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TAB

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Growth of *Escherichia coli* on amino sugars and the role of NagB allostery.

Crecimiento de *Escherichia coli* en aminoazúcares y el papel de la alostería de NagB.

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Resumen

Los amino azúcares son una importante fuente de carbono y nitrógeno para las bacterias y son los principales componentes de las paredes celulares bacterianas. La enzima alostérica glucosamina-6P desaminasa, NagB, es la enzima clave que vincula el metabolismo de los amino azúcares con la glucólisis, la ruta general de producción de energía. Debido a su rol en la confluencia de las rutas de biosíntesis y catabolismo de los amino azúcares, NagB está sujeta a varios mecanismos de control. Su nivel de expresión está controlado por el represor transcripcional, NagC. La señal de inducción para NagC es la GlcNAc6P que también es el efector alostérico de la enzima NagB. Hemos intentado comprender cómo la GlcNAc6P coordina los controles transcripcionales y enzimáticos de NagB y demostrar el papel de la regulación alostérica de NagB durante el crecimiento de *E. coli*.

Palabras clave: alostérico, glucosamina, *N*-acetilglucosamina, operon nag, metabolitos.

Abstract

Amino sugars are important sources of carbon and nitrogen for bacteria and major components of bacterial cell walls. The allosteric enzyme glucosamine-6P deaminase, NagB, is the key enzyme, which links the specific metabolism of amino sugars to the general energy-generating glycolytic pathway. As fitting its role at the junction of biosynthetic and catabolic routes for amino sugars, NagB is subject to various control mechanisms. Its expression level is controlled by the transcriptional repressor, NagC. The inducing signal for NagC is GlcNAc-6P, which is also the allosteric effector of the NagB enzyme. We have attempted to understand how GlcNAc6P co-ordinates the transcriptional and enzymatic controls of NagB and to demonstrate the role of the allosteric regulation of NagB during growth of *E. coli*.

Key words: allosteric, glucosamine, *N*-acetylglucosamine, nag operon, metabolites.

Introduction

The enzyme glucosamine-6P (GlcN6P) deaminase is the key enzyme for the metabolism of amino sugars in all species. The work of Mario Calcagno and his laboratory has established GlcN6P deaminase as a model enzyme for studies of a homohexameric allosteric system. Binding of the substrate (GlcN6P) induces positive cooperativity, while binding of *N*-acetylglucosamine-6P (GlcNAc6P) produces allosteric activation at low substrate concentrations. The allosteric effector GlcNAc6P, is the acetylated form of GlcN6P and the substrate of the preceding reaction in the pathway of GlcNAc utilisation (Fig. 1).

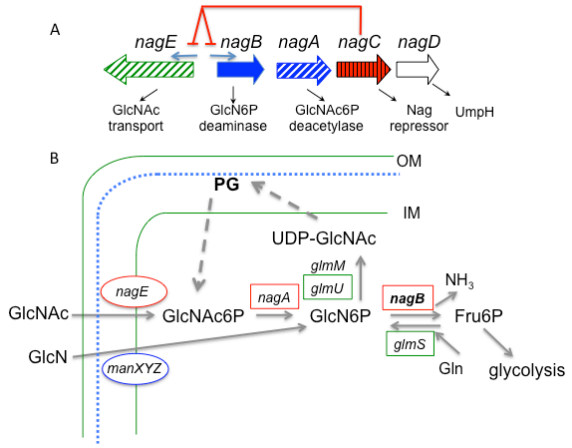

Figure 1. Amino sugar metabolism in *E. coli.* (A) Organisation of the *nag* operon in *E. coli*. The *nagE* and *nagBACD* genes are expressed from divergent promoters P*nagE* and P*nagB*, which are repressed by the *nagC*-encoded transcriptional repressor. The *nagD* gene encodes a putative phosphatase, UmpH. (B) Scheme for amino sugar metabolism in *E. coli*. GlcNAc and GlcN are taken up by the *nagE*- and *manXYZ*-encoded PTS transporters, producing the corresponding phosphorylated sugars. The phosphorylated sugars are converted to fructose-6P by the sequential action of the *nagA* and *nagB* encoded enzymes, GlcNAc6P deacetylase and GlcN6P deaminase. In the absence of extracellular amino sugars GlcN6P is synthesised by the *glmS*-encoded GlcN6P synthase. GlcN6P is either directed towards UDP-GlcNAc for synthesis of cell wall components, peptidoglycan (PG) and lipopolysaccharides or degraded to Fru6P, which enters glycolysis. Recycling of PG occurs via a series of periplasmic, membrane-bound and soluble components and generates GlcNAc6P (reviewed in [31]). A mutation in *nagA* causes accumulation of mM concentrations of GlcNAc6P from interruption of the PG recycling pathway [29, 30, 54]. Expression of genes shown in red ovals (*nagE-BA*) are repressed by NagC, those in green ovals (*glmUS*) activated by NagC while expression of *manXYZ* (blue oval) is repressed by Mlc [53]. Grey dotted lines indicate multistep pathways. OM - outer membrane, IM - inner membrane.

Their work was initiated, and the purification and the kinetic characterisation of this enzyme published [1] before the DNA or protein sequence was known. The DNA sequencing of the *E. coli nag* operon was started in Dieter Söll's lab (Yale, New Haven, USA) [2] and finished in Paris [3] and then the operon was also sequenced in Canada [4]. The DNA sequence provided valuable and confirmatory information about the protein composition of the Nag enzymes.

The Mexican-Paris collaboration was initiated in 1990 and it was quickly obvious that this was a mutually beneficial arrangement. The existence of the cloned *nagB* gene on an overexpressing plasmid greatly facilitated purification of the enzyme and the availability of larger amounts of protein allowed more protein-avid biophysical studies to be undertaken [5, 6] and more importantly permitted the crystallography studies [7, 8]. The enzymatic studies were also extended to the preceding enzyme of the GlcNAc utilisation pathway, the *nagA*-encoded GlcNAc-6P deacetylase [9]. In Paris we used the NagB and NagA proteins purified in Mexico, to produce antibodies and start quantifying levels of these proteins in *E. coli* [10]. At the same time, based on the protein studies and the DNA sequence and using oligonucleotide-directed mutagenesis, we produced a series of mutations in NagB. These modified enzymes facilitated the dissection of the kinetic and allosteric mechanisms of NagB *in vitro* [5, 11-14]. This is an on-going investigation, which continues to probe the finer points of the mechanism and associated structural changes [15-18].

These *in vitro* studies raised the question of what happens *in vivo* and what are the consequences of an allosteric NagB for the bacteria? Allosteric enzymes and proteins have long been the subjects of studies by enzymologists *in vitro*. The effects of the allosteric regulation could be extrapolated to the *in vivo* situation but at the time (nearly 30 years ago) very little work had tried to directly see the effects of allostery during bacterial cell growth. Here we present the efforts of the Paris-Mexico collaboration to investigate NagB allostery *in vivo*.

The *nag* genes, their regulation and growth on amino sugars

Amino sugars are important biomolecules with pharmaceutical and biotechnology applications [19]. The two most common amino sugars are glucosamine (GlcN) and *N*-acetylglucosamine (GlcNAc) and they are widely distributed in all living organisms, from components of glycosoamino-glycans and glycoproteins of vertebrate tissues to the peptidoglycan (PG) of bacterial cell walls. GlcNAc is the monomer constituent of chitin, the second most abundant biopolymer in nature after cellulose. Chitin forms the exoskeletons of insects and crustaceans

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and the cell walls of fungi and thus is widely distributed on land and especially in marine environments. A vast array of secreted chitinases are produced by both the chitin-producing organisms as well as some bacteria inhabiting the same biological niches [20-23]. These enzymes reduce polymeric chitin to shorter chain and monomeric forms of the constituent amino sugars, which can then be reabsorbed by the surrounding organisms. Amino sugars are valuable sources of energy and supply both carbon and nitrogen to cells. In bacteria they can be directly incorporated into the cell wall components like peptidoglycan (PG), thus bypassing the need for the otherwise essential enzyme, glucosamine-6P synthase (GlmS) (Fig. 1B).

Both amino sugars are taken up by phosphotransferase (PTS) systems in *E. coli* producing the phosphorylated form of the sugar. GlcNAc is taken up by *nagE*-encoded gene, which is part of divergent *nagE-BACD* operon (Fig. 1A) to give GlcNAc6P. GlcNAc6P is then deacetylated by the product of the *nagA* gene, giving GlcN6P. GlcN6P is also the product of GlcN transport by the *manXYZ*-encoded transporter (a generic PTS transporter for hexoses, including mannose and glucose). Further degradation of both GlcN- and GlcNAc-derived GlcN6P depends on the *nagB*-encoded GlcN6P deaminase, which, in converting GlcN6P to fructose-6P and ammonia, allows amino sugars to enter the glycolytic pathway (Fig. 1B). NagB thus controls the gateway between the biosynthetic pathway to cell wall components and the energy generating glycolytic pathway.

Growth of *E. coli* on amino sugars

E. coli grows more rapidly on GlcNAc (doubling time (DT) about 52 min) compared to GlcN (DT about 100 min) (Fig. 2A). As growth on GlcNAc generates GlcNAc6P, the allosteric activator of NagB, which growth on GlcN does not, our initial question was whether the GlcNAc6P-provoked allosteric activation of NagB is involved in the differential growth rates on GlcN and GlcNAc? GlcNAc6P has two regulatory functions in *E. coli*, in addition to being the allosteric activator of the NagB enzyme, it is also the allosteric regulator for the NagC transcriptional repressor of the divergent *nagE-BACD* operon (Fig. 1A). Thus, growth on GlcNAc generates GlcNAc6P, which binds to NagC causing its displacement from its operator sites and thus induction of expression of the *nag* genes. In consequence the protein levels of the NagE, NagB and NagA enzymes all increase significantly. Secondly the GlcNAc6P can allosterically activate the NagB produced from the derepressed *nag* operon. Western blots with antibodies against NagB and NagA, as well as gene fusions (see below Fig. 4B) showed that growth on GlcNAc increases the level of NagB more than 20-fold but growth on GlcN resulted in only about a four-fold increase. This lower level of NagB protein could, per se, account for slower growth on GlcN. We also showed that there was a reciprocal regulation between GlmS, the enzyme synthesising GlcN6P, and NagB [10]. NagC, as well as acting as a repressor for the *nagE-BACD* operons, is an activator of the *glmUS* operon and thus helps to coordinate the biosynthetic and catabolic metabolism of amino sugars (Fig. 1B) [24]. More recent work, notably from the laboratory of Boris Görke, has shown that the level of GlmS is subject to a complex post-transcriptional control involving sRNAs, GlmY and GlmZ, and a specialised RNA adaptor protein, which precisely links the GlmS level to the GlcN6P availability [25-27] reviewed in [28].

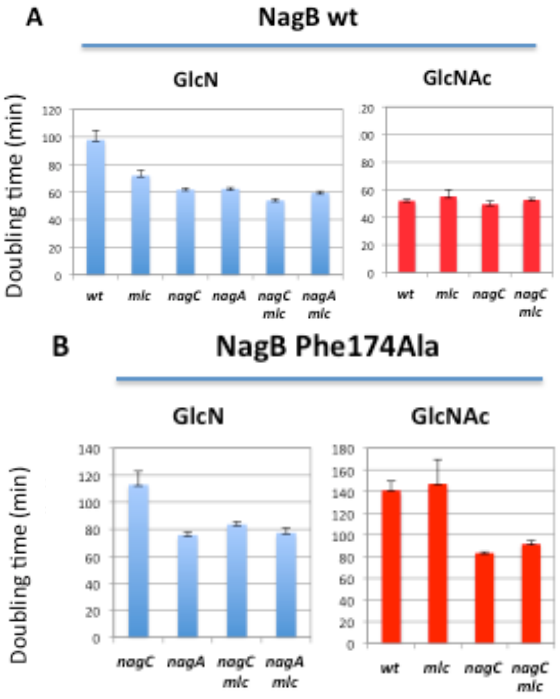

Figure 2. Growth of E. coli on amino sugars. (A) Growth rates (measured as doubling times in min) for wild type E. coli and (B) E. coli with the Phe174Ala mutant form of NagB replacing the chromosomal nagB gene. The effect of nagC, nagA and mlc mutations was tested on growth rates of bacteria growing in minimal medium with GlcN or GlcNAc at 37°C [32]. The Phe174Ala mutant does not grow on GlcN in the absence of a nagA or nagC mutation. The nagA mutation improves the growth rate more than the nagC, implying that the high level of GlcNAc6P in the nagA mutant is allosterically activating and stabilising the enzyme [34].

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NagB enzyme level is limiting growth on GlcN

The fact that there is much more NagB in the cell during growth on GlcNAc than on GlcN is a likely explanation for faster growth with GlcNAc than GlcN. A loss-of-function mutation in *nagC* also induces expression of the *nagE-BACD* genes, but without generating any GlcNAc6P. On the other hand, a mutation in *nagA,* encoding GlcNAc6P deacetylase, provokes induction of the *nag* genes, via an accumulation of the inducer for NagC, GlcNAc6P. NagA has an essential role in recycling the amino sugar components of the PG (Fig. 1B) [29, 30] reviewed [31], so that in a *nagA* strain, there is a high concentration of GlcNAc6P from the interruption of this pathway. This GlcNAc6P acts as an inducer for NagC, thus mimicking the effect of a *nagC* mutation, and increasing expression of the *nag* enzymes. However, the GlcNAc6P in the *nagA* strain can also act as the allosteric activator for NagB. Thus, a greater effect of a *nagA* mutation compared to a *nagC* mutation during growth on GlcN, would indicate allosteric activation of NagB by GlcNAc6P. When we tested this hypothesis the *nagA* mutation was as effective as a *nagC* mutation at improving the growth rate on GlcN but did not have any greater effect (Fig. 2A), implying that when NagB is strongly overproduced by loss of the repressor, no additional allosteric activation by GlcNAc6P is detectable. Thus, these experiments showed that the level of deaminase protein was limiting growth rate on GlcN but gave no indication of any effect of allosteric activation [32]. In addition, the effect of an *mlc* mutation was also tested. The *mlc* mutation increased the growth rate on GlcN and the double *nagC mlc* mutant strain grew almost as rapidly on GlcN as on GlcNAc (Fig. 2A). Mlc is the repressor for the GlcN transporter and an *mlc* mutation was expected to increase GlcN uptake. The fact that an *mlc* mutation also enhanced growth rates, we interpreted as signalling that GlcN transport, and by extension the concentration of the NagB substrate, GlcN6P, was also limiting growth.

Allosteric activation of mutant deaminases *in vivo*

A series of mutations affecting allosteric regulation of NagB had been characterised by the laboratory of Mario Calcagno. To investigate the performance of these modified NagB enzymes *in vivo,* we transferred the mutant *nagB* alleles to the *nagB* locus on the *E. coli* chromosome by the technique of λ*red*-mediated recombineering [33]. Many of the mutants were considerably defective for growth on GlcN and some also on GlcNAc. However, for two mutants, Tyr121Ser and Phe174Ala, the growth rates on GlcN with the *nagA* mutation were distinctly faster than strains with the *nagC* mutation (Fig. 2B), implying that excess GlcNAc6P present in the *nagA* strain was activating the mutant NagB enzymes. [32]. This was most easily interpreted for the Phe174Ala mutant, where the loss of Phe174 produced a protein, which was essentially inactive *in vitro* in the absence of GlcNAc6P [34]. Thus, allosteric activation was detectable *in vivo* for these modified enzymes.

Allosteric activation with limiting concentrations of NagB

The preceding experiments showed that the higher level of NagB gene expression during growth on GlcNAc, compared to GlcN, is the primary reason for better growth on GlcNAc. However, during growth on GlcN the levels of NagB are only increased 3-4 fold. Our inability to observe any allosteric activation in a strain with derepressed levels of NagB did not exclude a role of allostery affecting NagB activity when it was present at low levels during growth on GlcN. In an approach to investigate this question, we separated the effects of GlcNAc6P on NagC regulation of the *nagE-BACD* operons, and hence NagB protein levels, from the effects of GlcNAc6P on NagB enzyme activity. The *nagB* gene was deleted on the *E. coli* chromosome and placed on a single copy plasmid, where it was expressed from a constitutive plasmid promoter. The amount of NagB was varied by using an improved Shine and Dalgarno (S&D) sequence to increase translational initiation and/or a mRNA stabilising structure (Fig. 3A). For each of these plasmid constructions, the amount of NagB did not vary more than 2-fold in the 3 media - glucose, GlcN or GlcNAc (Fig. 3B). The L1, L2 and L3 constructs produced amounts of NagB comparable to the chromosomal copy during growth on GlcN, while the L4 construct, with both better S&D and mRNA stabilising structure, produced 20-fold more NagB in GlcN, similar to that present during growth on GlcNAc (Fig. 3B). As expected for these constructs, where the level of NagB is fixed, there was no effect of a *nagC* mutation and, significantly, even in the L1, L2 and L3 constructs where NagB levels are low, there was no effect of a *nagA* mutation (Fig. 3C). Thus a high concentration of the allosteric activator does not improve use of GlcN with limiting levels of NagB. NagB was behaving as if it was already fully active. Interestingly for constructs L2 and L3, the presence of an *mlc* mutation allowed similar growth rates on GlcN to that on GlcNAc, implying that transport of GlcN was also a limiting factor while GlcNAc uptake is more efficient [35].

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As a control the Phe174Ala mutation was also transferred to a plasmid construct. In this plasmid context, GlcNAc6P, generated by the *nagA* mutation improved the growth rate on GlcN compared to a *nagC* mutation [35]. The simplest interpretation of these data was that the wild-type NagB enzyme is already in the high activity state during growth on GlcN in the plasmid context and, by extension, in the chromosomal context.

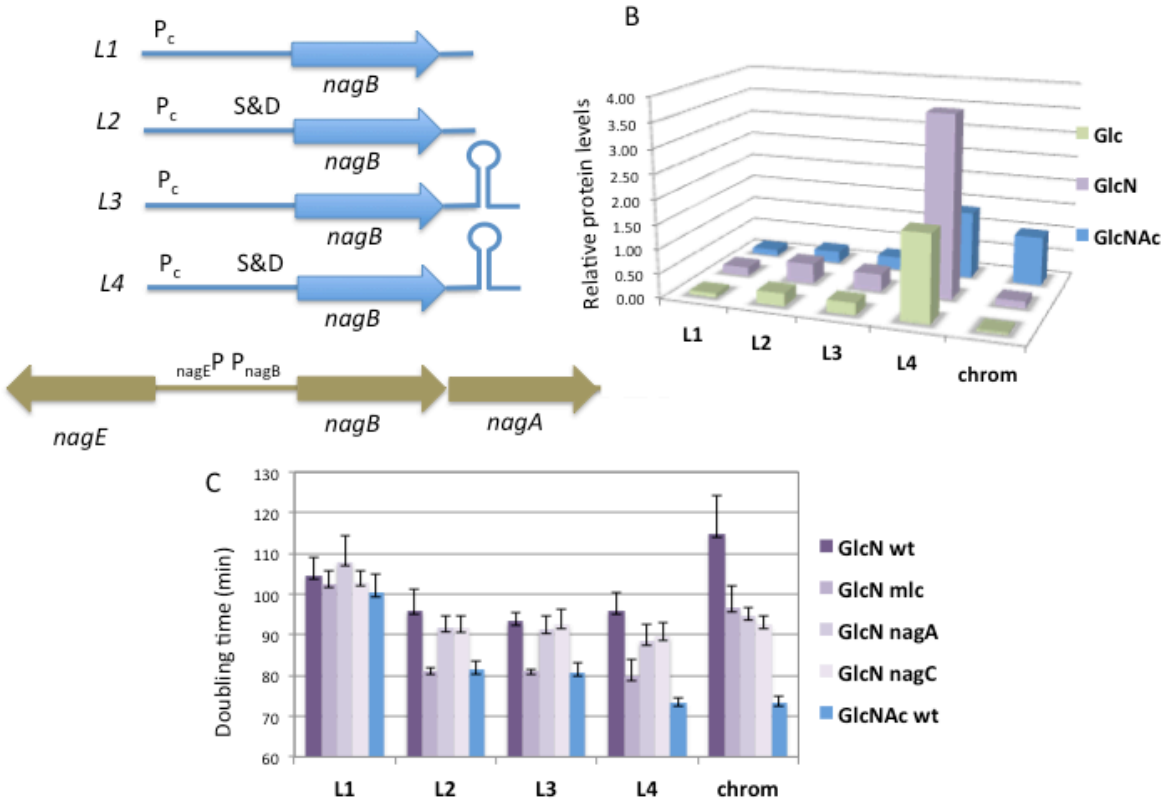

Figure 3. Growth of *E. coli* on GlcN with limiting amounts of NagB. (A) Organisation of *nagB* genes on single copy plasmids expressing low levels of NagB from a constitutive promoter (Pc) compared to the chromosome. S&D indicates an improved Shine-Dalgarno sequence to enhance translational initiation. A stem-loop after the gene indicates a structure stabilising the *nagB* mRNA. The organisation of the chromosomal *nagB* gene is shown for comparison. (B) Relative levels of NagB protein in glucose (Glc), GlcN and GlcNAc measured by Western blotting compared to that of NagB from the chromosome during growth on GlcNAc. For the chromosomal copy NagB levels increase strongly during growth on GlcNAc compared to Glc or GlcN. Note that for the plasmid constructs the level of expression of NagB is similar in all three media, and actually higher in GlcN (due to a lower level of catabolite repression of the "constitutive" plasmid promoter in GlcN). (C) Growth rates (measured as doubling times in min) in minimal GlcN and GlcNAc at 30°C of strains carrying the four plasmid constructs compared to the chromosomal copy are shown. Note that neither *nagA* nor *nagC* mutations affect growth on GlcN when NagB is at limiting fixed concentrations (constructs L1, L2 and L3). Bacteria were grown at 30°C because of the thermosensitive replicon of the single copy plasmids [35].

Growth on GlcN generates high intracellular GlcN6P

The conclusion at this point was that growth on GlcN allowed synthesis of only low levels of NagB, but that the NagB, which was present in the cell was fully active and could not be further allosterically activated. The low-level induction of the NagC-repressed *nagB* expression (Fig. 4B) implied that the concentration of GlcNAc6P, the NagC inducer, was low during growth on GlcN. To determine if this concentration was sufficient to fully activate NagB, we undertook some metabolomics experiments to quantitate the amino sugar pools during growth on GlcN and GlcNAc. Total soluble metabolites were extracted from *E. coli* growing on glucose, GlcN and GlcNAc. Hannes Link (laboratory of Uwe Sauer, ETH, Zurich) measured the levels of GlcNAc6P, GlcN6P and a range of other low molecular weight metabolites by targeted LC-MS [36, 37]. These results were somewhat of a surprise (Fig. 4A).

During growth on Glc the intracellular levels of GlcN6P (derived from de novo synthesis by GlmS) and of GlcNAc6P (due to PG recycling), were both quite low (63 ± 14 µM and 14 ± 6 µM). Growth on

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GlcNAc increased both GlcN6P and GlcNAc6P levels, 10 to 20-fold (830 ± 160 and 270 ± 180 µM respectively). However, during growth on GlcN the intracellular GlcN6P increased a 100-fold (9.04 ± 4.49 mM), while the GlcNAc6P level was only increased about 2-fold (Fig. 4A).

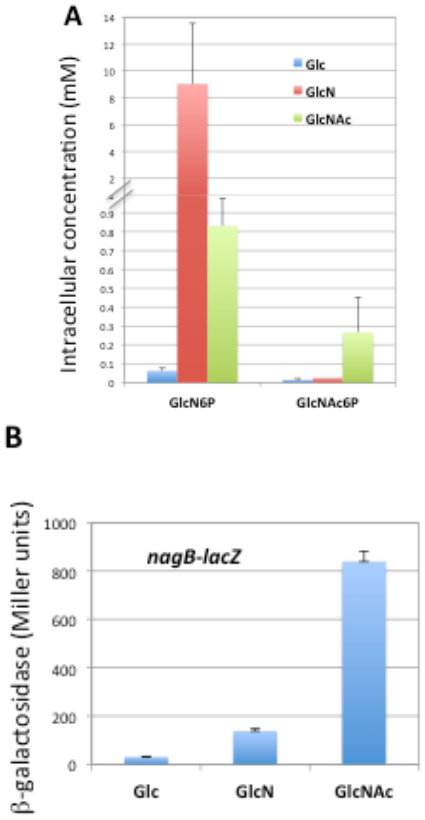

Figure 4. Intracellular concentrations of GlcN6P and GlcNAc6P. (A) GlcN6P and GlcNAc6P concentrations in metabolite extracts from wild-type *E. coli* growing on glucose (Glc), GlcN and GlcNAc were measured using targeted LC-MS. (B) β-galactosidase activities of a NagC-regulated *nagB-lacZ* fusion were measured in wild-type *E. coli* growing in Glc, GlcN and GlcNAc [38].

In the absence of GlcNAc6P, binding of the substrate, GlcN6P, induces positive cooperativity in NagB but previous *in vitro* measurements implied that very high concentrations (>10 mM) were necessary for full activity e.g. [17, 18]. The high intracellular concentration of GlcN6P measured during growth on GlcN suggested that, in fact, it was positive cooperativity that was fuelling the allosteric transition to the R state and not GlcNAc6P binding. A classic kinetic analysis of NagB with varying concentrations of substrate and activator under conditions of the metabolomics cultures at 37°C showed that this was indeed the case (Fig. 5A). The grey-lined area indicates the range of substrate and activator concentrations during growth on GlcN, as measured by metabolomics. Using these measured kinetic data, we could calculate that during growth on either GlcNAc or GlcN, NagB is almost totally in the high activity R conformation (Fig. 5B). During growth on GlcNAc, NagB is 84% saturated with GlcNAc6P but only 45% saturated with substrate. During growth on GlcN the site occupation is reversed, with 88% of NagB saturated with the substrate GlcN6P but only 33% saturated with activator GlcNAc6P.

These data confirm that it is positive cooperativity that is responsible for NagB being in the fully active state and thus insensitive to the high levels of GlcNAc6P produced by a *nagA* mutation [38].

The data also posed another question that is not yet resolved. The introduction of an *mlc* mutation enhanced growth on GlcN when NagB was either present on the chromosome or on a low copy plasmid (Fig. 2A and 3C). The Mlc repressor controls various genes related to glucose uptake by the PTS, including the GlcN transporter *manXYZ*. Initially we hypothesised that loss of Mlc repression enhanced PTS transport of GlcN via ManXYZ. Considering the high intracellular concentrations of GlcN6P during growth on GlcN, this hypothesis seems less tenable and we need an additional function for Mlc.

Growth of *E. coli* with a non-allosteric NagB

NagB enzymes belong to one of two protein families. Most, at least of those identified in genome databases, including those from eukaryotes, are homologous to the *E. coli* enzyme. However, in some bacteria and archaea (like *Xanthomonas* [39] *and Thermococcus* [40]), enzymes with GlcN6P deaminase activity belong to the SIS sugar isomerase family. These are now called NagBII and the *E. coli* homologues are named NagBI. Interestingly, at least some members of this family e.g. the *Shewanella oneidensis* enzyme, are also subject to allosteric activation by GlcNAc6P [41]. However, within the NagBI family not all the enzymes are allosteric. Of the enzymes, which have been studied, the mammalian (e.g. two human enzymes [42, 43]) are allosteric while those from fungi (e.g. *Candida albicans* [44]) are not. Amongst prokaryotes some NagB enzymes are allosteric and some are not. In *Bacillus subtilis* there are two genes encoding GlcN6P deaminases, called *nagB* and *gamA,* and neither is allosteric [38, 45-47]. In another approach to assess the impact of allostery on *E. coli*, we precisely replaced the ORF of the *E. coli nagB* gene

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with the two *B. subtilis* genes, *nagB*$_{Bs}$ and *gamA*, and also the allosteric NagBII$_{So}$ enzyme from *S. oneidensis,* so that the heterologous *nagB* genes were subject to the same transcriptional and translational signals as native *nagB*$_{Ec}$. However, the *gamA* DNA replacement was found to provoke some transcriptional effects on the downstream *nagA* gene expression, which affected growth rates and so was not useful for our analysis [38].

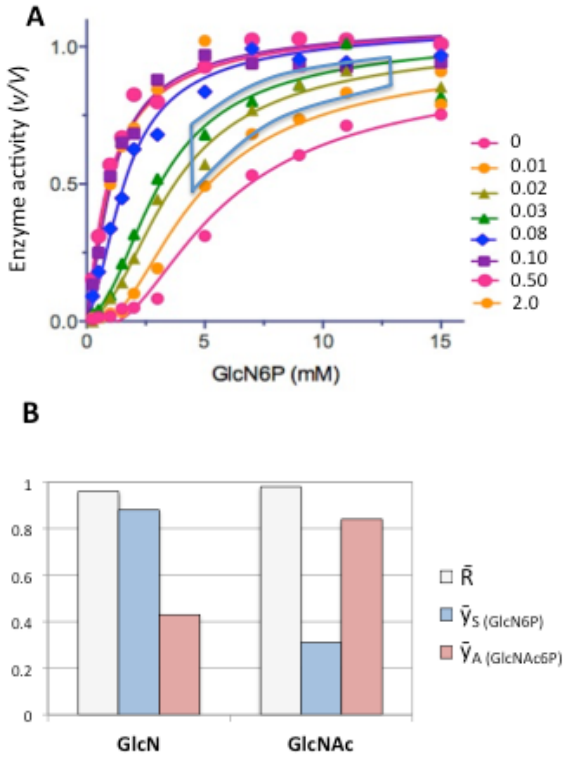

Figure 5. Activation of NagB by subsaturating amounts of its substrate, GlcN6P, and allosteric activator, GlcNAc6P. (A) Enzyme activity is expressed as the fraction of Vmax (v/Vmax) and is equivalent to the substrate fraction ($\bar{y}_S$) according to the known kinetic mechanism of NagB. Assays were conducted at 37°C and pH 7.4 with the concentrations of GlcN6P (abscissa) and GlcNAc6P (coloured lines) shown. The area designated by grey lines indicates the range of concentrations of GlcN6P and GlcNAc6P measured in the metabolomics experiments (Fig. 4A). (B) Fraction of NagB in the R conformer ($\bar{R}$), and fractional saturation with substrate ($\bar{y}_{S(GlcN6P)}$) and with allosteric activator ($\bar{y}_{A(GlcNAc6P)}$). See [38] for further details.

The other NagB recombinant strains were tested for growth on GlcN and GlcNAc (Fig. 6). The strain with the non-allosteric *nagB*$_{Bs}$ enzyme replacement grew at essentially the same rate as wt *E. coli* on GlcN and GlcNAc. Moreover, the presence of either a *nagC* or *nagA* mutation, to increase NagB$_{Bs}$ expression, also gave identical growth rates on GlcN, showing that loss of allostery did not affect the ability of *E. coli* to grow on GlcN. The strain with the *nagBII*$_{So,}$ replacement grew slowly on GlcN. This was undoubtedly due to low expression of the heterologous *Shewanella* protein. However, either a *nagC* or *nagA* mutation increased NagB$_{So}$ levels considerably (as shown by Western blots) and allowed growth rates only slightly slower than the NagB$_{Ec}$ or NagB$_{Bs}$ strains (Fig. 6). Again, there was no differential effect of a *nagA* compared to a *nagC* mutation, implying that, as in the case of the *E. coli* enzyme, NagBII$_{So}$ was in the fully active form during growth on GlcN. Interestingly a *mlc* mutation had no effect on the growth rate of either the *nagB*$_{Bs}$ or *nagBII*$_{So}$ recombinant on GlcN (Fig. 6), which again raises the question of the role of Mlc in the use of GlcN by *E. coli*.

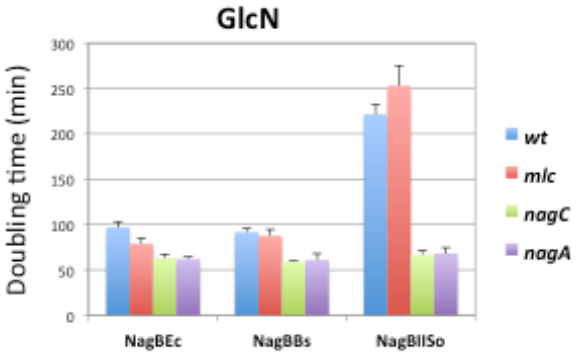

Figure 6. Growth of *E. coli* with heterologous NagB enzymes on GlcN. Growth rates (measured as doubling times (min)) of wild-type *E. coli* and *E. coli* with precise replacements of the ORF of the gene for *nagB* with those of *nagB*$_{Bs}$ and *nagBII*$_{So}$ were measured in minimal medium with 0.2% GlcN at 37°C. The effects of mutations in *nagC*, *nagA* and *mlc* on growth rates were measured.

NagB allostery and amino sugar homeostasis

A final question that we tried to answer was whether the allosteric regulation had a role in maintaining basal amino sugar pools in the absence of an exogenous source of GlcN or GlcNAc. As described above, GlcN6P synthesized by the GlmS enzyme is destined for cell wall components and it is important to prevent a futile cycle, where the newly-synthesized GlcN6P is rapidly degraded by the catabolic NagB enzyme (Fig. 1B). If the presence of an allosteric enzyme is important for maintaining the GlcN6P pool, then we can imagine that bacteria with a non-allosteric enzyme would be outcompeted by bacteria with its allosteric homologue. We performed competition growth experiments between recombinant bacteria with NagB$_{Bs}$ and wt NagB$_{Ec}$ and also with the *nagB* deletion strain and could detect no fitness advantage for any of the strains during growth on glucose. Measurements of metabolites showed only a minor decrease in GlcN6P pools in the NagB$_{Bs}$ strain compared to wt *E. coli.*

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Indeed there was only a 30% increase in GlcN6P in *E. coli* where *nagB* had been deleted [38]. These observations confirm that the control of amino sugar pools is robust and probably is largely dependent upon the tightly regulated expression of *glmS* [25-27] reviewed in [28].

Discussion

The metabolite measurements have allowed us to understand why the *E. coli* NagB enzyme behaves *in vivo* as if it is fully active, even when the concentration of GlcNAc6P is low. During growth on GlcN, the concentration of GlcNAc6P is too low to fully displace NagC from its operators, thus limiting *nag* operon derepression to about 4-fold, and also too low to allosterically activate NagB by itself. However, the massive increase in GlcN6P during growth on GlcN produces positive cooperativity, which is mostly responsible for driving NagB to the fully-activated state, as during growth on GlcNAc (Fig. 4). Thus, the NagB enzyme does change its conformation from the low activity T state, present during growth on glucose, to the high activity R form during growth on GlcN and GlcNAc. However, we have no evidence that the ability to pass from a low activity state to a high activity form is important for growth since the strain with the non-allosteric $NagB_{Bs}$ replacement exhibited identical growth characteristics compared to wt *E. coli*. It must be admitted that growth rate is a rather crude parameter and more precise measurements associated with an integrated systems biology approach might reveal new aspects to amino sugar metabolism.

When we started this work there were few examples where allosteric activation had been observed *in vivo* [48]. Recent advances, applying metabolomics and other high throughput techniques have demonstrated functional allosteric regulation in growing bacteria e.g. regulation of PEP flow into the TCA cycle (anapleurosis) via the known allosteric effectors of PEP carboxylase [49]. Another study identified new allosteric regulatory interactions of glycolytic enzymes by mathematical modelling of flux reversal data during switching between different carbon sources [50]. Future studies of NagB would benefit from these multi-omics and systems analysis approaches.

Based on protein-protein interactome data, Rodinova *et al*. have claimed that NagB has additional protein allosteric effectors including the unphosphorylated form of the PTS phosphate carrier protein, HPr, also Nitrogen Regulatory PII protein (GlnB) and GlcNAc6P epimerase (NanE) [51, 52]. These *in vitro* studies were carried out in the presence of GlcNAc6P so NagB was already in the activated state and any significance for growth of bacteria needs further investigation. It can be noted that HPr (gene *ptsH*) is expressed from the *ptsHIcrr* operon, which is regulated by Mlc [53]. If HPr really can activate NagB during growth on GlcN, with low levels of GlcNAc6P, then this might be an explanation of the effect of an *mlc* mutation on growth. Finally, it has to be mentioned that although some bacteria and yeast exist quite successfully without an allosteric NagB enzyme, all mammalian enzymes have conserved the allosteric homohexameric format and so it seems probable that an allosteric enzyme must confer a fitness benefit to those organisms, which have maintained it throughout evolution.

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