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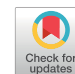
# Metabolic engineering of *Corynebacterium glutamicum* S9114 based on whole-genome sequencing for efficient *N*-acetylglucosamine synthesis



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ABSTRACT

Glucosamine (GlcN) and its acetylated derivative *N*-acetylglucosamine (GlcNAc) are widely used in the pharmaceutical industries. Here, we attempted to achieve efficient production of GlcNAc via genomic engineering of *Corynebacterium glutamicum*. Specifically, we ligated the *GNA1* gene, which converts GlcN-6-phosphate to GlcNAc-6-phosphate by transferring the acetyl group in Acetyl-CoA to the amino group of GlcN-6-phosphate, into the plasmid pJYW4 and then transformed this recombinant vector into the *C. glutamicum* ATCC 13032, ATCC 13869, ATCC 14067, and S9114 strains, and we assessed the GlcNAc titers at 0.5 g/L, 1.2 g/L, 0.8 g/L, and 3.1 g/L from each strain, respectively. This suggested that there were likely to be significant differences among the key genes in the glutamate and GlcNAc synthesis pathways of these *C. glutamicum* strains. Therefore, we performed whole genome sequencing of the S9114 strain, which has not been previously published, and found that there are many differences among the genes in the glutamate and GlcNAc synthesis pathways among the four strains tested. Next, *nagA* (encoding GlcNAc-6-phosphate deacetylase) and *gamA* (encoding GlcN-6-phosphate deaminase) were deleted in *C. glutamicum* S9114 to block the catabolism of intracellular GlcNAc, leading to a 54.8% increase in GlcNAc production (from 3.1 to 4.8 g/L) when grown in a shaker flask. In addition, lactate synthesis was blocked by knockout of *ldh* (encoding lactate dehydrogenase); thus, further increasing the GlcNAc titer to 5.4 g/L. Finally, we added a key gene of the GlcN synthetic pathway, *glmS*, from different sources into the expression vector pJYW-4-ceN, and the resulting recombinant strain CGGN2-*GNA1*-*CgglmS* produced the GlcNAc titer of 6.9 g/L. This is the first report concerning the metabolic engineering of *C. glutamicum*, and the results of this study provide a good starting point for further metabolic engineering to achieve industrial-scale production of GlcNAc.

## 1. Introduction

*N*-acetylglucosamine (GlcNAc) is widely present in nature as a derivative of glucose. This monosaccharide is mainly used as an additive in medicine, food, health care, and other related fields, and has been shown to effectively treat arthritis and participate in liver and kidney detoxification and liver protection [1,2]. Moreover, GlcNAc has been widely used as a food ingredient in many developed countries. At present, the production methods of GlcNAc mainly include chitin hydrolysis, biotransformation, and microbial synthesis. Compared with the former two, the production method via microbial synthesis has many advantages, including a short production time, high yield, high efficiency, and limited environmental impacts.

To date, the strains used for glucosamine (GlcN) and GlcNAc production include the fungi *Aspergillus* sp. BCRC 31742 [2,3] as well as recombinant *Escherichia coli* [4,5] and *Bacillus subtilis* [6–8] strains. By pathway design and modular optimization, the titer of GlcNAc can reach as high as 17 g/L and 13.2 g/L by recombinant *E. coli* and *B. subtilis* in shake flasks [6–8]. For fermentation of filamentous fungal, GlcN production from the fungal cell wall requires acid hydrolysis and a long culture period; the low productivity of this approach weakens its economic competitiveness when compared to conventional extraction approaches. In addition to filamentous fungal fermentation, GlcN and GlcNAc can also be produced using engineered *E. coli* or *B. subtilis*. *E. coli* is not generally recognized as safe (GRAS) for production of food additives, which limits the applications of the GlcN and GlcNAc produced from this bacteria in regard to the nutraceutical and pharmaceutical industries [9]. Moreover, *B. subtilis* typically produces spores in

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the late stage of fermentation, and this affects the fermentation production process [10]. Therefore, in this study, we selected an alternative host bacterium, *Corynebacterium glutamicum*, and engineered these strains to generate production levels of GlcNAc.

*C. glutamicum* is currently the most widely used corynebacterium in industrial fermentation for the production of amino acids, including the strains ATCC 13032, ATCC 13869, ATCC 14067, and other subspecies [11,12]. In recent years, the entire genomes of *C. glutamicum* ATCC 13032, *C. glutamicum* ATCC 13869, and *C. glutamicum* ATCC 14067 have been sequenced, creating favorable conditions for the metabolic engineering of *C. glutamicum* in regard to high-yield amino acid production [13–16]. However, none of the three strains have the capability to produce glutamate, which is the prerequisite for GlcNAc production [17–23]. Therefore, we selected the strain S9114, with its high-yield glutamate capability, for comparison with the three previously mentioned strains. This strain has been registered and preserved in the China Center for Type Culture Collection, and the collection number is CCTCCAB2019183. However, the complete genome sequence of S9114 has not been previously released.

Although *C. glutamicum* can absorb glucose as a carbon source, the pathway for glucose synthesis of GlcNAc in *C. glutamicum* is incomplete. Therefore, we first exogenously expressed the *GNA1* gene (encoding glucosamine-6-phosphate acetyltransferase, which converts GlcN-6-phosphate to GlcNAc-6-phosphate by transferring the acetyl group in Acetyl-CoA to the amino group of GlcN-6-phosphate, and not present in *C. glutamicum* genome) from *Caenorhabditis elegans*, making *C. glutamicum* capable of producing GlcNAc. The *GNA1* gene was ligated into the plasmid pJYW4 [20], and the recombinant vector was verified. We transformed this recombinant vector into the *C. glutamicum* ATCC 13032, ATCC 13869, ATCC 14067, and S9114 strains, and initially selected the host strain expressing the highest levels of GlcNAc. Second, in order to improve the expression levels of *glmS* (encoding GlcN-6-phosphate synthase, which is the first rate-limiting enzyme in GlcNAc synthesis pathway and converts fructose-6-phosphate to GlcN-6-phosphate with glutamine acting as an amino donor), we added *glmS* genes from different sources downstream of the *GNA1* gene and selected for the *glmS* gene with the highest expression. Third, in order to better perform genome editing of the S9114 strain, we performed genome-wide sequencing. The *nagA* (encoding GlcNAc-6-phosphate deacetylase) and *gamA* (encoding GlcN-6-phosphate deaminase) genes were knocked out, as based on their genomic sequences, to block the degradation of GlcNAc by the *C. glutamicum* host strain. In addition, to eliminate the formation of major acidic by-products, we completely blocked the lactate synthesis by knocking out *ldh* (encoding lactate dehydrogenase). After a series of transformations, we successfully introduced a heterologous synthetic pathway for GlcNAc and optimized the endogenous GlcNAc related metabolic pathways of *C. glutamicum* (Fig. 1). Importantly, this study is the first to reveal the whole genome sequence of *C. glutamicum* S9114, and this will prove useful for the metabolic engineering of *C. glutamicum* S9114 for the production of other products in the future.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids, and materials

The primers, strains, and plasmids used in this study are shown in Tables 1 and 2. The *C. glutamicum* ATCC 13032, ATCC 13869, ATCC 14067, and S9114 strains were used as initial host bacteria. *C. glutamicum* ATCC 13032, ATCC 13869, and ATCC 14067 are laboratory-preserved strains, and S9114 strain was obtained from Dr. Liming Liu (State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi, P. R. China). Plasmid pJYW-4 was a gift from Dr. Xiaoyuan Wang (State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi, P. R. China). The recombinant plasmid pJYW-4-ceN, which was used as the expression vector, was constructed as detailed in our previous work [19].

All microorganisms were grown at 30 °C in LBB broth (Luria–Bertani broth with 18.5 g/L brain heart infusion) or on LBB agar plates. LB consists of 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl). The seed medium contained the following components in units of g/L: glucose, 25.0; corn syrup, 20.0; KH2PO4, 1.0; (NH4) 2SO4, 0.5; and urea, 1.25; and was adjusted to a final pH of 7.0. The fermentation medium contained the following components in units of g/L: glucose, 100.0; corn syrup, 10.0; $KH_2PO_4$, 1.0; $(NH_4)\ _2SO_4$, 20.0; $MgSO_4$, 0.5; $CaCO_3$, 20.0; and $FeSO_4$, 0.18 adjusted to a final pH of 7.0. A total of 25 mg/L kanamycin was added to all media for detection of transformants or for recombinant culture. A single colony grown on the LBB plate was inoculated into the seed culture medium. After 16–18 h of culture, the bacterial culture medium of the seed culture was inoculated into the fermentation medium at an initial $OD_{562}$ of 1.6 and grown for 72 h. Samples were taken every 12 h.

The *E. coli* strain JM109 was used as the host for plasmid construction. The PrimeSTAR HS DNA polymerase, restriction endonucleases, PCR reagents, Genomic Extraction Kit, Blunting Kination Ligation (BKL) Kit, and DNA purification kit were purchased from Takara (Dalian, China). The ClonExpress II One Step Cloning Kit was purchased from Vazyme (Nanjing, China). All other chemicals and reagents were of analytical grade. Sequence determinations were completed by Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China).

### 2.2. DNA manipulation and construction of plasmids

The isolation and manipulation of recombinant DNA were performed using standard protocols. *C. glutamicum* transformation was performed via electrotransformation. The modified DNA fragments and plasmids were sequenced by Sangon Biotech Co., Ltd. (China). The *C. glutamicum* ATCC 13032, ATCC 13869, ATCC 14067, and S9114 strains were prepared into competence according to the preparation method of *C. glutamicum* competence. The recombinant plasmid pJYW-4-ceN, containing the *Caenorhabditis elegans* gene *GNA1*, was transformed into competent *C. glutamicum* cells, and transformants were selected to perform the fermentation. After 72 h of fermentation, we assessed the accumulation of GlcNAc in the different strains over time during the fermentation process.

### 2.3. De novo genome assembly of C. glutamicum S9114

The genome of *C. glutamicum* S9114 was sequenced by integrating Illumina paired-end sequencing and PacBio single-molecule real-time sequencing technologies. Terminal degrees and long readings were obtained on the Illumina HiSeq 2000 platform and the PacBio sequencing platform, respectively. The Illumina readings were processed using NGS QC Toolkit [24] version 2.3 software for quality inspection and low-quality data filtering. The PacBio reads were corrected with high-quality Illumina reads using PacBioToCA [25] and then assembled using Celera Assembler [26] version 8.0. All coding genes were annotated using Prokaryotic Genome Annotation Pipeline (PGAP) version 2.9 software at the NCBI (http://www.ncbi.nlm.nih.gov/genome/annotation prok/) and Rapid Annotation using the Subsystem Technology (RAST; http://rast.nmpdr.org/) server [27]. Additional functional annotations were performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) database [28]. The metabolic pathways were established using the KEGG automatic annotation server (KAAS; http://www.genome.jp/tools/kaas/).

### 2.4. Gene knockouts

The method for constructing knockout mutations in *C. glutamicum* is described in Hu et al. [29]. The pBluscriptII SK (+) plasmid was preserved in the laboratory as a starting plasmid. This plasmid contained

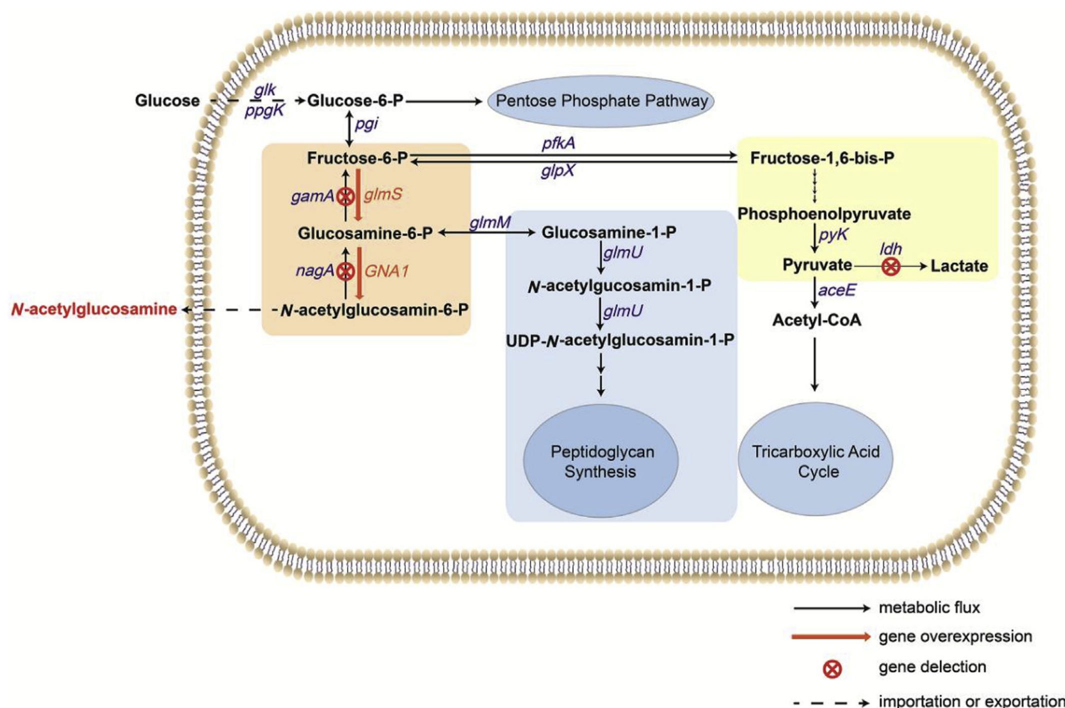


**Fig. 1.** The metabolic pathway of *N*-acetylglucosamine (GlcNAc) synthesis in *C. glutamicum*.

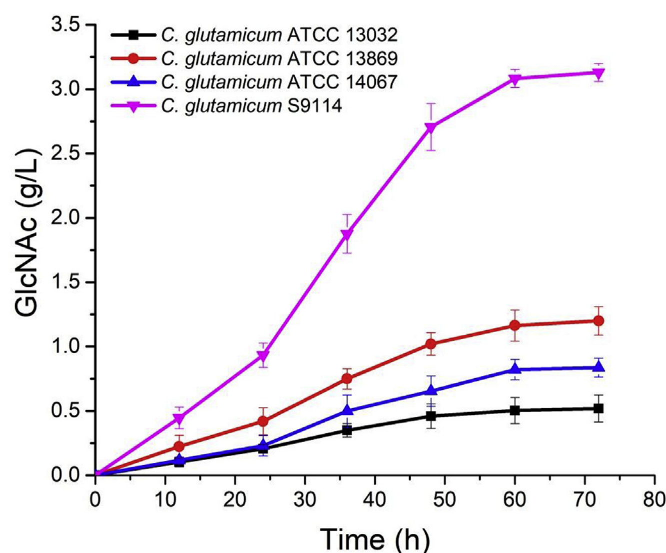


**Fig. 2.** The GlcNAc titers of *C. glutamicum* ATCC 13032-*GNA1* (black line), *C. glutamicum* ATCC 13869-*GNA1* (red line), *C. glutamicum* ATCC 14067-*GNA1* (blue line), and *C. glutamicum* S9114-*GNA1* (pink line). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

**Table 1**
Primers used in this study.

| Primer | Sequence (5'→3′) |
|---|---|
| na-galoxPU F | ATCTCGAGGGTCAAAGGTGATTTCACCGGCGAATT |
| na-galoxPU R | ACCTCTAGAGGAAACGGCCACATCGCTTTCAATGAGC |
| na-galoxPD F | ACGGATCCAGGAAACGCGCCACCGTTTCC |
| na-galoxPD R | CCGGAATTCTCCTTGGTGCCTGCAAGAACGCCA |
| kan-loxp-F | ACCTCTAGAGCGCAATTAACCCTCACTAAAG |
| kan-loxp-R | ATGGATCCAATACGACTCACTATAGGGCG |
| ldhloxPU F | ATCTCGAGGACCTTAATTGCATCGACTGCTTGT |
| ldhloxPU R | ACCTCTAGACCCCAACCCATTACGTTGGTG |
| ldhloxPD F | ACGGATCCCGGCGAATTAACCCAGCAC |
| ldhloxPD R | CCGGAATTCCTTGCTTGGGAGTTTTCAACCATTC |
| loxpy F | AACCGCTGGCAGGTCCGTCGATA |
| loxpy R | CCAGCAGTTACGGGAACGCGG |
| C.gl-*glmS* F | GCGCTTTTAAGCTGCAACTTAATTATGGTCCTCCC |
| C.gl-*glmS* R | CTTTGCTAGTTTATTCGACGGTGACAGACTTTGC |
| B.su-*glmS* F | CAGCGCTTTTAACCAAAAAACATGTAGGAGGGGACG |
| B.su-*glmS* R | TCCTTTGCTAGTTTACTCCACAGTAACACTCTTCGCAAG |
| E.co-*glmS* F | GCGCTTTTAAATCCCGCGAAATTAATACGACTCAC |
| E.co-*glmS* R | TCCTTTGCTAGTTTACTCAACCGTAACCGATTTTGCC |
| P4–C.gl-*glmS* F | CGTCGAATAAACTAGCAAAGGAGAAGAAAAGCCG |
| P4–C.gl-*glmS* R | TAAGTTGCAGCTTAAAAGCGCTGGGTCATAAAATTACAGTCA |
| P4–B.su-*glmS* F | CTGTGGAGTAAACTAGCAAAGGAGAAGAAAAGCCGGA |
| P4–B.su-*glmS* R | CATGTTTTTTGGTTAAAAGCGCTGGGTCATAAAATTACAGTCA |
| P4-E.co-*glmS* F | CGGTTGAGTAAACTAGCAAAGGAGAAGAAAAGCCGGA |
| P4-E.co-*glmS* R | TTCGCGGGATTTAAAAGCGCTGGGTCATAAAATTACAGT |

only *E. coli* replicons and no *C. glutamicum* replicon. Therefore, the plasmid could not be autonomously replicated in *C. glutamicum*, facilitating the removal of the plasmid in subsequent procedures.

First, we designed knockout cassettes for the *nagA* and *gamA* genes to block the pathway by which the host strain *C. glutamicum* degrades GlcNAc. To increase the knockout efficiency, the length of the front and back regions was designed to be approximately 1000 bp. Using the *C. glutamicum* S9114 genomic sequence as a template, primers na-galoxPU F, na-galoxPU R, na-galoxPD F, and na-galoxPD R were used in PCR to amplify the homologous arm fragments na-gaU and na-gaD of the *nagA-gamA* gene.

The DNA fragment was recovered using a gel recovery kit. The plasmid pDTW202 carrying the Kana resistance gene fragment loxp-kan-loxp cassette was used as a template, and the kan-loxp-F and kan-loxp-R primers were used to amplify the loxp-kan-loxp cassette. The *E. coli*-derived pBluscriptII SK(+) plasmid was extracted using a plasmid extraction kit, and the DNA concentration was determined. The fragment na-gaU was digested with *Xho*I and *Xba*I, the fragment loxp-kan-loxp cassette was digested with *Xba*I and BamHl, the fragment na-gaD was digested with *Bam*HI and *Eco*RI, and the plasmid pBluscriptII SK (+) was digested with *Xho*I and *Eco*RI. All the digested fragments were recovered using a gel recovery kit, and the DNA concentrations were determined. The enzymatically purified fragments na-gaU, na-gaD, and

**Table 2**
Strains and plasmids used in this study.

| Name | Description | Source |
|---|---|---|
| Strains | | |
| *C. glutamicum* ATCC 13032 | Wild type *C. glutamicum* | Laboratory stock |
| *C. glutamicum* ATCC 13869 | Wild type *C. glutamicum* | Laboratory stock |
| *C. glutamicum* ATCC 14067 | Wild type *C. glutamicum* | Laboratory stock |
| *C. glutamicum* S9114 | Wild type *C. glutamicum* | Laboratory stock |
| *C. glutamicum* ATCC 13032-*GNA1* | *C. glutamicum* ATCC 13032 derivate, overexpression of *C. elegans GNA1* gene | This work |
| *C. glutamicum* ATCC 13869-*GNA1* | *C. glutamicum* ATCC 13869 derivate, overexpression of *C. elegans GNA1* gene | This work |
| *C. glutamicum* ATCC 14067-*GNA1* | *C. glutamicum* ATCC 14067 derivate, overexpression of *C. elegans GNA1* gene | This work |
| *C. glutamicum* s9114-*GNA1* | *C. glutamicum* S9114 derivate, overexpression of *C. elegans GNA1* gene | This work |
| CGGN1 | *C. glutamicum* S9114 derivate: Δ*nagA*-Δ*gamA* | This work |
| CGGN1-*GNA1* | CGGN1 derivate, overexpression of *C. elegans GNA1* gene | This work |
| CGGN2 | CGGN1 derivate: Δ*nagA*-Δ*gamA*-Δ*ldh* | This work |
| CGGN2-*GNA1* | CGGN2 derivate, overexpression of *C. elegans GNA1* gene | This work |
| CGGN2-*GNA1-CgglmS* | CGGN2-*GNA1* derivate, overexpression of *C. glutamicum glmS* gene | This work |
| CGGN2-*GNA1-BsglmS* | CGGN2-*GNA1* derivate, overexpression of *B. subtilis glmS* gene | This work |
| CGGN2-*GNA1-EcglmS* | CGGN2-*GNA1* derivate, overexpression of *E. coli glmS* gene | This work |
| **Plasmids** | | |
| pJYW-4-ceN | pJYW-4 derivate, Ptac-*GNA1*,Kan$^r$ | [19] |
| pTYW-4-ceN-C.gl*glmS* | pJYW-4-ceN derivate with C.g*glmS* cloned | This work |
| pTYW-4-ceN-B.su*glmS* | pJYW-4-ceN derivate with B, s*glmS* cloned | This work |
| pTYW-4-ceN-E.co*glmS* | pJYW-4-ceN derivate with E.c*glmS* cloned | This work |
| pBluescript IISK(+) | Cloning vector | [18] |
| pDTW-109 | Vector carry *Cre* | [18] |
| pDTW-202 | pBluescript IISK(+) carry the segment loxp-kan-loxp, Amp$^r$ | [18] |
| pDTW-NG | pBluescript IISK(+) carry the *nagA/gamA* genes knockout cassette | This work |
| pDTW-LDH | pBluescript IISK(+) carry the *ldh* gene knockout cassette | This work |

Amp$^r$, ampicillin resistance; Kan$^r$, kanamycin resistance.

loxp-kan-loxp cassette were ligated to the pBluscriptII SK(+) plasmid using T4 DNA ligase at a molar ratio of 1:3 (plasmid: fragment). The ligation was then transformed into competent *E. coli* JM109 cells and plated onto Kana-resistant LB plates for screening. The resulting correct transformants carried the plasmid pDTW-NG, which harbored the *nagA/gamA* knockout cassette.

The correctly sequenced pDTW-NG plasmid was transformed into the *C. glutamicum* S9114 strain and then coated onto Kana-resistant LBB plates. The upstream and downstream homologous arms of *nagA/gamA* underwent homologous recombination with the upstream and downstream homologous arms of the *C. glutamicum* S9114 genome *nagA/ gamA* genes, with the loxp-kan-loxp cassette replacing the *nagA/ gamA* gene fragment within the genome, thereby achieving the goal of deletion of these genes in the genome. The obtained recombinant genome carried the loxp-kan-loxp cassette fragment, and the lox sequence at both ends of the Kana resistance gene could be specifically recognized and cleaved by the Cre enzyme. The plasmid pDTW-109 carrying the *Cre* gene was further electroporated into the mutant strain. In addition to carrying the *Cre* gene, this plasmid also carried a temperature-sensitive replicon (normal copy at 25 °C, cannot copy at 37 °C or higher) and a chloramphenicol resistance gene. LBB plates containing kanamycin and chloramphenicol were separately streaked at 25 °C, and transformants that grew only on the chloramphenicol plates were selected and further cultured in a 37 °C incubator to remove the pDTW-109 plasmid. Finally, primers loxpy F and loxpy R were used to verify the strains containing both homologous arms bound to the genome. The resulting correct transformant was named *C. glutamicum* S9114-Δ*nagA*-Δ*gamA* (CGGN1).

The *ldh* gene was knocked out using the same method as for the *nagA* and *gamA* genes. The primers for amplifying the left and right arms of the *ldh* gene knockout frame were: ldhloxPU F, ldhloxPU R, ldhloxPD F, and ldhloxPD R. The plasmid with the *ldh* gene knockout cassette was named pDTW-LDH. The transformant with the further knock out of the *ldh* gene was named *C. glutamicum* S9114-Δ*nagA*-Δ*gamA*-Δ*ldh* (CGGN2).

### 2.5. Enhancing gene expression

In our previous work, the expression vector pTYW-4 was used to clone the gene encoding glucosamine deacetylase, *GNA1,* of *C. elegans*, which was ligated into plasmid pTYW-4-ceN, which was constructed as an expression vector for the *GNA1* gene. Additionally, in order to increase the expression level of another key gene, *glmS*, in the GlcNAc synthesis pathway, we added the *glmS* gene derived from *C. glutamicum* S9114, *B. subtilis* 168, or *E. coli* K-12 MG1655 to the 3′ untranslated region of the *GNA1* gene, allowing it to be co-expressed with *GNA1* in the same expression frame.

The *glmS* genes were amplified from *C. glutamicum* S9114, *B. subtilis* 168, and *E. coli* K-12 MG1655 using the primers C.gl-*glmS* F and C.gl-*glmS* R, B.su-*glmS* F and B.su-*glmS* R, and E.co-*glmS* F and E.co-*glmS* R, respectively. The linearized pTYW-4-ceN vectors were amplified with the primers P4–C.gl-*glmS* F and P4–C.gl-*glmS* R, P4–B.su-*glmS* F and P4–B.su-*glmS* R, and P4-E.co-*glmS* F and P4-E.co-*glmS* R. The *glmS* genes and the linearized pTYW-4-ceN vector were then ligated together via Gibson assembly, yielding the recombinant plasmids pTYW-4-ceN-C.gl*glmS*, pTYW-4-ceN-B.sul*glmS*, and pTYW-4-ceN-E.co*glmS*. All of the recombinant plasmids were then transformed into the *C. glutamicum* S9114-Δ*nagA-gamA* strain via electroporation.

### 2.6. Analytic methods

The concentrations of GlcNAc and lactate in the fermentation broth were measured via high-performance liquid chromatography on an instrument equipped with an HPX-87H column (Bio-Rad Hercules, CA, USA) and a refractive index detector. High-performance liquid chromatography was carried out with 5 mM $H_2SO_4$ as the mobile phase with a flow rate of 0.6 mL/min at 35 °C. To assess for intracellular GlmS and *GNA1* activities, cells were harvested during fermentation via centrifugation at 10,000 × *g* and 4 °C for 10 min and then disrupted with ultrasonic oscillation (Ultrasonic processor, Sonics Vibra-Cell, Ultrasonic processor VCX 750 W; Sonics & Materials, Newtown, CT, USA; amplitude 30%, time 30 min). After centrifugation at 10,000 × *g* and 4 °C for 10 min, the supernatant was used to determine the GlmS

activity levels [8]. One unit of GlmS activity was defined as the amount of enzyme that produced 1 pmoL/min of GlcN-6-phosphate under the conditions assayed. Protein concentrations were detected using the protein concentration kit SK3041 from Sangon Biotech Co., Ltd. (China). The glucose concentration in the supernatant was measured using a glucose-glutamate analyzer (SBA-40C; Biology Institute of Shandong Academy of Sciences, Jinan, China).

### *2.7. Statistical analysis*

All the experiments were performed independently at least three times and the results were expressed in means ± standard deviations (SD).

## 3. Results

### *3.1. Production of GlcNAc by the different C. glutamicum strains*

After culture of the different *C. glutamicum* strains, it was found that the GlcNAc production of the *C. glutamicum* ATCC 13032, ATCC 13869, ATCC 14067, and s9114 strains were 0.5 g/L, 1.2 g/L, 0.8 g/L, and 3.1 g/L, respectively, after 72 h (Fig. 3B). Since the GlcNAc production of *C. glutamicum* S9114 was quite different from that of the other three strains, we would like to have compared and analyzed the genomic differences between the S9114 strain and the ATCC 13032, ATCC 13869, ATCC 14067 strains. However, the complete genome sequence of strain S9114 has not been previously published. As such, we sequenced the complete genome of strain S9114 by way of *de novo* genome assembly.

### *3.2. Whole genome sequencing of C. glutamicum S9114*

The whole genome sequences of *C. glutamicum* ATCC 13032, ATCC 13869, and ATCC 14067 are all known, but the continuous whole genome sequence of *C. glutamicum* strain S9114 has not been published, making subsequent genome editing of the *C. glutamicum* strain S9114 difficult. Therefore, we utilized the new generation of bacterial genome *de novo* sequencing for generating the genome sequence of the S9114 strain. Bacterial genome *de novo* sequencing involves the *de novo* assembly of the bacterial genome after sequencing. The procedure first involves randomly cleaving the stained DNA of the bacteria into fragments of a certain relative molecular mass range and then to construct two sets of libraries for large-scale sequencing. The gaps in the sequence are filled after preliminary assembly. Gap filling is a key step, and it usually takes several combinations of assembly methods to complete, which can be time-consuming and require significant effort. The ability to complete the gap filling can often be the key to a complete genome-wide sequence. The final assembly level is based on the needs of the study and the characteristics of the bacteria itself. The highest indicator is a contig, which is the complete sequence of the genome. On the basis of the assembly, subsequent genomic component analysis, functional annotation, and other analyses are performed to determine whether an ORF is a real protein coding sequence as well as to check for functional sites, analyze consensus or characteristic sequences, assess for single gene or inter-gene interactions, and to define expression regulation and other functions. Bacterial *de novo* sequencing has replaced other traditional methods and is an important tool for studying the genetic mechanisms of bacterial evolution and identifying key functional genes.

The complete genome of *C. glutamicum* S9114 consists of a circular chromosome of 3,353,693 bp with a GC content of 54.86%. In total, 3097 identified protein-coding sequences (CDS) were encoded by 86.52% of the whole genomic DNA. A total of 59 tRNAs and 18 rRNA gene copies were also detected (Table 3). The genome data have been deposited in the NCBI Sequence Read Archive (SRA) (accession number: SRP198722).

We made a brief comparative analysis of genome sequence of ATCC 13032, ATCC 13869, ATCC 14067, and *C. glutamicum* S9114. The preliminary results showed that some notable sequences of some genes related to the glutamate, lysine, valine, serine and arginine synthesis pathway differences in these *C. glutamicum* strains, which might lead to difficulties in amino acid production rates. Genome analysis revealed that the genome of *C. glutamicum* S9114 had a complete set of genes for glutamate biosynthesis. Many base alterations were found within the key genes potentially relevant to GlcNAc synthesis, including key genes in the GlcNAc biosynthesis pathway (*pgi*, *glmS*), cell wall biosynthesis and glutamate transport (*murE*, *ftsI*, and *dtsR*), NADPH generation (*zwf*, *gnd*, *malE*,*ppnk*, etc.), biosynthesis pathways (*pyc*, *pdh*, *pepC*, *odhA*, and *gltB*), and key genes in the glutamate biosynthesis pathway (*gdh*, *gltD*, etc.) (Fig. 3A). According to the alignment of the genome, it was determined that the nucleotide sequences proximal the catalytic center of *glmS* of the three *C. glutamicum* strains, ATCC 13032, ATCC 13869, and ATCC 14067, differed from those of *C. glutamicum* S9114 (Fig. 3B). In particular, *glmS* of *C. glutamicum* ATCC 13032 and *C. glutamicum* S9114 have distinctly different sequences, and at least some of the base changes detected in this analysis may contribute to the high glutamate production capacity and high GlcNAc production capacity of S9114.

### *3.3. Effects of nagA/gamA deletion on the accumulation of GlcNAc in C. glutamicum*

In *C. glutamicum* S9114, intracellular GlcNAc could still be degraded by *N*-deacetylation and deaminated by GlcNAc deacetylase (NagA) and GlcN deaminases (GamA) (Fig. 1) [6]. These intracellular catabolic reactions would hamper GlcNAc accumulation and should be eliminated to block GlcNAc catabolism. Therefore, we deleted the *nagA* and *gamA* genes in S9114 strain by homologous recombination, yielding the recombinant strain CGGN1, in which the GlcNAc and GlcN catabolic pathways were completely deleted. The deletion mutant strain CGGN1 was verified by colony PCR using the corresponding primers loxpy F and loxpy R (Fig. 4A), and the vector pJYW-4-ceN was transformed into CGGN1 to strengthen the GlcNAc biosynthetic pathway during the fermentation process. Fig. 4B shows the GlcNAc production of the metabolically engineered strains *C. glutamicum* S9114-*GNA1* and CGGN1-*GNA1*, and it is apparent that the deletion of the *nagA* and *gamA* genes significantly enhanced GlcNAc production, indicating that deletion of amino sugar catabolism was highly effective for promoting the accumulation of GlcNAc. Deletion of the *nagA* and *gamA* genes thoroughly blocked the *N*-deacetylation and deamination processes and improved the GlcNAc production of CGGN1-*GNA1* to 4.8 g/L, which was 54.8% higher than that produced by *C. glutamicum* S9114. In addition, it was determined that there was only a slight decrease in the $OD_{562}$ with the knockout of the *nagA* and *gamA* genes, indicating that cell growth was not significantly impacted when the degradation pathway for GlcNAc was blocked in *C. glutamicum* (Fig. 4C).

### *3.4. Effects of blocking lactate formation on GlcNAc production*

The accumulation of lactic acid, a by-product of the GlcNAc synthesis pathway, is toxic to cellular growth and competes with the synthesis of GlcNAc for carbon resources. To enhance GlcNAc synthesis, we used gene knockout to block the formation of lactate in *C. glutamicum* completely. First, the *ldh* gene encoding L-lactate dehydrogenase, which catalyzes the formation of lactate from pyruvate, was knocked out. As expected, no lactate was detected during fermentation of CGGN2-*GNA1* (Fig. 5A). After blocking the lactate synthesis pathway, the $OD_{562}$ was significantly enhanced, and the titer of GlcNAc was also increased to 5.4 g/L (Fig. 5B).

### *3.5. Influence of GNA1 and glmS overexpression on GlcN and GlcNAc production*

Our laboratory has employed metabolic engineering to construct a

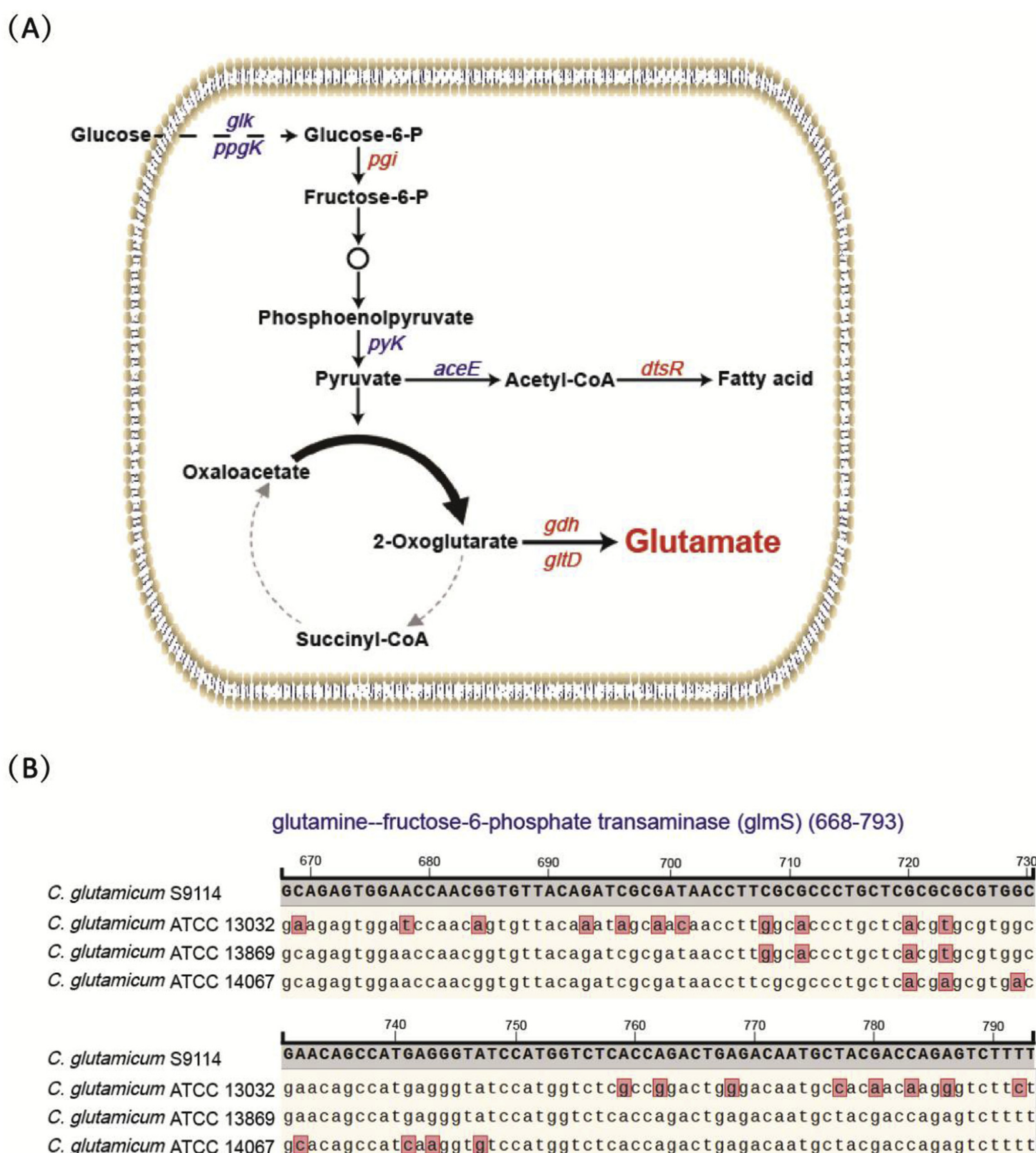


**Fig. 3.** Key genes in the glutamate synthesis pathway in *C. glutamicum* and a homologous alignment of *glmS* genes. (A) Overview of the glutamate production mechanism in *C. glutamicum.* (B) A partial nucleotide sequence alignment map of the *glmS* genes from different sources. Bases within the red box represent non-conserved bases.

**Table 3**
Basic features of the *C. glutamicum* S9114 genome.

| Features | Chromosome |
|---|---|
| Length [bp] | 3,353,693 |
| Total number of genes | 3162 |
| G + C content [%] | 54.86% |
| CDS | 3097 |
| 5s_rRNA (De novo) | 6 |
| 16s_rRNA (De novo) | 6 |
| 23s_rRNA (De novo) | 6 |
| sRNA | 7 |
| tRNA | 59 |

recombinant *C. glutamicum* that can produce GlcNAc. Our previous study inserted the *GNA1* gene into the pJYW-4 plasmid. In this study, the *glmS* gene, a key enzyme of the GlcN synthesis pathway, was added to this expression vector. Recombinant *C. glutamicum* CGGN2 strain was constructed by over-expressing GlmS from different sources, and the accumulation of the target product GlcNAc was examined. The three different source genes that express GlmS that were assessed in this experiment were *CgglmS* (from *C. glutamicum*, NCBI CP004048.1), *BsglmS* (from B. subtiiis, NCBI NC_ 000964.3), and *EcglmS* (from *E. coli*. K12, NCBI CP032667.1). All of the *glmS* genes were amplified from the genome of their corresponding strains. Finally, the *glmS* genes were each individually inserted into the 3′ untranslated region of the *GNA1* gene.

Three recombinant strains CGGN2-*GNA1-CgglmS*, CGGN2-*GNA1-BsglmS,* and CGGN2-*GNA1-EcglmS* were cultured in shaker flasks, and the yields of GlcNAc were sampled and tested. After fermentation, the GlcNAc titer was substantially enhanced after blockage of the lactate synthesis pathway (Fig. 6A). During the entire fermentation process, the GlcNAc titer of the three recombinant strains increased, and the accumulation of extracellular GlcNAc reached a maximum in the three recombinant strains at 72 h. It can be seen from Fig. 6 that the titer of GlcNAc in the CGGN2-*GNA1-CgglmS* strain increased rapidly in the early stage, and reached the maximum value of 6.9 g/L at 72 h, and the GlcNAc yields of GlcNAc on glucose is 69 mg/g. The titer of GlcNAc and

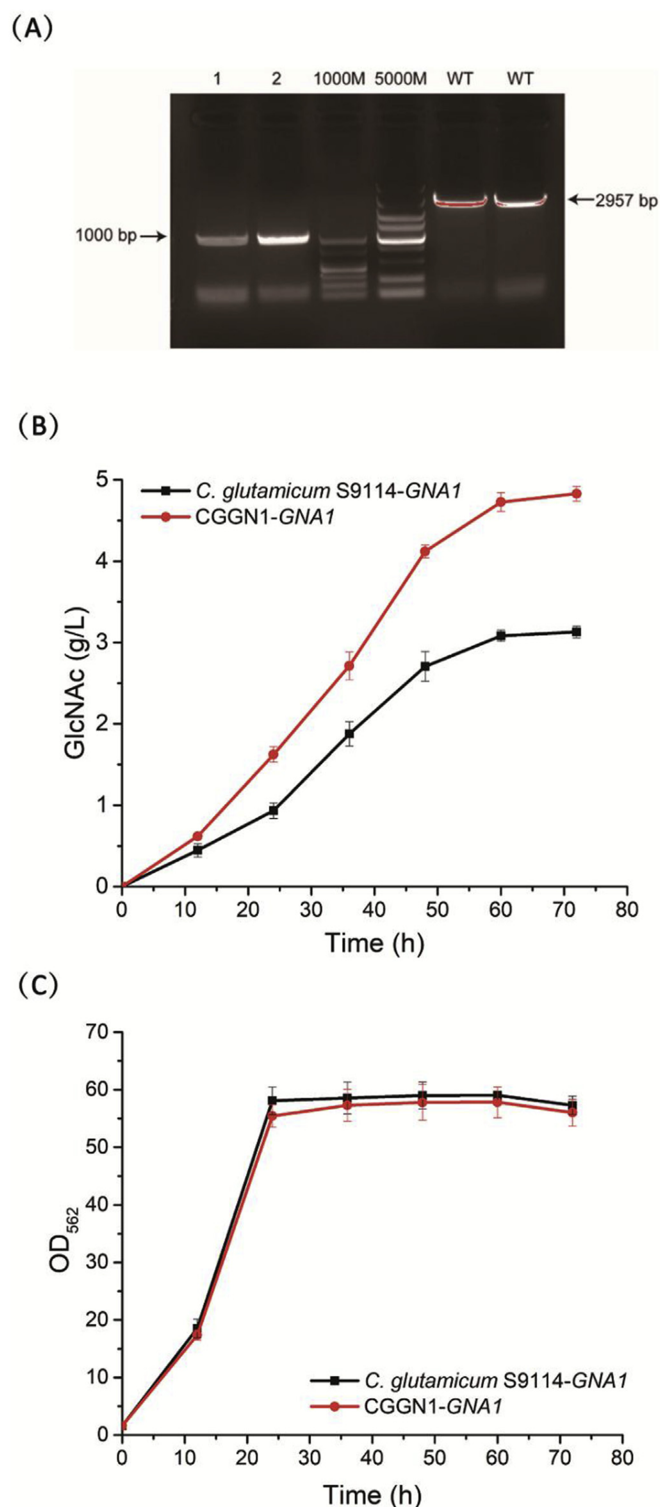


**Fig. 4.** Effects of the knockout of *nagA* and *gamA* genes on cell growth and GlcNAc production. (A) Confirmation of the knockout of *nagA* and *gamA*. 1000 M: DL1000 DNA marker; 5000 M: DL5000 DNA marker; all samples used loxpy F/loxpy R as validation primers; 1, 2: CGGN1Δ*nagA*Δ*gamA*:kan as the template; WT: original S9114 strain as the template. (B) GlcNAc titers of *C. glutamicum* S9114-*GNA1* (black line) and CGGN1-*GNA1* (red line). (C) Cell growth of *C. glutamicum* S9114-*GNA1* (black line) and CGGN1-*GNA1* (red line).

the GlcNAc yields of GlcNAc on glucose in the recombinant strain CGGN2-*GNA1-CgglmS* were 7.8%, 13.4%, and 27.8% higher than that of CGGN2-*GNA1-EcglmS* (6.4 g/L, 64 mg/g), CGGN2-*GNA1-BsglmS* (6.1 g/L, 61 mg/g), and CGGN2-*GNA1* (5.4 g/L, 54 mg/g) strains, respectively. The maximum GlcNAc volumetric productivity of strain CGGN2-*GNA1*, CGGN2-*GNA1-EcglmS*, CGGN2-*GNA1-BsglmS* and CGGN2-*GNA1-CgglmS* was 0.07 g/L/h, 0.11 g/L/h, 0.08 g/L/h, 0.13 g/L/h, respectively (Fig. 6B). Together, these observations indicated that the recombinant CGGN2-*GNA1-CgglmS* strain had the best cumulative production effect on the target product GlcNAc.

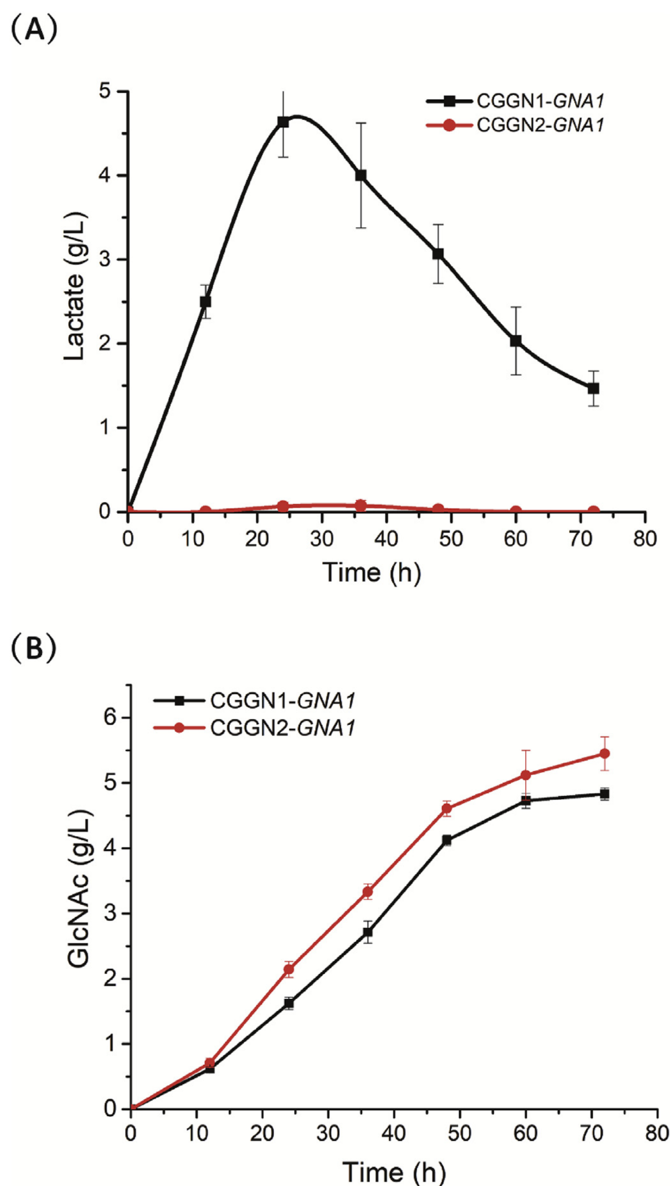


**Fig. 5.** Effects of the knockout of *ldh* gene on GlcNAc production. (A) Concentration of the acidic by-product lactate during CGGN1-*GNA1* (black line) and CGGN2-*GNA1* (red line) culture. (B) GlcNAc titers of CGGN1-*GNA1* (black line) and CGGN2-*GNA1* (red line). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The intracellular fractions collected after cell lysis of the three recombinant strains were subjected to enzyme activity analyses. The results of the GlmS enzyme activity assays are shown in Fig. 6C. The GlmS activity of the recombinant strain CGGN2-*GNA1-CgglmS* was 513.8 U/mg, which was higher than the recombinant strains CGGN2-*GNA1-EcglmS* (467.1 U/mg), CGGN2-*GNA1-BsglmS* (435.4 U/mg), or CGGN2-*GNA1* (204.6 U/mg), demonstrating an increase of 10%, 18%, and 104.6%, respectively. Together, these results demonstrated that the recombinant enzyme *CgglmS* from the CGGN2-*GNA1-CgglmS* strain

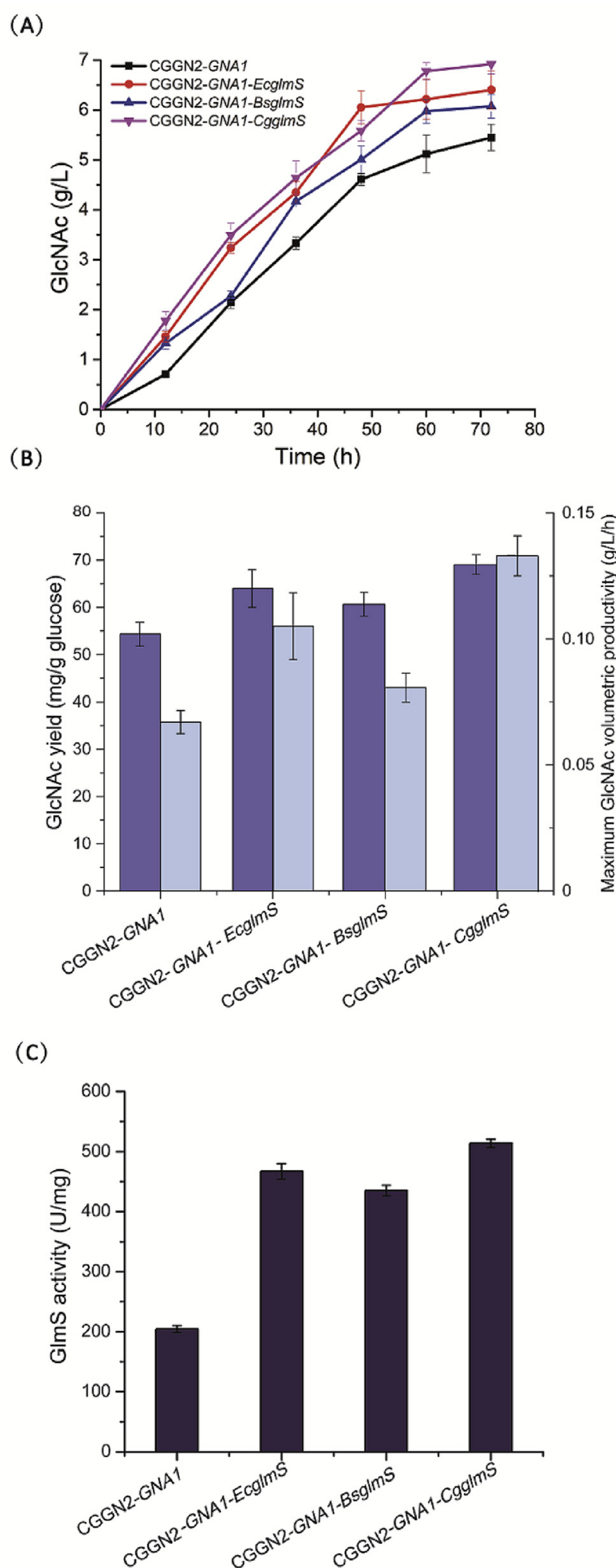


**Fig. 6.** Shake flask fermentation of recombinant strains containing *glmS* from different sources. (A) GlcNAc titers of CGGN2-*GNA1* (black line), CGGN2-*GNA1-CgglmS* (red line), CGGN2-*GNA1-BsglmS* (blue line), and CGGN2-*GNA1-EcglmS* (pink line). (B) Yields of GlcNAc on glucose (dark blue bar) and maximum GlcNAc volumetric productivity (wathet blue bar) in the recombinant strains. (C) The comparison of GlmS activity. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

demonstrated the best catalytic efficiency.

## 4. Discussion

*C. glutamicum* is a Gram-positive bacterium and has been widely used for the industrial production of amino acids, including monosodium glutamate, and lysine, and it can also utilize carbohydrate derivatives such as *N*-acetyl-glucosamine and sialic acid as carbon sources [13,30]. In recent years, with the gradual improvement of the genomic information and genome editing tools related to *C. glutamicum*, this bacterium has developed into a model microorganism capable of producing a variety of bio-based chemicals, materials, and fuels, including polygalacturonic acid, 2-ketoisovalerate, and succinate [31,32]. By way of metabolic engineering, *C. glutamicum* is also capable of producing amino acid derivatives, including amino sugars. Additionally, the production of non-amino–acid compounds by *C. glutamicum* has also been explored by a number of labs in recent years.

Table 4 shows the progress of GlcNAc production in different strains in recent years. *E. coli* produces endotoxin, which is not suitable for food and medicine, and lacks post-translational modification mechanism. Compared with *E. coli*, *C. glutamicum* has more advantages in food safety index and its metabolic pathway is simpler. In addition, the clarification of metabolic regulation mechanisms and the improvement of related vector systems in recent years have enabled us to carry out genome transformation of *C. glutamicum* by molecular biology methods. Although *B. subtilis* is a food safety strain, its physiological characteristics also have some deficiencies in industrial production. First of all, *B. subtilis* can form spores in the stable period, which increases the difficulty of controlling engineering bacteria in the production process and is easy to cause environmental pollution by engineering bacteria. Compared with *B. subtilis*, *C. glutamicum* has the advantages of rapid growth, no spore production, no secretion of extracellular protease, stable transcriptome, no odor generation during fermentation, etc. It is considered as a safe expression host and suitable for large-scale production and fermentation [33]. Compared to *B. subtilis*, *C. glutamicum* also has a superior ability to synthesize glutamate, and glutamate is an essential precursor of GlcNAc [17,34]. Therefore, this report details the construction of a *C. glutamicum* strain that can produce GlcNAc, and this was achieved via metabolic modification and optimization of genes expression.

The most commonly used *C. glutamicum* strains mainly include the three subspecies: ATCC 13032, ATCC 13869, and ATCC 14067. In recent years, the *C. glutamicum* ATCC 13032, *C. glutamicum* ATCC 13869, and *C. glutamicum* ATCC 14067 genomes have been sequenced and published. However, due to differences in their genomes, they each have the ability to produce different amino acids: ATCC 13032 has the ability to produce lysine [35], ATCC 13869 has the ability to produce valine and serine [15], and ATCC 14067 is capable of high-yield arginine production [16]. Since the synthesis goal of this study was GlcNAc, which requires glutamate to provide amino groups, we utilized a high-yield glutamate strain, S9114, as one possible expression host. After fermentation, it was apparent that the S9114 strain has the strongest ability to produce GlcNAc among the strains tested. By comparing the nucleotide sequences of the *glmS* genes, a key gene in GlcNAc synthesis, among the four strains mentioned above, we found that there were many different nucleotides encoded at its key catalytic sites, resulting in obvious differences in the amino acid composition of GlmS among the different strains, which was likely one of the reasons for the differences in GlcNAc production among these strains. Based on genomic sequence comparison, we also found that some synthesis pathways involved in amino acid production in these four strains, such as glutamate, lysine, and other amino acids, also had sequence differences in important key genes, which would likely lead to different amino acid production capacities. These results provide indications regarding the importance of choosing the most appropriate starting strain in selection breeding experiments for the production of amino acids or related substances. In

**Table 4**
Comparison of GlcNAc production by different strains.

| Strains | Culture method | Titer (g/L) | Source |
|---|---|---|---|
| *E. coli* 7107-18 | fed batch fermentation in 1-L fermentor | 110 | [9] |
| *Aspergillus* sp. BCRC 31742 | batch fermentation in 3-L fermentor | 14.37 | [3] |
| *Bacillus subtilis* 168 | fermentation in 500 mL shake flask culture | 5.19 | [6] |
| *B. subtilis* strain BSGN6-P xylA -*glmS* | fermentation in 500 mL shake flask culture | 13.2 | [7] |
| *B. subtilis* strain BNX122 | fed batch fermentation in 3-L fermentor | 103.1 | [40] |
| *B. subtilis* strain BSGN13 | fed batch fermentation in 3-L fermentor | 82.5 | [41] |
| *C. glutamicum* CGGN2-*GNA1-CgglmS* | fermentation in 500 mL shake flask culture | 6.9 | This work |

the future, we intend to produce other derivatives of amino acids or amino acids in *C. glutamicum*, and it will be important to rationally choose the starting strain according to the level of expression of key genes among the selected strains.

In previous studies, *C. glutamicum* has been used to produce amino acids and to produce other products, such as vitamins and succinic acid [12]. Because *C. glutamicum* has a good molecular basis for efficient transformation and a wide range of metabolic networks, this study attempted to produce saccharides using *C. glutamicum*. Based on alterations to the metabolic network, including knocking out the *nagA* and *gamA* genes, we were able to block the degradation of GlcNAc by the host strain, allowing intracellular GlcNAc to accumulate at increased levels.

In addition to the strength of the GlcNAc synthesis module, the formation of lactate and the competitive effects of glycolysis and peptidoglycan synthesis also affected the GlcNAc titer and yield. Therefore, only engineering the GlcNAc pathway was insufficient to enhance GlcNAc production substantially. For lactate synthesis pathways, which promote nonessential metabolism that competes for carbon resources and energy, we took a gene knockout approach to block the formation of acidic by-products completely. Reducing non-essential metabolism is a demonstrably effective approach for boosting cellular properties in optimized prokaryotic cell factories [36,37]. Therefore, further deletion of dispensable regions of *C. glutamicum* is a promising strategy for enhancing GlcNAc production. In follow-up experiments, we intend to continue to search for the GlcNAc-competitive pathways and knock out the non-essential genes of the growth of the bar in an effort to reduce production of by-products and, thus, optimize the production of GlcNAc.

Finally, overexpression of key genes is an important aspect of metabolic engineering. In *B. subtilis*, the metabolic pathway for GlcN production is strictly regulated, and the expression of GlmS, a key enzyme of the GlcN synthesis pathway, is controlled by a riboswitch [38,39]. When intracellular GlcN-6-P accumulates, GlcN-6-P binds to the GlmS riboswitch, and the transcription product of the GlmS-encoding gene is degraded, reducing the expression of GlmS. However, it is not clear whether a GlmS riboswitch exists in *C. glutamicum*, so we introduced additional *glmS* genes from different sources downstream of the *GNA1* gene in the pJYW-4-ceN expression vector. Ultimately, it was found that the GlcNAc yield following the introduction of *glmS* from *C. glutamicum* was the highest. Additionally, comparing the structures of *glmS* from different sources allowed us to further our understanding regarding the key aspects of its catalysis. By comparing the nucleotide sequences of the *glmS* genes, we found that there were many different nucleotides encoded at its key catalytic sites, resulting in obvious differences in the amino acid composition of glmS among the different strains, which was likely one of the reasons for the differences in GlcNAc production among these strains. Readers can compare the similarities and differences of related genes according to their needs.In subsequent research, we intend to determine if the GlmS riboswitch is functional in *C. glutamicum*.

In conclusion, this study provided a food-safe bacteria strain for microbial production of non-shellfish GlcNAc, and this may widen the application of GlcNAc in the nutraceutical and pharmaceutical industries. Moreover, additional genome comparisons may provide additional avenues for altering *C. glutamicum* S9114 for further optimization of GlcNAc production.

## Competing financial interests

The authors declare that they have no competing financial interests.

## Acknowledgments

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