Characterization of an *N*-Acetylmuramic Acid/*N*-Acetylglucosamine Kinase of *Clostridium acetobutylicum*^{†‡}

Jan Reith, Anne Berking,[‡] and Christoph Mayer*

Fachbereich Biologie, Molekulare Mikrobiologie, Universität Konstanz, 78457 Constance, Germany

Received 10 June 2011/Accepted 13 July 2011

We report here the cloning and characterization of a cytoplasmic kinase of *Clostridium acetobutylicum*, named MurK (for murein sugar kinase). The enzyme has a unique specificity for both amino sugars of the bacterial cell wall, *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc), which are phosphorylated at the 6-hydroxyl group. Kinetic analyses revealed K_m values of 190 and 127 μM for MurNAc and GlcNAc, respectively, and a k_{cat} value (65.0 s^{-1}) that was 1.5-fold higher for the latter substrate. Neither the non-*N*-acetylated forms of the cell wall sugars, i.e., glucosamine and/or muramic acid, nor epimeric hexoses or 1,6-anhydro-MurNAc were substrates for the enzyme. MurK displays low overall amino acid sequence identity (24%) with human GlcNAc kinase and is the first characterized bacterial representative of the BcrAD/BadFG-like ATPase family. We propose a role of MurK in the recovery of muropeptides during cell wall rescue in *C. acetobutylicum*. The kinase was applied for high-sensitive detection of the amino sugars in cell wall preparations by radioactive phosphorylation.

Clostridia are anaerobic, spore-forming, and A/T-rich Gram-positive rods that include several toxin-producing pathogens (e.g., *Clostridium difficile*, *C. botulinum*, *C. tetani*, and *C. perfringens*), as well as nonpathogenic members such as *C. acetobutylicum* ATCC 824. The latter is a biotechnologically important strain that is used for the production of solvents such as acetone, butanol, and ethanol by fermentation of a broad range of mono-, di-, and polysaccharides (13). Although solvent formation by the conversion of carbohydrates attracted interest in the physiology of carbohydrates in this organism (17) and despite its wide use in metabolic engineering and the recent sequencing of its genome (20), the cell wall (peptidoglycan) catabolism has not been investigated thoroughly in *C. acetobutylicum* or in any of its pathogenic relatives. For the related members of the *firmicutes*, *Bacillus subtilis*, *B. megaterium*, and *B. cereus*, it was shown that as much as 50% of the parental peptidoglycan is turned over in one generation (6, 16, 21). We have recently identified a pathway in *B. subtilis* for the rescue of peptidoglycan fragments, which involves the sequential processing of *N*-acetylglucosamine (GlcNAc)-*N*-acetylmuramic acid (MurNAc) peptides (muropeptides) by the secreted *B. subtilis* enzymes *N*-acetylglucosaminidase (NagZ_{Bs}) and *N*-acetylmuramyl-L-alanine amidase (AmiE_{Bs}) (14). Thereby, the amino sugars GlcNAc and MurNAc are generated extracellularly and are presumably further metabolized alike in *Escherichia coli*. The pathways involve the uptake by specific phosphotransferase system transporters, NagP (25) and MurP (8),

respectively, yielding, intracellularly, the respective 6-phosphate sugars, the cytoplasmic recycling enzyme MurNAc-6-phosphate etherase (MurQ), which converts MurNAc-6-phosphate to GlcNAc-6-phosphate (10, 14), and the *nag* genes (*nagA*-encoded GlcNAc-6-phosphate deacetylase and *nagB*-encoded glucosamine-6-phosphate deaminase), which finally yield fructose-6-phosphate (33, 34). We found orthologs of the latter genes also on the genome of *C. acetobutylicum* except for *murP*. Furthermore, a protein orthologous to the *nagZ*-encoded *N*-acetylglucosaminidase of *B. subtilis* appeared to lack a signal sequence and thus is probably not secreted as is the *Bacillus* enzyme but rather is cytoplasmic in *C. acetobutylicum*. Therefore, we concluded that the process of recovery of the amino sugars of the cell wall in *C. acetobutylicum* relies on an enzyme(s) that allows their specific phosphorylation within the cytoplasm prior to further degradation. We describe here the cloning and characterization of an amino sugar kinase of *C. acetobutylicum* that phosphorylates ATP dependently both cell wall sugars MurNAc and GlcNAc at the 6-position.

MATERIALS AND METHODS

Plasmid construction. For heterologous overexpression in *E. coli*, *murK* was cloned as a recombinant construct with a C-terminal His₆ tag. *C. acetobutylicum* chromosomal DNA (kindly provided by G. Bennett [Rice University, Houston, TX] and H. Bahl [University of Rostock, Rostock, Germany]) was used to amplify a 921-bp *murK* DNA fragment by PCR with the primer pair 5'-AAAA CCATGGGCAAGTATGTTATAGGAATAGACGGTGG-3' and 5'-AAAAG GATCCTTACTCGAGCTCACTTCTTGCTATAATTACAGCAC-3' (the recognition sites for endonucleases NcoI and XhoI that were used for cloning in *E. coli* strain DH5 α are underlined [2]). The PCR product was ligated into the pET28a expression vector (Kan^r; Novagen) using T4 DNA ligase (Fermentas). The resulting plasmid, pMurK, carried the *murK* gene under the control of the IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible T7 promoter.

Overexpression and purification of recombinant MurK. MurK was overproduced in *E. coli* BL21(DE3) (26) carrying pMurK. Cultures were grown at 25°C with vigorous shaking in 4 liter of LB medium supplemented with kanamycin at a final concentration of 50 $\mu\text{g}/\text{ml}$, starting from a 2% inoculum of an overnight culture. After growth to mid-log phase (optical density at 600 nm [OD₆₀₀] of 0.6), MurK expression was induced by the addition of IPTG at a final concentration of 0.2 mM, and incubation was continued for further 16 h. All of the following

* Corresponding author. Mailing address: Interfakultäres Institut für Mikrobiologie und Infektionsmedizin Tübingen IMIT, Mikrobiologie/Biotechnologie, Eberhard Karls Universität Tübingen, 72076 Tübingen, Germany. Phone: 49 7071 29-78866. Fax: 49 7071 29-5979. E-mail: christoph.mayer@biotech.uni-tuebingen.de.

[†] Supplemental material for this article may be found at <http://jb.asm.org/>.

[‡] Present address: Fachbereich Biologie, Zellbiologie, Universität Konstanz, 78457 Constance, Germany.

[‡] Published ahead of print on 22 July 2011.

FIG. 1. Schematic representation of the coupled enzyme ATPase assay. MurK phosphorylates GlcNAc and MurNAc using ATP. ATP is regenerated from the reaction product ADP by pyruvate kinase (PK), which thereby converts phosphoenolpyruvate (PEP) to pyruvate. This reaction is made thermodynamically favorable by coupling to the conversion of pyruvate to lactate by lactate dehydrogenase (LDH). This allows to monitor the reaction by quantifying NADH oxidation yielding NAD⁺ by assaying its absorption at 340 nm.

purification steps were performed at 4°C. Cells were harvested by centrifugation at $5,000 \times g$ for 45 min, washed once in 50 ml of buffer (20 mM Na₂HPO₄ · 2H₂O, 500 mM NaCl, 20 mM imidazole [pH 7.5]), and then resuspended in 30 ml of the same buffer. The cellular extract was obtained by disruption in a French press cell (three times). Afterward, cell debris and unbroken cells were removed by ultracentrifugation at $150,000 \times g$ for 1 h. The His-tagged MurK was purified by Ni²⁺ affinity chromatography on a 1-ml His-Trap column (GE Healthcare) according to the manufacturer's protocol. A linear gradient from 0 to 500 mM imidazole was applied (20 mM Na₂HPO₄ · 2H₂O, 500 mM NaCl, 500 mM imidazole [pH 7.5]), and MurK eluted from the column with 70 mM imidazole. The eluted fractions were analyzed for purity by SDS-PAGE. Fractions containing pure MurK protein were pooled and dialyzed against 20 mM Na₂HPO₄ · 2H₂O–500 mM NaCl (pH 7.5) and stored at –80°C. The protein concentration was estimated by the method of Bradford with bovine serum albumin as the standard (4). The protein yield was 43 mg of purified MurK from 4 liters of cell culture.

Nonradioactive and radioactive phosphorylation assays. The ability of MurK to phosphorylate various amino sugars (MurNAc, GlcNAc, GalNAc, ManNAc, anhydromurNAc [1,6-anhydro-*N*-acetylmuramic acid], GlcN, or MurN [muramic acid]), as well as non-amino sugars (Glc and anhg), was analyzed. MurNAc was obtained from Bachem, Bubendorf, Switzerland, and all other sugars were obtained from Sigma. Portion (20 µg [0.58 nmol]; 2.91 µM in the assay) of MurK were incubated with 50 mM sugar substrate in a 200-µl reaction mixture containing 100 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 100 mM ATP. The samples were incubated for up to 2 h at 37°C. At defined time intervals, 5-µl samples were taken from the assay and spotted onto a thin-layer chromatography (TLC) plate (silica gel 60 F₂₅₄; Merck, Darmstadt, Germany). After the separation of sugars and sugar phosphates in butanol-methanol-ammonia-water (5:4:2:1 [vol/vol/vol/vol]), the TLC plate was developed in methanol containing 2% (vol/vol) concentrated H₂SO₄, followed by drying and charring for 5 min at 180°C. No phosphorylation products were obtained with MurK in the absence of the substrate.

The more sensitive detection of phosphorylated amino sugars was possible by the use of radioactively labeled ATP as phosphate donor. A mixture of [γ -³²P]ATP (4,000 Bq) and 0.5 mM ATP was incubated for 60 min at 37°C with 1 mM substrate (GlcNAc, MurNAc, anhydromurNAc, MDP [*N*-acetyl-muramyl-L-alanyl-D-isoglutamine], or GlcNAc-MurNAc) in 20-µl reaction mixtures containing 25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 20 ng (0.58 pmol; 29.13 nM concentration in the assay) of purified MurK. Therefore, 2-µl samples were spotted onto a TLC plate and applied as described above. The radioactive phosphorylation products were detected and quantified by using a FLA-9000 phosphorimager (FujiFilm, software Multi Gauge).

Coupled enzyme ATPase assay. The kinetic parameters of MurK for the phosphorylation of amino sugars with ATP were determined by using a coupled enzyme assay (Fig. 1). In this assay, the formation of ADP was directly, stoichiometrically coupled to NADH oxidation by pyruvate kinase (PK) and lactate dehydrogenase (LDH). In a 1-ml cuvette, 100 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 5 mM ATP, 1 mM phosphoenolpyruvate, 0.2 mM NADH, 10 U of pyruvate kinase, and 7 U of lactate dehydrogenase (from rabbit muscle [Sigma-Aldrich], 0.7 U of PK/µl and 1 U of LDH/µl) were incubated with various substrates (GlcNAc, MurNAc, GalNAc, ManNAc, GlcN, MurN, and Glc) at final concentrations ranging from 0.01 to 2.5 mM. The hydrolysis of ATP was

initiated by addition of 0.5 µg (14.57 pmol) MurK. The reactions were then incubated for 5 min at 30°C. The change of NADH absorbance was monitored at 340 nm in a spectrophotometer (Pharmacia Biotech). Kinetic parameters were evaluated by fitting the experimental data to the Michaelis-Menten equation with nonlinear regression using the software Prism4 (GraphPad). We used a molar extinction coefficient of NADH at 340 nm of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. Similarly, the K_m and V_{max} values for ATP at various concentrations from 0.01 to 2.5 mM were determined at saturated concentration of 5 mM GlcNAc.

***E. coli* cell wall preparation.** Cell cultures of *E. coli* strain MG1655 (3) were harvested in exponential phase (OD₆₀₀ of 0.6) by centrifugation (5,000 × *g*, 45 min, 4°C). Approximately 5×10^9 cells were suspended in water and immediately boiled in a water bath for 10 min. The insoluble material was recovered by centrifugation (13,000 × *g*, 5 min), washed three times with distilled water, and then resuspended in 200 µl of Tris buffer (50 mM Tris-HCl [pH 7.0]). The peptidoglycan portion of the insoluble material was digested enzymatically at 30°C for 18 h at continuous slow rotation with a mixture of autolysins, each at 10 µg: mutanolysin (from *Streptomyces globisporus* [Sigma-Aldrich], 4,000 U/mg), *N*-acetylmuramic acid-L-alanine amidase AmiD (from *E. coli*, prepared as described previously [27]), and *N*-acetylglucosaminidase NagZ_{Bs} (from *Bacillus subtilis*, prepared as described previously [14]). After complete degradation, the enzymes were heat inactivated for 10 min at 95°C. Supernatants of the digestions were collected by centrifugation (13,000 × *g*, 5 min) and evaporated in a vacuum concentrator. Finally, the dried pellets were resuspended in 20 µl of water. Then, a 5-µl sample was incubated with 1.5 µg (43.71 pmol) of MurK in a 20-µl total volume further containing 25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, [γ -³²P]ATP (2,500 Bq), and 0.5 mM ATP. The reactions were incubated for 60 min at 37°C. At the beginning and end of the reactions a 2-µl sample was spotted onto a TLC plate, which was then developed and analyzed as described above (radioactive MurK phosphorylation assay).

Activity-pH dependency and pH stability. The activity-pH profile of MurK was determined with a total amount of 10 ng (0.3 pmol) of MurK in phosphate-citrate buffer (pH 4.5 to 8.0), Tris-HCl buffer (pH 7.5 to 9.0), and glycine-NaOH buffer (pH 9.0 to 10.5). The standard 20-µl reaction mixtures contained 2 mM GlcNAc, 10 mM MgCl₂, and a mix of [γ -³²P]ATP (4,000 Bq) and 1.5 mM ATP in the buffers described above. After 30 min of incubation at 37°C, the reactions were stopped by spotting a 2-µl sample onto a TLC plate, which was subsequently developed. The radioactively labeled reaction products were then quantified with a phosphorimager. The stability of the enzyme at different pHs was investigated. A 20-fold dilution of concentrated MurK in buffers at different pH values (0.2 M phosphate–0.1 M citrate, 0.2 M Tris-HCl, 0.2 M glycine-NaOH [pH 4.5 to 10.5]) was incubated for 30 min at 30°C. The protein solutions were then diluted 20-fold with 0.2 M phosphate–0.1 M citrate reaction buffer (pH 7.5). The enzyme reactions were initiated by the addition of 10 ng (0.3 pmol) of preincubated MurK to the standard reaction mixture (as described above). Changes in the activity of MurK were determined by quantification of radioactively labeled reaction products separated by TLC. MurK stability at various temperatures over a period of 6 h at 4 and 30°C, respectively, was also studied. To exclude decreases in MurK activity, caused by lower amounts of possibly copurified proteases, MurK was preincubated with or without protease inhibitors (Roche complete, EDTA-free protease inhibitor cocktail) for 1 h at 4°C. By applying the coupled enzyme ATPase assay with GlcNAc as the substrate the decrease in absorbance at 340 nm was measured hourly and the MurK activity was determined.

RESULTS

Identification, cloning, and heterologous expression of *murK* of *C. acetobutylicum*. We identified orthologs of mucopeptide recycling genes of *B. subtilis* being clustered on the genome of *C. acetobutylicum* ATCC 824. Putative proteins with 34, 30, and 52% amino acid sequence identity, respectively, with *N*-acetylglucosaminidase NagZ (NagZ_{Bs}), MurNAc-L-Ala amidase AmiE (AmiE_{Bs}), and MurNAc-6-phosphate etherase MurQ of *B. subtilis* (MurQ_{Bs}) indicated that a mucopeptide recovery pathway likely exists in *C. acetobutylicum*. Surprisingly, however, we found that NagZ and AmiE of *C. acetobutylicum* (NagZ_{Ca} and AmiE_{Ca}) both lack a signal sequence and thus apparently are located in the cytoplasm. Therefore, in contrast to *B. subtilis*, nonphosphorylated cell wall amino sugars are likely generated in *C. acetobutylicum* in the cytoplasm

FIG. 2. Purity of recombinant MurK analyzed by SDS-PAGE and Coomassie brilliant blue staining. The protein was overproduced in *E. coli* BL21(DE3) carrying pMurK. Lane 1, protein size standard; lane 2, *E. coli* cell extract before induction of MurK; lane 3, *E. coli* cell extract after induction of MurK; lane 4, 20 µg of purified MurK.

by the action of NagZ and AmiE and need to be phosphorylated for further metabolism. We identified an open reading frame (CA_C0183) downstream of *nagZ_{Ca}* that was classified as a member of the poorly characterized BcrAD/BadFG-like ATPase family (PF01869). This family includes *N*-acetylglucosamine kinases of eukaryotic origin (human, rat, or mouse), which display 24% amino acid sequence identity with CA_C0183. Neither the *N*-acetylglucosamine kinase (NagK) (28) nor the anhydro-*N*-acetylmuramic acid kinase (AnmK) of *E. coli* (29) have any significant amino acid sequence identity with CA_C0183, nor were orthologs of these genes found elsewhere on the chromosome of *C. acetobutylicum*. To analyze the enzymatic function of the putative sugar kinase, we cloned the gene and heterologously expressed it in *E. coli* as a C-terminal His₆-tagged fusion protein. Purification by Ni²⁺ affinity chromatography resulted in an apparently pure protein that appeared on SDS-PAGE consistently with the calculated molecular mass (*M_r*) of MurK-His₆ of 34,329 (Fig. 2).

FIG. 4. Identification of the products of the MurK reaction. The products were identified as GlcNAc-6-phosphate and MurNAc-6-phosphate by treatment with the lactyl etherase MurQ, which interconverts MurNAc-6-phosphate and GlcNAc-6-phosphate, yielding an equilibrium of the reaction intermediate Δ -2,3-GlcNAc-6-phosphate and GlcNAc-6-phosphate of 30 to 70%.

MurK is a kinase with specificity for GlcNAc and MurNAc.

We first showed that MurK is able to phosphorylate both cell wall amino sugars, GlcNAc and MurNAc. We used ATP as a phosphoryl donor in a time course experiment with MurK, separated the reaction products by TLC and visualized the sugar substrate depletion and sugar phosphate production on the plate by charring the sample with sulfuric acid (Fig. 3). Phosphorylation occurred at the 6 position, yielding GlcNAc-6-phosphate and MurNAc-6-phosphate, respectively, as revealed by treatment with MurNAc-6-phosphate etherase (10, 11), which converts MurNAc-6-phosphate and GlcNAc-6-phosphate, yielding an equilibrium of GlcNAc-6-phosphate and the reaction intermediate Δ -2,3-GlcNAc-6-phosphate (Fig. 4).

We determined the specificity of MurK, revealing its possible function in cell wall amino sugar recovery. Potential substrates that differ in the stereochemistry of hydroxyl groups at the C2 or C4 position in carbon ring and/or by the absence of the *N*-acetyl group or 3-O-lactyl group (Fig. 5A) were tested as substrates for MurK with the nonradioactive assay as described in Materials and Methods. MurK phosphorylated only GlcNAc and MurNAc at the 6-hydroxyl group (Fig. 5B), and even the structurally closely related amino sugars GalNAc, ManNAc, GlcN, and MurN were not substrates for MurK (Fig. 5B; see

FIG. 3. Time course of phosphorylation of GlcNAc (A) and MurNAc (B) by MurK, respectively, yielding GlcNAc-6-phosphate and MurNAc-6-phosphate. ATP was used as phosphate donor in a 2-fold excess relative to the amino sugar (250 nmol per assay). The amino sugars were separated by TLC as described in Materials and Methods.

FIG. 5. Substrate specificity of MurK. Chemical structures of selected amino sugars and sugars (A) that were tested as substrates for MurK by TLC with nonradioactive (B) and radioactive (C) kinase assays. Spots that can be assigned to phosphorylated sugars are only visible for GlcNAc and MurNAc upon incubation with MurK. Neither anhMurNAc, the disaccharide GlcNAc-MurNAc, nor the muramyl dipeptide (MDP) served as a substrate for MurK. After cleavage of GlcNAc-MurNAc disaccharide with *N*-acetylglucosaminidase NagZ_{Bs}, the products could be phosphorylated.

also Fig. S2B and Table S1 in the supplemental material). The activities determined with these sugars applying the coupled enzyme assay were <5% compared to GlcNAc, and the K_m values were higher than 1 mM. These results revealed that MurK of *C. acetobutylicum* is a kinase with specificity for the monosaccharides GlcNAc and MurNAc.

Characterization and kinetic parameters of MurK. The kinase MurK was found to keep activity for months at -80°C but lost ca. 50% of its activity within 2 months at 4°C . The pH dependency of MurK activity was analyzed by quantification of the reaction product GlcNAc-6-phosphate as described in Materials and Methods (Fig. 6). MurK had maximal activity at pH 7.5 to 9.0, retained half maximal activity at about pH 6.5 and 10.5 and was inactive at pH 5 and below. At 30°C , however, the purified protein lost activity within hours of incubation, regardless of the addition of protease inhibitors (data not shown). The MurK kinase activity required the presence of the divalent

FIG. 6. Activity-pH profile of MurK with GlcNAc substrate. A mixture of radioactively labeled and nonradioactive ATP was applied as described in Materials and Methods, and the enzyme activity was quantified by applied TLC. The data are means of three independent experiments; the standard errors are indicated.

TABLE 1. Kinetic parameters of MurK^a

Substrate	<i>V</i> _{max} (μmol/min/mg)	<i>K</i> _m (μM)	<i>k</i> _{cat} (1/s)	<i>k</i> _{cat} / <i>K</i> _m (1/s/mM)
GlcNAc	113.6	127.4	65.0	510.2
MurNAc	74.8	190.2	42.8	225.0
ATP	102.5	241.8	58.6	242.2

^a Kinetic parameters were determined in 100 mM Tris-HCl (pH 7.5) at 30°C using the coupled enzyme assay as described in Materials and Methods. Data are means of three independent experiments; standard errors were <5%.

metal ion Mg²⁺. A 3-fold excess of EDTA compared to the Mg²⁺ concentration strongly repressed the MurK activity. The addition of 2 mM MgCl₂ to the reaction mixture partially restored MurK activity (see Fig. S1 in the supplemental material). The kinetic parameters of MurK were determined with the coupled enzyme assay (see Materials and Methods and Fig. 1) and are summarized in Table 1. Michaelis-Menten plots of the data can be found in the supplemental material (see Fig. S2 in the supplemental material). MurK revealed a slightly lower *K*_m and a higher *V*_{max} value for GlcNAc compared to MurNAc, which is reflected in a catalytic efficiency that is 1.5-fold higher for GlcNAc than for MurNAc. Accordingly, when GlcNAc and MurNAc were simultaneously phosphorylated by MurK, GlcNAc appeared to be the preferred substrate and MurNAc phosphorylation was inhibited by GlcNAc, but not the other way around (see Fig. S3 in the supplemental material).

Radioactive phosphorylation assay. We applied a radioactive assay using [γ-³²P]ATP that allows a more sensitive product detection. The results obtained from the radioactive phosphorylation experiments confirmed the specificity of MurK for GlcNAc and MurNAc (data not shown). 1,6-Anhydro-MurNAc, which contains an intramolecular β-1,6-glycosidic linkage, and muramyl dipeptide (MDP) were also not phosphorylated by MurK. Furthermore, the enzyme did not phosphorylate the heterodisaccharide GlcNAc-MurNAc but the products GlcNAc and MurNAc after cleavage with NagZ_{Bs} (Fig. 5C).

It has not escaped our attention that this kinase is applicable for highly sensitive assay of cell wall sugars by radioactive phosphorylation. Both amino sugars can be reliably detected at amounts as low as 5 fmol (see Fig. S4 in the supplemental

material). This is at least 10⁵ times more sensitive than the colorimetric Morgan-Elson assay, which is the common assay to detect amino sugars (22). With this enzyme, it should be possible to identify cell wall fragments, as well as to detect minor bacterial contaminations in various specimens of bacteria after conversion into monosaccharides by specific radioactive phosphorylation. As a proof of principle, we applied MurK for the analysis of cell wall preparations from *E. coli*. These were degraded with mutanolysin (a muramidase from *Streptomyces globisporus* [5]), amidase AmiD (an *N*-acetylmuramic acid-*L*-Ala amidase [27]), and *N*-acetylglucosaminidase NagZ_{Bs} (14). In this way, the monosaccharides GlcNAc and MurNAc were released from cell wall preparations that could be subjected to radioactive phosphorylation with MurK (Fig. 7).

DISCUSSION

We present here the identification and characterization of MurK, a cytoplasmic 34-kDa GlcNAc/MurNAc kinase of the Gram-positive bacterium *C. acetobutylicum*. MurK can be assigned to the BadF/BadG/BcrA/BcrD ATPase family (BcrAD/BadFG) but reveals only low overall amino acid identity with the mammalian GlcNAc kinases of this family (e.g., 24% identity with human NagK). However, MurK harbors the highly conserved ATP-binding site (G⁶IDGGGSK¹²) near the N terminus that characterizes this kinase family. MurK has a novel specificity toward cell wall (peptidoglycan) sugars. The enzyme ATP dependently phosphorylated MurNAc and GlcNAc, as shown by TLC and coupled enzyme assays, yielding MurNAc-6-phosphate and GlcNAc-6-phosphate, respectively. All other tested sugar substrates, including the structurally similar ManNAc and GalNAc, as well as non-*N*-acetylated amino sugars were very poor substrates that revealed high *K*_m (1 to 4 mM) values and catalytic efficiencies lower than 3.5 s⁻¹ mM⁻¹ (cf. Table S1 in the supplemental material). Therefore, substrate specificity distinguishes *C. acetobutylicum* MurK from mammalian NagK. Besides GlcNAc, the preferred substrate, GlcNAc kinases from humans and rats are also able to phosphorylate ManNAc to a considerable degree and very inefficiently glucose. Both were very poor substrates for MurK. Thus far, only one prokaryotic member of the BcrAD/BadFG kinase family has been characterized, a hexokinase from the hyper-

FIG. 7. Assay of MurNAc and GlcNAc in cell wall preparation from *E. coli*. The cell wall sugars can be assayed only after degradation of the peptidoglycan with mutanolysin, AmiD, and/or NagZ_{Bs}. The monosaccharides GlcNAc and MurNAc were phosphorylated by MurK with [γ-³²P]ATP.

thermophilic archaeon *Sulfolobus tokodaii* (ST2354), which displays a broad substrate specificity for glucose, mannose, GlcN and, less efficiently, also phosphorylates 2-desoxyglucose and GlcNAc (18, 19). X-ray structures of this enzyme, which has 25% sequence identity with MurK, were recently solved (of *Sulfolobus tokodaii*, PDB codes 2E2N, 2E2O, 2E2P, and 2E2Q). Structures of three other prokaryotic family members with ca. 30% amino acid identity toward MurK were deposited to the structural database (CV2896 of *Chromobacterium violaceum*, PG1100 of *Porphyromonas gingivalis*, and BT3618 of *Bacteroides thetaiotaomicron*; PDB codes 1ZC6, 1ZBS, and 1ZXO), but the function and specificity of these proteins is unknown (9, 31, 32). MurK is conserved in most *Clostridium* species, including *C. phytofermentans*, *C. saccharolyticum*, and *Thermoanaerobacter tencongensis* (38, 36, and 43% amino acid identity, respectively) and other *Firmicutes*. Interestingly, putative orthologs of MurK were also found in cyanobacteria such as *Anabaena variabilis* (37% identity).

We propose a role of MurK in the rescue of the murein sugars GlcNAc and MurNAc released from mucopeptides during cell wall turnover in *C. acetobutylicum*. Cell wall recycling in Gram-positive bacteria remains enigmatic. However, orthologs of the MurNAc catabolic enzymes of *E. coli* have been recognized in most Gram-positive bacteria (24) (Table 1), including the MurNAc phosphotransferase system transporter MurP (8), the MurNAc-6-phosphate etherase MurQ (10), and the MurNAc-6-phosphate-sensitive transcriptional repressor MurR (12). This indicates that Gram-positive bacteria may have the capability to recycle their own cell wall, which we addressed in a recent review providing information regarding cell wall turnover and recycling in Gram-positives (24). Gram-positive bacteria apparently lack an AmpG permease that is required for the uptake of anhydromuropeptides in Gram-negatives and lytic transglycosylases that generate these cell wall fragments appear to play only a minor role in cell wall turnover during vegetative growth in Gram-positive bacteria. In contrast, lysozyme-like muramidases that liberate MurNAc-containing mucopeptides from cell wall peptidoglycan are widespread in Gram-positive bacteria. Non-*N*-acetylated peptidoglycan is a common modification of Gram-positive bacteria, particularly in *B. subtilis* and likely also in *C. acetobutylicum*, which accounts for high resistance against lysis due to cell wall hydrolysis by lysozyme (1, 30). At the beginning of the stationary growth phase, *C. acetobutylicum* produces an autolytic extracellular muramidase that is active on non-*N*-acetylated peptidoglycan (7). Thus, liberated mucopeptides containing GlcN residue at the nonreducing end are not substrates for NagZ *N*-acetylglucosaminidases, which are highly specific for *N*-acetylated substrates (15). Non-*N*-acetylated amino sugars in *C. acetobutylicum* need to be *N*-acetylated prior to further degradation. In an accompanying study, we report the identification and characterization of a glucosamine/glucosaminide *N*-acetyltransferase of *C. acetobutylicum* that is probably required for the acetylation of substrates released by the autolytic muramidase during the putative process of cell wall recycling in *C. acetobutylicum* (23). We recently showed that in *B. subtilis* mucopeptides are sequentially processed in the wall compartment (14). In contrast, catabolism of mucopeptides in *C. acetobutylicum* apparently occurs in the cytoplasm. *C. acetobutylicum* possesses orthologous enzymes of NagZ_{BS}

(CA_C0182) and AmiE_{BS} (CA_C0181) that lack a signal sequence and apparently are not secreted. Therefore, amino sugars are likely released from mucopeptides in the cytoplasm of *C. acetobutylicum*, where they can then be phosphorylated by the MurK kinase, yielding MurNAc- and GlcNAc-6-phosphate prior to further processing in the mucopeptide recovery pathway. Further studies are required to clarify the role of MurK in cell wall rescue in *C. acetobutylicum*. Its narrow substrate specificity and the highly sensitive radioactive detection suggest that MurK is a useful enzyme for the analysis of the cell wall sugars MurNAc and GlcNAc in environmental or clinical preparations. We showed that MurK also can be applied to quantify the cell wall sugar content after sequential digest (cf. Fig. 7).

ACKNOWLEDGMENTS

We thank G. Bennett (Rice University) and Hubert Bahl (University of Rostock) for kindly providing *C. acetobutylicum* genomic DNA.

This study was supported by grant MA2346/5 and Heisenberg stipend MA2346/3 to C.M. from the German Research Foundation (DFG).

REFERENCES

1. Atrih, A., G. Bacher, G. Allmaier, M. P. Williamson, and S. J. Foster. 1999. Analysis of peptidoglycan structure from vegetative cells of *Bacillus subtilis* 168 and role of PBP 5 in peptidoglycan maturation. *J. Bacteriol.* **181**:3956–3966.
2. Bethesda Research Laboratories. 1986. BRL pUC host: *Escherichia coli* DH5 α competent cells. Focus **8**:9.
3. Blattner, F. R., et al. 1997. The complete genome sequence of *Escherichia coli* K-12. *Science* **277**:1453–1462.
4. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
5. Calandra, G. B., and R. M. Cole. 1980. Lysis and protoplast formation of group B streptococci by mutanolysin. *Infect. Immun.* **28**:1033–1037.
6. Chaloupka, J., and P. Koecková. 1971. Turnover of mucopeptide during the life cycle of *Bacillus megaterium*. *Folia Microbiol.* **16**:372–382.
7. Croux, C., B. Canard, G. Goma, and P. Soucaille. 1992. Purification and characterization of an extracellular muramidase of *Clostridium acetobutylicum* ATCC 824 that acts on non-*N*-acetylated peptidoglycan. *Appl. Environ. Microbiol.* **58**:1075–1081.
8. Dahl, U., T. Jaeger, B. T. Nguyen, J. M. Sattler, and C. Mayer. 2004. Identification of a phosphotransferase system of *Escherichia coli* required for growth on *N*-acetylmuramic acid. *J. Bacteriol.* **186**:2385–2392.
9. Forouhar, F., et al. 2005. Crystal structure of the putative *N*-acetylglucosamine kinase (PG1100) from *Porphyromonas gingivalis*, northeast structural genomics target PgR18. PDB Data Bank. <http://www.wwPDB.org/>.
10. Jaeger, T., M. Arsic, and C. Mayer. 2005. Scission of the lactyl ether bond of *N*-acetylmuramic acid by *Escherichia coli* "etherase." *J. Biol. Chem.* **280**:30100–30106.
11. Jaeger, T., and C. Mayer. 2008. *N*-Acetylmuramic acid 6-phosphate lyases (MurNAc etherases): role in cell wall metabolism, distribution, structure, and mechanism. *Cell Mol. Life Sci.* **65**:928–939.
12. Jaeger, T., and C. Mayer. 2008. The transcriptional factors MurR and catabolite activator protein regulate *N*-acetylmuramic acid catabolism in *Escherichia coli*. *J. Bacteriol.* **190**:6598–6608.
13. Jones, D. T., and D. R. Woods. 1986. Acetone-butanol fermentation revisited. *Microbiol. Rev.* **50**:484–524.
14. Litzinger, S., et al. 2010. Mucopeptide rescue in *Bacillus subtilis* involves sequential hydrolysis by β -*N*-acetylglucosaminidase and *N*-acetylmuramyl-L-alanine amidase. *J. Bacteriol.* **192**:3132–3143.
15. Litzinger, S., et al. 2010. Structural and kinetic analysis of *Bacillus subtilis* *N*-acetylglucosaminidase reveals a unique Asp-His dyad mechanism. *J. Biol. Chem.* **285**:35675–35684.
16. Mauck, J., and L. Glaser. 1970. Turnover of the cell wall of *Bacillus subtilis* W-23 during logarithmic growth. *Biochem. Biophys. Res. Commun.* **39**:699–706.
17. Mitchell, W. J. 1998. Physiology of carbohydrate to solvent conversion by clostridia. *Adv. Microb. Physiol.* **39**:31–130.
18. Nishimasu, H., S. Fushinobu, H. Shoun, and T. Wakagi. 2007. Crystal structures of an ATP-dependent hexokinase with broad substrate specificity from the hyperthermophilic archaeon *Sulfolobus tokodaii*. *J. Biol. Chem.* **282**:9923–9931.

19. Nishimasu, H., S. Fushinobu, H. Shoun, and T. Wakagi. 2006. Identification and characterization of an ATP-dependent hexokinase with broad substrate specificity from the hyperthermophilic archaeon *Sulfolobus tokodaii*. *J. Bacteriol.* **188**:2014–2019.
20. Nolling, J., et al. 2001. Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*. *J. Bacteriol.* **183**:4823–4838.
21. Pooley, H. M. 1976. Layered distribution, according to age, within the cell wall of *Bacillus subtilis*. *J. Bacteriol.* **125**:1139–1147.
22. Reissig, J. L., J. L. Strominger, and L. F. Leloir. 1955. A modified colorimetric method for the estimation of *N*-acetylamino sugars. *J. Biol. Chem.* **217**:959–966.
23. Reith, J., and C. Mayer. 2011. Characterization of a glucosamine/glucosaminide *N*-acetyltransferase of *Clostridium acetobutylicum*. *J. Bacteriol.* **193**:5393–5399.
24. Reith, J., and C. Mayer. 2011. Peptidoglycan turnover and recycling in Gram-positive bacteria. *Appl. Microbiol. Biotechnol.* [Epub ahead of print.]
25. Reizer, J., et al. 1988. The phosphoenolpyruvate:sugar phosphotransferase system in Gram-positive bacteria: properties, mechanism, and regulation. *Crit. Rev. Microbiol.* **15**:297–338.
26. Studier, F. W., and B. A. Moffatt. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189**:113–130.
27. Uehara, T., and J. T. Park. 2007. An anhydro-*N*-acetylmuramyl-L-alanine amidase with broad specificity tethered to the outer membrane of *Escherichia coli*. *J. Bacteriol.* **189**:5634–5641.
28. Uehara, T., and J. T. Park. 2004. The *N*-acetyl-D-glucosamine kinase of *Escherichia coli* and its role in murein recycling. *J. Bacteriol.* **186**:7273–7279.
29. Uehara, T., et al. 2005. Recycling of the anhydro-*N*-acetylmuramic acid derived from cell wall murein involves a two-step conversion to *N*-acetylglucosamine-phosphate. *J. Bacteriol.* **187**:3643–3649.
30. Vollmer, W. 2008. Structural variation in the glycan strands of bacterial peptidoglycan. *FEMS Microbiol. Rev.* **32**:287–306.
31. Vorobiev, S. M., et al. 2005. Crystal structure of putative *N*-acetylglucosamine kinase from *Chromobacterium violaceum*. PDB Data Bank. <http://www.wwPDB.org/>.
32. Weihofen, W. A., M. Berger, H. Chen, W. Saenger, and S. Hinderlich. 2006. Structures of human *N*-acetylglucosamine kinase in two complexes with *N*-acetylglucosamine and with ADP/glucose: insights into substrate specificity and regulation. *J. Mol. Biol.* **364**:388–399.
33. White, R. J. 1968. Control of amino sugar metabolism in *Escherichia coli* and isolation of mutants unable to degrade amino sugars. *Biochem. J.* **106**:847–858.
34. White, R. J., and C. A. Pasternak. 1967. The purification and properties of *N*-acetylglucosamine 6-phosphate deacetylase from *Escherichia coli*. *Biochem. J.* **105**:121–125.