Allosteric Activation of *Escherichia coli* Glucosamine-6-Phosphate Deaminase (NagB) *In Vivo* Justified by Intracellular Amino Sugar Metabolite Concentrations

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**ABSTRACT**

We have investigated the impact of growth on glucosamine (GlcN) and N-acetylglucosamine (GlcNAc) on cellular metabolism by quantifying glycolytic metabolites in *Escherichia coli*. Growth on GlcNac increased intracellular pools of both GlcNAc6P and GlcN6P 10- to 20-fold compared to growth on glucose. Growth on GlcN produced a 100-fold increase in GlcN6P but only a slight increase in GlcNAc6P. Changes to the amounts of downstream glycolytic intermediates were minor compared to growth on glucose. The enzyme glucosamine-6P deaminase (NagB) is required for growth on both GlcN and GlcNac. It is an allosteric enzyme in *E. coli*, displaying sigmoid kinetics with respect to its substrate, GlcN6P, and is allosterically activated by GlcNAc6P. The high concentration of GlcN6P, accompanied by the small increase in GlcNAc6P, drives *E. coli* NagB (NagB6P) into its high activity state, as observed during growth on GlcN (L.I. Álvarez-Añorve, I. Bustos-Jaimés, M. L. Calcagno, and J. Plumbridge, *J Bacteriol* 191:6401–6407, 2009, [http://dx.doi.org/10.1128/JB.00633-09](http://dx.doi.org/10.1128/JB.00633-09)). The slight increase in GlcNac6P during growth on GlcN is insufficient to displace NagC, the GlcNAc6P-sensitive repressor of the nag genes, from its binding sites, so there is only a small increase in nagB expression. We replaced the gene for the allosteric NagB6P enzyme with that of the nonallosteric, *B. subtilis* homologue, NagB6ac. We detected no effects on growth rates or competitive fitness on glucose or the amino sugars, nor did we detect any effect on the concentrations of central metabolites, thus demonstrating the robustness of amino sugar metabolism and leaving open the question of the role of allostery in the regulation of NagB.

**IMPORTANCE**

Chitin, the polymer of N-acetylglucosamine, is an abundant biomaterial, and both glucosamine and N-acetylglucosamine are valuable nutrients for bacteria. The amino sugars are components of numerous essential macromolecules, including bacterial peptidoglycan and mammalian glycosaminoglycans. Controlling the biosynthetic and degradative pathways of amino sugar metabolism is important in all organisms to avoid loss of nitrogen and energy via a futile cycle of synthesis and breakdown. The enzyme glucosamine-6P deaminase (NagB) is central to this control, and N-acetylglucosamine-6P is the key signaling molecule regulating amino sugar utilization in *Escherichia coli*. Here, we investigate how the metabolic status of the bacteria impacts on the activity of NagB6ac and the N-acetylglucosamine-6P-sensitive transcriptional repressor, NagC.

The genes for use of N-acetylglucosamine (GlcNAc) as a carbon source in *Escherichia coli* form a typical inducible regulon, controlled by the NagC transcription factor. The divergent operon nagE-nagBACD (Fig. 1) encodes the transporter (NagE) and the enzymes N-acetylglucosamine-6P (GlcNAc6P) deacetylase (NagA) and glucosamine-6P (GlcN6P) deaminase (NagB) for the conversion of GlcNac6P to fructose-6P (F6P). NagD encodes a UMP phosphatase (UmpH) involved in pyrimidine homeostasis (1). The *E. coli* GlcN6P deaminase enzyme NagB (EC 3.5.99.6) has been extensively studied as a model allosteric enzyme. It is a homohexameric protein that displays positive cooperativity toward its substrate, GlcN6P, and is allosterically activated by GlcNAc6P (2, 3). GlcNAc6P is the substrate of the previous enzyme in the pathway of amino sugar assimilation, GlcNAc6P deacetylase (NagA) (Fig. 1B). Allosteric activation by the substrate of the previous reaction constitutes part of a feed-forward loop. In the case of glycolysis, the function of a feed-forward loop is to sense flux through the pathway (4), and feed-forward regulation in general speeds up the response time to extrinsic signals (5). Several of the enzymes of glycolysis and gluconeogenesis have been shown to...
respond allosterically to changes in concentrations of the metabolites of glycolysis and thus allow a rapid response to changes in metabolic flux (6, 7).

NagB enzymes have been purified and studied kinetically from a variety of sources: bacteria (2, 3, 8), fungi (9), protozoa (10), and mammals (11, 12). The crystallographic structures of the E. coli enzyme and several of its homologues, e.g., enzymes from Bacillus subtilis and Strepotoccus mutans and human isozyme I, have been determined (8, 13–16), and all share the same protein fold and tertiary structure. However, only some of these enzymes are allosteric. The nonallosteric enzymes, e.g., those from B. subtilis (8) and Candida albicans (9), exist as monomers or dimers. The allosteric enzymes, like E. coli NagB (NagB	extsubscript{Ec}), are hexameric.

In archaea and some bacteria, there is a second family of isofunctional but nonhomologous GlcN6P deaminases called NagB-II (17). This operon, unique to E. coli, is formed during growth on GlcN but also implies that the nonallosteric NagB-II enzyme is cooperative with respect to its substrate, GlcN6P.

**FIG 1** Use of amino sugars by E. coli. (A) Organization of the divergent nagE and nagBACD operons encoding the PTS transporter of GlcN6P (NagE), the enzymes for the metabolism of GlcN6P, GlcNAc6P deacetylase (NagA), and GlcN6P deaminase (NagB), the repressor of the operon (NagC) and NagD, a UMP phosphatase (1). (B) Metabolism of amino sugars. GlcNAc is synthesized by the phosphotransferase system (PTS) transporter, NagE, producing intracellular GlcNAc6P, which is metabolized by the NagA (GlcNAc6P deacetylase) and NagB (GlcN6P deaminase) enzymes to fructose-6P (F6P). GlcN is transported by the manXYZ-encoded PTS transporter, producing GlcN6P, which is also metabolized by NagB. Glucose is predominately transported by the ptsG-encoded PTS transporter giving G6P and is converted to F6P, the first common compound of the glycolytic pathway. GlcN6P is synthesized by the glmS-encoded enzyme and converted, by the sequential action of the glmM and glmU-encoded enzymes, to UDP-GlcNAc, the essential precursor to the amino sugar components of the peptidoglycan (PG) and lipopolysaccharide.

grows more rapidly on GlcN than GlcNAc. In this respect B. subtilis is unusual compared to other bacilli (21). In addition to the gene encoding the NagB enzyme, which is carried within the nag regulon in all bacilli, a second gene (gamA) encodes an isozyme of B. subtilis NagB (NagB	extsubscript{Bs}) and is part of the gamR-gamAP operon. This operon, unique to B. subtilis (and a few very close neighbors), is required for the better growth of B. subtilis on GlcN. GamA is also responsible for the majority of growth on GlcN6P in this organism (21). As shown below, it is nonallosteric.

We are interested in the biological role of the allosteric activation of NagB in E. coli and why an allosteric form has been conserved in some organisms (e.g., E. coli and mammals) but is absent in other species such as firmicutes (B. subtilis) and fungi (Candida and Aspergillus). Although we could demonstrate allosteric activation in vivo in the case of some mutant forms of NagB	extsubscript{Ec}, there was no evidence that a lack of allosteric activation by GlcNAc6P affected the growth rate of the wild-type strain on GlcN (19). The major effect of growth on GlcN6P was to increase expression of the nagE-BA operators to uncouple the two effects of GlcNAc6P, allosteric activation of NagB from induction of the nag operon via NagC, we put the nagB gene under the control of a constitutive promoter and adjusted the amount of the enzyme by changing the translational efficiency of the gene. In this case there was no effect of GlcNAc6P on the quantity of the deaminase, but we still could not show any contribution of the allosteric activation of wild-type E. coli NagB to growth on either GlcN or GlcNAc. In fact, the enzyme behaved as if it was fully active during growth on either sugar (22).

High enzymatic activity of NagB during growth on GlcN was an unexpected result, since it seemed unlikely that the intracellular concentration of GlcN6P could attain the concentrations required to saturate the enzyme with substrate. In the absence of the allosteric activator, the enzyme shows sigmoidal velocity versus substrate curves with a half maximal reaction rate ($K_{0.5}$) at 5.5 ± 0.2 mM. The allosteric effector, GlcNAc6P, activates the enzyme by decreasing $K_{0.5}$ which approaches the limit value of 0.75 mM (23, 24). The allosteric kinetics of NagB	extsubscript{Ec} can be accurately described within the framework of the allosteric symmetry model of Monod, Wyman, and Changeux (the MWC model) (2, 25–28). The allosteric activator GlcNAc6P binds exclusively to the R conformer with a microscopic dissociation constant of 0.035 ± 0.005 mM (values obtained at pH 7.7 and 30°C) (26, 29). GlcNAc6P binding occurs in a range of concentrations 2 orders of magnitude lower than that required for effective saturation of the enzyme by the substrate, GlcN6P.

The observation that NagB appeared to be fully activated, during growth on GlcN, suggested that some GlcNAc6P was also being synthesized. The nagE-BA genes, controlled by the GlcNAc6P-sensitive NagC repressor, are partially induced by growth on GlcN (22, 30). This low-level induction supports the idea that some GlcNAc6P is formed during growth on GlcN but also implies that it is only present in a low concentration.

In a first approach, using a liquid chromatography mass spectrometry (LC-MS) method (31, 32), we have measured the effects of growth on GlcN and GlcNAc on the pools of amino sugar phosphates and other glycolytic metabolites to investigate whether the metabolite levels truly reflect the effects on regulation of NagB and NagC previously observed. Growth of E. coli on GlcN was found to produce only a small increase in intracellular GlcNAc6P but a very large increase in GlcN6P levels, which together are sufficient to
account for the high enzyme activity previously deduced for NagB during growth on GlcN.

As a continuation of our study of the role of allosteric regulation of *E. coli* NagB, we made chimeric bacteria, where the *E. coli* nagB gene was precisely replaced by the two nonallosteric nagB homologues from *B. subtilis*. Using these constructions we have investigated the effect of the absence of allostericity on the composition of amino sugar pools and on amino sugar utilization in *E. coli*. The absence of allosteric regulation was found to have surprisingly little impact on bacterial physiology, either during growth on amino sugars or on glucose, at least under the laboratory conditions used.

**MATERIALS AND METHODS**

**Bacteriological methods.** (i) Construction of *E. coli* strains with replacements of *nagBE* with *nagBr* and *gama*. The replacement of nagB with a *cat-sacB* cassette was described previously (19). The *nagB-cat-sacB* allele was introduced into MC4100 to give strain LAA32. Using the oligonucleotides shown in Table S1 in the supplemental material, DNA fragments carrying *gama* and *B. subtilis nagB* (*nagBr*) genes with 50-bp extensions corresponding to the flanking sequences of *E. coli nagB* (*nagBr*) were amplified from *B. subtilis* chromosomal DNA using Advantage HD (Clontech). The purified DNA fragments were used for allelic replacement by the method of Datsenko and Warner (33) using LAA32 carrying pKD46 as a recipient. Loss of the *cat-sacB* cassette was selected by sucrose resistance and screening for chloramphenicol sensitivity. Suscrose sensitivity was tested on *BYE* plates (10 g/liter Bacto-tryptone, 5 g/liter yeast extract, 40 g/liter sucrose). Candidate bacteria were purified, and correct allelic replacements were verified by sequencing a 1.3-kb PCR fragment, *Nag1*-Nag53, encompassing the *nagB* chromosomal locus of *E. coli*. Bacterial strains are listed in Table 1.

Growth rates were measured in minimal morpholinepropanesulfonic acid (MOPS) medium supplemented with 10 mM glucose, GlcN, or GlcNac at 37°C as described previously (19). The *gama* and *nagBr* alleles were also introduced by P1 transduction into MC-B52 (a *ΔnagEBACD::Tc* strain carrying a *nagB-lacZ* fusion on a λ lysogen) (22), with selection for growth on GlcNac and screening for tetracycline sensitivity. The β-galactosidase activities were measured as described previously (22).

(ii) Metabolite extracts and measurements. Samples of bacterial cultures were collected by using a filter method, and metabolites were extracted in hot ethanol as described previously (32). Starting from a fresh bacterial colony on Luria-Bertani (LB) plates, precultures were grown in minimal MOPS medium with 0.4% glycerol as the carbon source overnight at 37°C. Bacteria were diluted into minimal MOPS medium with 10 mM glucose, GlcN, or GlcNac as carbon source at an optical density (A_{600}) of 0.02 and grown with vigorous shaking at 37°C. At an A_{600} of 0.5, three 2-ml aliquots were rapidly removed, filtered on polyvinylidene difluoride membranes (Durapore; 0.45-µm pore size, 2.5-cm diameter), washed with 4 ml of the same prewarmed medium at 37°C, and rapidly transferred to 4 ml of 60% ethanol prewarmed at 78°C. An aliquot of uniformly [13C]labeled metabolite extract, prepared as described below, was immediately added. The filters were held at 78°C for 3 min, agitated occasionally, and then transferred to ice. The ethanolic solution of bacteria was centrifuged to remove bacterial debris and the supernatants stored at −80°C prior to drying by speed evaporation. The dried pellets were stored at −80°C until analysis. Metabolites were detected using an ion-pairing ultrahigh-performance liquid chromatography-tandem mass spectrometry method as described previously (31, 32). Dry metabolite extracts were resuspended in 100 µl of water, 10 µl of which were injected on a Waters Acquity UPLC apparatus with a Waters Acquity T3 end-capped reversed-phase column (150 by 2.1 mm by 1.8 µm; Waters Corporation, Milford, MA). The UPLC system was coupled to a triple-quadrupole mass spectrometer (TSQ Quantum Triple Quadrupole with ElectroSpray Ionization; Thermo Scientific, Waltham, MA) for metabolite detection. Three filters were analyzed for each culture, and two independent, biological replicate, cultures were analyzed.

Uniformly [13C]glucose-labeled metabolites for an internal standard were made from an overnight microaerobic culture of bacteria growing in minimal MOPS medium in which the only carbon source was 0.1% [13C]glucose. Bacteria were filtered, and metabolites were extracted as described above by filtering 20 ml of bacteria at an optical density at 650 nm (OD_{650}) of 0.5 on 5.0-cm-diameter filters and extracting with 6 ml of 60% ethanol at 78°C. The dried pellets were resuspended so that bacteria were at an optical density of ~1 per 0.1 ml of water. Labeled extracts were made from MC-B1 (wild type) and MC-B174C (nagA mutant) in which GlcNac6P accumulates (34). The two [13C]-labeled extracts were mixed to ensure the presence of both labeled GlcN6P and GlcNac6P and stored in aliquots at −20°C. The calculated concentrations of metabolites in extracts were normalized to intracellular concentrations using the preestablished conversion factors, 0.5 mg/OD unit of bacteria and a specific cell volume of 2 µl/mg (32, 35).

(iii) Overproduction, purification, and kinetic properties of GlcN6P deaminases. The *nagBr* gene was obtained by total synthesis (Epoch Laboratories, Missouri City, TX) and subcloned into the T7 expression vector pET22b+, which was used to transform the *E. coli* strain BL21*ΔnagEBACD::pRARE. The *gama* gene was obtained by PCR using

**Table 1. Bacterial strains used**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
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<tr>
<td>MC4100</td>
<td>Δ e14 araD139 Δ(arg-lac)169 fliD5301 deoC1 relA1 rbsR22 rpsL150 Δ(fruK-yeiR) Δ(fimB-fimE)632</td>
<td>Lab stock (49)</td>
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<tr>
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<td>MC4100 ΔnagB::cat-sacB</td>
<td>19</td>
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<td>LAA193</td>
<td>MC4100 gamA</td>
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<td>This study</td>
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<td>LAA199</td>
<td>MC4100</td>
<td>Wild-type recombinant</td>
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<tr>
<td>MC-B1</td>
<td>MC4100 ΔRS/nagB-lacZ</td>
<td>34</td>
</tr>
<tr>
<td>MC-B52</td>
<td>MC-B1 ΔnagEBACD::Tc</td>
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<td>MC-B1 ΔnagB</td>
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<tr>
<td>MC-B174c</td>
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<tr>
<td>MC-B362</td>
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<td>This study</td>
</tr>
<tr>
<td>MC-B364</td>
<td>MC-B1 nagBr</td>
<td>This study</td>
</tr>
<tr>
<td>BL21ΔNagEBACD::pRARE</td>
<td>OmpT hisD5 gal dcm rne131 (DE3) ΔnagEBACD::Tc::pRARE</td>
<td>This study</td>
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*MC-B76c carries the nagB::FRT kanamycin mutation from the Kieso collection (50) and was then cured of the kanamycin cassette by transformation with pCP20 (33). The ΔnagA mutation in MC-B174c removes only the 5′ half of the nagA gene, leaving the nagC promoters intact (34). ΔnagEBACD::Tc was introduced into BL21Δ (51) (previously called BL21(DE3) (rne131)) by P1 transduction, and the resulting strain was transformed with the pRARE plasmid from Rosetta(DE3) (Novagen).*
the oligonucleotides GamA2NdeI and GamA3BamHI (see Table S1 in the supplemental material) using chromosomal DNA from B. subtilis strain SSB1002 (21). The PCR product was inserted into the T7 expression vector pJE8307 and also transformed into BL21ΔΔ(nagE)BAD/pRARE.

The transformed cells were grown in LB broth at 30°C, and enzyme expression was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 6 h before harvesting. The cells were disrupted by mild sonication, and the extract was centrifuged at 45,000 × g for 30 min. Both deaminases were purified by preparative column chromatography using the same procedure. The clarified extract was applied to a Source 15Q cation-exchange preparative column equilibrated at pH 7.8 at 25°C and eluted with a gradient of NaCl from 0 to 1 M prepared in 50 mM Tris-HCl buffer at the same pH. NagBBm, eluted at 0.35 M NaCl, whereas GamA appeared in the eluate at a 0.5 M salt concentration. To remove the remaining impurities, each preparation was refined by molecular sieving in a Superdex 7-50 column run in the same buffer solution. In both preparations a single peak of electrophoretically pure protein was obtained. E. coli NagB was purified by affinity chromatography on ninyhydrin chromatography as described previously (2).

Kinetic studies. GlcN6P deamination rate was measured at 37°C in 100 mM Tris buffer (pH 7.4), with the concentrations of GlcN6P and GlcNAc6P indicated in figures. The amount of Fructose6P formed at a fixed time was measured using the previously described procedure (3), keeping the reaction below 5% conversion of the initial substrate concentration. The kinetic mechanism of the NagBcatalyzed reaction is “rapid equilibrium random” (36). This allows equating $v/V$ (v = fractions of $V_{\text{max}}$ measured as initial velocity divided by maximum velocity) to kinetic experiments to fractions of substrate saturation ($f_{s}$) and $K_{m}$ values as true dissociation constants.

Data analysis. Data fitting and graph plotting were performed with Prism 6.0 for Mac OS X (GraphPad Software, Inc., USA). Nonlinear regression analyses were always checked by inspection of their residual plots. Errors given in the tables and text are the standard deviations of the regressions.

RESULTS

Effect of growth on amino sugars on intracellular pools of GlcN6P, GlcAc6P and downstream metabolism. Wild-type E. coli (MC-B1) was grown in minimal medium with glucose, GlcN, or GlcNAc as a carbon source, and metabolites were extracted and analyzed by targeted liquid chromatography-mass spectrometry (32) for a set of common glycolytic metabolites to measure the overall changes in the concentrations of the amino-sugar precursors of all cell wall PG and LPS amino sugar components, including GlcN and GlcNAc6P (33). The liquid chromatography-mass spectrometry metabolic measurements confirmed that NagB is not usually acting on the GlcN6P pool and/or is not affecting regulation of GlcN6P homeostasis. Since GlcN6P synthesis by GlnS occurs in the cell in the absence of exogenous amino sugars, it is synthesized during growth on glucose, GlcN6P, and GlcNAc6P deacetylase (nagA) prevents the use of GlcNAc as a carbon source. The $\Delta$agB mutant is known to provoke the accumulation of high concentrations of GlcNAc6P from interruption of the PG recycling pathway (34, 37, 38). The liquid chromatography-mass spectrometry metabolic measurements confirmed these results, determining an intracellular concentration of $\sim$2 mM GlcNAc6P within the E. coli $\Delta$agB cells growing on glucose (Fig. 3A; Table S2 in the supplemental material).

GlcN6P was at very low levels in the $\Delta$agB strain (Table S2 in the supplemental material). This is certainly due to two related but independent causes. The accumulation of GlcNAc6P in the $\Delta$agB mutant prevents induction of NagC-repressed genes, resulting in large overproduction of the proteins of the nag operon, including NagB (19, 20). The consequently high levels of NagB will be fully active, because of the high concentration of its allosteric activator, GlcNAc6P, and will convert any available GlcN6P to F6P. Second, since NagA is an integral part of the PG recycling pathway (37), the $\Delta$agB mutation prevents all PG recycling, so that no GlcN6P is regenerated from PG degradation, and all amino sugar containing cell wall components must be made from GlcN6P synthesized de novo by GlnS.

On the other hand, deletion of the nagB gene had no strong effect on the amino sugar pools. In particular, there was very little increase in the GlcN6P concentration (at most 30%), implying that NagB is not usually acting on the GlcN6P pool and/or is not affecting regulation of GlcN6P homeostasis. Since GlcN6P synthesized by GlnS during growth on glucose is the essential precursor of all cell wall PG and LPS amino sugar components, it is necessary to protect newly synthesized GlcN6P from degradation by NagB. This could be achieved by controlling GlnS activity (39, 40).

Activity of NagBBm in vitro with physiological concentrations of GlcNAc6P and GlcN6P. We performed in vitro enzyme assays using purified E. coli GlcN6P deaminase to investigate the effect of GlcN6P and GlcNAc6P, over the concentration ranges measured in vivo, on the kinetic parameters and activity of the enzyme. Figure 4A shows the kinetic pattern of velocity versus substrate concentration at pH 7.4 and 37°C. The data were fitted to the general MWC allosteric equation (25). The fitted parameter for GlcNAc6P binding was 66 ± 17 μM, which is somewhat higher than our previously reported values at pH 7.7 and 30°C by direct binding studies (36 ± 3 μM (41)) or from kinetic measurements (35 ± 5 μM (26)). The measured intracellular concentration of GlcNAc6P during growth on GlcN is less than this (25 ± 7 μM; Fig. 3; Table S2 in the supplemental material). The area within the gray lines on Fig. 4A covers the substrate concentrations 5 to 12 mM GlcN6P with 10 to 30 μM for the allosteric activator.
FIG 2. Effect of growth on amino sugars on glycolysis. A schematic view of glycolysis and the TCA cycle during growth on Glc, GlcN, or GlcNAc is shown. The intracellular concentrations of the compounds indicated are shown for the three strains: MC-B1 expressing wild-type NagBEc (strain 1), MC-B362 expressing GamA (strain 2), and MC-B364 expressing NagBBs (strain 3). The concentrations of metabolites during growth on glucose (Glc) are shown in blue, those on GlcN are shown in green, and those on GlcNAc are shown in brown/red. Glyceraldehyde-3P (GAP) and pyruvate (PYR) are unstable during the extraction process and were not quantified. The complete analysis was repeated twice. A representative experiment is shown with means and standard deviations of three replicate filters. Data for GlcN6P and GlcNAc6P are expanded in Fig. 3.
GlcNAc6P, i.e., the range of concentrations measured during growth on GlcN. The enzyme under these conditions is operating at between 50 and 90% of its maximum rate. These values are consistent with the high NagB activity deduced for the enzyme during growth on GlcN (22).

Characterization of the NagB isozymes from *B. subtilis*. The NagB enzyme of *B. subtilis* has been purified, and its crystallographic structure was determined previously (8), but no information was available in the literature for the GamA enzyme. Both GamA and the NagBs enzymes were purified, and their reaction kinetics were compared to those of NagBec under identical conditions. NagBec and GamA were both confirmed to be nonallosteric and were unaffected by the presence of GlcNAc6P (Fig. 5A). Their catalytic activities were compared to that of NagBec in its activated form, i.e., saturated with GlcNAc6P (Fig. 5B). The $K_m$ for GlcN6P for NagBec and GamA were nearly 2- and 4-fold higher than NagBec (in its high-affinity R allosteric state) while the $k_{cat}$ values for the two *B. subtilis* enzymes were ~2-fold lower than their *E. coli* counterpart (Fig. 5B). These differences are reflected in the lower specificity constants (also called catalytic efficiency) for the two *B. subtilis* enzymes.

Growth of *E. coli* with *nagBec* replaced by nonallosteric GlcN6P deaminase genes of *B. subtilis*. In an attempt to assess the impact that NagBec allostery has on growth of *E. coli* on amino

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**FIG 3** Effect of growth on glucose (Glc), GlcN, and GlcNAc on the pools of GlcN6P and GlcNAc6P in the wild type, in nagB recombinants expressing the *B. subtilis* GlcN6P deaminases, and in strains with the nagA and nagB genes deleted. Intracellular concentrations of GlcN6P and GlcNAc6P during growth of wild-type *E. coli* NagBec (MC-B1), *E. coli* expressing GamA (MC-B362), and *E. coli* expressing NagBec (MC-B364) in Glc (A), GlcN (B), and GlcNAc (C) and the ΔnagA (MC-B174c) and ΔnagB (MC-B76c) deletion strains in glucose are shown. The results are means with the standard deviations for two biological replicate cultures (six filters). Note the change in scales for concentrations. The same data are given in numerical form in Table S2 in the supplemental material.

**FIG 4** Activation of NagBec by subsaturating amounts of its allosteric activator, GlcNAc6P. (A) Enzyme activity expressed as $v/V$ (fraction of $V_{max}$ measured as initial velocity divided by maximum velocity) was plotted versus the GlcN6P concentration at the GlcNAc6P concentrations shown. The $v/V$ ratio is equivalent to the fraction of substrate saturation, $\gamma_s$, according to the known kinetic mechanism of NagBec. Assays were performed at 37°C and pH 7.4, conditions chosen to resemble the conditions of the bacterial cultures (Fig. 2, 3, and 6). The area bordered by a gray line indicates the range in GlcN6P and GlcNAc6P concentrations measured in vivo during growth on GlcN, where allosteric activation can be expected to be significant. The NagBec concentration was 5 nM (hexamer). The data were fitted by nonlinear global regression analysis using the MWC equation for an exclusive-binding allosteric activator (25). The fitted MWC parameters are $K_R(\text{GlcN6P}) = 0.85 \pm 0.07 \text{ mM}$, $K_A(\text{GlcNAc6P}) = 66 \pm 17 \text{ mM}$, $L_0$ (MWC allosteric constant, defined by the $T_j/R_j$ ratio) $= 5 \times 10^4$, and $c$ (defined as $K_s/K_c$) $= 0.069 \pm 0.005$. These values can be compared to the previously measured values at 30°C and pH 7.7 ($K_R(\text{GlcN6P}) = 0.55 \pm 0.05 \text{ mM}$, $K_A(\text{GlcNAc6P}) = 35 \pm 5 \text{ mM}$, $L_0 = 10^5$, and $c = 0.025 \pm 0.002$) (24, 26). Note that $K_A(\text{GlcNAc6P})$ is nearly 2-fold higher, and the parameter $c$, related to the free energy change of the T-R transition ($K_s/K_c$), is also 2-fold higher whereas $L_0$, the $T/R$ ratio of the ligand-free enzyme, is 20-fold lower than at 30°C. (B) Fraction of NagBec in the R conformer ($R$) and fractional saturation of the enzyme with substrate ($\gamma_s$) or allosteric activator ($\gamma_A$) during growth on GlcN and GlcNAc. $R$ was calculated with the parameters given in panel A using the following state equation: $R = ([1 + S/K_s])^b/[L_0/(1 + S/K_c)^b (1 + c S/K_c)^b + (1 + S/K_s)^b]$ (from reference 52, where $S$ and A are the concentrations of substrate [GlcN6P] and allosteric activator [GlcNAc6P] and $K_s$ and $K_c$ are their microscopic dissociation constants) and the measured metabolite concentrations (Fig. 3; see Table S2 in the supplemental material). The fraction of the enzyme active sites occupied by GlcN6P ($\gamma_A$) is the $v/V$ value (see above). The fraction of allosteric sites occupied by GlcNAc6P ($\gamma_A$) was calculated as $[\text{GlcNAc6P}]/(K_A + [\text{GlcNAc6P}])$. This is valid because only the R conformer of NagBec binds the allosteric effector, GlcNAc6P.
GlcN6P deaminase isozymes (regulation of growth rate of a interesting, especially since GamA is responsible for the higher improvement in growth of the strain in the absence of GlcNAc6P, which was fitted to the MWC equation for allosteric activation (2, 25). Enzyme concentrations were normalized to 40 nM per subunit. GlcNAc6P has no effect on the activities of the B. subtilis enzymes under the conditions used. (B) Kinetic parameters for the three enzymes in the presence of GlcNAc6P. Catalytic efficiency (also known as the specificity constant) was calculated as \( k_{cat}/K_m \). Substrate inhibition was previously reported for NagB \(_\text{Ec} \), when measured at pH 8.0 and at 22°C (8). Using our assay conditions (pH 7.4 and 37°C), we did not detect any inhibition by GlcN6P (up to 10 mM) or by GlcNAc6P (1 mM), but substrate inhibition was observed with our preparation when assayed under the conditions described previously (8).

FIG 5 (A) Kinetic properties of three GlcN6P deaminases, NagB\(_\text{Ec} \), GamA, and NagB\(_\text{Bs} \). The enzyme activity, expressed as \( v/v_0 \), of NagB\(_\text{Ec} \), GamA, and NagB\(_\text{Bs} \) (and equivalent to the substrate saturation fraction \( [\tilde{y}] \)), was measured in the absence (dotted lines) or presence (solid lines) of 1 mM GlcNAc6P at 37°C and pH 7.4. Data were fitted to the hyperbolic equation except for NagB\(_\text{Bs} \) in the absence of GlcNAc6P, which was fitted to the MWC equation for allosteric activation (2, 25). Enzyme concentrations were normalized to 40 nM per subunit. GlcNAc6P has no effect on the activities of the B. subtilis enzymes under the conditions used. (B) Kinetic parameters for the three enzymes in the presence of GlcNAc6P. Catalytic efficiency (also known as the specificity constant) was calculated as \( k_{cat}/K_m \). Substrate inhibition was previously reported for NagB\(_\text{Ec} \), when measured at pH 8.0 and at 22°C (8). Using our assay conditions (pH 7.4 and 37°C), we did not detect any inhibition by GlcN6P (up to 10 mM) or by GlcNAc6P (1 mM), but substrate inhibition was observed with our preparation when assayed under the conditions described previously (8).

sugars and on amino sugar homeostasis, we exactly replaced the native E. coli nagB gene (nagB\(_\text{Ec} \)) with the genes for the two B. subtilis GlcN6P deaminase isozymes (nagB\(_\text{Bs} \) and gamA). Although both NagB\(_\text{Ec} \) and GamA had higher \( k_m \) and lower \( k_{cat} \) values than NagB\(_\text{Bs} \) (when saturated with GlcNAc6P) in vitro, the replacement of the nagB\(_\text{Ec} \) open reading frame with nagB\(_\text{Bs} \) produced a strain that grew rather similarly to bacteria with the wild-type E. coli enzyme on glucose (Glcn), Glcn, and GlcNAc (Fig. 6A). The GamA strain also grew similarly to the wild-type E. coli strain on glucose and GlcNAc, but it grew significantly faster on Glcn (Fig. 6A). These observations show that the absence of allosteric regulation of NagB does not impair growth on GlcNAc. However, the improvement in growth of the gamA strain on Glcn was interesting, especially since GamA is responsible for the higher growth rate of B. subtilis on Glcn compared to GlcNAc (21).

### Amino sugar metabolite levels in bacteria with heterologous B. subtilis GlcN6P deaminase genes

Metabolites of the GamA and NagB\(_\text{Bs} \) recombinants during growth on glucose, Glcn, and GlcNAc were measured in parallel with the wild-type NagB\(_\text{Ec} \) strain (Fig. 2, strains 2 and 3). Remarkably, GlcN6P and GlcNAc6P levels were about 2- and 4-fold higher, respectively, in the NagB\(_\text{Bs} \) strain during growth on GlcNAc (Fig. 6 and see Table S2 in the supplemental material), even though the growth rates are comparable.

The presence of the gamA allele (strain 2, in Fig. 2) had major effects on the amino sugar pools in all three media. In particular, the levels of GlcN6P were ~2-fold lower in the GamA strain compared to those with E. coli or B. subtilis NagB, while the GlcNAc6P concentrations were strongly increased in all three media (Fig. 3; see Table S2 in the supplemental material). The GlcNAc6P concentrations were 2-fold higher on glucose, ~7-fold higher on Glcn, and nearly 20-fold higher during growth on GlcNAc. However, there were no significant effects on the downstream glycolytic intermediates in the GamA strain compared to NagB\(_\text{Ec} \) or NagB\(_\text{Bs} \) strains, except for a small effect on FBP and DHAP. Confirmation that the GlcNAc6P levels were significantly higher in the GamA strain came from measurements of the NagC repression of a nagB-lacZ fusion (Fig. 6B). Expression from the nagB promoter is 2-fold higher in the GamA strain than in the wild-type NagB\(_\text{Ec} \) or NagB\(_\text{Bs} \) recombinant.
In the GamA recombinant, NagC is controlling expression of the gamA gene (from the nagB promoter in cis), as well as the nagB-lacZ fusion (located in trans on a lambda lysogen). A higher GlcNAc6P concentration, in the GamA recombinant would lead to less NagC repression of the gamA gene, and hence more GlcN6P deaminase activity, and would account for the higher growth rate on GlcN. This scenario was supported by measurements of the intracellular levels of the three deaminases and NagA (see Fig. S1 in the supplemental material). Effectively, the relative level of intracellular levels of the three deaminases and NagA (see Fig. S1 on GlcN. This scenario was supported by measurements of the deaminase activity, and would account for the higher growth rate on GlcN. The nature of the signal provoking activity and higher GlcNAc6P levels (see Fig. S2 in the supplemental material). On the other hand, the NagA protein level was much lower in the gamA strain than in the strains with the other two nagB alleles (see Fig. S1 in the supplemental material). A smaller amount of NagA protein (and hence GlcNAc6P deacytase activity) would account for the higher concentration of its substrate, GlcNAc6P. We conclude that the replacement of the nagB$_{Ec}$ gene with the B. subtilis gamA gene has introduced a polarity into expression of the nagBA genes, resulting in lower nagA expression and hence less GlcNAc6P deacytase activity and higher GlcNAc6P levels (see Fig. S2 in the supplemental material for more details). The nature of the signal provoking the polarity in the gamA gene has not been investigated.

These unexpected transcripational effects, produced by the replacement of the nagB$_{Ec}$ gene with the gamA gene, meant that this strain was not suitable to study the effects of loss of allosteric regulation on growth of E. coli. We thus focused our attention on the nagB$_{Ec}$ replacement. As shown in Fig. 2, 3, and 6, the loss of alloster in the NagB$_{Ec}$ recombinant has only minor consequences on the amino sugar metabolite pools and downstream glycolytic metabolites and no appreciable effect on the growth rates.

**DISCUSSION**

**Effect of growth on amino sugars on the E. coli metabolome.** Growth on GlcN or GlcNac produced large increases in the concentrations of GlcN6P or GlcNac6P (Fig. 2 and 3; see Table S2 in the supplemental material). However, the glucose-6P (G6P) concentration was not much higher during growth on glucose than on GlcN or GlcNac, presumably due to its synthesis by the gluconegenesis pathway and reflecting its pleiotropic roles in cell metabolism and glycosyltransferase reactions. The concentration of F6P, and most downstream glycolytic intermediates, was rather constant in all media for the three strains. The main exception was the concentration of fructose-1,6-bis-phosphate (FBP), which was slightly lower on GlcNac than on glucose and 2- to 3-fold lower on GlcN. FBP has been described as a metabolic flux sensor (4) and so its lower concentration reflects the lower growth rate on GlcN, compared to glucose or GlcNac (Fig. 6A). It is interesting that in the GamA recombinant, which does grow more rapidly on GlcN than the NagB$_{Ec}$ or NagB$_{Bs}$ strains, the FBP concentration was only 2-fold lower than on glucose, whereas in the NagB$_{Ec}$ and NagB$_{Bs}$ strains the FBP was 3-fold lower, corroborating its role in metabolic flux monitoring (Fig. 2).

**Intracellular pools of amino sugar phosphates and allosteric regulation of E. coli GlcN6P deaminase.** The high intracellular pool of GlcN6P, measured during growth on GlcN, explains why NagB$_{Ec}$ appeared in its high activity R state in our previous growth tests in GlcN (22). Using the measured GlcN6P and GlcNac6P concentrations during growth on glucose, GlcN, and GlcNac, together with the MWC allosteric parameters, derived from the data of Fig. 4A, we can calculate the allosteric status and occupancy of the active and allosteric sites of the enzyme, during growth on GlcN and GlcNac (Fig. 4B). The fraction of enzyme in the high-affinity R conformer (R) is close to unity (0.96 to 0.98) in both GlcN and GlcNac media. The fraction of enzyme saturated with substrate (y$_{s}$) is also very high (0.88) in GlcN but much lower (0.45) in GlcNac. On the other hand the fraction of enzyme with bound GlcNAc6P (y$_{y}$) is high in GlcNac (0.84) but much lower during growth on GlcN (0.33). The measured intracellular GlcNAc6P concentration during growth on GlcN (25 ± 10 μM) is lower than the K$_{y}$ value for GlcNAc6P (66 ± 17 μM), accounting for the partial occupancy. Thus, we can see that during growth on GlcN the high enzyme activity is driven by positive cooperative binding of the substrate, GlcN6P, producing the high y$_{s}$ and R values. On the other hand, during growth on GlcNac, it is the binding of the allosteric activator, GlcNac6P, which shifts the enzyme to its R conformer with higher substrate affinity (high R value) but with a much lower substrate occupancy (y$_{s}$), due to the lower GlcN6P concentration (Fig. 4B). During growth in glucose, the intracellular concentrations of both amino sugar phosphates are so low that the small amount of NagB$_{Ec}$ present is almost all in the ligand-free T state.

**Intracellular pool of GlcNac6P and induction of the nag regulon.** The partial induction of the NagC repressed nagB genes during growth on GlcN, as measured by the nagB$_{Ec}$-lacZ fusion in trans (Fig. 6B), implies that the concentration of GlcNac6P present during growth on GlcN is insufficient for complete displacement of the NagC repressor from the nagE-B operators. Interestingly, the measured GlcNac6P concentrations during growth of the GamA recombinant on glucose and GlcN are higher than in the other strains, which can account for the higher induction of nagB promoter activity and increased GamA protein level measured in this strain (Fig. 3 and 6B; see also Table S2 and Fig. S1 in the supplemental material). Even during growth on GlcNac, expression of the nagB-lacZ fusion was 2-fold higher in the GamA strain than in the NagB$_{Ec}$ strain, implying that the concentration of GlcNac6P in the NagB$_{Ec}$ strain during growth on GlcNac was not leading to complete loss of NagC repression.

**NagB allostery and GlcN6P homeostasis.** GlcN6P is the key starting compound for synthesis of all the amino sugar containing constituents of the cell wall and outer membrane. It might be expected that this pool should be protected from a cytoplasmic catalytic enzyme. One possible function of the allosteric regulation of NagB$_{Ec}$ could be to maintain the enzyme in its state with low substrate affinity (T state), during growth in the absence of exogenous amino sugars. The measured intracellular concentrations of de novo-synthesized GlcN6P and of GlcNac6P (produced by peptidoglycan recycling) during growth on glucose, are both below the concentrations required to displace the conformational equilibrium of NagB$_{Ec}$ toward the active R state (Fig. 4A). However, the absence of allosteric regulation in the NagB$_{Ec}$ recombinant had apparently no, or only a minor effect, on the basal level of GlcN6P levels (maximum 20% decrease; see Table S2 in the supplemental material). Indeed, even in the nagB deletion strain, the intracellular GlcN6P concentration did not change very much (30% increase; see Table S2 in the supplemental material). Thus, the absence of a GlcN6P deaminase or the presence of a constitutively active version did not significantly affect the GlcN6P levels. Amino sugar homeostasis is achieved by a sophisticated posttranscriptional regulation of the expression of the glmS gene, encoding
GlCN6P synthase. At high GlCN6P concentrations, expression of glms is low, but when the GlCN6P concentration falls, a regulatory cascade is initiated, resulting in higher GlmS levels (40, 43). This mechanism must maintain GlCN6P levels despite a potentially active deaminase (39). How the level of GlCN6P is monitored by the bacteria and how regulation of GlCN6P synthase and deaminase affects nitrogen metabolism are currently unknown.

We attempted to determine whether the glms regulatory loop was activated in the nagBke strain. We looked for evidence of any loss of GlCN6P by futile cycling by performing a growth yield experiment, using limiting amounts of glucose or GlcNAc as carbon source, and so any futile cycle resulting in loss of energy from converting glutamine to ammonia cannot be seen with this simple technique. We also carried out competitive growth experiments between the gamA or nagBke recombinants and wild-type nagBke strains (44, 45). These experiments confirmed the growth advantage of the GamA strain in GlcN (fitness = 1.37 ± 0.16) and also suggested a small gain of fitness of the NagBke strain in GlcN (1.13 ± 0.14). However, we detected no significant change in fitness in glucose, even after 40 generations, for either of the recombinant strains, compared to the wild-type nagBke strain, or indeed compared to a strain carrying a nagB deletion. So, under our laboratory conditions, there is no evidence that an allosteric NagB enzyme is conferring a fitness advantage (or disadvantage) to the bacteria. The lack of any physiological response to loss of NagB allosterically demonstrates the robustness of the amino sugar regulon and that other layers of regulation are involved in ensuring GlCN6P homeostasis. Other strategies could exist which prevent the establishment of a futile cycle involving NagB and GlmS in bacteria. For example, the enzymes of the early steps of cell wall amino sugar components, including GlmS, GlmM and GlmU (Fig. 2), could be part of a specialized bacterial microcompartment (46), which would direct the newly synthesized GlCN6P toward the next step in the biosynthetic pathway and prevent it from being available for immediate degradation by GlCN6P deaminase.

**Concluding remarks.** GlCNAc6P has dual regulatory functions in *E. coli*, as the allosteric effector of both NagC and NagB. Quantitation of the cellular concentrations of amino sugar-phosphates has allowed us to explain the anomaly that growth on GlcN produces strong activation of the enzyme GlCN6P deaminase (NagBke) but only very weak induction of the NagC-repressed nagE-BACD genes when both NagBke and NagC are sensitive to the same allosteric effector, GlCNAc6P. Strong cooperative binding of the substrate GlCN6P mostly drives the high activation of NagBke, whereas the minimal increase in GlCNAc6P results in just a partial displacement of NagC from its operators. Thus, despite having the same allosteric effector, GlCNAc6P, NagB and NagC are activated to very different levels during growth on GlcN.

In the absence of a suitable nonallosteric *E. coli* GlCN6P deaminase, we have studied two heterologous, authentic, nonallosteric enzymes, within the context of the *E. coli nagE-BACD* operon. The *B. subtilis* genes precisely replaced the *E. coli nagB* open reading frame, so that the heterologous genes are expressed from and are subject to the same transcriptional regulatory signals as the native *E. coli* gene. Indeed regulated induction by growth on GlcN and GlCNAc was verified by Western blotting (see Fig. S1 in the supplemental material). The loss of an allosteric deaminase seemed to have no significant effect on the growth rate or competitive fitness for the nagBke recombinant, at least under our laboratory conditions (Fig. 6). (The growth advantage of the GamA recombinant on GlcN was shown to be an artifact due to an indirect transcriptional effect on nagA expression and not an effect of the GamA enzyme per se [see Fig. S2 in the supplemental material for further details].)

Finally, it must be admitted that we have not identified a role for the allosteric regulation of NagBke, nor have we determined why it has been maintained through evolution in *E. coli* and in some other species, including humans. The ability to use amino sugars is an important criterion in the infection and colonization of mammalian hosts by both commensal and pathogenic bacteria (47, 48). *E. coli* and other *Enterobacteriaceae* have to endure periods of “feast and famine” as part of a dual life cycle, alternating between habitats rich in nutrients in mammalian hosts, or in aquatic or terrestrial habitats, usually habitats poor in nutrients. Allosterol could be advantageous for survival when bacteria are competing in a dynamic environment for a limited amount of nutrients.

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**REFERENCES**


42. Plumbridge J. 1998. Control of the expression of the mnrX2 operon in...


