

# The *N*-Acetyl-D-Glucosamine Kinase of *Escherichia coli* and Its Role in Murein Recycling

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***N*-Acetyl-D-glucosamine (GlcNAc) is a major component of bacterial cell wall murein and the lipopolysaccharide of the outer membrane. During growth, over 60% of the murein of the side wall is degraded, and the major products, GlcNAc-anhydro-*N*-acetylmuramyl peptides, are efficiently imported into the cytoplasm and cleaved to release GlcNAc, anhydro-*N*-acetylmuramic acid, murein tripeptide (L-Ala-D-Glu-*meso*-diaminopimelic acid), and D-alanine. Like murein tripeptide, GlcNAc is readily recycled, and this process was thought to involve phosphorylation, since GlcNAc-6-phosphate (GlcNAc-6-P) is efficiently used to synthesize murein or lipopolysaccharide or can be metabolized by glycolysis. Since the gene for GlcNAc kinase had not been identified, in this work we purified GlcNAc kinase (NagK) from *Escherichia coli* cell extracts and identified the gene by determining the N-terminal sequence of the purified kinase. A *nagK* deletion mutant lacked phosphorylated GlcNAc in its cytoplasm, and the cell extract of the mutant did not phosphorylate GlcNAc, indicating that NagK is the only GlcNAc kinase expressed in *E. coli*. Unexpectedly, GlcNAc did not accumulate in a *nagK nagEBACD* mutant, though both GlcNAc and GlcNAc-6-P accumulate in the *nagEBACD* mutant, suggesting the existence of an alternative pathway (presumably repressed by GlcNAc-6-P) that reutilizes GlcNAc without the involvement of NagK.**

In 1966, Asensio and Ruiz-Amil reported the properties of GlcNAc kinase activity in *Escherichia coli* and purified the enzyme about 150-fold (1). No further work on the enzyme has been reported, and the *nagK* gene has not been identified heretofore. The metabolism of GlcNAc and D-glucosamine (GlcN) has been studied for decades. The divergently transcribed *nagE* and *nagBACD* operons are involved in the uptake and degradation of the amino sugars. When *E. coli* grows on GlcNAc-containing medium, GlcNAc is transported by a phosphotransferase system, either NagE or ManXYZ, to generate GlcNAc-6-phosphate (GlcNAc-6-P) (26). GlcNAc-6-P is converted by NagA deacetylase to GlcN-6-P (38). GlcN-6-P enters either the glycolysis pathway via conversion to fructose-6-P by the NagB deaminase (38) or the biosynthesis pathways for murein and lipopolysaccharide by conversion to UDP-GlcNAc by GlmM and GlmU (15–17) (Fig. 1). Hence, NagK is not required for the metabolism of GlcNAc derived from the medium. The other proteins encoded by the *nag* gene cluster are NagC, a repressor controlling the expression of *nagE* and the *nagBACD* operon (24), and NagD, a putative hydrolase, whose activity is not essential for the metabolism of the amino sugars.

However, significant amounts of nonphosphorylated GlcNAc are released in the cytoplasm via the murein recycling pathway (4, 37) (Fig. 1). Over half of side-wall murein is broken down each generation by lytic transglycosylases and endopeptidases (7, 9, 11). The principal degradation product is the anhydro-muropeptide GlcNAc- $\beta$ -1,4-anhydro-*N*-acetylmuramyl-(anhMurNAc)-L-alanyl- $\gamma$ -D-glutamyl-*meso*-diaminopimelyl-D-alanine. Muropeptides are imported into the cytoplasm via a specific permease,

AmpG (5, 11), and thereafter are cleaved into GlcNAc, anhMurNAc, murein tripeptide, and D-alanine by the combined actions of the NagZ protein ( $\beta$ -*N*-acetylglucosaminidase), the AmpD protein (anhMurNAc-L-Ala amidase), and the LdcA protein (DL-carboxypeptidase) (4, 10, 12, 33, 37). Murein tripeptide reenters the murein synthesis pathway following direct ligation to UDP-MurNAc by Mpl (18). To a lesser extent, murein tripeptide is cleaved by MpaA, YcjJ, and PepD to yield the individual amino acids (30, 36). Interestingly, the roles of all eight of these enzymes, except PepD, are concerned exclusively with the recycling process.

Recently, both cytoplasmic GlcNAc and anhMurNAc derived from cell wall recycling were shown to be reused (23). Cytoplasmic GlcNAc must be phosphorylated to enter the known metabolic pathway of GlcNAc-6-P (Fig. 1). Though one possibility for utilization of murein-derived GlcNAc is for GlcNAc to be exported and then taken up by a specific phosphotransferase system, it was reasonable to assume that NagK is responsible for the phosphorylation of murein-derived GlcNAc. In this communication, the *nagK* (*ycfX*) gene is identified, and NagK is characterized and shown to be the only GlcNAc kinase expressed in *E. coli* to phosphorylate GlcNAc in the cytoplasm. On the basis of the observation that GlcNAc does not accumulate in a *nagK nagEBACD* mutant, we suggest that an unknown pathway must exist to utilize GlcNAc in the absence of NagK and NagA and that the pathway may be regulated by GlcNAc-6-P.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The bacterial strains and plasmids used here are listed in Table 1. The strains were grown aerobically at 37°C in Luria-Bertani (LB) broth (20), except as indicated. Ampicillin (100  $\mu$ g/ml), kanamycin (25  $\mu$ g/ml), tetracycline (10  $\mu$ g/ml), and chloramphenicol (20  $\mu$ g/ml) were used as required.

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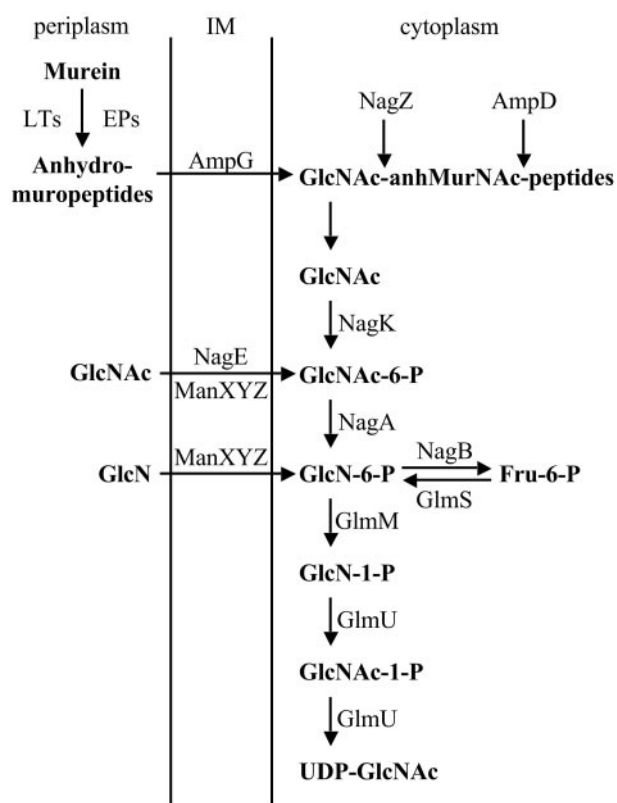


FIG. 1. Scheme of murein recycling and metabolism of GlcNAc. UDP-GlcNAc is the main cytoplasmic precursor of murein and lipopolysaccharide. AmpG is a specific permease for muropeptides. NagE and ManXYZ are phosphoenolpyruvate-dependent phosphotransferases. IM, inner membrane; LTs, lytic transglycosylases; EPs, endopeptidases; Fru-6-P, fructose-6-phosphate.

**Purification and identification of NagK from *E. coli* wild-type cell extract.** All purification steps were performed at 4°C or the temperature described. *E. coli* strain MG1655 was grown with vigorous shaking in 5 liters of LB medium at 37°C overnight. Cells (wet weight, 21.1 g) were harvested, washed with PEND50 buffer

(50 mM potassium phosphate [pH 7.6], 1 mM EDTA, 0.1 mM GlcNAc, and 1 mM dithiothreitol), and suspended in 50 ml of PEND50, to which phenylmethylsulfonyl fluoride was added (final concentration, 1 mM). The sample was sonicated by a sonifier (Branson Ultrasonics Corp., Danbury, Conn.), and then centrifuged ( $27,000 \times g$  for 30 min) to remove debris and unbroken cells. Streptomycin sulfate was added to the sample (final concentration, 1.4%), and insoluble material was removed by ultracentrifugation ( $100,000 \times g$  for 60 min). The supernatant was loaded onto a DEAE-Sephacel column (Amersham Pharmacia, Uppsala, Sweden) which had been equilibrated with PEND50. The column was washed with PEND50 and developed with a linear gradient of 0 to 0.3 M NaCl in PEND50. NagK activity was eluted around 230 mM NaCl. All NagK active fractions were combined, concentrated by Ultrafree 4, 10K (Millipore Corp., Bedford, Mass.), and desalted with PEND1 (the same mixture as PEND50, except it has 1 rather than 50 mM potassium phosphate). The active concentrate was loaded onto a hydroxyapatite column (BIO-GEL HT; Bio-Rad, Hercules, Calif.). The column was washed with PEND1 and developed with a linear gradient of PEND1 to PEND50. NagK was eluted at 20 mM potassium phosphate. Active fractions were combined and loaded onto a MonoQ HR 5/5 column (Amersham Pharmacia) and fractionated by fast pressure liquid chromatography at room temperature. The column was washed with PEND50, and proteins were eluted with a linear gradient of 0 to 0.6 M NaCl. The peak of NagK activity was at 220 mM NaCl. NagK fractions were concentrated and loaded onto a Sephacryl S-200 column (Amersham Pharmacia), and the proteins were separated with 0.15 M NaCl in PEND50 by the fast pressure liquid chromatography system. The retention volume of the peak of the active fractions was 58 ml, and the active fractions were combined and concentrated. Protein concentration was measured by the Bradford assay (Bio-Rad) with bovine serum albumin as a standard. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using gels stained with Coomassie brilliant blue R250 (CBB) (29). To determine the N-terminal amino acid sequence of a protein, the protein band of interest was transferred to a Sequi-Blot polyvinylidene difluoride membrane (Bio-Rad) after SDS-PAGE, followed by light staining with CBB to facilitate excision of the protein band. The N-terminal amino acid sequence of the protein was determined with an ABI477 protein sequencer (Applied Biosystems, Foster City, Calif.) at the Tufts University Core Facility.

**GlcNAc kinase activity.** The two assay methods described by Asensio and Ruiz-Amil (1) were employed with the following modifications. For method 1 (for crude mixtures), each enzyme fraction was added to a reaction mixture (final volume, 0.1 ml) containing 1.9 mM GlcNAc, 20 mM ATP, 20 mM MgCl<sub>2</sub>, and 100 mM Tris-HCl (pH 7.8). A control without ATP was used to correct for GlcNAc deacetylase activity (28). Samples were incubated at 37°C for 1 h. To stop the reaction and precipitate GlcNAc-P, 0.4 ml of 5% ZnSO<sub>4</sub> was added, followed by 0.4 ml of 0.15 N Ba(OH)<sub>2</sub>. Phosphorylated GlcNAc was removed by low-speed centrifugation ( $300 \times g$  for 5 min), and the remaining GlcNAc was measured by the Morgan-Elson assay (27). The supernatant (0.1 ml) was mixed with 0.1 ml of 10% Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub> and heated in a boiling-water bath for 7 min. A 0.8

TABLE 1. *E. coli* K-12 strains and plasmids used in this work

<i>E. coli</i> strain or plasmid	Genotype and/or phenotype	Source or reference
<b>Strains</b>		
MG1655	F <sup>-</sup> <i>ilvG rfb-50 rph-1</i>	34
XL1-Blue	F <sup>'</sup> ::Tn10 <i>proA<sup>+</sup> proB<sup>+</sup> relA1 lacI<sup>s</sup> endA1 Δ(lacZ)M15 recA1 thi gyrA96 (Nal<sup>r</sup>) hsdR17(r<sub>K</sub><sup>-</sup> m<sub>K</sub><sup>+</sup>) supE44 lac</i>	Stratagene
TP71	F <sup>-</sup> mutant <i>lysA opp araD139 rpsL150 deoC1 ptsF25 fibB5301 rbsR relA1 Δ(argF-lac)</i>	11
TP71B	TP71 <i>nagB</i> ::Kan	23
TP71BK	TP71 <i>nagB</i> ::Kan <i>nagK</i> ::Cm	This work
TP80	TP71 <i>nagEBACD</i> ::Tet	23
TP80K	TP71 <i>nagEBACD</i> ::Tet <i>nagK</i> ::Cm	This work
IBPC524	<i>thi-1 argG6 argE3 his-4 xyl-5 mtl-1 tsx-29 rpsL ΔlacX74 nagA</i> ::Cm	24
TP71A	TP71 <i>nagA</i> ::Cm	This work
TP71AK	TP71 <i>nagA</i> ::Cm <i>ΔnagK</i>	This work
<b>Plasmids</b>		
pGEM-T	Amp <sup>r</sup>	Promega
pKD3	Cm <sup>r</sup> Amp <sup>r</sup>	6
pKD46	Amp <sup>r</sup> P <sub>ara</sub> -Red recombinase	6
pCP20	Amp <sup>r</sup> Cm <sup>r</sup> <i>ori</i> (Ts) FLP	6
pNagK2	pGEM-T carrying <i>nagK</i> under the control of the P <sub>lac</sub> promoter	This work
pNagK11	pNagK2 <i>Δ</i> (SacI-PstI)	This work

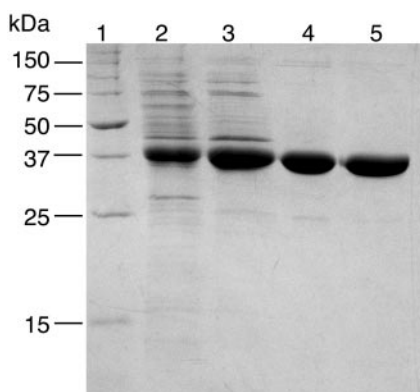


FIG. 2. SDS-PAGE gel (stained with CBB) of samples obtained during purification of recombinant NagK overexpressed by adding the inducer to *E. coli* strain XL1-Blue carrying pNagK11. Lane 1, molecular mass markers; lane 2, streptomycin sulfate precipitation; lane 3, DEAE-Sephacel; lane 4, hydroxyapatite; lane 5, MonoQ. Lanes 2 to 5 each contained 5  $\mu$ g of protein.

ml-portion of Ehrlich's reagent (91 mg of *p*-dimethylamino benzaldehyde [Fisher Scientific, Fair Lawn, N.J.], 91  $\mu$ l of HCl, and 9.909 ml of glacial acetic acid) was then added, and color was developed by incubation for 20 min at 37°C followed by 15 min at room temperature. The absorbance of the sample was measured at 585 nm. For method 2 (for purified NagK), the assay mixture (0.5 ml) contained 100 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 1 mM phosphoenol-pyruvate, 4 mM ATP, 0.2 mM NADH, 4 U of lactate dehydrogenase, 4 U of pyruvate kinase from rabbit muscle (Sigma, St. Louis, Mo.), and 0.23  $\mu$ g of purified NagK protein. The assay mixture was incubated at 37°C. The reaction was started by the addition of the substrate. The formation of NAD<sup>+</sup> was monitored at a wavelength of 340 nm. The apparent  $K_m$  value was calculated by direct fit to the Lineweaver-Burk plot. One unit is defined as the amount of NagK that phosphorylates 1  $\mu$ mol of GlcNac per min at 37°C.

**Cloning of NagK.** The *nagK* DNA fragment from the *E. coli* chromosome was amplified by PCR using 5'-CATTAAACAAGGAGCGGC and 5'-AGCATA GCAACTCTGTG as primers. The amplified DNA was ligated into the pGEM-T vector (Promega, Madison, Wis.), generating plasmid pNagK2, which carried the *nagK* gene under the control of the  $P_{lac}$  promoter. Though the DNA sequence of the *nagK* gene on pNagK2 was verified, induction of expression from pNagK2 by the addition of 1 mM isopropyl- $\beta$ -D-thiogalactoside (IPTG) led to overproduction of a protein larger than expected. The larger protein proved to be the product of an in-frame fusion of *lacZ* to *nagK*. To make it out of frame, pNagK2 was digested with two restriction endonucleases, SacI and PstI, which cleave between the start codons of *lacZ* and *nagK*. The overhangs of the linear DNA after digestion were filled with deoxynucleoside triphosphate by the Klenow fragment, followed by self-ligation, generating plasmid pNagK11.

**Overexpression and purification of recombinant NagK.** *E. coli* strain XL1-Blue, carrying pNagK11, was grown at 37°C with vigorous shaking in 1 liter of LB medium containing ampicillin from a 2% inoculum of overnight culture. After the culture grew to a Klett value of 50, IPTG was added to a final concentration of 1 mM, and incubation was continued for 3 h. The cells were harvested by centrifugation, washed with 20 ml of PEND50, suspended in 8 ml of PEND50, broken by sonication, and centrifuged to remove the debris. The supernatant was ultracentrifuged after the addition of streptomycin sulfate (final concentration, 1.4%). SDS-PAGE revealed one overproduced protein present in the cell extract (Fig. 2). The molecular weight of the protein appeared to be the same as that of NagK. The protein was purified to >95% by sequential chromatographic steps with DEAE-Sephacel, hydroxyapatite, and MonoQ as described above for the purification and identification of NagK. Aliquots of purified NagK were frozen in dry ice-ethanol and stored at -80°C. NagK was fully active for months. Thawed samples of NagK retained full activity in 40% glycerol at -20°C for a few months.

**Deletion of NagK.** A *nagK* null mutant (*nagK*::Cm) was constructed by one-step inactivation of the *E. coli* chromosomal gene as described before (6, 36), using the primers 5'-CCAGTAAAGGCGAGTACATTAAACAAGGAGCGGCA ATGTAGGCTGGAGCTGCTTCG and 5'-AAGCCACCACCAATGACGACCA GGTCAGGGTCAACGATGAATATCCTCCTTAGTTC (the italicized sequences are homologous to the *nagK*-flanking sequence, and the underlined sequences are homologous to the pKD3 vector sequence flanking the Cm<sup>r</sup> gene)

TABLE 2. NagK activities in *nagK*::Cm mutants of *E. coli*

Strain	Genotype	Sp act <sup>a</sup>
TP80	<i>nagEABACD</i>	18.3
TP80K	<i>nagEABACD nagK</i>	0.0
TP71A	<i>nagA</i>	17.1
TP71AK	<i>nagA nagK</i>	0.3
TP71B	<i>nagB</i>	17.3
TP71BK	<i>nagB nagK</i>	3.4

<sup>a</sup> The NagK activity (in units of activity [10<sup>-3</sup>] per milligram of protein) was measured by method 1 (see Materials and Methods) in cell extracts of cells grown overnight in LB medium at 37°C.

and pKD3 as the template to synthesize the PCR product used for one-step inactivation. The *nagK*::Cm mutation was confirmed by PCR with the primers used for cloning NagK, as described in Materials and Methods. *nagK*::Cm was transduced into strains TP71B and TP80 with T4gt7 phage (20). To construct TP71AK, the Cm<sup>r</sup> gene in TP71BK was eliminated as described previously (6), i.e., by transforming the strain with the plasmid pCP20, which expresses FLP recombinase, followed by screening for the loss of chloramphenicol resistance. The *nagA*::Cm mutation from IBPC524 was transduced into the *nagB*::Kan  $\Delta$ *nagK* strain with T4gt7 phage, generating a *nagA*::Cm  $\Delta$ *nagK* strain, TP71AK.

**Labeling cells with [6-<sup>3</sup>H]GlcN and preparation of hot-water extract.** The radioactive labeling and preparation of the cytoplasmic amino sugars derived from cell wall recycling were performed as described previously (23). All strains used in this study carry the *nagB* mutation, which prevents conversion of GlcN-6-P to fructose-6-P and hence facilitates efficient incorporation of [6-<sup>3</sup>H]GlcN into murein and lipopolysaccharide (Fig. 1). *nagB* mutant strains were grown at 37°C in 4 ml of M9 minimal medium containing 0.6% glycerol, 0.1% Casamino Acids, 1 mM MgCl<sub>2</sub>, 1  $\mu$ g of thiamine per ml, 20  $\mu$ g of lysine per ml, and 1  $\mu$ Ci of [6-<sup>3</sup>H]GlcN (21.6 Ci/mol; NEN Life Science Products, Boston, Mass.) per ml. [6-<sup>3</sup>H]GlcN is transported by a phosphotransferase system, ManXYZ, to form GlcN-6-P. At mid-log phase, the [6-<sup>3</sup>H]GlcN-labeled cells were harvested, washed with water, resuspended in water, incubated at 90°C for 5 min, and centrifuged (4,300  $\times$  g for 7 min at 4°C). The supernatant (hot-water extract) containing soluble [6-<sup>3</sup>H]GlcN derivatives was lyophilized and suspended with water.

**Analysis of hot-water extracts by HPLC and TLC.** anhMurNAc and UDP-MurNAc-pentapeptide were separated by high-pressure liquid chromatography (HPLC) (23). A mixture of GlcN, GlcN-P, GlcNAc, GlcNAc-P, and UDP-GlcNAc, which did not bind to the HPLC column, was recovered and analyzed by thin-layer chromatography (TLC) as described previously (23). UDP-GlcNAc was defined as that material that remained at the origin in TLC with the basic solvent (80% ethanol, 0.4 N NH<sub>4</sub>OH) and was resistant to calf intestinal phosphatase. The amount of each compound was normalized with the turbidity of the cell culture.

## RESULTS

**Identification of NagK.** Confirming the report by Asensio and Ruiz-Amil (1), we detected NagK activity in cell extracts of the *E. coli* K-12 wild-type strain MG1655. Since NagK was active in the *nag* gene cluster deletion mutant TP80 (*nagEABACD*) (Table 2),

TABLE 3. Purification of NagK enzyme from *E. coli* wild-type cell extract

Purification step	Total protein (mg)	Total activity <sup>a</sup> (U)	Sp act (U/mg of protein)	Yield (%)	Purification factor
Cell extract	2,551	101	0.04	100	1
DEAE	33.9	5.9	0.17	5.9	4
HA <sup>b</sup>	1.27	3.5	2.7	3.4	68
MonoQ	0.10	1.7	17.0	1.7	428
Sephacryl	0.006	0.2	33.3	0.2	842

<sup>a</sup> Activities were measured by the Morgan-Elson assay (method 1).

<sup>b</sup> HA, hydroxyapatite.



TABLE 4. Substrate specificity of *E. coli* NagK

Substrate	Relative activity <sup>b</sup>	$K_m^a$ (mM)	$V_{max}^a$ ( $\mu\text{M}/\text{min}/\text{mg}$ )
GlcNAc	100	0.34	118
ATP		0.90	108
Glucose	21	37.2	24
Mannose	0.3 (100)	ND	ND
Galactose	<0.1 (50)	ND	ND
GlcN	0.6 (50)	ND	ND
D-Mannosamine	<0.1 (20)	ND	ND
ManNAc <sup>c</sup>	0.4 (50)	ND	ND
GalNAc <sup>d</sup>	<0.1 (1)	ND	ND

<sup>a</sup> To determine the apparent  $K_m$  and  $V_{max}$ , different amounts of substrate were incubated with a 14 nM concentration (0.46 mg/liter) of purified NagK and 4 mM ATP at 37°C and activities measured by method 2 were plotted according to the Lineweaver-Burk diagram. Values of  $K_m$  and  $V_{max}$  for ATP were measured with 1 mM GlcNAc. ND, not determined.

<sup>b</sup> The concentrations in parentheses (in millimolar units) were those tested to measure the kinase activities.

<sup>c</sup> ManNAc, *N*-acetyl-D-mannosamine.

<sup>d</sup> GalNAc, *N*-acetyl-D-galactosamine.

*nagD*, the unidentified member of the *nag* gene cluster, could not be *nagK*. To identify *nagK*, the purification of the NagK enzyme from MG1655 cell extract was performed as described in Materials and Methods. NagK was purified over 800-fold by a sequence of four chromatographic steps (Table 3). The final fraction contained two proteins detected by SDS-PAGE gels stained with CBB. The N-terminal sequence of the ~34-kDa protein band was MYYGFD. A homology search of the *E. coli* genome with the TagIdent tool (<http://us.expasy.org/tools/tagident.html>) showed that only the N-terminal sequence of YcfX is identical to the 6-amino-acid sequence. The predicted molecular mass of YcfX is 32.9 kDa. YcfX is coded in a putative *lolC-lolD-lolE-ycfX-cobB* operon present at 25.4 min on the *E. coli* chromosome. *ycfX* appears to be transcribed from a putative  $\sigma^{70}$ -dependent promoter upstream of *lolC*, and a predicted  $\sigma^{54}$ -dependent promoter exists within the *ycfX* gene to transcribe *cobB*. LolCDE is an essential ABC transporter for lipoprotein transport from the inner membrane to the outer membrane (21, 39). YcfX has been shown not to be involved in lipoprotein transport (39). CobB is homologous to the NAD<sup>+</sup>-dependent protein deacetylase Sir2 (40).

To confirm that YcfX is NagK, the *ycfX* gene was cloned into the expression vector pGEM-T under the control of the  $P_{lac}$  promoter. XL1-Blue carrying the plasmid grew slowly even in the absence of inducer, presumably because of an accumulation of a semitoxic level of GlcNAc-6-P (2). Nonetheless, YcfX was overexpressed after induction at mid-log phase. The soluble fraction contained 1,000-fold more NagK activity than was found in wild-type extracts. We conclude that YcfX is NagK. The plasmid containing *nagK* was named pNagK11.

**Characterization of NagK.** To characterize NagK enzymatically, NagK was overproduced in XL1-Blue carrying pNagK11 and purified as described above (Fig. 2). After incubation with purified NagK in the presence of ATP and MgCl<sub>2</sub>, [6-<sup>3</sup>H]GlcNAc was converted to a compound which remained at the origin during TLC with a basic solvent. This compound was converted to GlcNAc by treatment with calf intestinal phosphatase, demonstrating the direct phosphorylation of GlcNAc by NagK (data not shown). The activity of the kinase was

TABLE 5. Amino sugar-containing compounds present in the cytoplasm of the mutants

Compound	Amt of 6- <sup>3</sup> H-labeled compound (cpm [10 <sup>3</sup> ]) in hot-water extracts			
	<i>nagB</i> mutant	<i>nagB nagK</i> mutant	<i>nagEBACD</i> mutant	<i>nagEBACD nagK</i> mutant
GlcN	3.8	3.1	<0.1	2.3
GlcN-P	4.4	0.7	<0.1	0.5
GlcNAc	13.4	12.2	139.4	13.6
GlcNAc-P	24.0	0.7	129.9	<0.1
anhMurNAc	<0.1	<0.1	1.4	0.9
UDP-MurNAc-pentapeptide	5.4	7.3	2.9	23.9
UDP-GlcNAc	16.8	27.3	<0.1	42.5

constant from pH 6.5 to 10. The substrate specificity and kinetics of NagK were determined by method 2, described in Materials and Methods. NagK phosphorylated GlcNAc and glucose. The kinase reaction followed the Michaelis-Menten equation. The calculated  $K_m$ s for GlcNAc, ATP, and glucose were 342  $\mu\text{M}$ , 896  $\mu\text{M}$ , and 37 mM, respectively, while  $V_{max}$ s were 118  $\mu\text{mol}$  of GlcNAc and 24  $\mu\text{mol}$  of glucose per mg of NagK per min. This value for GlcNAc is equivalent to the phosphorylation of 3,900 molecules of GlcNAc by 1 molecule of NagK per min. NagK had very slight activity with GlcN, D-mannose, and *N*-acetyl-D-mannosamine and no activity with D-galactose, D-mannosamine, or *N*-acetyl-D-galactosamine (Table 4). While 10 mM GlcNAc-6-P did not inhibit NagK activity when 2 mM GlcNAc was incubated with 14 nM NagK, ADP was a strong inhibitor ( $K_i$  was approximately 1.4 mM).

**Deletion of *nagK*.** A *nagK* null mutation (*nagK::Cm*) which retained the promoter of *cobB* within the C-terminal region of *nagK* was constructed and transduced into the *nagB* strain TP71B and the *nagEBACD* strain TP80, generating TP71BK and TP80K, respectively. The two NagK null mutants grew with about the same doubling times as their parent strains and had normal morphology (data not shown), so under these conditions, NagK is not essential for *E. coli* growth. TP80 ceased growth in early stationary phase and produced ghost cells (data not shown), presumably due to the toxicity of the accumulated GlcNAc-6-P (2, 38), whereas TP80K, which lacked GlcNAc-6-P (see below), grew normally. As shown in Table 2, TP80K totally lacked NagK activity. TP71AK had a level of activity equal to 2% of that of TP71A, but TP71BK appeared to have a level of activity equal to about 20% of that of the wild type. Since GlcN would register as GlcNAc-P in method 1, which depends on measuring the GlcNAc remaining following precipitation of GlcNAc-P, this apparent activity of TP71BK may be caused by the deacetylation of GlcNAc. We have confirmed that the cell extract of TP71BK, but not that of TP71AK or TP80K, deacetylated [6-<sup>3</sup>H]GlcNAc by detecting the formation of [<sup>3</sup>H]GlcN by TLC in the solvent containing *n*-butanol-acetic acid-water (2:1:1), which separates GlcN from GlcNAc (data not shown). Since NagA is expressed in the cells containing *nagB::Km* (24), NagA apparently must deacetylate GlcNAc as well as GlcNAc-6-P.

**Analysis of [6-<sup>3</sup>H]GlcN-derived compounds in *nagK* mutants.** We have observed that in strains lacking *nagEBACD*, GlcNAc and GlcNAc-6-P derived from murein recycling accu-

multate (23). If NagK is required for utilization of GlcNAc, then the *nagK::Cm* mutants should accumulate GlcNAc, lack GlcNAc-6-P, and not recycle GlcNAc. To analyze the cytoplasmic compounds derived from the amino sugars of murein, the wild type and the mutants were labeled with [6-<sup>3</sup>H]GlcN, and the hot-water extracts of the *nagK* mutants and their parent strains were analyzed. Table 5 shows the amounts of the various radioactive compounds present in the cytoplasm of the strains examined. As expected, GlcNAc-P was absent or barely detected in the *nagK* mutants. Though GlcNAc was expected to accumulate in the cytoplasm, neither the *nagK nagB* mutant nor the *nagK nagEBACD* mutant accumulated GlcNAc. On the contrary, compared to the *nagEBACD* mutant, which accumulated GlcNAc and GlcNAc-P, the *nagK nagEBACD* mutant contained a normal amount of GlcNAc and no GlcNAc-P. Puzzlingly, the amounts of UDP-MurNAc-pentapeptide and UDP-GlcNAc in the *nagK nagEBACD* mutant were significantly higher than those in the *nagEBACD* mutant, whereas the amounts in the *nagK nagB* mutant were less than twofold higher than those in the *nagB* strain. No other radioactive compound accumulated in the two *nagK* mutants. These results confirm that cytoplasmic GlcNAc derived from murein recycling is phosphorylated by NagK and also suggest that the GlcNAc is utilized or released by cells lacking NagK.

To examine whether the lack of accumulation of GlcNAc was due to leakage from the cells, chase experiments were done as described in reference 23. The increase in the amount of radioactivity in the spent medium from the *nagEBACD nagK* mutant during the chase was similar to that from the *nagEBACD* cells (data not shown), indicating that GlcNAc was not released to the medium. We can also exclude the possibility that as soon as GlcNAc is leaked out, it is imported by ManXYZ or NagE, because GlcNAc-P, the product of the uptake of GlcNAc, is not detectable in the *nagK* mutants. Hence, these results strongly suggest that an alternative pathway to utilize GlcNAc in the absence of NagK must exist.

## DISCUSSION

During exponential growth, GlcNAc is released in the cytoplasm of *E. coli*, due to murein recycling. Here, we have identified the kinase which is responsible for phosphorylation of free GlcNAc in the cytoplasm, NagK. The absence of GlcNAc kinase activity and the total or near-total absence of GlcNAc-P from the two *nagK* mutants proves that NagK is the only GlcNAc kinase expressed in *E. coli*.

NagK is a 303-amino-acid protein which belongs to the ROK (repressor, open reading frame, kinase) family (35). NagK lacks the N-terminal DNA-binding helix-turn-helix motif which is conserved in the transcription regulators of the ROK family (35), such as NagC from *E. coli* (25) and XylR from *Bacillus subtilis* (13). NagK contains the typical ATP-binding site (G<sup>4</sup>FDIGGT<sup>10</sup>) of glucokinase near the N terminus (19) and has a very low level of similarity to mammalian GlcNAc kinase (8, 14). NagK is conserved in most gamma-proteobacteria, such as species of the *Salmonella*, *Vibrio*, and *Haemophilus* genera. Since those gram-negative bacteria contain the homologous enzymes required for murein recycling (4), the NagK homologs presumably phosphorylate GlcNAc.

Recombinant NagK was overproduced, purified, and characterized. Of the candidate sugars tested here, NagK phosphorylated only GlcNAc and glucose. Since the  $K_m$  for glucose was 100-fold higher than that for GlcNAc, NagK should be specific for GlcNAc under physiological conditions. The properties of NagK, such as its substrate specificity, its inhibition by ADP, and its behavior during purification steps, indicate that the protein studied here is identical to the enzyme (EC 2.7.1.59) partially purified and studied 38 years ago by Asensio and Ruiz-Amil (1). Though no other GlcNAc kinase gene has been reported to occur in bacteria, C. Brigham and M. Malamy (personal communication) have recently identified a ROK family kinase, Roka, in *Bacteroides fragilis*, which phosphorylates both glucose and GlcNAc with approximately the same affinity: the  $K_m$  for glucose is 0.6 mM, and the  $K_m$  for GlcNAc is 0.8 mM. Thus, compared to Roka (23% identity to *E. coli* NagK), NagK has a much more strict substrate specificity.

A strain lacking the *nag* cluster *nagEBACD* cannot metabolize GlcNAc because it lacks NagA, the GlcNAc-6-P deacetylase required to form the essential intermediate, GlcN-6-P. As a result, GlcNAc-6-P and GlcNAc accumulate in the cells, and growth into late log phase results in the lysis of many cells, a possible indirect effect of the high level of GlcNAc-6-P in the cells. The accumulation of GlcNAc in the *nagEBACD* mutant in log phase is not due to the inhibition of NagK by GlcNAc-6-P, because GlcNAc-6-P does not inhibit NagK. Since the activity of NagK is much greater than the rate of the production of GlcNAc by murein recycling, the large pool of GlcNAc suggests that GlcNAc-6-P is being dephosphorylated. There is other indirect evidence for the dephosphorylation of GlcNAc-6-P. The *nagEBACD* mutant lysed upon addition of GlcNAc to cells growing in the glycerol minimal medium, whereas the *nagEBACD nagK* mutant was able to grow under these conditions. GlcNAc from the medium is phosphorylated by the mannose phosphotransferase system during uptake. If GlcNAc-6-P is the cause of cell lysis, then the pool of GlcNAc-6-P must remain relatively low in the *nagEBACD nagK* mutant when GlcNAc is added to the culture, which implies dephosphorylation. In the absence of NagA deacetylase, GlcNAc-6-P is likely to be dephosphorylated to GlcNAc and subsequently utilize the alternative pathway postulated here. On the other hand, in the *nagEBACD* mutant, GlcNAc is (re)phosphorylated by NagK, resulting in cells with a near-lethal concentration of GlcNAc-6-P. The mechanism of killing by GlcNAc-6-P remains to be determined but is presumably related to the lack of UDP-GlcNAc in the *nagEBACD* mutant cells (Table 5).

### How does *E. coli* utilize GlcNAc without NagK and NagA?

When *nagK* was deleted from the *nag* cluster mutant, not only did GlcNAc-P disappear, but the cells grew normally and the GlcNAc level returned to normal, indicating that an alternate pathway for recycling GlcNAc had been derepressed. Examination of Table 5 reveals that a significant amount of GlcN was detected in both the *nagK nagB* mutant and the *nagK nagEBACD* mutant relative to that in the *nagEBACD* mutant. This suggests that when phosphorylation of GlcNAc is not possible due to the lack of NagK, the first step in utilization of cellular GlcNAc may be deacetylation. In 1956, Roseman detected GlcNAc deacetylase activity in an *E. coli* cell extract (28). A gene coding GlcNAc deacetylase, however, has not

been reported for any organism. To be metabolized further following the deacetylation of GlcNAc, the resulting GlcN must be phosphorylated. A GlcN-specific kinase has been identified in *Vibrio cholerae* (22), but no homolog exists in the *E. coli* genome. However, it has been reported that GlcN is phosphorylated by mannose-fructose kinase, although its GlcN kinase activity is only 5% of that of mannose-fructose kinase activity and the  $K_m$  for GlcN is 5 mM (31). Recently, mannose-fructose kinase has been shown to be encoded by *yajF* (32). Thus, one possible hypothetical pathway would involve deacetylation of GlcNAc followed by phosphorylation to produce the normal intermediate, GlcN-6-P.

**Is the alternative pathway regulated?** Considering the large accumulation of GlcNAc and GlcNAc-P in the *nagEBACD* mutant (23) and the lack of accumulation of GlcNAc in the *nagK nagEBACD* mutant, the alternative pathway must be inactive in the *nagEBACD* mutant and become active only in the *nagK nagEBACD* mutant lacking GlcNAc-6-P. This observation suggests that the pathway is repressed by NagK or GlcNAc-6-P. NagK belongs to the ROK family but lacks an N-terminal DNA-binding domain, indicating that NagK is unlikely to bind DNA and regulate genes involved in the pathway directly. GlcNAc-6-P is known to be not only an inducer of the *nagBACD* operon (24) but also the allosteric activator of NagB deaminase (3). Hence, the pathway is likely to be repressed transcriptionally or enzymatically by the high concentration of GlcNAc-6-P present in normal cells and especially in the *nagEBACD* mutant. Efforts are under way to determine the alternative pathway for utilization of free GlcNAc and its regulation.

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