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# Engineering of *Corynebacterium glutamicum* for growth and L-lysine and lycopene production from *N*-acetyl-glucosamine

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**Abstract** Sustainable supply of feedstock has become a key issue in process development in microbial biotechnology. The workhorse of industrial amino acid production *Corynebacterium glutamicum* has been engineered towards utilization of alternative carbon sources. Utilization of the chitin-derived aminosugar *N*-acetyl-glucosamine (GlcNAc) for both cultivation and production with *C. glutamicum* has hitherto not been investigated. Albeit this organism harbors the enzymes *N*-acetylglucosamine-6-phosphatedeacetylase and glucosamine-6P deaminase of GlcNAc metabolism (encoded by *nagA* and *nagB*, respectively) growth of *C. glutamicum* with GlcNAc as substrate was not observed. This was attributed to the lack of a functional system for GlcNAc uptake. Of the 17 type strains of the genus *Corynebacterium* tested here for their ability to grow with GlcNAc, only *Corynebacterium glycinophilum* DSM45794 was able to utilize this substrate. Complementation studies with a GlcNAc-uptake deficient *Escherichia coli* strain revealed that *C. glycinophilum* possesses a *nagE*-encoded EII permease for GlcNAc uptake. Heterologous expression of the *C. glycinophilum nagE* in *C. glutamicum* indeed enabled uptake of GlcNAc. For efficient GlcNac utilization in *C. glutamicum*, improved expression of *nagE* with concurrent overexpression of the endogenous *nagA* and *nagB* genes was found to be necessary. Based on this strategy, *C. glutamicum* strains for the efficient production of the amino acid L-lysine as well as the carotenoid lycopene from GlcNAc as sole substrate were constructed.

**Keywords** *Corynebacterium glutamicum* · *N*-Acetyl-glucosamine · NagA · NagE · PTS · L-Lysine · Lycopene · *Corynebacterium glycinophilum*

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## Introduction

The non-pathogenic, Gram-positive *Corynebacterium glutamicum* is traditionally employed in the industrial production of amino acids, in particular L-glutamate and L-lysine (about 2.2 and 1.5 million tons/year) (Wendisch 2007). This bacterium has been genetically engineered to become a versatile cell factory for the production of various bulk chemicals apart from amino acids (Becker and Wittmann 2012; Zahoor et al. 2012). Nowadays, the production of diamines (e.g., cadaverine and putrescine; Mimitsuka et al. 2007; Schneider and Wendisch 2010), organic acids (e.g., succinate, 2-ketoisovalerate, 2-ketoisocaproate, and pyruvate; Bückle-Vallant et al. 2014; Krause et al. 2010; Litsanov et al. 2012; Okino et al. 2008; Wieschalka et al. 2012), biofuels (e.g., ethanol and isobutanol; Blombach et al. 2011; Inui et al. 2004), compatible solutes (ectoine; Becker et al. 2013), and carotenoids (e.g., lycopene, β-carotene, and zeaxanthin;

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Heider et al. 2012, 2013) have been achieved using engineered *C. glutamicum* strains. *C. glutamicum* can use a variety of carbon sources for its growth and energy supply, such as sugars (e.g., glucose, fructose, sucrose, maltose, ribose), alcohols (ethanol and *myo*-inositol), and organic acids (acetate, propionate, L-lactate, gluconate, pyruvate) (Arndt and Eikmanns 2008; Blombach and Seibold 2010; Claes et al. 2002; Krings et al. 2006; Stansen et al. 2005). Feedstock costs affect to a large extent the overall fermentation costs, therefore further development of sustainable biorefinery processes relies apart from the optimization of endogenous substrate utilization pathways also on developing access to alternative carbon sources mostly derived from cheap, renewable resources (Blombach and Seibold 2010; Buschke et al. 2013). Although *C. glutamicum* possesses the capacity to grow with D-lactate unlike other corynebacteria (Kato et al. 2010) and even grows on sialic acid (Gruteser et al. 2012), its substrate spectrum is somewhat restricted as compared to, e.g., *Escherichia coli*. In some instances such as utilization of dicarboxylates or glucosamine endogenous genes had to overexpressed in *C. glutamicum* since their expression levels were too low to sustain efficient growth (Uhde et al. 2013; Youn et al. 2008; Youn et al. 2009). Therefore, various approaches to increase the substrate spectrum and flexibility of *C. glutamicum* strains have been assessed (Buschke et al. 2013; Zahoor et al. 2012): Apart from enabling direct utilization of glycerol (Meiswinkel et al. 2013b; Rittmann et al. 2008) and polymeric carbohydrates such as starch (Seibold et al. 2006; Tateno et al. 2007) by *C. glutamicum*, strains with a broadened substrate spectrum towards utilization of the lignocellulosic biomass derived sugars arabinose and xylose have been developed (Gopinath et al. 2011; Kawaguchi et al. 2006, 2008; Meiswinkel et al. 2013a; Schneider et al. 2011).

The amino sugar *N*-acetyl-D-glucosamine (GlcNAc) is the monomer of chitin, the second most abundant polysaccharide in nature after cellulose (Chen et al. 2010). Chitin is found, e.g., in the exoskeleton of crustaceans. In shrimp production, the shells of these animals make up as much as 75 % of the waste with roughly half being chitin (Bhattachrya et al. 2007). With an annual harvest of about 6 million tons of shrimp (Gillett 2008), chitin from shellfish waste represents a large resource of substrate for potential applications in biorefinery (Bhattachrya et al. 2007; Hayes et al. 2008). Acid hydrolysis of chitin releases GlcNAc (Chen et al. 2010), which has been recently used as a substrate for bioethanol production by *Mucor* species (Inokuma et al. 2013) and for growth of genetically modified *Saccharomyces cerevisiae* strains (Wendland et al. 2009). To our knowledge utilization of GlcNAc by Corynebacterianeae has not been investigated so far. In *E. coli*, GlcNAc is taken up and concomitantly phosphorylated by the *nagE*-encoded EII of the GlcNAc-specific phosphoenolpyruvate: carbohydrate phosphotransferase system (PTS) yielding *N*-acteyl-glucosamine-6-phosphate (Lengeler 1980; White 1970), which is then deactelylated by the *nagA*-encoded deactylase to glucosamine 6-phosphate (White 1968). Glucosamine-6-phosphate is in turn converted to the glycolysis intermediate fructose-6-phosphate by the *nagB* encoded deaminase (Comb and Roseman 1956; Nakada and Wolfe 1956). The *nagA* and *nagB* genes are located adjacent to each other) and form an operon together with *scrB* in *C. glutamicum* (Kalinowski et al. 2003; Engels et al. 2008a). Overexpression of *nagB* either achieved by ectopic expression or caused by a suppressor mutation upstream of the *nagA* gene in the strain *C. glutamicum* M4 was shown to be essential for efficient utilization of the amino sugar glucosamine as a combined source of carbon, energy and nitrogen in *C. glutamicum* (Uhde et al. 2013). Glucosamine is taken up and phosphorylated in *C. glutamicum* by the *ptsG*-encoded EII permease of the PTS that is mainly responsible for glucose uptake, the formed glucosamine-6-phosphate is then converted by the *nagB*-encoded glucosamine 6-phosphate deaminase (NagB) to fructose-6-P (Uhde et al. 2013). Furthermore, *C. glutamicum* can utilize *N*-acetylneuaminic acid (Neu5Ac) as a substrate for growth (Gruteser et al. 2012). Following uptake by the *cg2937* to *cg2940* encoded ABC-transporter, Neu5Ac is catabolized to fructose-6-P in *C. glutamicum* by a pathway which involves formation of the intermediate *N*-acetyl-glucosamine 6-phosphate and requires activities of both the *nagA*-encoded deactelyase and NagB (Gruteser et al. 2012). Thus, we concluded that *C. glutamicum* possesses at least a functional metabolic pathway for GlcNAc utilization. The existence of a functional uptake system for GlcNAc in *C. glutamicum* remains to be investigated. Here, members of the genus *Corynebacterium* were investigated for their ability to use GlcNAc for growth. We observed efficient GlcNAc utilization by *C. glycinophilum* DSM45794 but not by *C. glutamicum* strains. We describe identification of the *nagE*-encoded EII permease for GlcNAc uptake in *C. glycinophilum* and establish GlcNAc utilization in *C. glutamicum*. Finally we employ heterologous expression of *C. glycinophilum nagE* together with overexpression of *C. glutamicum nagAB* in *C. glutamicum* model production strains and demonstrate production of the amino acid L-lysine and the carotenoid lycopene from a novel carbon source.

## Materials and methods

### Microorganisms, plasmids, and cultivation conditions

The corynebacterial strains used in this study were *C. glutamicum* ATCC13032, *C. glutamicum* ATCC14751, *C. glutamicum* ATCC14752, *C. glutamicum* ATCC15455, *C. glutamicum* ATCC31830, *C. glutamicum* ATCC31832, *C. glutamicum* DSM20137, *Corynebacterium glycinophilum*

DSM45794, *Corynebacterium melassecola* ATCC17965, *C. melassecola* ATCC22243, *C. melassecola* ATCC22220, *Brevibacterium divariatum* ATCC14020, *Brevibacterium immariophilum* ATCC14068, *Brevibacterium thiogenitalis* ATCC19240, *Brevibacterium chang-fua* ATCC14017, *Microbacterium ammoniaphilum* ATCC15354 and *Corynebacterium* sp. ATCC14747. All these strains were obtained from the American Type Culture Collection (ATCC) or from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen). The lysine-producing strain *C. glutamicum* DM1729 (*lysC*$^{P458S}$, *hom*$^{V59A}$, *pyc*$^{T311I}$) (Georgi et al. 2005), the lycopene-producing strain *C. glutamicum* Δ*crtYEB* (Heider et al. 2012) as well as the spontaneous mutant strain *C. glutamicum* M4 (Uhde et al. 2013) are derived from *C. glutamicum* ATCC13032. The strain *E. coli* DH5α (Hanahan 1983) was used for plasmid construction. *E. coli* LR2-168 (Lengeler et al. 1981) was provided by J. Plumbridge (Institut de Biologie Physico-Chimique, CNRS UPR9073, Paris, France). The plasmids used in this study are listed in Table S1. The CGXII minimal medium used for *C. glutamicum* has been described previously (Eggeling and Reyes 2005) and contained glucose, acetate and/or GlcNAc in concentrations indicated in the Results section, to prepare CGGXII minimal medium agar plates for growth tests with Corynbacterianeae 16 g/l Difco select agar (Becton, Dickinson and Company, Le Ponte de Caix, France) were added. LB medium (Sambrook and Russell 2001) was used as complex medium for *C. glutamicum* and for *E. coli*. MacConkey indicator plates containing 1 % (w/v) GlcNAc were prepared as described (Lengeler 1975). When appropriate, kanamycin (50 μg ml$^{-1}$), spectinomycin (20 μg ml$^{-1}$) and/or isopropyl-ß-D-thio-galactopyranoside (IPTG; 100 μM) were added to the medium. If not stated otherwise, *C. glutamicum* was grown aerobically at 30 °C, *E. coli* at 37 °C as 50-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 120 rpm. Growth of *E. coli* and of *C. glutamicum* in liquid cultures was followed by measuring the optical density at 600 nm ($OD_{600}$). For strain optimization *C. glutamicum* batch cultivations were performed in the Biolector® cultivation system (m2pLabs, Baesweiler) using 1 ml medium microtiter plates (FlowerPlate®; m2pLabs, Baesweiler) at 1,100 rpm at 30 °C and CGXII medium containing glucose or GlcNAc. L-Lysine and lycopene production experiments were performed in triplicates as 1 ml batch cultures in FlowerPlates® in the Biolector® cultivation system, presented data are from at least three independent cultivation experiments.

## Quantification of glucose, GlcNAc, lycopene, and L-lysine

For quantification of the glucose, L-lysine, lycopene, and GlcNAc concentrations in the culture fluid, aliquots of the culture were withdrawn, the cells were removed by centrifugation (5 min at 13,000×*g*) and the supernatants were analysed. Quantifications of glucose, lycopene, and L-lysine concentrations in culture supernatants were performed by HPLC as described previously (Heider et al. 2012; Lindner et al. 2011). GlcNAc concentrations were determined essentially as described by Reissig et al. 1955. In detail, 100 μl of saturated $NaHCO_3$ were added to 200 μl of the sample and incubated for 3 min at 95 °C. Afterwards samples were cooled down to room temperature before 50 μl of $Na_2CO_3$ were added. After incubation for 3 min at 95 °C 1.5 ml of Ehrlich reagent (1.25 ml 1 M HCl and 1 g *p*-dimethylaminobenzaldehyde solved in 100 ml acetic acid) were added and the samples then incubated at 37 °C for 1 h. After the incubation step the absorption of the samples was measured at 585 nm in an Ultrospec 2100 pro Spectrophotometer (GE Healthcare). Quantification of GlcNAc was done by calculation of the concentration using a six point calibration curve.

## DNA preparation, manipulation and transformation

Standard procedures were employed for chromosomal as well as plasmid DNA isolation and for molecular cloning and transformation of *E. coli*, as well as for electrophoresis (Sambrook and Russell 2001). Isolation of plasmids and chromosomal DNA of *C. glutamicum* was performed as described (Eikmanns et al. 1994). Transformation of *C. glutamicum* was performed by electroporation as described (Tauch et al. 2002). PCR experiments were performed in a Flexcycler (Analytik Jena) with Phusion DNA Polymerase (New England Biolabs) or KOD Hot Start DNA Polymerase (Novagen) and with oligonucleotides obtained from Eurofins MWG Operon listed in Table S2. All restriction enzymes, T4-DNA ligase, and shrimp alkaline phosphatase were obtained from New England Biolabs and used according to the manufacturer's instructions.

## Construction of plasmids and strains

For construction of pEKEx2-*nagEBA*, the *nagEBA* operon of *C. glycinophilum* was amplified by PCR using primers nagEBA_fw and nagEBA_rv, the 4218 bp PCR product was then cut using *Nsi*I and *Eco*RI and cloned into the *Pst*I/*Eco*RI cut plasmid pEKEx2. For construction of pEKEx2-*nagE* the transporter gene *nagE* from *C. glycinophilum* was amplified with the primer pair nagEBA_fw and nagE_rv and the 2184 bp PCR product cloned into pEKEx2. For construction of pVWEx1-*nagE*, the *nagE* gene was PCR amplified using KOD Hot Start polymerase and the primers nagE-gibs-rbs_fw and nagE-gibs_rv from genomic DNA of *C. glycinophilum* and the resulting 2178 bp PCR product ligated into the *Pst*I/*Bam*HI digested plasmid pVWEx1 via Gibson DNA assembly (Gibson et al. 2009). For overexpression of *nagAB*, the genes were PCR-amplified using genomic DNA from

*C. glutamicum* ATCC13032 as template with the primers nagAB-Ex-fw and nagAB-Ex-rv (listed in Table S1). The amplified 2003 bp product was cloned into the *Sma*I blunt end cut pEKEx3 leading to pEKEx3-*nagAB*, which allows the IPTG-inducible expression of the *nagA* and *nagB* genes in *C. glutamicum*. The recombinant plasmids were isolated from *E. coli* DH5α, controlled by sequencing (GATC Biotech AG, Konstanz, Germany) and transformed into *E. coli* LR2-168 and/or *C. glutamicum* strains.

### [$^{14}$C]-*N*-cetylglucosamine uptake studies

*C. glutamicum* cells were grown to early-exponential growth phase (3 h), harvested by centrifugation, washed twice with ice cold CGXII medium, suspended to an $OD_{600}$ of 2 with CGXII medium, and stored on ice until the measurement. Before the transport assay, cells were incubated for 3 min at 30 °C; the reaction was started by addition of 0.1 μM to 50 mM [$^{14}$C]-*N*-acetyl-glucosamine (specific activity 45 mCi mmol$^{-1}$; Hartmann Analytik, Braunschweig). At given time intervals (15, 30, 45, 60, and 90 s), 200-μl samples were filtered through glass fibre filters (Typ F; Millipore, Eschborn, Germany) and washed twice with 2.5 ml of 100 mM LiCl. Radioactivity of the samples was determined using scintillation fluid (Rotiszinth; Roth, Germany) and a scintillation counter (LS 6500; Beckmann, Krefeld, Germany).

### NagA and NagB activity assays

Exponentially growing cells of *C. glutamicum* strains were harvested by centrifugation (10 min, 3,200×*g*, and 4 °C) and washed twice with 50 mM Tris–HCl pH 8.0. Cells were disrupted by ultrasonic treatment (UP 200S; Dr. Hielscher GmbH, Teltow, Germany) with an amplitude of 50 % and a duty circle of 0.5 for 7 min. The cell suspension was centrifuged 1 h, 4 °C, and 16,000 rpm. Glucosamine 6-phosphate deaminase activity of the supernatant was determined accordingly to the assay described by Uhde et al. (2013). *N*-Acetylglucosamine-6P deacetylase activity was determined by applying a coupled assay containing 50 mM Tris–HCl pH 7.5, 1 mM $MnSO_4$, yeast glucose-6-phosphate dehydrogenase (0.5 U/ml), yeast phosphoglucoisomerase (0.7 U/ml), 0.5 mM NADP, NagB purified accordingly to Uhde et al. (2013) (1 U/ml) and 1 mM*N*-acetyl-glucosamine-6-P as substrate, monitoring spectrophotometrically the NADPH formation at 340 nm.

### Computational analysis

Databank searches and alignments were carried out by using BLAST (Altschul et al. 1990) and CLUSTAL W (Thompson et al. 1994). The Genbank accession number for the annotated genome sequences of *C. glutamicum* ATCC13032 and *C. glycinophilum* DSM45794 are NC_006958 (Kalinowski et al. 2003) and CP006842 (A. Al-Dilaimi, J. Kalinowski, C. Rückert, Bielefeld University, unpublished results), respectively. The following NCBI-GI accession numbers for protein sequences were retrieved from the KEGG database (Kanehisa et al. 2008): 16128655 — *E. coli* NagE, 16129082 — *E. coli* NagK, 19553842 — *C. glutamicum* NagB, and 62391484 — *C. glutamicum* NagA.

## Results

### The *nagA* gene encodes the *N*-acetylglucosamine-6-phosphate deacetylase in *C. glutamicum*

The *nagA* gene is co-transcribed in an operon with *nagB* which codes for glucosamine deaminase (NagB) (Engels et al. 2008a; Uhde et al. 2013). The strain *C. glutamicum* (pEKEX3-*nagAB*) overexpressing the *nagAB* operon not only showed 30-fold increased NagB activity, but also 20-fold higher NagA activity as compared to the empty vector control (Table 1). Thus, *nagA* from *C. glutamicum* encodes a functionally active *N*-acetylglucosamine-6-phosphate deacetylase (NagA).

### Overexpression of *nagAB* does not allow for growth of *C. glutamicum* on GlcNAc

Based on the described utilization of Neu5Ac as substrate for *C. glutamicum* ATCC 13032 (Gruteser et al. 2012) and the presence of NagA activity, we concluded that this organism probably possesses enzymatic capabilities to metabolize GlcNAc. However, even after prolonged incubation, no growth of *C. glutamicum* ATCC13032 was observed in cultivations in minimal medium with 1 % (w/v) GlcNAc as sole carbon source. Furthermore, no GlcNAc consumption was

**Table 1** Specific activities of *N*-acetyl-glucosamine deacetylase (NagA) and glucosamine deaminase (NagB) in various *C. glutamicum* strains and in *C. glycinophilum*

| Strain | Medium | NagA (U/mg) | NagB (U/mg) |
|---|---|---|---|
| *C. glutamicum* WT (pEKEx3) | BHI, IPTG | 0.01±0.00 | 0.01±0.01 |
| *C. glutamicum* WT (pEKEx3-*nagAB*) | BHI, IPTG | 0.20±0.02 | 0.29±0.03 |
| *C. glutamicum* M4 | BHI, IPTG | 0.45±0.05 | 0.15±0.03 |
| *C. glycinophilum* | BHI | 0.02±0.01 | 0.01±0.00 |
| *C. glycinophilum* | MM,[a] GlcNAc | 0.20±0.8 | 0.06±0.01 |

[a] CgXII minimal medium with 100 mM of the indicated carbon source was used

detected, when GlcNAc was added as additional carbon source to cultivations of *C. glutamicum* in minimal medium with 1 % (w/v) glucose or 1 % (w/v) acetate (data no shown). Since our previous work showed that *C. glutamicum* WT cannot efficiently utilize glucosamine unless endogenous *nagB* was overexpressed (Uhde et al. 2013), growth of *C. glutamicum* WT overexpressing the IPTG-induced *nagAB* operon with GlcNAc was tested. However, *C. glutamicum* WT (pEKEx3-*nagAB*) could not grow on GlcNAc, while it grew well on glucosamine (Fig. S1). In the recently isolated strain *C. glutamicum* M4, expression of the *nagAB-scrB* operon is increased due to a point mutation within the *nagA* promoter region, causing a significantly improved utilization of glucosamine as carbon source when compared to the parental strain (Uhde et al. 2013). *C. glutamicum* M4 exhibits not only higher NagB activities than the parental strain, but also about 45-fold higher NagA activities (Table 1). Nevertheless, neither growth on nor consumption of GlcNAc (data not shown) was observed for *C. glutamicum* M4. In accordance, no uptake of $^{14}$C-labelled GlcNAc was detected in *C. glutamicum* ATCC 13032 and its derivates *C. glutamicum* WT (pEKEx3-*nagAB*) and *C. glutamicum* M4 (measured at concentration of 50 μM GlcNAc). No genes for putative GlcNAc uptake systems were identified within the *C. glutamicum* genome (Kalinowski et al. 2003; Winnen et al. 2005). Blast analyses using the amino acid sequence of the *E. coli* GlcNAc kinase NagK as template (Uehara and Park 2004) revealed that *C. glutamicum* probably possesses no gene encoding such an enzyme. Taken together, *C. glutamicum* probably lacks enzymes for both GlcNAc uptake and GlcNAc phosphorylation.

## Utilization of GlcNAc as substrate in *Corynebacterium* species

Expression of genes for GlcNAc uptake systems derived from a closely related bacterium might be a strategy to obtain efficient GlcNAc utilization in *C. glutamicum* ATCC 13032. GlcNAc utilization has hitherto not been investigated within the family Corynebacteriaceae. Therefore, various strains of *Corynebacterium* species (see Materials and methods section) were pre-grown in LB complex medium and then tested for growth on solid minimal medium containing 1 % (w/v) GlcNAc. Even after prolonged incubation for 96 h growth on GlcNAc was observed for only one of the 16 species tested, namely, *C. glycinophilum* DSM45794. Growth of *C. glycinophilum* DSM45794 in liquid CGXII minimal medium cultures with either 1 % (w/v) GlcNAc or 1 % (w/v) glucose as sole substrate proceeded with comparable growth rates (growth rates of 0.14±0.01 and 0.15±0.02 $h^{-1}$, respectively; Fig. 1a) and similar final ODs of 7.9±0.7 and 8.2±1.1

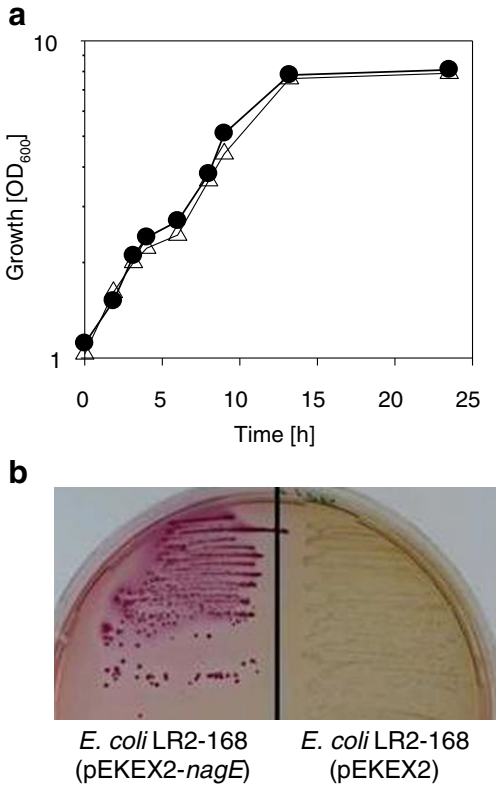


**Fig. 1** **a** Growth of *C. glycinophilum*DSM45794 in CGXII minimal media supplemented with 1 % (w/v) GlcNAc (*white triangles*) or 1 % (w/v) glucose (*black circles*). **b** Growth and coloration of *E. coli* LR2-168 (pEKEx2-*nagE*) and *E. coli* LR2-168 (pEKEx2) on MacConkey indicator plates containing 1 % (w/v) GlcNAc

were reached with the two substrates. Analyses of the *C. glycinophilum* DSM45794 genome by BLAST searches using amino acid sequences from *C. glutamicum* NagA and NagB as well as the *E. coli* NagE as templates led to the identification of a putative *nagEBA* operon in *C. glycinophilum*: The genes encoding a putative EII permease for GlcNAc (CGLY_01500; 40 % identity to *E. coli* NagE), a putative GlcNAc 6-P deacetylase (CGLY_01490; 38 % identity to *C. glutamicum* NagA) and a putative glucosamine 6-phosphate deaminase (CGLY_01495, 46 % identity to *C. glutamicum* NagB) are located adjacent to each other and orientated identically in the *C. glycinophilum* genome. To analyze its function as putative GlcNAc transporter the *nagE* gene from *C. glycinophilum* was cloned into the pEKEx2 expression vector and the resulting plasmid pEKEx2-*nagE* was transformed into the GlcNAc-uptake deficient strain *E. coli* LR2-168. The resulting strain *E. coli* LR2-168 (pEKEx2-*nagE*) was able to utilize the GlcNAc provided in MacConkey agar plates (Fig. 1b): red colonies were visible after incubation for 48 h, while no GlcNAc utilization was observed for the control strain *E. coli* LR2-168 (pEKEx2). These results show that the *C. glycinophilum nagE* gene encodes a functional EII permease for GlcNAc uptake and phosphorylation.

Heterologous expression of *nagEBA* from *C. glycinophilum* in C. *glutamicum* M4 leads to GlcNAc utilization

Expression of the *nagE*-encoded EII permease from *C. glycinophilum* in *C. glutamicum* using the plasmid pEKEx2-*nagE* allowed only marginal growth of the strain *C. glutamicum* (pEKEx2-*nagE*) in CGXII minimal medium with 1 % GlcNAc as sole substrate (Fig. 2a, final OD 2.3±

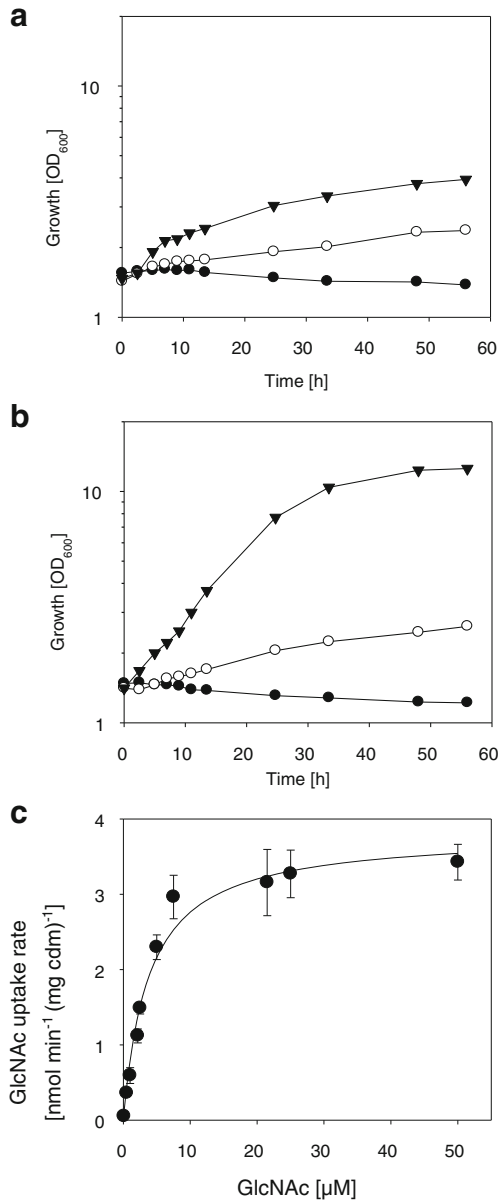


**Fig. 2** Growth of *C. glutamicum* ATCC 13032 (**a**) and *C. glutamicum* M4 (**b**) strains carrying the plasmids pEKEx2 (*filled circles*), pEKEx2-*nagE* (*open circles*), and pEKEx2-*nagEBA* (*filled triangles*) in CGXII minimal medium with 1 % (w/v) GlcNAc as sole source of carbon and energy. Kinetics of GlcNAc uptake in *C. glutamicum* M4 (pEKEx2-*nagEBA*), different concentrations of [$^{14}$C]-GlcNAc were tested (0.5–30 μM) (**c**). Data represent mean values from three independent measurements from two independent cultivations in LB medium, data were fitted according to the Michaelis–Menten equation

0.4, growth rate $\mu$ >0.01 h$^{-1}$). To establish uptake and phosphorylation of GlcNAc and to simultaneously improve metabolisation of GlcNAc-6-P the whole *nagEBA* operon from *C. glycinophilum* DSM45794 was cloned into pEKEx2 and the derived plasmid pEKEx2-*nagEBA* was then transformed into *C. glutamicum*. Indeed growth of the resulting strain C. *glutamicum* (pEKEx2-*nagEBA*) on GlcNAc was slightly improved ($\mu$=0.02±0.01 h$^{-1}$, final OD 3.8±0.3) when compared to growth of *C. glutamicum* (pEKEx2-*nagE*) (Fig. 2a). In addition pEKEx2-*nagE* was introduced in *C. glutamicum* M4, which has an increased expression of the *nagAB* operon due to a point mutation in its promoter region (Uhde et al. 2013). Growth of this strain *C. glutamicum* M4 (pEKEx2-*nagE*) on 1 % (w/v) GlcNAc was improved (μ 0.02± 0.01 h$^{-1}$, final OD 2.5±0.3) when compared to *C. glutamicum* pEKEx2-*nagE* but still too slow for any application. Nevertheless, overexpression of the *nagEBA* operon in *C. glutamicum* M4 resulted in even better growth on GlcNAc as sole carbon source. Growth of the strain *C. glutamicum* M4 (pEKEx2-*nagEBA*) proceeded with a rate of 0.07±0.02 h$^{-1}$ and a final OD of 12.3±1.3 was reached after 48 h of cultivation (Fig. 2b). Characterization of GlcNAc uptake in *C. glutamicum* M4 (pEKEx2-*nagEBA*) using [$^{14}$C] labeled substrate as a tracer revealed a Michaelis–Menten type kinetics for the NagE mediated uptake of GlcNAc with a $K_M$ of 3.8±0.6 μM and a $V_{max}$ of 3.8±0.2 nmol min$^{-1}$ (mg cdm$^{-1}$) (Fig. 2c). The data show that the uptake of GlcNAc in *C. glutamicum* M4 (pEKEx2-*nagEBA*) proceeds rather slowly but the substrate affinity of NagE for GlcNAc is high.

Taken together, we conclude that NagE from *C. glycinophilum* is suited to catalyze GlcNAc uptake in *C. glutamicum*; however, for efficient GlcNAc utilization in *C. glutamicum* strains expression of *nagE* from *C. glycinophilum* alongside overexpression of *C. glutamicum nagA* and *nagB* must be accomplished. To test this hypothesis, a *C. glutamicum* strain able to use GlcNAc efficiently as sole source of carbon and energy was rationally designed. For overexpression of both *nagA* and *nagB* in *C. glutamicum*, the plasmid pEKEx3-*nagAB* was used, which allows ectopic expression of both *nagA* and *nagB* under the control of an IPTG-inducible promoter. To improve expression of *C. glycinophilum nagE* in *C. glutamicum* strains the *nagE* start codon was exchanged from GTG to ATG as well as the 5′-GAAAAGGAGG-3′ ribosomal binding site of the16S rRNA (Martin et al. 2003) introduced upstream of the *nagE* ATG start codon via PCR. The PCR product was cloned in pVWEx1 under the control of an IPTG-inducible promoter and the resulting plasmid pVWEx1-*nagE* introduced in *C. glutamicum* (pEKEx3-*nagAB*). The resulting two-plasmid strain *C. glutamicum* (pEKEx3-*nagAB*)(pVWEx1-*nagE*) grew well with 100 mM GlcNAc as sole source of carbon and energy ($\mu$=0.12±0.01 h$^{-1}$, Fig. 3) and the initially provided GlcNAc was completely consumed. The final OD of

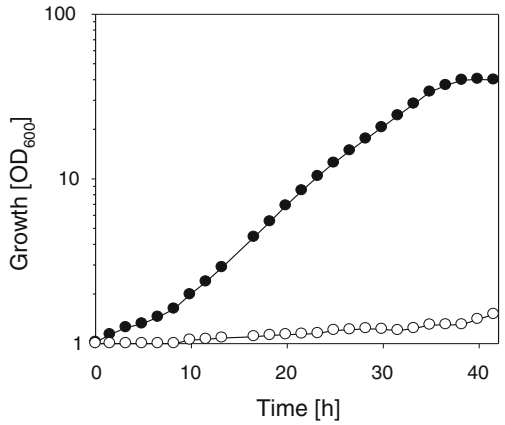


**Fig. 3** Growth of *C. glutamicum* (pEKEx3-*nagAB*)(pVWEx1-*nagE*) (*filled symbols*) and *C. glutamicum* (pEKEx3)(pVWEx1) (*open symbols*) in CGXII minimal medium with 100 mM GlcNAc as sole source of carbon and energy. Data represent mean values from three independent cultivations performed in a Biolector® cultivation system

40.0±2.3 reached by *C. glutamicum* (pEKEx3-*nagAB*)(pVWEx1-*nagE*) when cultivated with 100 mM GlcNAc as substrate was found to be even slightly higher when compared to cultivations with 100 mM glucose (OD 36.6±2.0, Fig. S2). Using $^{14}$C-labelled GlcNAc an uptake rate of 11.1±3.9 nmol min$^{-1}$ (mg cdm$^{-1}$) was determined for *C. glutamicum* (pEKEx3-*nagAB*)(pVWEx1-*nagE*). As expected from the results for *C. glutamicum* (pEKEx2-*nagE*) no growth on GlcNAc was observed for *C. glutamicum* (pEKEx3)(pVWEx1-*nagE*) (data not shown).

In order to test whether GlcNAc can also serve as a nitrogen source for *C. glutamicum* (pEKEx3-*nagAB*)(pVWEx1-*nagE*), three modified CGXII media were used which all contained 100 mM glucose as carbon source and either no nitrogen source, or 150 mM ammonium sulfate, or 300 mM GlcNAc as nitrogen source. No growth was observed in the absence of a nitrogen source for both *C. glutamicum* (pEKEx3-*nagAB*)(pVWEx1-*nagE*) and the control strain *C. glutamicum* (pEKEx3)(pVWEx1), while both strains grew well and completely utilized the glucose provided as carbon source in cultivations with ammonium sulfate (final ODs of 31.14±2.22 and 33.32±2.14 were measured for *C. glutamicum* (pEKEx3-*nagAB*)(pVWEx1-*nagE*) and *C. glutamicum* (pEKEx3)(pVWEx1), respectively). In CgXII medium with GlcNAc as sole nitrogen source no growth of the empty vector control strain *C. glutamicum* (pEKEx3)(pVWEx1) was observed, while the recombinant strain *C. glutamicum* (pEKEx3-*nagAB*)(pVWEx1-*nagE*) grew well and reached a final OD of 37.74±1.33, which clearly shows that GlcNAc can be used by the here designed *C. glutamicum* strains as a nitrogen source.

Taken together, these results show that the introduction of both pEKEx3-*nagAB* and pVWEx1-*nagE* in *C. glutamicum* and overexpression of the *nagAB* and *nagE* genes can generate a GlcNAc utilizing strain.

### Application of GlcNAc for L-lysine and lycopene production by *C. glutamicum*

The suitability of GlcNAc as substrate for the production of L-lysine and lycopene by *C. glutamicum* was tested. Strain *C. glutamicum* DM1729 is a lysine producer developed by the Evonik AG (Georgi et al. 2005) and strain *C. glutamicum* Δ*crtYEB* has been engineered for the production of lycopene (Heider et al. 2012), a key intermediate in the biosynthesis of many carotenoids. The two production strains that were transformed with pEKEx3-*nagAB* and pVWEx1-*nagE* in order generated two-plasmid strains able to use GlcNAc as sole carbon source for growth and production. Batch fermentations were carried out in a Biolector® microfermentation system in microtiter plates utilizing CgXII plus 100 μM IPTG as minimal medium, with 100 mM glucose or 100 mM GlcNAc as carbon sources. Cell growth was faster on glucose for both strains than on GlcNAc, with a specific growth rate of about 0.25±0.03 h$^{-1}$ on glucose for *C. glutamicum* DM1729 (pEKEx3-*nagAB*)(pVWEx1-*nagE*) as well as for *C. glutamicum* Δ*crtYEB* (pEKEx3-*nagAB*)(pVWEx1-*nagE*) and growth rates of 0.17±0.03 and 0.21±0.01 h$^{-1}$ on GlcNAc for *C. glutamicum* DM1729 (pEKEx3-*nagAB*)(pVWEx1-*nagE*) and *C. glutamicum* Δ*crtYEB* (pEKEx3-*nagAB*)(pVWEx1-*nagE*), respectively (Fig. S3). The amino sugar GlcNAc allowed higher L-lysine titers, with a final concentration of about 25.7±1.5 mM and a yield of 0.17±0.01 g Lys (g substrate)$^{-1}$, while using glucose about 17.0±2.7 mM of lysine were produced, corresponding to a yield of 0.14±0.02 g Lys (g substrate)$^{-1}$. In the *C. glutamicum* Δ*crtYEB*-based-strain, lycopene production from GlcNAc was observed, however, the lycopene titre of 17.4±0.4 mg (g CDW)$^{-1}$ was lower than that of 51.5±7.6 mg (g CDW)$^{-1}$ using glucose.

## Discussion

The *C. glutamicum* strains constructed in this study are able to utilize GlcNAc as carbon source due to overexpression of the endogenous *nagAB* genes encoding the *N*-acetyl-glucosamine-6-phosphate deacetylase and glucosamine-6-phosphate deaminase and heterologous overexpression of the gene *nagE*, encoding the PTS element specific for GlcNAc uptake in *C. glycinophilum*. As the overexpression of *nagB* gene alone is sufficient for growth on glucosamine (Uhde et al. 2013), the constructed strains (*C. glutamicum* (pEKEx3-*nagAB*)(pVWEx1-*nagE*), *C. glutamicum* DM1729 (pEKEx3-*nagAB*)(pVWEx1-*nagE*), and *C. glutamicum* Δ*crtYEB* (pEKEx3-*nagAB*)(pVWEx1-*nagE*)) are able to utilize both aminosugars. GlcNAc and glucosamine are the constituents of chitin and chitosan, the major waste generated from shrimp and crab processing industries (Chen et al. 2010;

Kurita 2006). In the perspective of feedstock flexibility used in fermentations, this is a promising step, provided that the degradation of chitin to its acetylated (GlcNAc) or deacetylated (GlcN) forming monomers is achieved in an inexpensive and environmentally acceptable way. For the moment, the chemical breakdown of shrimp waste to its forming units is still an environmental issue, due to the utilization of concentrated acids and bases (Kandra et al. 2012). Enzymatic degradation of chitin and chitosan by chitinases is a widespread process in nature (Beier and Bertilsson 2013), and properties of the involved enzymes from *Serratia marcenscens* and *Streptomyces* sp. have been studied in detail (Bhattacharya et al. 2007; Eijsink et al. 2008, 2010). For utilization of the glucose-polymer starch by *C. glutamicum* strains, the heterologous expression of genes encoding amylases has been successfully applied (Seibold et al. 2006; Song et al. 2013; Tateno et al. 2007, 2009; Yao et al. 2009). By this means, the efficient degradation of the starch polymer to sugars was achieved, allowing the utilization of starch as a feedstock for growth and amino acid production in *C. glutamicum*. In a similar way strains for the direct utilization of chitin and chitosan might by developed by heterologous expression of genes encoding chitinases in the here obtained *C. glutamicum* strains able to use aminosugars.

Application of GlcNAc as substrate for the L-lysine production with the *C. glutamicum* resulted in a significantly increased product yield when compared to cultivations with glucose as substrate. In difference to glucose, which serves only as source of carbon and energy, amino sugars additionally can serve as a nitrogen source. Production of L-lysine, which contains two nitrogen atoms, was higher with GlcNAc than with glucose, while biomass formation did not differ, which might be due to increased nitrogen supply from GlcNAc. By contrast, production of the nitrogen-free compound lycopene was lower, but biomass formation higher as compared to glucose (final ODs of 52.0±3.2 and 30.2±2.1 were measured for *C. glutamicum* Δ*crtYEB* (pEKEx3-*nagAB*)(pVWEx1-*nagE*) cultivated on GlcNAc or glucose, respectively). Biosynthesis of lycopene in the methylerythritol phosphate (MEP) pathway in *C. glutamicum* does not start from acetyl-CoA (Heider et al. 2012), but from glyceraldehyde 3-phosphate and pyruvate. Likely, increased provision of nitrogen from GlcNAc shifted carbon flux from lycopene production towards biomass formation. Alternatively, carbon source-dependent regulation by regulators such as RamA, RamB, SugR, or GlxR (Arndt and Eikmanns 2008) may have influenced the expression of lycopene biosynthesis genes. However, currently it is unknown if these genes show carbon source-dependent regulation. Therefore, in addition to the optimal genetic design required for the efficient utilization of alternative feedstocks also the consequences on the formation of the desired products need to be taken into account.

As shown here for GlcNAc and as observed in our previous work in which we investigated glucosamine utilization (Uhde et al. 2013), the growth rate of *C. glutamicum* strains on amino sugars is slower when compared to glucose. In addition to their role as a source of carbon, nitrogen, and energy, amino sugars are essential precursors for the synthesis of the major bacterial cell wall compound peptidoglycan (Johnson et al. 2013; Typas et al. 2012). Thus, tight control of anabolic and catabolic pathways for amino sugar metabolism is essential in bacteria and is brought about by transcriptional regulation of genes for biosynthesis, degradation, and uptake (Bertram et al. 2011; Nothaft et al. 2010; Plumbridge 1995). The observed consecutive utilization of glucose and Neu5Ac by *C. glutamicum* (Gruteser et al. 2012) and the identification of suppressor mutations in the promoter region of *nagAB* leading to improved growth on glucosamine are two hints for the existence of control mechanisms for amino sugar metabolism in *C. glutamicum*, which have not been investigated. In contrast to cultivation on glucosamine, in which final OD and titer of lysine were the same as for cultivation on glucose, use of GlcNAc as a substrate entails both higher biomass and L-lysine yields than use of glucose. Those higher yields are probably a consequence of the acetate moiety of GlcNAc: acetate has been shown to be simultaneously consumed together with glucose, resulting in an increased energy metabolism for *C. glutamicum* (Wendisch et al. 2000). This particular trait of substrate co-consumption present in *C. glutamicum* requires tight regulatory control, which is exerted by transcriptional regulators such as SugR (Engels and Wendisch 2007). SugR represses genes encoding PTS components and glycolytic enzymes in the presence of gluconeogenic substrates such as acetate (Engels et al. 2008b; Gaigalat et al. 2007; Toyoda et al. 2008). In consequence the NagA catalyzed formation of acetate in the course GlcNAc metabolisation might cause a reduced expression of *ptsI*, *ptsH*, *pfkA*, *fba*, and *gapA*, which encode the general PTS components EI and Hpr, the 6-phosphofructokinase, the fructose-1,6-bisphosphate aldolase, and the glyceraldehyde-3-phosphate dehydrogenase, respectively. Thus GlcNAc utilization probably limits its own PTS mediated uptake as well as metabolisation of its own sugar residue, which results in the observed slower growth on GlcNAc than on glucosamine. However, studies on both the regulation of amino sugar metabolism and central metabolism in *C. glutamicum* are required to further elucidate the underlying regulatory mechanisms.

In conclusion, the concomitant overexpression of the *C. glutamicum nagAB* genes and the *C. glycinophilum nagE* gene in *C. glutamicum* resulted in strains that are able to grow on glucosamine and GlcNAc. This allows us to utilize these chitin-derived amino sugars as alternative sources of carbon, nitrogen and energy for production with *C. glutamicum*.

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