains generated were controlled by

PCR experiments, which verified correct gene disruption and absence of wild-type alleles.

Gel retardation experiments. To investigate AmtR binding, gel shift assays were carried out. For the upstream region of the *urtA* and *ureA* genes, PCR-generated DNA fragments spanning the corresponding putative AmtR binding sites were labeled with digoxigenin using the DIG oligonucleotide 3'-end labeling kit, second generation (Roche, Mannheim, Germany). The labeled DNA fragments were incubated for 20 min on ice with different amounts of AmtR-containing *E. coli* cell extract (DH5 α *mcr* pUC11-1.8) or control extract lacking AmtR (DH5 α *mcr* pUC18). The formation of unspecific DNA-protein complexes was minimized by adding sheared salmon sperm DNA (Eppendorf, Hamburg, Germany) and bovine serum albumin (AppliChem, Darmstadt, Germany). DNA-protein complexes were separated from free DNA on a non-denaturing 6% polyacrylamide-Tris-borate-EDTA gel by electrophoresis at 20 mA. The DNA was blotted onto a positively charged nylon membrane (Roche), and the digoxigenin-labeled DNA was detected with a Fuji luminescent image analyzer LAS1000 (Raytest, Straubenhardt, Germany).

RNA preparation and hybridization. Total RNA was prepared after disruption of the *C. glutamicum* cells with glass beads by using the NucleoSpin RNA II kit as recommended by the supplier (Macherey-Nagel, Düren, Germany). The RNA was blotted onto positively charged nylon membranes (BioBond Sigma, Taufkirchen, Germany) using a Minifold I dot blotter (Schleicher & Schuell, Dassel, Germany). Hybridization of digoxigenin-labeled RNA probes was detected with a Fuji luminescent image analyzer LAS1000 (Raytest) or Kodak X-Omat X-ray films (Sigma-Aldrich, Darmstadt, Germany) using alkaline phosphatase-conjugated antidigoxigenin Fab fragments and CSPD as light-emitting substrate as recommended by the supplier (Roche Diagnostics, Mannheim, Germany). In order to quantify hybridization signals, the total amount of RNA applied was determined using the RiboGreen RNA quantitation kit (Molecular Probes, Eugene, Oreg.).

For the generation of antisense probes, internal DNA fragments of the corresponding genes were amplified by PCR and cloned into plasmid pGEM plasmids (Promega, Mannheim, Germany) using restriction sites added to the primer sequence. For cloning of the *ureA* gene fragment EcoRI and HindIII sites were introduced (primer sequences, 5'-GCGCGCAAITCCGTCGCCGTAAGATC G-3' and 5'-GCGCGCAAGCTTATCAAACGTTGCTTCAACC-3'), and for the *urtA* probe EcoRI and HindIII restriction sites were added (5'-GCGCGCAAITCGGCTTGGACTTTGAAGACG-3' and 5'-GCGCGCAAGCTTACCGTGATC AACAAATGG-3') (restriction sites are shown in bold) to insert the PCR products into plasmid pGEM-3z. The hybridization probes were produced by in vitro transcription with the SP6 polymerase.

RT-PCR. For reverse transcriptase (RT)-PCR the OneStep RT-PCR kit was used as recommended by the supplier (QIAGEN) together with the following primers: 5'-AGCAACACCGCAGCTTCC-3' (*urtA*); 5'-GCGCCTTGTCGCGA TTCC-3' (*urtE*); 5'-GCGATCTTTACGCGACGTGCAA-3' (*ureA*); 5'-AGC AACCGCACTGAGGAGTTGAT-3' (*ureD*).

Real-time PCR. For real-time PCR, a TaqMan device (Applied Biosystems), the QuantiTect SYBR Green RT-PCR kit (QIAGEN), 100 ng of template RNA, and the following primer combinations were used: 5'-TCGCCCTCATCACGT ATGAA-3' and 5'-TCAAATGGTGCTTCCCCAG-3' (*urtA*); 5'-CGATGCC TCCGACTTCTTCTT-3' and 5'-TTCTCCGCCAGTACAACAGC-3' (*ureA*). Reverse transcription was carried out at 50°C for 30 min, the RT was inactivated and the polymerase was activated by 15 min of incubation at 94°C, and PCR was carried out for 40 cycles of the following program: DNA denaturation for 15 s at 94°C, primer annealing for 30 s at 60°C, and DNA polymerization for 30 s at 72°C. All experiments were carried out at least in triplicate with templates from independent cultures. For an exact quantification of real-time PCR results, dilution series were tested for each pair of primers to determine the PCR efficiency, and the total amount of RNA applied in the real-time PCR experiments was determined using the RiboGreen RNA quantitation kit (Molecular Probes). This method allows normalization of the results without additional control reactions with housekeeping genes or 16S rRNA (8).

Two-dimensional polyacrylamide gel electrophoresis and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). *C. glutamicum* cells were disrupted using glass beads and a Q-BIOgene FastPrep FP120 instrument (Q-BIOgene, Heidelberg, Germany) by lysing the cells four times for 30 s and 6.5 m/s in the presence of Complete proteinase inhibitor as recommended by the supplier (Roche, Basel, Switzerland). Proteins were separated in the cytoplasmic and membrane-associated protein fractions by ultracentrifugation (10–12). In this study, only the cytoplasmic proteins were further analyzed. Protein concentrations were determined using the Roti-Nanoquant assay (Roth).

For isoelectric focusing, 24-cm precast IPG strips of pI 4 to 7 and an IPGphor isoelectric focusing unit (Amersham Biosciences, Freiburg, Germany) were used as described elsewhere (11). A 120- μ g aliquot of protein was focused for 68,000 V \cdot h in a sample buffer containing 6 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 0.5% Pharmalyte (3-10), and 0.4% dithiothreitol. For the second dimension, electrophoresis was carried out using precast 12-to-14% polyacrylamide linear gradient gels (ExcelGel Gradient XL 12-14; Amersham Biosciences, Freiburg, Germany) in a Multiphor II apparatus as described previously (11, 12). After electrophoresis, two-dimensional gels were stained with colloidal Coomassie brilliant blue. The Coomassie-stained gels were aligned using the Delta2D software, version 3.1 (Decodon, Greifswald, Germany).

MALDI-TOF-MS for peptide mass fingerprint analyses was carried out by the bioanalytics service unit at the Center for Molecular Medicine Cologne.

RESULTS

Identification of the *C. glutamicum* urea uptake system.

While urea transport has been characterized biochemically in a wide variety of microorganisms, including *C. glutamicum*, only a few urea uptake systems have been characterized at the genetic level. These transporters can be differentiated into those facilitating diffusion (9, 26, 30) and active, energy-dependent uptake systems (19, 29). Since we were interested in identifying the *C. glutamicum* urea uptake system, we used the known genes as query sequences for searches in the recently published *C. glutamicum* genome (15). Genes with high similarity to *ureI* from *Helicobacter pylori* (30) or *yut* from *Yersinia pseudotuberculosis* (26) were not observed, but a gene cluster similar to the *fmd* genes of *Methylophilus methylotrophus* (19) and the *urt* gene cluster from *Anabaena* (29) was found. The amino acid sequence deduced from the NCgl0893 gene exhibited 43.3% identical amino acids with the *fmdD* gene product and 49.2% identity with the UrtA protein. The identity of the putative NCgl0894 protein was 40.8% to FmdE and 49.3% to UrtB, while the NCgl0895 gene product showed an identity of 35.2% with FmdF and 40.4% with UrtC. The NCgl0893,

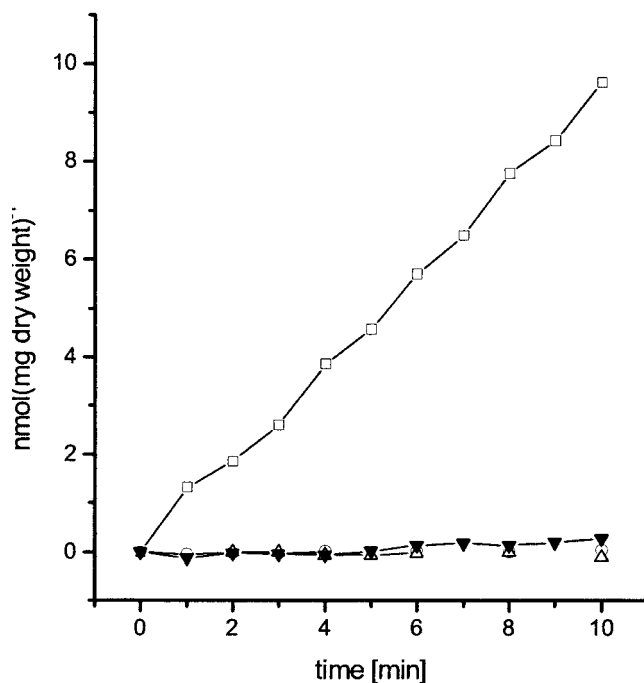


FIG. 1. Urea consumption in *C. glutamicum* wild type (open squares) and *urtA*, *urtC*, and *urtE* mutant strains (open triangles, open circles, and inverted closed triangles, respectively) grown in minimal medium with urea as single nitrogen source. Under the experimental conditions used (urea concentration, 4 μ M), diffusion of urea across the cytoplasmic membrane was negligible.

NCgl0894, and NCgl0895 genes encode components of an ABC-type transport system, namely, a lipid-anchored substrate binding protein and two membrane-integral permease subunits. Downstream, two open reading frames, NCgl0986 and NCgl0987, code for two putative ATP binding proteins, completing the proteins necessary for a typical ABC-type transporter. The proteins encoded by the latter two genes showed an identity of 34.0 and 38.7% with the corresponding Urt subunits. Besides the Fmd and Urt system, the transporter encoded by the NCgl0893-NCgl0897 cluster showed similarity (between 21.6 and 37.0% identical amino acids depending on the different subunits) to the LIV transporter of *E. coli* for the uptake of L-leucine, L-isoleucine, and L-valine. Based on the highest homologies observed, the NCgl0893-NCgl0897 genes are designated as *urtABCDE* in the following text.

To demonstrate that the *urtABCDE* gene cluster encodes the *C. glutamicum* urea uptake system, the *urtA*, *urtC*, and *urtE* genes encoding the substrate binding protein, one permease subunit, and one ATP binding protein were inactivated by insertion mutagenesis. Each single gene disruption resulted in loss of urea transport activity (0.01 to 0.04 nmol min⁻¹ mg [dry weight]⁻¹ compared to 0.8 \pm 0.2 nmol min⁻¹ mg [dry weight]⁻¹ determined for the wild type) (Fig. 1), indicating a crucial role of the ABC transporter for urea uptake. Polar effects of the mutations on downstream genes cannot be excluded; however, these are unlikely due to the chromosomal organization of the gene cluster.

To exclude that the loss of urea transport is the result of a secondary effect, i.e., that the ABC transporter takes up Ni²⁺

ions, which are essential for urease function, urease activities in the different mutant strains were determined. For the wild type a urease activity of 0.32 ± 0.13 U min⁻¹ was measured; the mutant strains showed activities of 0.24 ± 0.10 (*urtA*), 0.48 ± 0.12 (*urtC*), and 0.25 ± 0.07 (*urtE*) U min⁻¹. Hence, urease activity is not greatly influenced by *urt* mutations.

Because the *C. glutamicum* urea transporter showed similarity to the Fmd and LIV systems, competition of urea uptake was tested with Fmd and LIV substrates as well as other nitrogen sources. While addition of a 10-fold excess of unlabeled urea reduced the apparent uptake rate as expected by about 90%, 10-fold concentrations of ammonium, formamide, γ -amino butyric acid, L-glutamate, L-glutamine, L-histidine, L-isoleucine, L-alanine, L-threonine, and L-valine had no significant impact on urea uptake (data not shown). Furthermore, when uptake of labeled L-arginine (50 or 500 μ M final concentration), L-serine (0.5 to 500 μ M final concentration), or L-leucine (50 to 500 μ M final concentration) was tested in the wild type and the *urtA* and *urtC* mutant strains, no differences in their uptake rates were observed (data not shown).

Since the *urt* genes encode an ABC-type urea uptake system, an ATP-dependent transport mechanism was expected. However, an influence of the protonophore CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) on urea transport was reported previously (27). Addition of this uncoupler leads to immediate dissipation of the membrane potential, while in *C. glutamicum* the intracellular ATP pool is only marginally affected by this substance for several minutes (27). As a consequence, ATP-driven transport systems should not be affected by CCCP addition. However, as described before (27), here also an influence of CCCP on urea transport was observed. Urea uptake rates decreased from 0.9 ± 0.3 nmol min⁻¹ mg (dry weight)⁻¹ to 0.5 ± 0.1 nmol min⁻¹ mg (dry weight)⁻¹ when 5 μ M CCCP was added to the transport assay mixture 30 s prior to the start of the measurement. To characterize whether this CCCP effect was specific for the urea transporter, L-glutamate uptake via the GluABCD ABC transporter (18) was determined. These measurements showed that the uptake of L-glutamate via the GluABCD transport system was significantly less affected by the addition of the uncoupler than was urea uptake by the UrtABCDE transporter. An L-glutamate uptake rate of 1.3 nmol min⁻¹ mg (dry weight)⁻¹ was obtained without the addition of CCCP, and addition of 5 μ M CCCP hardly decreased this rate (1.2 nmol min⁻¹ mg [dry weight]⁻¹).

To investigate the CCCP sensitivity of the urea transporter in more detail, the intracellular ATP pool of the cells was determined before CCCP addition and after 6 min of incubation with the uncoupler. Without the addition of CCCP, the internal ATP concentrations decreased from 4.8 to 4.1 mM after 6 min. Addition of 5 μ M CCCP had no effect on the ATP pool compared to the control (4.6 mM before addition, 4.1 mM after 6 min of CCCP treatment). Consequently, a decrease of the ATP concentration can be excluded as a reason for the CCCP effect on urea transport.

Regulation of *urtABCDE* transcription. *C. glutamicum* exhibits active urea transport only under conditions of nitrogen starvation, and synthesis of this transporter can be suppressed by the addition of chloramphenicol, indicating a regulation on the level of gene expression (27). Therefore, we investigated transcription of the *urtABCDE* genes.

In our first approach, RT-PCR experiments were carried out using RNA extracted from nitrogen-starved cells as template to investigate the transcriptional coupling suggested by the genetic organization of the *urt* genes. Control reactions carried out without RT gave no PCR product, showing that the RNA preparation was DNA free. Primers annealing to *urtA* and *urtE* sequences led to a DNA fragment of 4.1 kb in an RT-PCR (Fig. 2A), indicating the presence of a joint transcript spanning the complete *urtABCDE* gene cluster. The result was confirmed by Northern hybridization experiments which showed an approximately 4,000-base-long mRNA which hybridized with a *urtA* probe (data not shown).

RNA slot blot hybridizations were carried out to investigate the regulation of *urtABCDE* expression. An antisense *urtA* probe as a representative of the complete operon was hybridized to total RNA isolated from cells grown in nitrogen-rich medium and then incubated for different time periods in nitrogen-free minimal medium. As shown, *urtA* expression was enhanced within 5 min of nitrogen starvation and stayed at a high level for at least 30 min (Fig. 2B). To determine the factor by which transcription of *urtA* was increased more accurately, real-time PCR experiments were carried out in triplicate with independent RNA preparations. These revealed that *urtA* expression in the wild type was upregulated after 30 min of N starvation by a factor of 344 ± 2 .

In *C. glutamicum*, a repressor protein designated AmtR controls the nitrogen starvation-dependent expression of several genes (3, 14, 22; for review, see references 4 to 6). We tested if AmtR were involved in the regulation of the *urtABCDE* operon. Two AmtR binding sites with the sequences CTATAG and CTAT-N₄-CTATAGA were observed at positions 63 to 58 and 37 to 23 upstream of the *urtA* gene. To prove AmtR-dependent regulation, RNA hybridization experiments were carried out with a *urtA* probe and total RNA isolated from *amtR* deletion strain MJ6-18. In the *amtR* deletion strain, high and unregulated expression of *urtA* was observed (Fig. 2B). In contrast to the wild-type ATCC 13032, transcription of *urtA* in the *amtR* deletion strain MJ6-18 was found even in nitrogen-rich growth medium, and transcription stayed constant when strain MJ6-18 was incubated in nitrogen-free minimal medium. These results were quantified in real-time PCR experiments. Expression of *urtA* under nitrogen excess was increased by a factor of $1,504 \pm 2$ compared to the wild-type transcription level, and after 30 min of nitrogen deprivation a factor of $1,882 \pm 2$ was observed, verifying the RNA hybridization experiments. The higher factors obtained for upregulation of *urtA* transcription in strain MJ6-18 compared to the wild type might be the result of another level of transcriptional regulation independent from AmtR.

The binding of AmtR to the *urt* operon promoter DNA was verified by gel retardation experiments. A 122-bp fragment of the *urtA* upstream region was changed in its electrophoretic mobility depending on the AmtR protein. In the presence of the repressor, two additional bands with lower mobility were detectable, indicating that the two putative AmtR binding sites upstream of *urtA* in fact bind the repressor protein (Fig. 2C).

Transcription of urease-encoding genes. Although the expression of the *urtABCDE* operon, which codes for the urea uptake system, is nitrogen starvation dependent, the regulation of the urease-encoding *ureABCEFGD* cluster was less clear. In

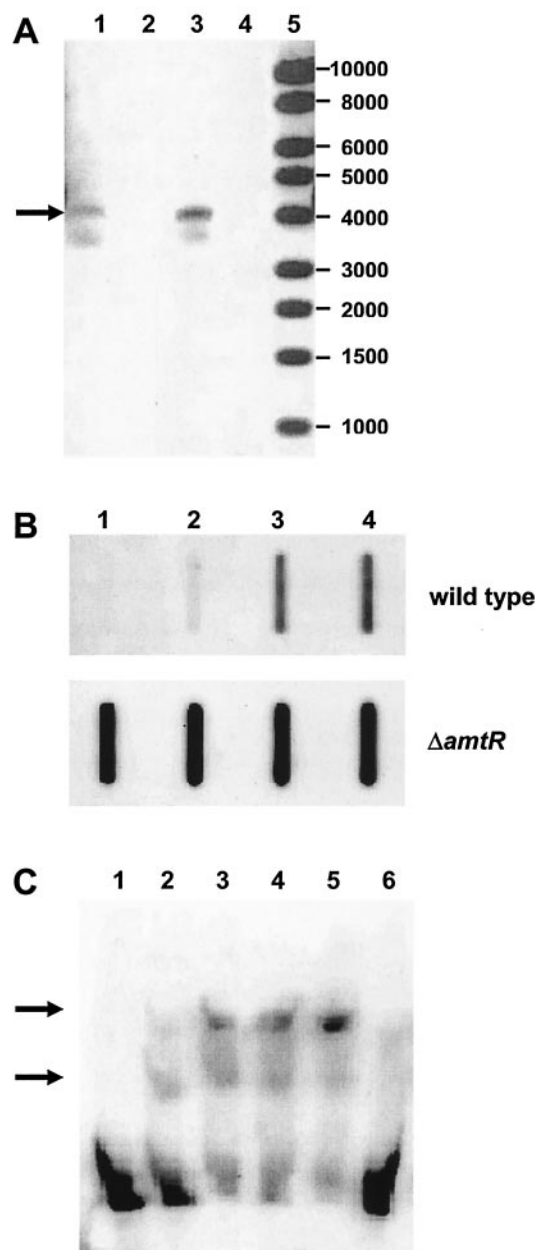


FIG. 2. Transcription of the genes encoding the *C. glutamicum* urea uptake system. (A) RT-PCR using primers annealing to *urtA* and *urtE*. Total RNA isolated from wild-type strain ATCC 13032 after 30 min of nitrogen starvation (lane 1) and from *amtR* deletion strain MJ6-18 grown under nitrogen surplus (lane 3) was used as template. The RT-PCR products (indicated by the arrow) had an expected size of 4.0 kb. Control reactions without the addition of RT (lanes 2 and 4) gave no PCR product, validating that the RNA preparations used were DNA free (lane 5). Lane M, marker DNA (1-kb DNA ladder; New England Biolabs). (B) RNA hybridization experiments with an *urtA* probe and total RNA (1 μ g per slot) prepared from the wild type and from the *amtR* deletion strain MJ6-18 grown in nitrogen-rich minimal medium and after 5, 15, and 30 min of nitrogen limitation (lanes 1 to 4). (C) Gel retardation experiment. Increasing amounts of AmtR-containing *E. coli* cell extracts (lanes 1 to 5: 0, 5, 10, 20, and 30 μ g of protein, respectively) were added to a digoxigenin-labeled 122-bp DNA fragment from the *urtA* promoter region spanning the two identified putative AmtR binding sites. As a control, cell extract lacking AmtR was used (lane 6; 30 μ g). DNA fragments with shifted mobilities depending on AmtR are indicated by arrows.

a previous study, regulation of expression depending on the urea or ammonium concentration in the growth medium was not observed (21). However, urease activity was found to increase during nitrogen starvation (21, 27). Therefore, the transcriptional control of the *ureABCEFGD* cluster was revisited in this study.

RT-PCR experiments were carried out using total RNA extracted from nitrogen-starved cells as template to reveal the organization of the *ure* genes. Control reactions carried out without RT gave no PCR product and thus showed that the RNA preparation used was DNA free. RT-PCR with primers annealing to *ureA* and *ureD* sequences gave a DNA fragment of 5.1 kb (Fig. 3A), which proved that a common mRNA of the complete *ureABCEFGD* gene cluster is transcribed. This result was validated by Northern hybridization experiments. Using a *ureA* probe, an mRNA of approximately 5,000 bases was observed, spanning the complete gene cluster, in addition to at least two smaller transcripts which corresponded to 3'-truncated forms (data not shown).

RNA hybridization experiments were carried out using the *ureA* gene as a probe and total RNA isolated from cells grown in nitrogen-rich medium and incubated for different time periods in nitrogen-free minimal medium (Fig. 3B). Transcription of *ureA* increased within 5 min of nitrogen starvation and stayed constant for at least 30 min. As in the case of the *urtA* gene, real-time PCR experiments were carried out to quantify the data obtained by RNA hybridization. Again, this approach showed a clear upregulation of *ureA* transcription by a factor of 14 ± 2 when cells were incubated for 30 min without nitrogen source compared to the basal level during nitrogen-rich growth.

For the regulation of the *ure* cluster, two proteins might function as expression regulators, the putative *ureR* gene product and the global regulator AmtR. The *ureR* gene lies adjacent to *ureA* but is transcribed in the opposite direction. Due to its location, UreR might function in the regulation of *ureABCEFGD* expression. To investigate its importance, a *ureR* mutant was constructed and tested for *ureA* transcription. In comparison to the wild type, no significant changes were observed with the *ureR* mutant when RNA preparations from cells grown in nitrogen-rich standard minimal medium and those from nitrogen-starved cells were studied (Fig. 3B). Additionally, no influence of UreR on transcription of the *ureABCEFGD* operon was seen when cells were grown in the presence of different urea concentrations.

Since mutagenesis of *ureR* had no influence on the nitrogen starvation response of *ureABCEFGD* expression, the *amtR* deletion strain MJ6-18 was investigated. In this strain, constitutive transcription of *ureA* was observed, demonstrating an important function of AmtR in expression control of the *ureABCEFGD* operon. Compared to the wild type, expression of *ureA* was high during nitrogen-rich growth, and no further increase during incubation in nitrogen-free medium for 5 to 30 min was observed (Fig. 3B). Real-time experiments confirmed these results. Compared to the basal level of transcription observed in the wild type during growth under nitrogen surplus, expression of *ureA* in strain MJ6-18 was increased by a factor of 20 ± 3 under nitrogen excess and by a factor of 12 ± 4 after 30 min of nitrogen shortage.

The binding of AmtR to the *ure* operon promoter region was

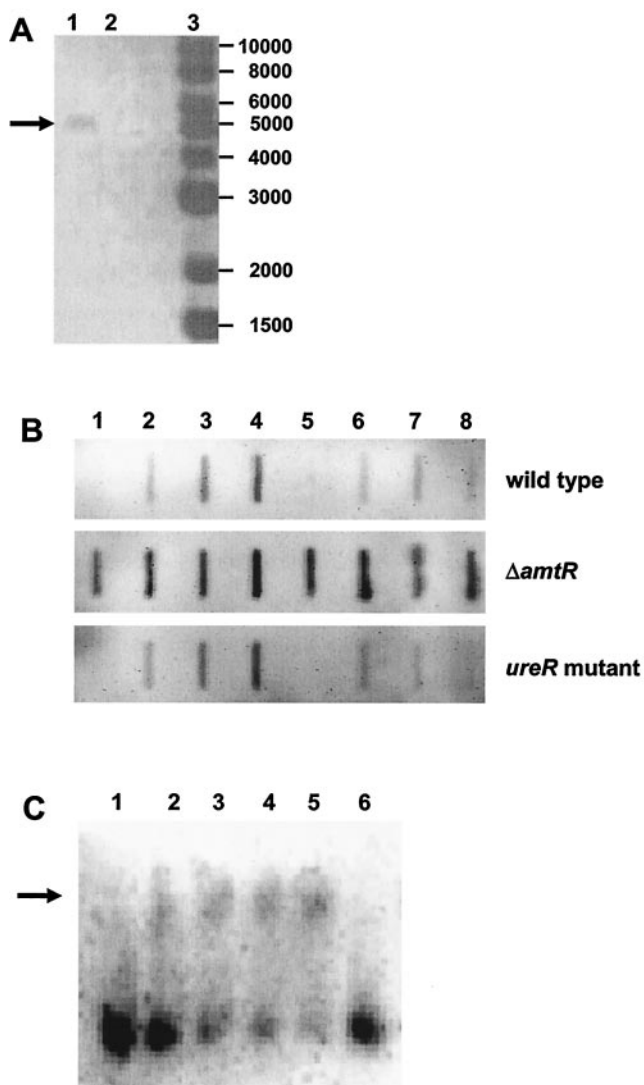


FIG. 3. Transcription of the urease-encoding genes in *C. glutamicum*. (A) RT-PCR with primers annealing to *ureA* and *ureD* cluster. As template, total RNA isolated from *amtR* deletion strain MJ6-18 was used. The RT-PCR product (lane 1; indicated by an arrow) had an expected size of 5.1 kb. A PCR control without addition of RT (lane 2) showed no signal, indicating DNA-free RNA preparation; lane 3, marker DNA (1-kb DNA ladder; New England Biolabs). (B) RNA hybridization experiments with a *ureA* probe and total RNA (1 μ g per slot) prepared from the wild type, from *amtR* deletion strain MJ6-18, and from the *ureR* gene disruption mutant Res167::pDRIVE-*ureR* under nitrogen surplus (lanes 1 and 5); after 5, 15, and 30 min of incubation with no nitrogen source starvation (lanes 2 to 4); and after 5, 15, and 30 min of incubation in the presence of 15 mM urea (lanes 6 to 8). (C) Gel retardation experiment using increasing amounts of AmtR-containing *E. coli* cell extracts (lanes 1 to 5: 0, 5, 10, 20, and 30 μ g of protein, respectively) and a digoxigenin-labeled 216-bp DNA fragment from the *ure* promoter region spanning the identified putative AmtR binding site. As a control, cell extract lacking AmtR was used (lane 6; 30 μ g). The DNA fragment that shifted in its electrophoretic mobility due to the presence of AmtR is indicated by an arrow.

verified by gel retardation assays. A 216-bp fragment of the *ureA* upstream region was retarded in its electrophoretic mobility in the presence of the AmtR protein (Fig. 3C). In accordance with the presence of only one putative AmtR binding

site, only one band with lower mobility was observed in the presence of AmtR.

Two-dimensional gel electrophoresis studies. In this study, transcriptional regulation of the *ureABCEFGD* genes has been shown which should, in principle, be detected at the protein level as well. To investigate putative changes in the amount of urease subunits, two-dimensional gel electrophoresis experiments and MALDI-TOF MS fingerprint analyses were carried out with protein extracts from cells grown under nitrogen surplus and incubated in nitrogen-free minimal medium. In fact, protein spots with molecular masses and pI values in the range expected for different urease subunits were detected in cytoplasmic protein fractions from nitrogen-starved wild-type cells (Fig. 4), while these spots were not detectable in protein extracts from ATCC 13032 cells grown in nitrogen-rich standard minimal medium (data not shown). The corresponding spots were excised from the gel, and proteins were subjected to tryptic in-gel digest followed by MALDI-TOF MS and peptide mass fingerprint analyses. Using this approach, five out of seven urease subunits were identified, namely, UreA, UreB, UreC, UreE, and UreG; the UreD and UreF proteins were not detected. These results on the protein level validate the data obtained for transcription control.

DISCUSSION

In this communication, we describe the molecular identification of the *C. glutamicum* urea uptake system. This ABC-type transporter is specific for urea; additionally, it has a low affinity to thiourea and hydroxyurea (27). The protein complex is encoded by the *urtABCDE* operon, which is transcribed in response to nitrogen limitation. Expression of this operon is controlled by the global nitrogen regulator AmtR, and an *amtR* deletion strain shows a constitutive expression of the *urtABCDE* genes.

In a previous communication (27), urea uptake in *C. glutamicum* was biochemically described as most likely linked to the symport of protons, since addition of CCCP, an uncoupler of the membrane potential, significantly reduced the transport rate while the internal ATP pool was only marginally affected. Changes in the membrane potential caused by the addition of valinomycin and potassium ions impaired urea uptake, indicating a secondary active transport system (27). However, in this communication, we have demonstrated that an ATP-driven ABC-type transporter is responsible for urea uptake. The reason for the decrease in urea uptake upon CCCP addition is unclear and might be due to secondary effects of the uncoupler on the internal pH or an unknown regulatory mechanisms.

Once inside the cell, urea is hydrolyzed by the urease enzyme complex, which is encoded by the *ureABCEFGD* gene cluster (21, 24). While coupling of transcription was previously shown for the *ureA*, *ureB*, and *ureC* genes (21), the operon structure of *ureABCEFGD* was demonstrated here. Moreover, while regulation of the urease-encoding gene cluster *ureABCEFGD* on the level of transcription had not been observed in RNA hybridization experiments or RT-PCR in a previous study, most likely due to problems with the preparation of total RNA (21), we showed upregulation of transcription levels in response to nitrogen starvation. The latter data are in accordance with urease activity measurements. These

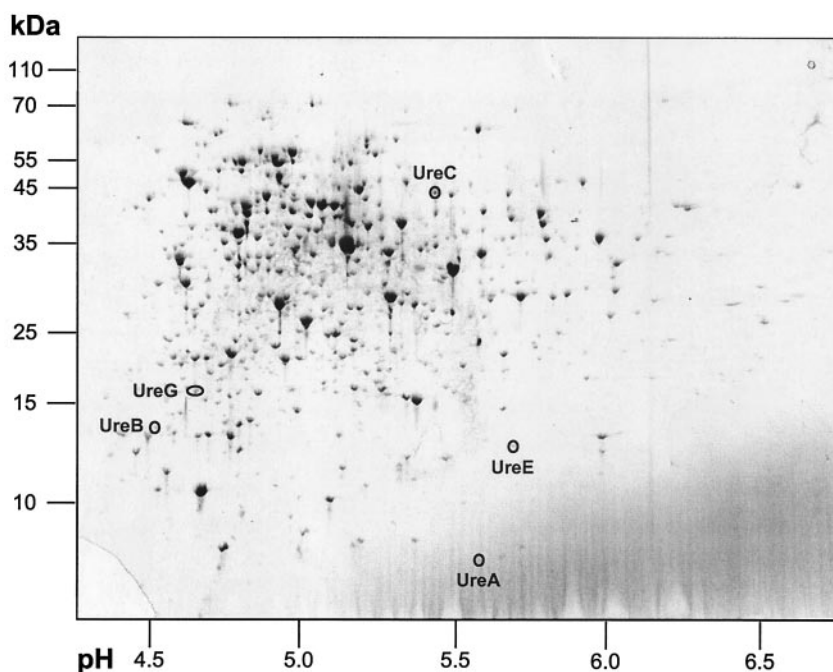


FIG. 4. Identification of urease subunits in the protein extract of nitrogen-starved cells. Cytoplasmic proteins (120 μ g) of *C. glutamicum* ATCC 13032 grown in standard minimal medium and incubated for 3 h in nitrogen-free minimal medium were separated by two-dimensional gel electrophoresis and stained with colloidal Coomassie brilliant blue. Marked protein spots, which were not detectable in extracts from cells grown in nitrogen-rich medium (data not shown), were identified by MALDI-TOF MS and peptide mass fingerprint analysis. Experiments were carried out independently in duplicate; molecular mass markers and pH values of the gel section are indicated.

data show a low activity of 0.9 U mg of protein⁻¹ when cells are grown in nitrogen-rich complex medium and a high activity of 7.8 U mg of protein⁻¹ in extracts from nitrogen-starved cells (21). The results of the transcription analysis were validated on the protein level for five of the seven urease subunits.

Mutant analyses showed that AmtR regulates transcription of the *ureABCEFGD* operon, and no influence of *ureR* was observed. This gene, which encodes a putative transcriptional regulator, lies adjacent to *ureA* but is transcribed in the opposite direction. Our experiments indicate that AmtR alone is sufficient to regulate expression of the urease-encoding genes under the conditions tested. However, a function of UreR for a fine tuning of transcription or responding to different environmental conditions cannot be excluded, especially in the light of the fact that multiple regulators of urease-encoding operons have been described in other bacteria, namely, *Bacillus subtilis* (31) and *Proteus mirabilis* (23).

ACKNOWLEDGMENTS

We thank U. Hildebrandt and H. Bothe for help with the real-time PCR experiments.

This work was supported by the Bundesministerium für Forschung und Technologie (GenoMik program) and the Deutsche Forschungsgemeinschaft (BU894/1-3).

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