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Engineering a new pathway for *N*-acetylglucosamine production: Coupling a catabolic enzyme, glucosamine-6-phosphate deaminase, with a biosynthetic enzyme, glucosamine-6-phosphate *N*-acetyltransferase

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Abstract

A metabolic pathway for high level production of *N*-acetylglucosamine has been engineered in *Escherichia coli* by overexpressing *E. coli* glucosamine synthase (GlmS) and *Saccharomyces cerevisiae* glucosamine-6-phosphate acetyltransferase (GNA1). GlmS catalyzes the synthesis of glucosamine-6-phosphate from fructose-6-phosphate and glutamine. GNA1 converts glucosamine-6-phosphate into *N*-acetylglucosamine, which is dephosphorylated and secreted into the growth medium. In the present work, *E. coli* glucosamine-6-phosphate deaminase (NagB) was evaluated as an alternative to GlmS for the production of glucosamine and *N*-acetylglucosamine. NagB is a catabolic enzyme that converts glucosamine-6-phosphate to fructose-6-phosphate and ammonia. The reverse biosynthetic reaction forming glucosamine-6-phosphate is kinetically unfavorable. In a *glmS* deletion strain requiring glucosamine supplement to survive and grow, NagB overexpression resulted in the synthesis of glucosamine-6-phosphate. This supported cell growth, but little or no glucosamine accumulated in the medium. Overexpression of both NagB and GNA1 resulted in production of *N*-acetylglucosamine at levels comparable to strains overexpressing both GlmS and GNA1. This indicates that the overexpression of GNA1 played a critical role in determining the direction and efficiency of the reaction catalyzed by NagB. These data demonstrate that a catabolic enzyme can be utilized in a biosynthetic pathway by coupling with an efficient downstream reaction.

Keywords: Glucosamine; *N*-Acetylglucosamine; *Escherichia coli*; Glucosamine synthase; Glucosamine deaminase; Glucosamine-6-phosphate *N*-Acetyltransferase; Metabolic engineering

1. Introduction

Glucosamine (GlcN) and *N*-acetylglucosamine (GlcNAc) are precursors for the synthesis of peptidoglycan and lipopolysaccharide, two essential constituents in the *Escherichia coli* outer membrane and cell wall (Fig. 1, [1,2]). Glucosamine synthase (EC 2.6.1.16, GlmS) catalyzes the first committed step in the pathway for amino sugar synthesis: the formation of glucosamine-6-phosphate (GlcN-6-P) from fructose-6-phosphate (Fru-6-P) and glutamine [3,4]. GlcN-6-P is converted to GlcN-1-P by phosphoglucosamine mutase (encoded by *glmM*). This product is further converted by a bifunctional enzyme, GlcN-1-P *N*-acetyltransferase/GlcNAc-1-P uridyltransferase (*glmU*), to GlcNAc-1-P and then to UDP-GlcNAc, an activated building block for the synthesis of peptidoglycan and lipopolysaccharide. *glmS* deficient mutants require an exogenous supply of GlcN or GlcNAc for survival and growth [5–7]. GlcN and GlcNAc also serve as alternative carbon sources for *E. coli* [5,8]. Both amino sugars are transported and phosphorylated through phosphotransferase systems (PTS): a mannose PTS (II^{Man}, encoded by the operon *manXYZ*) and a glucose PTS (II^{Glc}, encoded by *ptsG*) for GlcN; II^{Man} and a GlcNAc PTS (II^{NAG}, encoded by *nagE*) for GlcNAc [9]. The mannose PTS transports GlcN, GlcNAc as well as other hexoses. GlcNAc-6-P is hydrolyzed to GlcN-6-P by a deacetylase encoded by *nagA*, and further converted into Fru-6-P and ammonia by GlcN-6-P deaminase (encoded by *nagB*).

Expression of the genes involved in the synthesis and degradation of GlcN and GlcNAc are coordinated to avoid a futile cycle of the two pathways [3]. In the *E. coli* genome, *glmS* and *glmU* form the operon *glmUS* while genes for GlcNAc uptake (*nagE*) and catabolism (*nagBA*) constitute a *nag* regulon (two divergent operons *nagE-nagBACD*). The function of *nagD* has

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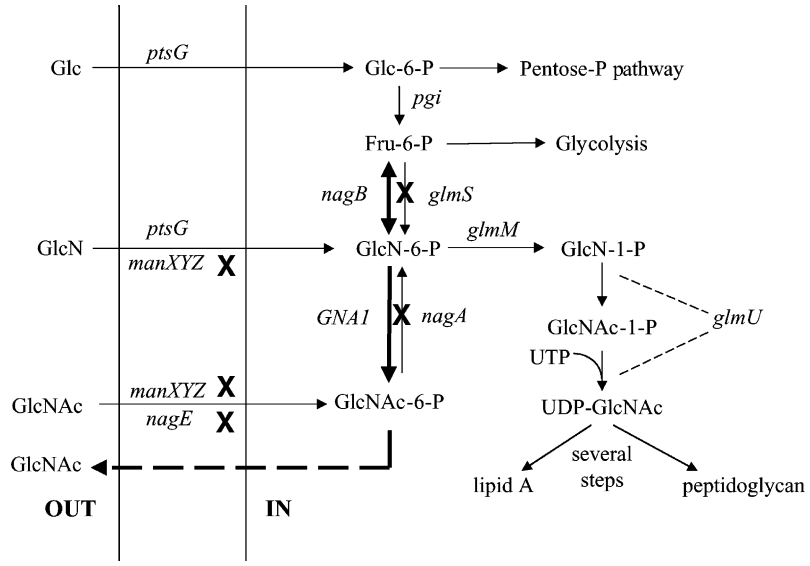

Fig. 1. Pathway engineering for GlcNAc production in *E. coli*. Crosses indicate metabolic flux blocked by gene inactivation or deletion. Thick lines indicate introduction and/or increase of the metabolic flux by gene overexpression. Dephosphorylation and secretion of GlcNAc-6-P are indicated as a dotted line. Glc: glucose, Glc-6-P: glucose-6-phosphate, Fru-6-P: fructose-6-phosphate, GlcN: glucosamine, GlcNAc: *N*-acetylglucosamine, *ptsG*: gene encoding for glucose transporter, *manXYZ*: mannose transporter, *nagE*: GlcNAc transporter, *pgi*: phospho-glucose isomerase, *glmS*: glucosamine synthase, *nagB*: GlcN-6-P deaminase, *nagA*: GlcNAc-6-P deacetylase, *GNA1*: GlcN-6-P *N*-acetyltransferase, *glmM*: phosphoglucosamine mutase, *glmU*: GlcN-1-P acetyltransferase/GlcNAc-1-P uridyltransferase, in: intracellular space, out: growth medium.

not been defined. *nagC* encodes a regulatory protein that functions as a repressor of the *nag* operons as well as both an activator and repressor of the *glmUS* operon. In addition to transcriptional control, GlmS, an enzyme with unidirectional activity, is inhibited by GlcN-6-P [5,10,11]. The enzyme from eukaryotic organisms (commonly referred as GFA1, glutamine:fructose-6-phosphate amidotransferase) is also inhibited by UDP-GlcNAc (see review [4]). The deaminase, although reversible, has an equilibrium strongly favoring the catabolic reaction. The turnover number (K_{cat}) was 455 s^{-1} for GlcN-6-P formation and 1800 s^{-1} for Fru-6-P formation [12]. GlcNAc-6-P is an allosteric activator of NagB, activating both the forward and reverse reactions by decreasing the K_m for the phosphorylated sugar substrates [12,13]. Vogler et al. isolated $GlmS^+$ suppressor mutants from a non-reverting *glmS*-negative strain [14]. An overexpressed or deregulated deaminase was shown to produce amino sugars at levels sufficient to support growth of *glmS*-negative mutants.

GlcN has been shown in clinical trials to relieve the symptoms of osteoarthritis and may also slow down the progression of the disease [15,16]. Current supply of GlcN comes from acid digestion of chitin (a linear polymer of GlcN), a shellfish waste product. The supply is limited by the amount of waste available and potentially carries the risk of contamination from allergenic shellfish proteins. A fermentation-based production process was developed recently by means of an *E. coli* modified using metabolic engineering [17]. The main feature of the engineered pathway is the overexpression of the *E. coli glmS* and yeast *GNA1* genes. The latter encodes for GlcN-6-P *N*-acetyltransferase that converts GlcN-6-P into GlcNAc-6-P. Overexpression of *glmS* alone led to an increased synthesis of GlcN. Since *E. coli* GlmS is inhibited by GlcN-6-P, variants were generated by error-prone PCR and screened for reduced sensitivity to product [18]. GlcN production was significantly improved by overexpression of product-resistant enzymes (such as GlmS*54). However, GlcN titer remained relatively low due to spontaneous degradation of GlcN at pH 5 or above. High levels of GlcN and degradation products also inhibited host cells. On the other hand, GlcNAc is stable and non-inhibitory to the host. By expressing both *glmS* and *GNA1*, high levels of GlcNAc were produced in the culture medium. GlcNAc could be chemically hydrolyzed to GlcN under relatively mild acidic conditions.

In the present work, we evaluated overexpression of *E. coli* GlcN-6-P deaminase (NagB) as an alternative to GlmS for the production of GlcN and GlcNAc. Although NagB has unfavorable kinetics for GlcN-6-P formation, simultaneous overexpression of both NagB and GNA1 formed an efficient pathway for GlcNAc-6-P synthesis, leading to a high-level accumulation of GlcNAc in the growth medium (Fig. 1).

2. Materials and methods

E. coli K-12 (ATCC 25947, a derivative of W3110, see [19]) was modified by metabolic engineering to produce GlcN and GlcNAc [17]. Briefly, the *manXYZ* operon and *nag* regulon were deleted or inactivated to minimize uptake and catabolism of GlcN and GlcNAc; a defective λDE3 prophage containing a T7 RNA polymerase gene under the *lacUV5* promoter control was introduced, resulting in strain 7101-17(DE3). GlcN production strain 7107-18 was developed from this strain by the integration of an expression cassette containing the *T7lac* promoter and a mutant *E. coli glmS* gene (P_{T7lac}-*glmS**54) at the *galK* locus. For GlcNAc production, the strain was further modified by integration of an expression cassette containing the *Saccharomyces cerevisiae GNA1* gene (P_{T7lac}-*GNA1*) at the *manXYZ* locus, generating strain 7107-92. A second copy of *GNA1* cassette was integrated at the *fucIK* locus, generating strain 7107-607. Genotypes of various *E. coli* strains are listed in Table 1.