Engineering a Novel Pathway for *N*-Acetylglucosamine in *E. coli* Using a Catabolic Enzyme

Bio-Technical Resources (BTR)

www.biotechresources.com

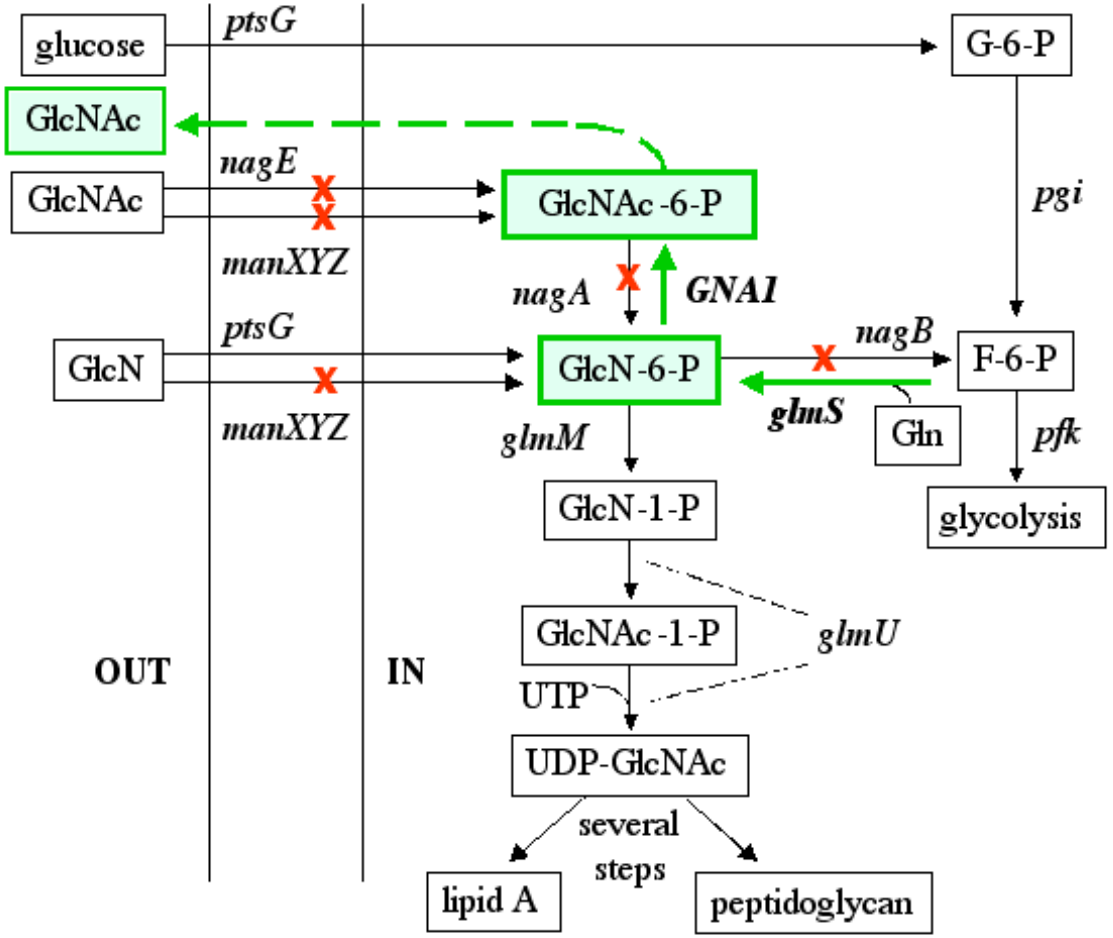
1035 S 7th Street, Manitowoc, WI 54220

Phone: (920) 684-5518

Fax: (920) 684-5519

Email: info@biotechresources.com

Metabolic Engineering for *N*-Acetylglucosamine Production in *E. coli*



Metabolic Engineering for *N*-Acetylglucosamine Production in *E. coli*

Abbreviations for Genes:

ptsG: glucose transporter

nagE: *N*-acetylglucosamine transporter

manXYZ: mannose transporter (also transports GlcN and GlcNAc)

pgi: phosphoglucoisomerase

pfk: phosphofructokinase

glmS: glucosamine synthase

nagB: glucosamine-6-P deaminase

nagA: *N*-acetylglucosamine-6-P deacetylase

GNA1: glucosamine N-acetyltransferase

glmM: phosphoglucosamine mutase

glmU: glucosamine-1-P acetyltransferase/*N*-acetylglucosamine-1-P uridyltransferase

Legends:

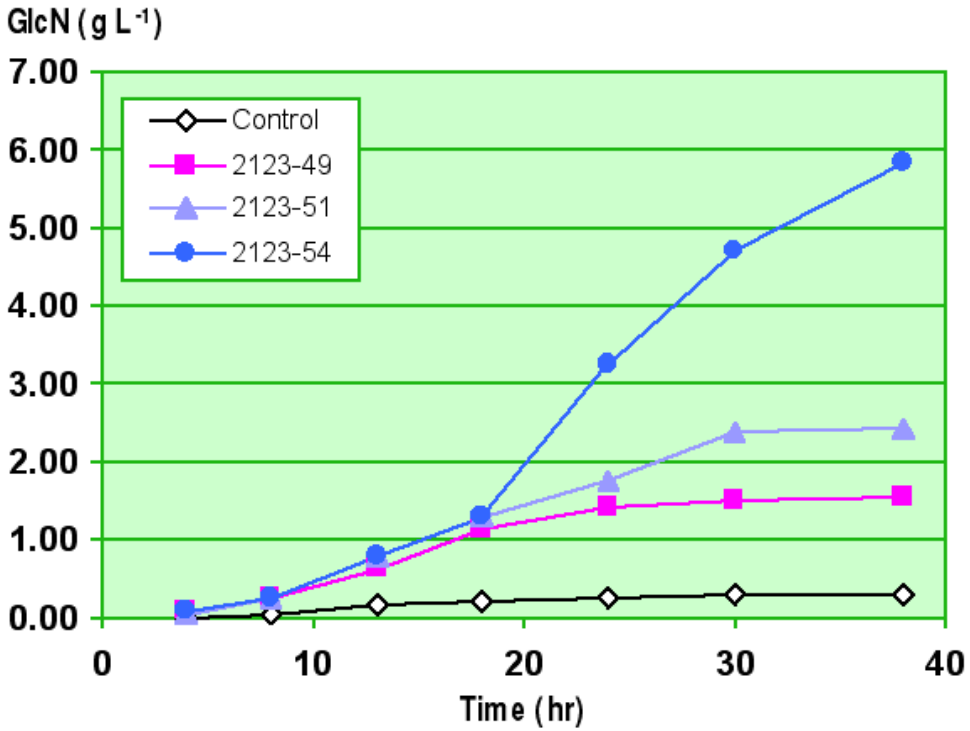
- Crosses: metabolic flux blocked by gene deletion.
- Thicker lines: introduction and/or increase of the metabolic flux by gene over-expression.
- Dotted lines: dephosphorylation and secretion of *N*-acetylglucosamine-6-P.
- Gln: glutamine, GlcN: glucosamine, GlcNAc: *N*-acetylglucosamine.

Expression of Recombinant *glmS* Genes in *E. coli*

Strain	Expression Construct	Enzyme Activity (nmol min^{-1} mg^{-1})	GlcN (mg L^{-1})
7107-22	pET24d(+)(control)	trace	5
7107-24	pET24d(+)/*T7-B. subtilis glmS*	637	128
7107-101	pET24d(+)/*T7-S. cerevisae GFA1*	trace	47
7107-23	pET24d(+)/*T7-C. albicans GFA1*	trace	23
7107-58	pET23b(+)/*T7-C. albicans GFA1*	trace	54
7107-214	pET24d(+)/*T7-E. coli glmS*	297	37
2123-12	*lacZ::T7-E. coli glmS*	613	75

- Glucosamine (GlcN) is detected in growth medium of shake flask cultures.
- Highest levels of enzyme activity and GlcN production with the *Bacillus glmS* gene, which encodes an enzyme resistant to inhibition by GlcN-6-P.
- GlcN production was higher with an integrated expression cassette than with a free-replicating plasmid.

GlcN Production by Over-Expressing GlmS Enzymes Resistant to Product Inhibition



- Wild type *E. coli* GlmS is strongly inhibited by GlcN-6-P. Activity was 80% inhibited at 6 mM GlcN-6-P
- Directed Evolution generated *E. coli* GlmS mutants resistant to product inhibition
- Strain expressing mutant enzymes produced much higher levels of GlcN than the control (wild-type enzyme) in shake flasks

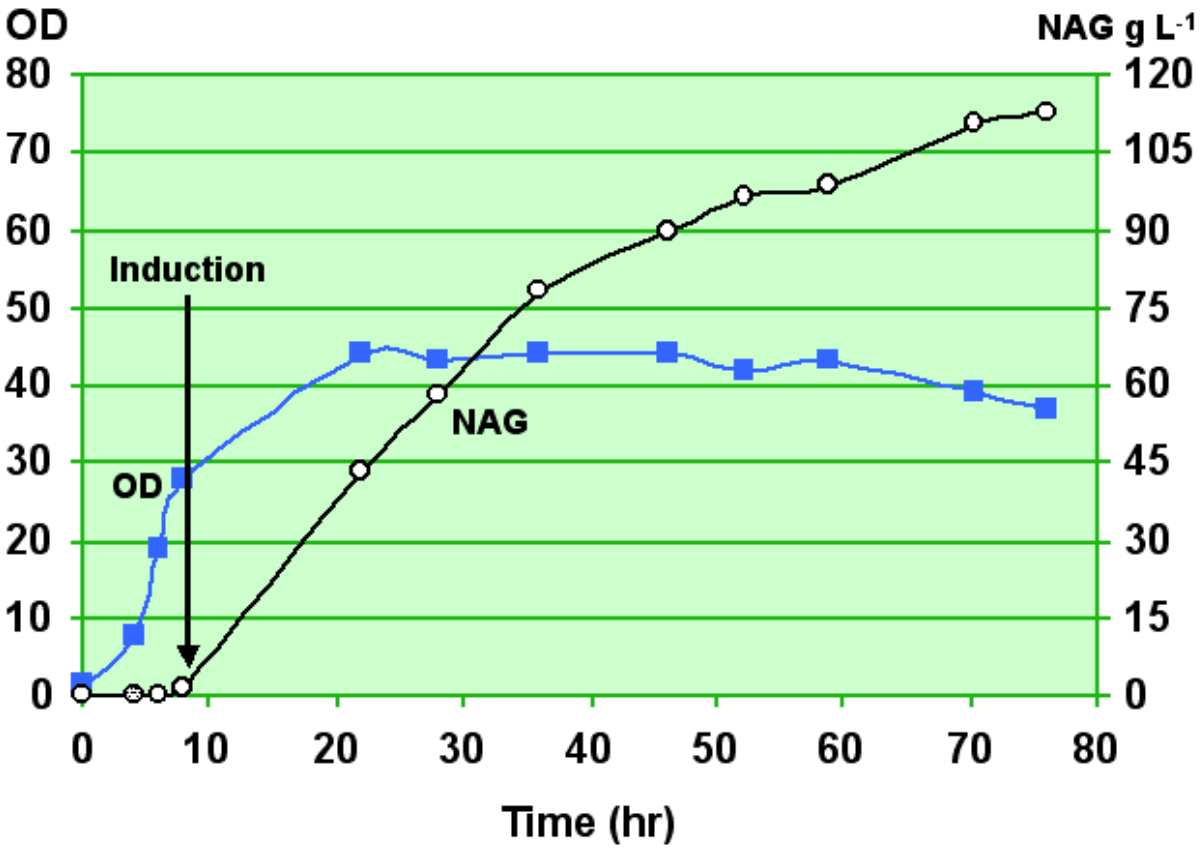
GlcNAc Production by Over-Expressing GlmS and GNA1

Strain	Construct	Activity (μmol min^{-1} mg^{-1} protein) GlmS	Activity (μmol min^{-1} mg^{-1} protein) GNA1	OD_{600}	Acetate (g L^{-1})	GlcNAc (g L^{-1})
7107-88	Vector	0.53	0.1	3.75	4.4	ND*
7107-87	*S. cerevisiae GNA1*	0.48	19.0	7.80	ND	11.7
7107-117	*C. albicans GNA1*	0.36	22.3	10.00	0.5	5.1
7107-93	*A. thaliana GNA1*	0.32	6.0	8.70	ND	8.0

*Not detectable

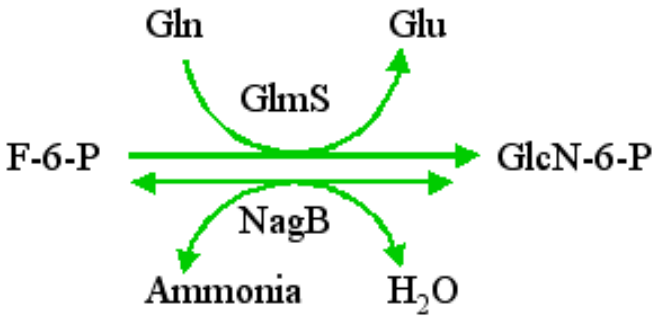
- Glucosamine-6-P N-acetyltransferase (GNA1) converts glucosamine-6-P to *N*-acetylglucosamine-6-P (GlcNAc-6-P).
- Heterologous *GNA1* genes were co-expressed with an improved *glmS* gene encoding product-resistant glucosamine synthase in *E. coli.*
- GlcNAc were produced at high levels in growth medium.

Lactose-Induced GlcNAc Production



- Efficient fermentation process was developed for GlcNAc (NAG) production using an *E. coli* strain containing integrated *T7lac-glmS*54* and *T7lac-GNA1* expression cassettes.
- Fermentation in one-liter fermentors was run in a fed batch mode. Cells were grown to an OD_{600} of about 28 and induced with lactose.
- NAG levels in broth reached 110 g L^{-1} at 72 hrs.

Construction of *nagB* Over-Expression *E. coli* Strains



GlmS: catalyzes GlcN-6-P synthesis - irreversible

NagB: reacts in both directions - catabolic reaction strongly favored

***nagB*: encoding glucosamine-6-P-deaminase**

W3110

↓ *nag* deletion, *manXYZ* mutation, Lysogenization of a DE3 element

7101-17 (DE3)

↓ Integration of *T7lac-nagB* cassette at the Δ*pfkB* site

7107-645

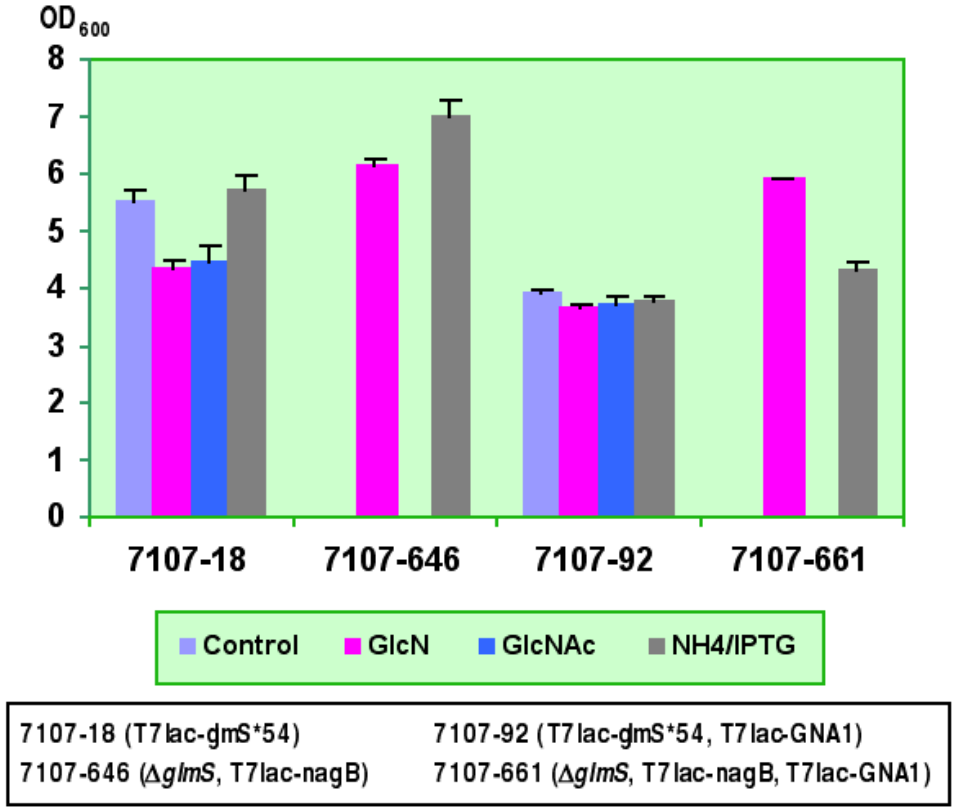
↓ *glmS* deletion

7101-646

↓ Integration of *T7lac-GNA1* cassette at the Δ*manXYZ* site

7107-660
7107-661

Suppression of GlcN Auxotrophy in *glmS* Mutants by *nagB* Over-Expression



- *glmS* deletion mutants were GlcN auxotrophs, no growth in control medium, no growth with GlcNAC due to deletion of the transporter genes *nagE* and *manXYZ*.
- NagB over-expression resulted in GlcN-6-P synthesis to support growth of *glmS* deletion mutants.
- Little or no GlcN was detected due to the catabolic nature of NagB.

Production of GlcNAc by Over-Expressing both *nagB* and *GNA1* Genes

Strain*	Relevant Genotype	Enzyme Activity (µmol min^{-1} mg^{-1} protein)			GlcNAc ($g\ L^{-1}$)
		NagB	GlmS	GNA1	
7101-17	Control	ND**	0.08	ND	ND
7107-607	*T7-glmS*54* + *T7-GNA1*	ND	0.22	3.8	30.0
7107-660(1)	*T7-nagB* + T7-*GNA1*	2	ND	3.5	5.5
7107-660(4)	*T7-nagB* + T7-*GNA1*	42	ND	2.9	18.7
7107-661(1)	*T7-nagB* + T7-*GNA1*	52	ND	3.5	22.0
7107-661(2)	*T7-nagB* + T7-*GNA1*	62	ND	4.1	22.1
7107-661(3)	*T7-nagB* + T7-*GNA1*	58	ND	3.9	24.2

*Numbers in parentheses indicate different siblings.
**Not detectable.

- Strains were grown for 72 hrs in M9B medium in shake flasks and induced with lactose from time of inoculation.
- GlcNAc was produced at high levels in the medium of strains with *nagB+GNA1* expressing cassettes, reaching as high as 80% of the level reached by *glmS*54+GNA1* expression strain.

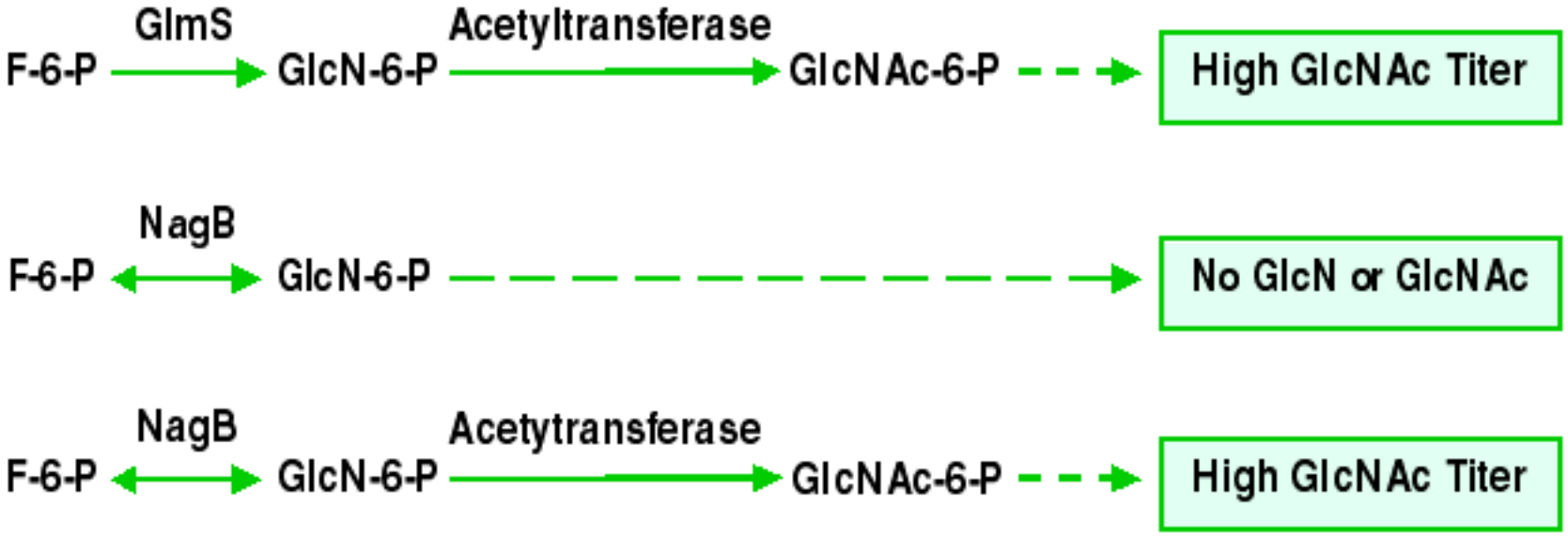
in vitro Synthesis of GlcN and GlcNAc

Strain	Relevant Genotype	GlcN/GlcNAc (μmol min^{-1} mg^{-1})		
		None	NH_4^+	Glutamine
7107-18	*T7lac-glmS*54*	0	0	0.22
7107-92	*T7lac-glmS*54, T7lac-GNA1*	0	0	0.31
7107-646	*T7lac-nagB*	0	0.19	0
7107-661	*T7lac-nagB, T7lac-GNA1*	0	0.23	0

- Crude enzyme extracts were prepared from IPTG-induced cultures and incubated with NH_4Cl (200 mM) and glutamine (20 mM) as amino donors. Amounts of amino sugars formed were determined.
- As expected, NagB enzyme uses only ammonia as the amino donor in GlcN-6-P formation.

Conclusions

- Over-expression of GNA1 played a critical role in determining the direction and efficiency of the upstream reaction catalyzed by NagB.
- A kinetically unfavorable enzyme could be used in constructing a desired metabolic pathway by coupling with an efficient downstream reaction.



Reference: Deng, Wassink and Grund. Enzyme & Microbial Tech. **39**:828-834 (2006)