APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 2005, p. 4339–4344 Vol. 71, No. 8
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.8.4339–4344.2005

Phosphate Starvation-Inducible Gene *ushA* Encodes a 5′ Nucleotidase Required for Growth of *Corynebacterium glutamicum* on Media with Nucleotides as the Phosphorus Source

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Received 10 December 2004/Accepted 28 February 2005

Phosphorus is an essential component of macromolecules, like DNA, and central metabolic intermediates, such as sugar phosphates, and bacteria possess enzymes and control mechanisms that provide an optimal supply of phosphorus from the environment. UDP-sugar hydrolases and 5′ nucleotidases may play roles in signal transduction, as they do in mammals, in nucleotide salvage, as demonstrated for UshA of *Escherichia coli*, or in phosphorus metabolism. The *Corynebacterium glutamicum* gene *ushA* was found to encode a secreted enzyme which is active as a 5′ nucleotidase and a UDP-sugar hydrolase. This enzyme was synthesized and secreted into the medium when *C. glutamicum* was starved for inorganic phosphate. UshA was required for growth of *C. glutamicum* on AMP and UDP-glucose as sole sources of phosphorus. Thus, in contrast to UshA from *E. coli*, *C. glutamicum* UshA is an important component of the phosphate starvation response of this species and is necessary to access nucleotides and related compounds as sources of phosphorus.

Phosphorus is one of the major constituents of the cell, accounting for about 1.5 to 2.1% of the cell dry weight. Cells contain inorganic phosphates, like orthophosphate, pyrophosphate, or polyphosphate, as well as organophosphates, such as nucleotides, sugar phosphates, or phospholipids. The major phosphorus-containing components are polymers, like RNA, DNA, or polyphosphate. Typically, cells take up phosphate as their preferred phosphorus source. Many organisms have developed mechanisms that allow them to adapt phosphate utilization to phosphate availability. When phosphate is scarce, many bacteria can use organophosphates as phosphorus sources. Either organophosphates are taken up into the cell directly, as in the case of glycerol 3-phophate or hexose phosphates, or they are degraded extracellularly by enzymes, such as alkaline phosphatases, and the inorganic phosphate formed is taken up into the cell subsequently.

Corynebacterium glutamicum, a gram-positive amino acid-producing bacterium, responds to phosphate starvation by changing expression of a number of genes involved in phosphorus metabolism (14, 32). Among the genes of the phosphate starvation stimulon are genes that putatively code for an uptake system for inorganic phosphate, an uptake system for glycerol 3-phosphate, an extracellular nuclease (*nucH*), and a 5′ nucleotidase or related esterase (*ushA*). While in *Bacillus subtilis* phosphate starvation also induced genes for the biosynthesis of teichuronic acids and genes for the general stress response (1, 12), in *C. glutamicum* genes for the general stress response were not induced by phosphate starvation (14, 32). In addition, the cell wall of *C. glutamicum* lacks teichoic and teichuronic acids, and its genome lacks genes for the biosynthesis of these compounds (15). The phosphate starvation stimulons of *C. glutamicum* and *Escherichia coli* are similar, but unlike *E. coli* (31), *C. glutamicum* cannot utilize phosphonates and its genome lacks genes for phosphonate metabolism (14, 15, 32). A further difference between the responses of *E. coli* and *C. glutamicum* to phosphate starvation is that the 5′ nucleotidase gene *ushA* is not a known member of the phosphate starvation stimulon of *E. coli* (3, 31), while its *C. glutamicum* homolog showed increased RNA levels in phosphate-starved cells (14).

In *E. coli*, the 5′ nucleotidase UshA has an important function in nucleotide salvage (21, 33). In addition, it is required for growth on 5′-AMP as a sole carbon source (21, 33). Animal 5′ nucleotidases exhibit homology with *E. coli* UshA (17), and their physiological role is termination of the action of ATP and other nucleotides that serve as extracellular messengers and neurotransmitters in the brain, as well as in the periphery (34). The aims of this study were to characterize the UshA protein of *C. glutamicum* and *ushA* gene expression and to determine the physiological role of UshA in this bacterium.

MATERIALS AND METHODS

Bacteria, plasmids, and growth conditions. Strain ATCC 13032 of *C. glutamicum* was used as the wild type (WT), and an *ushA* deletion mutant was generated from it as described below. *E. coli* DH5α was used for cloning (9). Luria-Bertani (LB) medium was used as the standard medium for *E. coli* (26), while brain heart infusion medium (Difco) was used as the complex medium for *C. glutamicum*. CGXII was used as a minimal medium for *C. glutamicum* (16), but it contained 30 mg/liter protocatechuic acid. Glucose (40 g liter^{-1}) was used as the carbon source. When appropriate, *E. coli* strains were cultured with ampicillin (100 μg ml^{-1}) or kanamycin (50 μg ml^{-1}), and *C. glutamicum* strains were cultured with kanamycin (50 μg ml^{-1}). A reduced concentration of kanamycin (15 μg ml^{-1}) was used to obtain transformants of *C. glutamicum*. *E. coli* was grown at 37°C, and *C. glutamicum* was grown at 30°C in 60 ml of medium in 500-ml baffled shake flasks at 130 rpm.

The phosphate starvation conditions used for growth of *C. glutamicum* have been described previously (14). For growth of *C. glutamicum* on different sources of phosphorus, CgXII medium lacking potassium phosphate was prepared and used for a 24-h preculture to exhaust the intracellular stored phosphorus. In the main cultures, CgXII medium lacking potassium phosphate but containing 1 mM UDP-glucose, 1 mM AMP, or a limiting concentration of phosphate (0.065 mM) as the sole source of phosphorus was used (14).

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Construction of plasmids and strains. Plasmids were constructed in *E. coli* DH5α from PCR-generated fragments (Expand High Fidelity PCR kit; Roche Diagnostics) by using *C. glutamicum* WT DNA prepared as described by Eikmanns et al. (7) as a template. *E. coli* was transformed by the $RbCl_2$ method (11). *C. glutamicum* was transformed via electroporation (30). All transformants were analyzed by plasmid analysis and/or PCR with appropriate primers.

For overexpression of *C. glutamicum ushA*, primers ushA(5′-AAGGAGAATTTAATGAAGAGGCTTTCC-3′) and ushArev (5′-ATTACATGAACTGCGCAAACATAGCC-3′) (the underlined nucleotides correspond to bp 343625 and 345721, respectively, of the accession no. NC003450 sequence) were used to amplify *ushA* from genomic DNA. The PCR product was subcloned into pGEM-T (Promega, Mannheim, Germany), and after NcoI/SpeI restriction and blunt ending it was cloned into EcoRI-restricted and blunt-ended pEKEx2 (6).

To amplify flanking regions of *ushA*, primers ush1 (5′-TGG**TCTAGA**ATCCAACAAGATTGTTGC-3′; the underlined nucleotide corresponds to bp 343379 of the NC003450 sequence, and the XbaI site is indicated by boldface type), ush2rev (5′-**CCCATCCACTAAACTTAAACA**ACGGGAAAGCCTCTTCAT-3′; the underlined nucleotide corresponds to bp 343653 of the NC003450 sequence, and the linker sequence is indicated by boldface type), ush3 (5′-**TGTTTAAGTTTAGTGGATGGG**CTCAGCTTCAGGCTATG-3′; the underlined nucleotide corresponds to bp 345686 of the NC003450 sequence, and the linker sequence is indicated by boldface type), and ush4rev (5′-CAGT**AAGCTT**GAGAATCGTGTTGTTC-3′; the underlined nucleotide corresponds to bp 345955 of the NC003450 sequence, and the HindIII site is indicated by boldface type) were used. The crossover PCR product obtained with primers ush1 and ush4rev and both of the PCR fragments generated as templates was cloned into pK19*mobsacB* (27) via its primer-attached XbaI and HindIII sites. Gene deletion mutagenesis with pK19*mobsacB*Δ*ushA* was carried out as described previously (24). The correct genotype of the deletion mutant was verified by PCR analysis using primers ushA-k-for (5′-GCAGATCCTCGGTGTGAACCT-3′; the underlined nucleotide corresponds to bp 343166 of the NC003450 sequence) and ushA-k-rev (5′-CTCACTGCGGACATTAAAACAGCC-3′; the underlined nucleotide corresponds to bp 346217of the NC003450 sequence), and the mutant was designated WTΔ*ushA*.

Preparation of cell extracts, membrane fractions, and supernatants. For determination of enzyme activities, cells and medium supernatant were separated by centrifugation (5,000 × *g*, 10 min, 4°C). Supernatants were passed through a 0.2-μm sterile filter and concentrated about 50-fold by ultrafiltration using Amicon Ultra 10,000-molecular-weight-cutoff membranes according to the manufacturer's recommendations. The cell pellet was washed with ice-cold 100 mM Tris-HCl, pH 7.6, resuspended in 1 ml of the same buffer, and disrupted by ultrasonic treatment at 4°C with a Branson 250 Sonifier (Branson, Danbury, CT) at an output of 2.5 and a duty cycle of 25% for 10 min. After centrifugation for 30 min at 16,000 × *g* and for 2 h at 190,000 × *g*, the supernatant was used as the cleared cell extract, and the pellet was resuspended in 50 mM Tris, pH 7.6, 5 mM $MgCl_2$, 5 mM cysteine, 30% (vol/vol) glycerol and used as the membrane fraction.

5′ Nucleotidase assay. 5′ Nucleotidase activity was determined using a modification of the assay described by Edwards et al. (5). The reaction mixtures (1 ml) contained 75 mM sodium acetate, pH 6.0, 4.5 mM $CoCl_2$, and 14.5 mM $CaCl_2$, and the reactions were started by addition of 1 mM 5′-AMP. The reaction mixtures were incubated at 37°C for 0, 1, 5, 10, and 15 min before the reactions were stopped by addition of 0.04 N HCl and the phosphate released by the activity of 5′ nucleotidase was determined. Then, after incubation of the stopped reaction mixtures on ice for 5 min and centrifugation at 4°C and 16,000 × *g* for 5 min, 1% (wt/vol) ascorbic acid, 0.25% (wt/vol) ammonium molybdate, and 0.6 NH_2SO_4 were added. After 20 min of incubation at 45°C and twofold dilution in water, the absorbance at 820 nm was determined. Phosphate concentrations were determined with appropriate standards. The 5′-AMP concentration was varied from 0.2 mM to 20 mM to determine the kinetic constants. To determine the pH optimum, the pH was varied from 4.5 to 6.0 with increments of 0.5 U. To determine the substrate spectrum of UshA, instead of 5′-AMP, 1 mM or 5 mM ADP, ATP, dATP, CMP, GMP, UMP, IMP, XMP, 2′,3′-cAMP, or 3′,5′-cAMP was used.

UDP-sugar hydrolase assay. UDP-sugar hydrolase activity was determined at 30°C by a coupled spectrophotometric assay as described previously (5). The reaction mixtures (1 ml) contained 35 mM Tris-HCl, pH 8.0, 35 mM $MgCl_2$, 3.1 μM glucose 1,6-bisphosphate, 0.7 mM $NADP^+$, rabbit muscle phosphoglucomutase (1 U/ml), and *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase (2.5 U/ml), and the reactions were started by addition of 1.4 mM UDP-glucose. Glucose 1-phosphate formed by the reaction of UDP-sugar hydrolase was converted to glucose 6-phosphate and subsequently to 6-phosphogluconate by coupling to phosphoglucomutase and glucose-6-dehydrogenase, and concomitant formation of NADPH ($\varepsilon_{340\ nm}$ = 6.3 $mM^{-1}\ cm^{-1}$) was monitored at 340 nm.

Assay for alkaline or acid phosphatase activity. Acid and alkaline phosphatase activities were determined using *p*-nitrophenylphosphate as the substrate, and the phosphate released during 15 to 60 min of incubation was quantified as described above after the reaction was stopped by addition of 0.5 ml of 2 N NaOH. In assays for alkaline phosphatase activity, the reaction mixtures contained 2 mM $MgCl_2$, 1 M Tris-HCl, pH 8.0, and 5 mg/ml *p*-nitrophenylphosphate. The reaction mixtures for acid phosphatase assays contained 2 mM $MgCl_2$, 100 mM sodium acetate, pH 5.0, and 5 mg/ml *p*-nitrophenylphosphate.

Primer extension analysis. Total RNA was prepared as described by Lange et al. (19). Nonradioactive primer extension analysis was performed using IRD800-labeled oligonucleotides (MWG Biotech) as described previously (8). In short, total RNA (20 μg) was transcribed to cDNA using the IRD800-labeled oligonucleotide 5′-GCGAAAGCTGGAACTGCGAGTGCGC-3′ (the underlined nucleotide corresponds to bp 343717 of the NC003450 sequence). The primer extension products were separated by denaturing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the lengths of the reaction products were determined by running the four lanes of a sequencing reaction setup using the same oligonucleotide that was used for reverse transcription alongside the primer extension products. The template for the sequencing reaction was generated by PCR amplification of genomic DNA using primers 5′-TTAACACCCCATAAAGAGGGTGAAGAT-3′ and 5′-CCACTGGCACCCATTTCTGAGTAAC-3′ (the underlined nucleotides correspond to bp 343450 and 343843, respectively, of the NC003450 sequence).

SDS-PAGE and mass spectrometry. SDS-PAGE and determination of protein concentrations were performed using standard techniques (26) as described previously (25). To identify proteins separated by SDS-PAGE, the bands of interest were excised and digested with trypsin, and the peptide masses determined by matrix-assisted laser desorption ionization—time of flight (MALDI-TOF) mass spectrometry were compared to the masses in a tryptic digest database of 3,746 predicted *C. glutamicum* proteins as described previously (28). To determine the length of UshA from supernatants, the bands of interest were excised and electroeluted using a DiaEx midi kit (Serva, Heidelberg, Germany) according to the manufacturer's recommendations, and the protein mass was determined using MALDI-TOF mass spectrometry as described previously (28).

RESULTS

***C. glutamicum* gene *ushA* encodes a secreted enzyme with 5′ nucleotidase and UDP-sugar hydrolase activities.** The *ushA* (NCgl0322) gene is located 181 bp downstream of an operon putatively encoding RNase H and a glycosyltransferase (NCgl0320 and NCgl0321) and is located 708 bp upstream of NCgl0323, which codes for a hypothetical protein. The *ushA* gene is predicted to code for a protein consisting of 694 amino acids and having a molecular mass of 72,288 Da. Upon cleavage of the putative signal peptide consisting of 28 amino acids, a 666-amino-acid protein with a molecular mass of 69,485 Da can be predicted. This protein might be secreted or might be an integral membrane protein, as suggested by a possible transmembrane helix at the C terminus.

Based on the fact that 29% of the amino acids of the encoded protein are identical to amino acids of the periplasmic UshA protein from *E. coli*, it was suggested that these two proteins have similar enzymatic activities. Thus, we examined whether *C. glutamicum* UshA, like *E. coli* UshA, is active as a secreted 5′ nucleotidase, a UDP-sugar hydrolase, and an alkaline phosphatase. To overexpress *ushA*, *C. glutamicum* strain WT(pEKEx2-*ushA*) was constructed. This strain and the control strain WT(pEKEx2) were grown on LB complex medium with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). SDS-PAGE of supernatants of *C. glutamicum* WT(pEKEx2-*ushA*) revealed a protein with a molecular mass of about 70 kDa that was not present in WT(pEKEx2) (Fig. 1, lanes 1 and 2). The band was excised and digested with trypsin, and the peptide

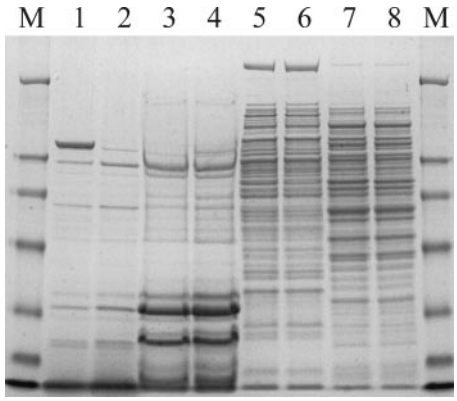

FIG. 1. SDS-PAGE analysis of supernatant fractions from *C. glutamicum* strains WT(pEKEx2) and WT(pEKEx2-*ushA*) and of cytoplasmic, membrane, and supernatant fractions from strains WT and WTΔ*ushA*. The SDS-PAGE analysis was performed with 3 μg of protein from supernatants of WT(pEKEx2-*ushA*) (lane 1) and WT(pEKEx2) (lane 2) after growth on LB medium containing 1 mM IPTG and with 10 μg of protein from supernatants, membrane fractions, and cell extracts from phosphate-starved WTΔ*ushA* (lanes 3, 5, and 7, respectively) and from phosphate-starved WT (lanes 4, 6, and 8, respectively). Lane M contained molecular mass standards (191 kDa, 64 kDa, 51 kDa, 39 kDa, 28 kDa, 19 kDa, and 14 kDa).

masses determined by MALDI-TOF mass spectrometry were compared to the masses in a tryptic digest database of 3,746 predicted *C. glutamicum* proteins as described previously (28). Thus, we verified that supernatants of *C. glutamicum* WT(pEKEx2-*ushA*) contained UshA. The specific activities of 5′ nucleotidase and UDP-sugar hydrolase were higher in supernatants of WT(pEKEx2-*ushA*) than in supernatants of control strain WT(pEKEx2) (Table 1). On the other hand, the specific activities of alkaline phosphatase and acid phosphatase were not increased noticeably (Table 1). Thus, *C. glutamicum* WT(pEKEx2-*ushA*) secreted UshA, which lacked activity as an acid or alkaline phosphatase but was active as a 5′ nucleotidase (EC 3.1.4.1) and a UDP-sugar hydrolase (EC 3.1.3.5).

To further characterize UshA from *C. glutamicum* as a 5′ nucleotidase, the optimum pH and the substrate specificity were determined using supernatants of WT(pEKEx2-*ushA*). UshA was active as a 5′ nucleotidase with AMP as a substrate at a pH range from 4.5 to 6.0 and showed maximal activity at pH 5.0 (data not shown). *C. glutamicum* 5′ nucleotidase followed Michaelis-Menten kinetics for the substrate AMP, and at 30°C the K_m and V_{max} values were 0.18 mM and 7 μmol min^{-1} mg^{-1}, respectively (data not shown). To determine the substrate specificity of *C. glutamicum* 5′ nucleotidase, various 5′ nucleoside monophosphates, adenosyl phosphates, dATP, and cyclic adenosyl monophosphates were tested (Table 2). Among the 5′-nucleoside monophosphates, GMP, IMP, and XMP, which contain 6-oxopurines, showed higher activities than the 6-aminopurine-containing AMP and the pyrimidine nucleoside monophosphates UMP and CMP (Table 2). *C. glutamicum* 5′ nucleotidase also hydrolyzed ADP, ATP, and dATP, and the observed specific activities were higher for the di- and triphosphates than for the monophosphates. However, neither 2′,3′-cAMP nor 3′,5′-cAMP was a substrate of *C. glutamicum* 5′ nucleotidase. Thus, *C. glutamicum* 5′ nucleotidase exhibits broad substrate specificity, accepting 5′-nucleoside phosphates as well as 5′-deoxynucleoside phosphates, but not cyclic nucleotides.

TABLE 1. Specific activities of 5′ nucleotidase, UDP-sugar hydrolase, alkaline phosphatase, and acid phosphatase in *C. glutamicum* WT(pEKEx2) and WT(pEKEx2-*ushA*)

Enzyme	Sp act (μmol min^{-1} mg^{-1})[a]	
	WT(pEKEx2)	WT(pEKEx2-*ushA*)
5′ Nucleotidase (EC 3.1.3.5)	0.04	2.40
UDP-sugar hydrolase (EC 3.6.1.45)	<0.01	0.55
Alkaline phosphatase (EC 3.1.3.1)	0.01	0.02
Acid phosphatase (EC 3.1.3.2)	0.01	0.01

[a] Specific activities were measured in supernatants after growth on LB complex medium with 1 mM IPTG.

TABLE 2. Specific activities of 5′ nucleotidase from *C. glutamicum* with various substrates

Substrate	Sp act of 5′ nucleotidase (μmol min^{-1} mg^{-1})[a]
AMP	7
GMP	15
IMP	15
XMP	17
CMP	6
UMP	6
ADP	27
ATP	37
dATP	26
2′,3′-cAMP	ND[b]
3′,5′-cAMP	ND

[a] The substrate specificity of *ushA*-encoded 5′ nucleotidase was determined using supernatants from *C. glutamicum* WT(pVWEx1-*ushA*). Substrates were tested at a concentration of 5 mM. Similar results were obtained at a substrate concentration of 1 mM.

[b] ND, not detected. The detection limit was <0.1 μmol min^{-1} mg^{-1}.

Based on its predicted amino acid sequence, UshA of *C. glutamicum* is likely to be secreted into the medium, and supernatants of *C. glutamicum* WT(pEKEx2-*ushA*) contained UshA protein active as a 5′ nucleotidase and a UDP-sugar hydrolase (Table 1). However, analysis of the amino acid sequence of UshA by the programs TopPred II and TMPred predicted a possible C-terminal transmembrane helix. Although the UshA sequence lacked cell wall sorting signals identified in staphylococci, the possibility that UshA is processed by a cell wall sortase and targeted to the cell wall could not be excluded because in *C. glutamicum* cell wall sorting has not been characterized. MALDI-TOF mass spectrometry of UshA from supernatants of WT(pEKEx2-*ushA*) revealed a molecular mass of 70,460 ± 1,000 Da. The determined molecular mass agreed well with the molecular mass of 69,485 Da predicted for secreted UshA after cleavage of the signal peptide, which comprises the first 28 amino acids. For independent confirmation of the results obtained with WT(pEKEx2-*ushA*), we analyzed the supernatants as well as the cytoplasmic and membrane fractions of phosphate-starved *C. glutamicum* WT and WTΔ*ushA*. While differences between cytoplasmic and membrane fractions of the two strains were not apparent (Fig. 1, lanes 5 to 8), the supernatant of *C. glutamicum* WT contained a protein with a molecular mass of about 70 kDa (Fig. 1, lane 4) that was not detectable in the supernatant of WTΔ*ushA*

(Fig. 1, lane 3). To estimate which fraction contained most of active UshA, the distribution of the 5′ nucleotidase activities, expressed in μmol ml^{-1} min^{-1}, in the cytoplasmic, membrane, and supernatant fractions was determined. While 5′ nucleotidase activity could not be detected in any of the fractions of WTΔ*ushA*, the cytoplasmic fraction contained 18%, the membrane fraction contained 5%, and the supernatant contained 77% of the 5′ nucleotidase activity present in phosphate-starved *C. glutamicum* WT. Taken together, these results indicate that UshA is a secreted enzyme.

The *ushA* deletion mutant WTΔ*ushA* exhibited no detectable 5′ nucleotidase activity before and after the onset of phosphate starvation in the cytoplasmic, membrane, and supernatant fractions. However, UDP-sugar hydrolase activity could be detected in cell extracts of phosphate-starved WTΔ*ushA* cells. This suggests that *C. glutamicum* possesses a cytoplasmic UDP-sugar hydrolase(s) in addition to UshA. On the other hand, *ushA* encodes the only 5′ nucleotidase of *C. glutamicum* active under the conditions tested.

Expression of *ushA* is phosphate starvation inducible. The phosphate starvation stimulon of *C. glutamicum* included 25 genes which showed increased mRNA levels after phosphate starvation compared to the levels after growth with sufficient phosphate (14). Among these genes was *ushA*, whose *E. coli* homolog is not a known member of the phosphate starvation stimulon (31). To confirm that expression of *ushA* is phosphate starvation inducible in *C. glutamicum*, we performed primer extension analysis of *C. glutamicum* WT before and after the onset of phosphate starvation. Primer extension products could be detected only after the onset of phosphate starvation and not before (Fig. 2). The primer extension analysis also revealed the transcriptional start site of *ushA* and indicated that transcription starts at the cytosine 60 bp upstream of the translational start codon, which is preceded by a ribosome binding site (Fig. 2). The promoter of *ushA* contains a conserved −10 region but lacks a conserved −35 region (Fig. 2); this was shown to be typical for *C. glutamicum* promoters (22, 23).

In order to assess whether increased *ushA* mRNA levels resulted in increased amounts of UshA protein as well as increased specific activities of 5′ nucleotidase and UDP-sugar hydrolase, supernatants of *C. glutamicum* WT before and after the onset of phosphate starvation were analyzed by SDS-PAGE and used to determine enzyme activities. The supernatant of phosphate-starved *C. glutamicum* WT cells contained small amounts of UshA protein (Fig. 1, lane 4), which could not be detected before the onset of phosphate starvation (data not shown). The specific activities of 5′ nucleotidase and UDP-sugar hydrolase in supernatants of *C. glutamicum* WT were not detectable before the shift to phosphate-free medium (<0.01 μmol min^{-1} mg^{-1}). The specific activities of 5′ nucleotidase were 0.07 and 0.39 μmol min^{-1} mg^{-1} 6 and 24 h, respectively, after the shift to phosphate-free medium. The specific activity of UDP-sugar hydrolase was 0.076 μmol min^{-1} mg^{-1} 24 h after the onset of phosphate starvation. Thus, phosphate starvation-inducible expression of *ushA* resulted in increased levels of secreted UshA protein and increased 5′ nucleotidase and UDP-sugar hydrolase activities in the supernatants.

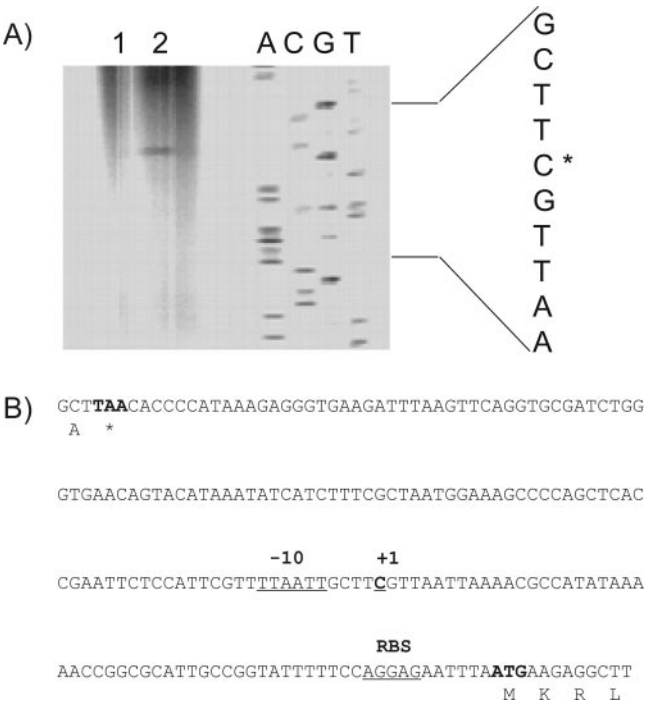

FIG. 2. Expression analysis and determination of the transcriptional start sites of the *C. glutamicum ushA* gene. (A) For primer extension analysis, 20 μg of total RNA isolated from *C. glutamicum* WT at zero time (lane 1) and 90 min after the shift to a medium without phosphate (lane 2) was used. The transcriptional start site is indicated by an asterisk. The corresponding sequencing reactions (lanes A, C, G, and T) were performed using the same IRD800-labeled oligonucleotide that was used in the primer extension reactions and PCR products which covered the region of the corresponding transcriptional start site as the template DNA. (B) Sequence of the intergenic region between NCgl0321 and *ushA*. The stop codon of NCgl0321 and the start codon of *ushA* are indicated by boldface type, and the transcriptional start site (+1), the −10 promoter region, and the ribosome binding site (RBS) are underlined.

***ushA* is required for growth of *C. glutamicum* on the phosphorus sources AMP and UDP-glucose.** To determine the physiological role of UshA in *C. glutamicum*, the abilities of *C. glutamicum* WT and WTΔ*ushA* to utilize AMP and UDP-glucose as sole sources of phosphorus were examined. To exhaust intracellular sources of phosphorus (14, 18), both strains were starved for phosphate before inoculation into phosphate-free CgXII mineral medium containing either 1 mM AMP, 1 mM UDP-glucose, or 0.065 mM phosphate as a sole source of phosphorus. *C. glutamicum* WT and WTΔ*ushA* could grow with the limiting concentration of phosphate (0.065 mM) (Fig. 3). However, while *C. glutamicum* WT could utilize AMP and UDP-glucose as sole sources of phosphorus, *C. glutamicum* WTΔ*ushA* could not grow on these phosphorus sources (Fig. 3 and data not shown). Thus, *ushA* is required for growth of *C. glutamicum* on UDP-glucose and nucleotides such as AMP as phosphorus sources.

While AMP could serve as a sole source of phosphorus, *C. glutamicum* could not utilize 100 mM AMP as a carbon source or as a combined carbon and phosphorus source regardless of whether the cells were starved for phosphate in the preculture or not (data not shown).

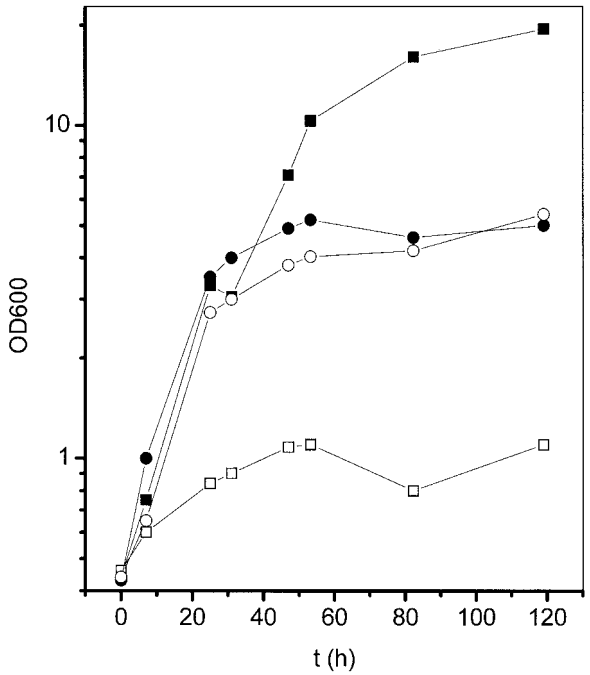

FIG. 3. Growth of *C. glutamicum* WT and WTΔ*ushA* on UDP-glucose and phosphate as sole sources of phosphorus. *C. glutamicum* WT (solid symbols) and WTΔ*ushA* (open symbols) were precultured under phosphate-limiting conditions for 24 h before inoculation into phosphate-free CgXII minimal medium containing 40 g/liter glucose and either 1 mM UDP-glucose (squares) or 0.065 mM phosphate (circles). OD600, optical density at 600 nm.

DISCUSSION

C. glutamicum UshA belongs to a family of bacterial and animal 5′ nucleotidases that have a common structure (17). UshA from *C. glutamicum* hydrolyzes ribonucleotides, deoxyribonucleotides, and UDP sugars, as do other 5′ nucleotidases. However, the physiological roles of 5′ nucleotidases differ considerably. Animal 5′ nucleotidases in the brain hydrolyze nucleotides to terminate their action as neurotransmitters and mediate cell-matrix and cell-cell adhesion independent of their catalytic activity (10). In enteric bacteria, the main physiological role of 5′ nucleotidase is nucleotide salvage. *E. coli*, but not *C. glutamicum*, can grow on 5′-AMP as a sole carbon source, and *E. coli* requires UshA for utilization of 5′-AMP (21, 33). Moreover, the 5′ nucleotidase UshA confers sensitivity of *E. coli* to 5′-fluorouracil under certain conditions. As 5′-fluorouracil is converted to toxic 5′-fluorouridylate in the purine salvage pathways either by uracil phosphoribosyl trasferase (encoded by *upp*) or, if ribose-1-phosphate is available, by uridine phosphorylase and uridine kinase, *ushA* mutations render *E. coli upp* mutants resistant to 5′-fluorouracil with AMP as a source of ribose 1-phosphate (21, 33). In *Salmonella enterica* serotype Typhimurium, in which the nonhomologous *ushB* gene encodes a functional 5′ nucleotidase, the *ushA* gene is cryptic due to a missense mutation (13). Hydrolysis of extracellular nucleotides either by UshA or by UshB in enteric bacteria yields inorganic phosphate and may contribute to the availability of extracellular free phosphate. Expression of neither *ushA* nor *ushB* is phosphate starvation inducible, and phenotypic manifestations of *ushA* or *ushB* mutations for phosphorus metabolism have not been described (31). Therefore, 5′ nucleotidases of enteric bacteria do not function primarily in phosphorus metabolism.

In *C. glutamicum*, *ushA* expression is phosphate starvation inducible, and UshA is required for growth on UDP-glucose or 5′-AMP as a sole source of phosphorus. Although the possibility of a role in nucleotide salvage or signal transduction cannot be excluded, the clear phenotype of the *ushA* deletion mutant and the phosphate-dependent control of *ushA* expression argue for a primary role of *C. glutamicum* UshA in phosphorus metabolism. *C. glutamicum* occurs in soil in which the turnover of microbial biomass phosphorus has been estimated to be about 25 kg per hectare per year (2). This turnover results in the release of large amounts of phosphorus in the form of mobile and labile nucleic acids, phospholipids, and sugar phosphates into the soil (2). The breakdown of nucleic acids to nucleotides is catalyzed by extracellular nucleases, such as NucH from *Aeromonas hydrophila* (4). Expression of the homologous *nucH* genes from *C. glutamicum* and also from *Synechocystis* sp. increased upon phosphate starvation (14, 29). Thus, the transcriptional program of *C. glutamicum* to adapt to phosphorus limitation includes induction of the nuclease gene *nucH* and the 5′ nucleotidase gene *ushA* and allows the organism to access nucleic acids and nucleotides in soil as sources of phosphorus for growth.

C. glutamicum lacks a gene homologous to the *ushB* gene in *S. enterica* serotype Typhimurium (15). However, two *C. glutamicum* genes, NCgl0021 and NCgl0022, encode proteins which have amino acid sequences similar to those of the larger *C. glutamicum* UshA protein (694 amino acids). It is likely that an *ushA* paralogous gene was mutated and now carries a stop codon in its open reading frame, giving rise to NCgl0021 and NCgl0022. Similarly, in *S. enterica* serotype Typhimurium the *ushA* gene has been inactivated by a single missense mutation (13). The UshA protein contains two conserved domains: the PF00149 domain conserved in 5′ nucleotidases and other calcineurin-type phosphoesterases at its N terminus (amino acids 36 to 252) and the PF02872 domain found in C termini of 5′ nucleotidases (amino acids 334 to 501). NCgl0021 encodes a 176-amino-acid polypeptide which contains the PF00149 domain and is 55% identical to amino acids 73 to 250 of UshA. On the other hand, the NCgl0022-encoded polypeptide, which contains the PF02872 domain, is 412 amino acids long and 37% of its amino acids are identical to amino acids of the C terminus of UshA (amino acids 269 to 633). The absence of detectable 5′ nucleotidase and UDP-sugar hydrolase activities from supernatants of the *C. glutamicum ushA* deletion mutant showed that the proteins encoded by NCgl0021 and NCgl0021 cannot functionally replace UshA.

The genome of the related organism *Corynebacterium efficiens* lacks homologs of NCgl0021 and NCgl0022, but it carries a homolog of *ushA* (CE0337). *Corynebacterium ammoniagenes* is another member of the *Corynebacteriaceae* and is used for biotechnological production of flavor enhancers, such as 5′-IMP (20). In such a process, inosine is synthesized de novo by intracellular dephosphorylation of 5′-IMP. Inosine secreted into the culture medium can be converted to 5′-IMP chemically or enzymatically, and efficient producing strains need to be devoid of extracellular 5′ nucleotidase activity (20). It re-

mains to be determined whether *C. ammoniagenes* possesses genes homologous to *ushA* and, if so, whether defined *ushA* deletion mutants perform better in the 5′-IMP production process. In addition to *C. glutamicum* and *C. efficiens*, UshA is found in *Streptomyces coelicolor* (SCO4152), while the genomes of *Corynebacterium diphtheriae*, *Mycobacterium tuberculosis*, *Mycobacterium bovis*, and *Mycobacterium leprae* do not carry homologs of UshA. This suggests that mycobacteria and *C. diphtheriae* either cannot utilize nucleotides as sources of phosphorus or do so by a pathway(s) that is an alternative to hydrolysis by 5′ nucleotidase.

ACKNOWLEDGMENTS

We thank Hermann Sahm for continuous support, Michael Bott for fruitful discussions, and Christina Mack, Steffen Schaffer, and Axel Niebisch for mass spectrometry analyses.

The support of the Bundesministerium für Bildung und Forschung (BMBF) through grant 031U113D/031U213D is gratefully acknowledged. Ulrike Sorger-Herrmann was supported by a fellowship from the Friedrich-Ebert-Stiftung.

Some of the work described here is from the planned Ph.D. dissertation of Ulrike Sorger-Herrmann at the faculty of Mathematics and Natural Sciences of Heinrich-Heine-Universität Düsseldorf.

REFERENCES

1. **Antelmann, H., C. Scharf, and M. Hecker.** 2000. Phosphate starvation-inducible proteins of *Bacillus subtilis*: proteomics and transcriptional analysis. J. Bacteriol. **182:**4478–4490.
2. **Bieleski, R. L.** 1973. Phosphate pools, phosphate transport and phosphate availability. Annu. Rev. Plant Physiol. **24:**225–252.
3. **Burns, D. M., and I. R. Beacham.** 1986. Nucleotide sequence and transcriptional analysis of the *E. coli ushA* gene, encoding periplasmic UDP-sugar hydrolase (5′-nucleotidase): regulation of the *ushA* gene, and the signal sequence of its encoded protein product. Nucleic Acids Res. **14:**4325–4342.
4. **Dodd, H. N., and J. M. Pemberton.** 1996. Cloning, sequencing, and characterization of the *nucH* gene encoding an extracellular nuclease from *Aeromonas hydrophila* JMP636. J. Bacteriol. **178:**3926–3933.
5. **Edwards, C. J., D. J. Innes, D. M. Burns, and I. R. Beacham.** 1993. UDP-sugar hydrolase isozymes in *Salmonella enterica* and *Escherichia coli*: silent alleles of *ushA* in related strains of group I *Salmonella* isolates, and of *ushB* in wild-type and K12 strains of *E. coli*, indicate recent and early silencing events, respectively. FEMS Microbiol Lett. **114:**293–298.
6. **Eikmanns, B. J., E. Kleinertz, W. Liebl, and H. Sahm.** 1991. A family of *Corynebacterium glutamicum*/*Escherichia coli* shuttle vectors for cloning, controlled gene expression, and promoter probing. Gene **102:**93–98.
7. **Eikmanns, B. J., N. Thum-Schmitz, L. Eggeling, K. U. Lüdtke, and H. Sahm.** 1994. Nucleotide sequence, expression and transcriptional analysis of the *Corynebacterium glutamicum gltA* gene encoding citrate synthase. Microbiology **140:**1817–1828.
8. **Engels, S., J. E. Schweitzer, C. Ludwig, M. Bott, and S. Schaffer.** 2004. *clpC* and *clpP1P2* gene expression in *Corynebacterium glutamicum* is controlled by a regulatory network involving the transcriptional regulators ClgR and HspR as well as the ECF sigma factor sigmaH. Mol. Microbiol. **52:**285–302.
9. **Grant, S. G., J. Jessee, F. R. Bloom, and D. Hanahan.** 1990. Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. Proc. Natl. Acad. Sci. USA **87:**4645–4649.
10. **Gutensohn, W., R. Resta, Y. Misumi, Y. Ikehara, and L. F. Thompson.** 1995. Ecto-5′-nucleotidase activity is not required for T cell activation through CD73. Cell. Immunol. **161:**213–217.
11. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
12. **Hulett, F. M.** 2002. The Pho regulon, p. 193–201. *In* A. L. Sonenshein, J. A. Hoch, and R. M. Losick (ed.), *Bacillus subtilis* and its closest relatives: from genes to cells. ASM Press, Washington, D.C.
13. **Innes, D., I. R. Beacham, C. A. Beven, M. Douglas, M. W. Laird, J. C. Joly, and D. M. Burns.** 2001. The cryptic *ushA* gene (*ushA*^{c}) in natural isolates of *Salmonella enterica* (serotype Typhimurium) has been inactivated by a single missense mutation. Microbiology **147:**1887–1896.
14. **Ishige, T., M. Krause, M. Bott, V. F. Wendisch, and H. Sahm.** 2003. The phosphate starvation stimulon of *Corynebacterium glutamicum* determined by DNA microarray analyses. J. Bacteriol. **185:**4519–4529.
15. **Kalinowski, J., B. Bathe, D. Bartels, N. Bischoff, M. Bott, A. Burkovski, N. Dusch, L. Eggeling, B. J. Eikmanns, L. Gaigalat, A. Goesmann, M. Hartmann, K. Huthmacher, R. Krämer, B. Linke, A. C. McHardy, F. Meyer, B. Möckel, W. Pfefferle, A. Pühler, D. A. Rey, C. Rückert, O. Rupp, H. Sahm, V. F. Wendisch, I. Wiegräbe, and A. Tauch.** 2003. The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. J. Biotechnol. **104:**5–25.
16. **Keilhauer, C., L. Eggeling, and H. Sahm.** 1993. Isoleucine synthesis in *Corynebacterium glutamicum*: molecular analysis of the *ilvB-ilvN-ilvC* operon. J. Bacteriol. **175:**5595–5603.
17. **Knöfel, T., and N. Strater.** 1999. X-ray structure of the *Escherichia coli* periplasmic 5′-nucleotidase containing a dimetal catalytic site. Nat. Struct. Biol. **6:**448–453.
18. **Lambert, C., D. Weuster-Botz, R. Weichenhain, E. W. Kreutz, A. A. de Graaf, and S. M. Schoberth.** 2002. Monitoring of inorganic polyphosphate dynamics in *Corynebacterium glutamicum* using a novel oxygen sparger for real time ^{31}P in vivo NMR. Acta Biotechnol. **22:**245–260.
19. **Lange, C., D. Rittmann, V. F. Wendisch, M. Bott, and H. Sahm.** 2003. Global expression profiling and physiological characterization of *Corynebacterium glutamicum* grown in the presence of L-valine. Appl. Environ. Microbiol. **69:**2521–2532.
20. **Mori, H., A. Iida, T. Fujio, and S. Teshiba.** 1997. A novel process of inosine 5′-monophosphate production using overexpressed guanosine/inosine kinase. Appl. Microbiol. Biotechnol. **48:**693–698.
21. **Neuhard, J., and R. A. Kelln.** 1996. Biosynthesis and conversion of pyrimidines, p. 580–599. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
22. **Patek, M., B. J. Eikmanns, J. Patek, and H. Sahm.** 1996. Promoters from *Corynebacterium glutamicum*: cloning, molecular analysis and search for a consensus motif. Microbiology **142:**1297–1309.
23. **Patek, M., J. Nesvera, A. Guyonvarch, O. Reyes, and G. Leblon.** 2003. Promoters of *Corynebacterium glutamicum*. J. Biotechnol. **104:**311–323.
24. **Peters-Wendisch, P. G., B. J. Eikmanns, G. Thierbach, B. Bachmann, and H. Sahm.** 1993. Phosphoenolpyruvate carboxylase in *Corynebacterium glutamicum* is dispensable for growth and lysine production. FEMS Microbiol Lett. **112:**269–274.
25. **Rittmann, D., S. Schaffer, V. F. Wendisch, and H. Sahm.** 2003. Fructose-1,6-bisphosphatase from *Corynebacterium glutamicum*: expression and deletion of the *fbp* gene and biochemical characterization of the enzyme. Arch Microbiol. **180:**285–292.
26. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
27. **Schäfer, A., A. Tauch, W. Jager, J. Kalinowski, G. Thierbach, and A. Pühler.** 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene **145:**69–73.
28. **Schaffer, S., B. Weil, V. D. Nguyen, G. Dongmann, K. Günther, M. Nickolaus, T. Hermann, and M. Bott.** 2001. A high-resolution reference map for cytoplasmic and membrane-associated proteins of *Corynebacterium glutamicum*. Electrophoresis **22:**4404–4422.
29. **Suzuki, S., A. Ferjani, I. Suzuki, and N. Murata.** 2004. The SphS-SphR two component system is the exclusive sensor for the induction of gene expression in response to phosphate limitation in *Synechocystis*. J. Biol. Chem. **279:**13234–13240.
30. **van der Rest, M. E., C. Lange, and D. Molenaar.** 1999. A heat shock following electroporation induces highly efficient transformation of *Corynebacterium glutamicum* with xenogeneic plasmid DNA. Appl. Microbiol. Biotechnol. **52:**541–545.
31. **Wanner, B. L.** 1996. Phosphorus assimilation and control of the phosphate regulon, p. 1357–1381. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
32. **Wendisch, V. F., and M. Bott.** 2005. Phosphorus metabolism of *Corynebacterium glutamicum*, p. 377–396. *In* L. Eggeling and M. Bott (ed.), Handbook on *Corynebacterium glutamicum*. CRC Press, Boca Raton, Fla.
33. **Zalkin, H., and P. Nygaard.** 1996. Biosynthesis of purine nucleotides, p. 561–579. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
34. **Zimmermann, H.** 1996. Biochemistry, localization and functional roles of ecto-nucleotidases in the nervous system. Prog. Neurobiol. **49:**589–618.