



Production of Food and Feed Additives From Non-food-competing Feedstocks: Valorizing *N*-acetylmuramic Acid for Amino Acid and Carotenoid Fermentation With *Corynebacterium glutamicum*

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Corynebacterium glutamicum is used for the million-ton-scale production of food and feed amino acids such as L-glutamate and L-lysine and has been engineered for production of carotenoids such as lycopene. These fermentation processes are based on sugars present in molasses and starch hydrolysates. Due to competing uses of starch and sugars in human nutrition, this bacterium has been engineered for utilization of alternative feedstocks, for example, pentose sugars present in lignocellulosic and hexosamines such as glucosamine (GlcN) and *N*-acetyl-D-glucosamine (GlcNAc). This study describes strain engineering and fermentation using *N*-acetyl-D-muramic acid (MurNAc) as non-food-competing feedstock. To this end, the genes encoding the MurNAc-specific PTS subunits MurP and Crr and the etherase MurQ from *Escherichia coli* K-12 were expressed in *C. glutamicum* $\Delta nanR$. While MurP and MurQ were required to allow growth of *C. glutamicum* $\Delta nanR$ with MurNAc, heterologous Crr was not, but it increased the growth rate in MurNAc minimal medium from 0.15 h⁻¹ to 0.20 h⁻¹. When in addition to *murP-murQ-crr* the GlcNAc-specific PTS gene *nagE* from *C. glycinophilum* was expressed in *C. glutamicum* $\Delta nanR$, the resulting strain could utilize blends of GlcNAc and MurNAc. Fermentative production of the amino acids L-glutamate and L-lysine, the carotenoid lycopene, and the L-lysine derived chemicals 1,5-diaminopentane and L-pipecolic acid either from MurNAc alone or from MurNAc-GlcNAc blends was shown. MurNAc and GlcNAc are the major components of the bacterial cell wall and bacterial biomass is an underutilized side product of large-scale bacterial production of organic acids, amino acids or enzymes. The proof-of-concept for valorization of MurNAc reached here has potential for biorefinery applications to convert non-food-competing feedstocks or side-streams to valuable products such as food and feed additives.

Keywords: L-lysine, diamino pentane, lycopene, L-glutamate, biorefinery, food additives, peptidoglycan, *N*-acetylmuramic acid

Abbreviations: μ_{max} , maximal specific growth rate; ΔS , variation of substrate concentration; gCDWL⁻¹, gram of Cell Dry Weight per liter; q_S, specific substrate uptake rate; Y_{P/S}, yield coefficient of cell product per used substrate; Y_{P/X}, yield coefficient of product per cell dry weight; Y_{X/S}, yield coefficient of cell dry weight per used substrate.

INTRODUCTION

Corynebacterium glutamicum is a predominantly aerobic, rod-shaped, Gram-positive soil bacterium which is generally recognized as safe (GRAS). Since the 1960s, *C. glutamicum* was first used for the production of the flavor enhancer (Kinoshita et al., 1957) under biotin limiting conditions (Shiio et al., 1962). *C. glutamicum* was developed into an important organism for the biotechnological industry, producing amino acids on a million-ton scale (Wendisch, 2014). *C. glutamicum* has also been engineered to produce diamines, organic acids, carotenoids, proteins and biopolymers (Wendisch et al., 2016). Recently, metabolic engineering of *C. glutamicum* to expand its substrate scope allowed to use alternative carbon sources that do not have competing uses in the food industry (Zahoor et al., 2012). Access to the hexosamines GlcN (Uhde et al., 2013) and GlcNAc (Matano et al., 2014) has been reported, but utilization of the hexosamine MurNAc as alternative carbon source by *C. glutamicum* has not been described (Dominguez et al., 1997; Cramer and Eikmanns, 2007).

GlcN and GlcNAc can be gained by hydrolysis of chitin and chitosan that make up the arthropod exoskeleton and are present in fungal cell walls. Every year, circa 100 billion tons of chitin are produced in Nature and GlcNAc and GlcN can be obtained by acid hydrolysis (Chen et al., 2010; Zhang and Yan, 2017) and are available, e.g., from shrimp shell waste, an abundant side stream of the fishery industry. MurNAc and GlcNAc are the hexosamine constituents of peptidoglycan which makes up about 5% of the cell mass of Gram-negative bacteria and up to 20% of the cell mass of Gram-positive bacteria (Munoz et al., 1967; Reith and Mayer, 2011). The peptidoglycan constituents that can be found in all bacterial habitats have been used as indicators of bacterial biomass content in soils (Domsch, 1982). The Gram-positive *C. glutamicum* is the main producing organism for the annual production of 5 million tons of amino acids (Wendisch et al., 2016). Under the assumptions that (a) the same amount of cell dry weight is produced, (b) 20% of the cell dry weight is peptidoglycan and (c) about half of peptidoglycan is GlcNAc and MurNAc, about 500.000 tons of MurNAc and GlcNAc would be available from the amino acid fermentation industry. Biotechnological processes with bacterial hosts are used at the million-ton scale to produce secreted compounds such as organic acids, amino acids and enzymes. The spent biomass may be used in waste-to-energy applications either by thermal (e.g., incineration), thermochemical (e.g., torrefaction) or by biochemical treatments (e.g., anaerobic digestion). However, it is desirable to make use of the carbon and nitrogen containing hexosamine fraction of peptidoglycan in food and feed fermentation processes. The hexosamines may function both as carbon and nitrogen source for bacterial fermentations.

In chemical hydrolysis, the hexosamine fraction of peptidoglycan is accessible via enzymes of bacterial cell wall recycling. The degradation of the own cell wall by autolytic enzymes as a part of cell wall recycling is a common pathway in bacteria. When an *Escherichia coli lys dap* mutant was labeled with [³H]diaminopimelate for two generations and

then chased, about 45% of its cell wall peptidoglycan was recycled per generation (Goodell and Schwarz, 1985; Uehara et al., 2006; Uehara and Park, 2008). The Gram-positive *Bacillus subtilis* can degrade, uptake and metabolize the cell wall component MurNAc in the stationary phase (Borisova et al., 2016). While all bacteria require cell wall peptidoglycan remodeling during growth and cell division, not all can utilize the monomeric components as carbon or nitrogen sources for growth. *C. glutamicum* may possess a minimal set of autolytic enzymes, however, many orthologs of the peptidoglycan degradation machinery from *E. coli* are absent (Dahl et al., 2004; Reith and Mayer, 2011). Catabolism of MurNAc in *E. coli* involves uptake and phosphorylation of MurNAc and GlcNAc via the phosphoenolpyruvate dependent phosphotransferase system (PTS). The MurNAc-specific PTS subunits are MurP and Crr (Figure 1). MurP, a two-domain protein that lacks a PTS-EIIA domain, is phosphorylated by EIIA^{Glc}, a kinase encoded by the *crr* gene (carbohydrate repression resistance), which interacts with several members of the glucose PTS family (Nuoffer et al., 1988; Tchieu et al., 2001; Dahl et al., 2004). MurNAc-6-phosphate is further catabolized by the etherase MurQ (Figure 1) that cleaves the lactyl ether bond yielding GlcNAc-6-phosphate and D-lactate (Jaeger et al., 2005; Hadi et al., 2008). GlcNAc and GlcN are also taken up via the PTS with the specific subunits NagE (Plumbridge, 2009) and PTS^{Man} (Curtis and Epstein, 1975). NagA deacetylates GlcNAc-6-phosphate to GlcN-6-phosphate which is deaminated by NagB to the glycolytic intermediate fructose-6-phosphate (Figure 1).

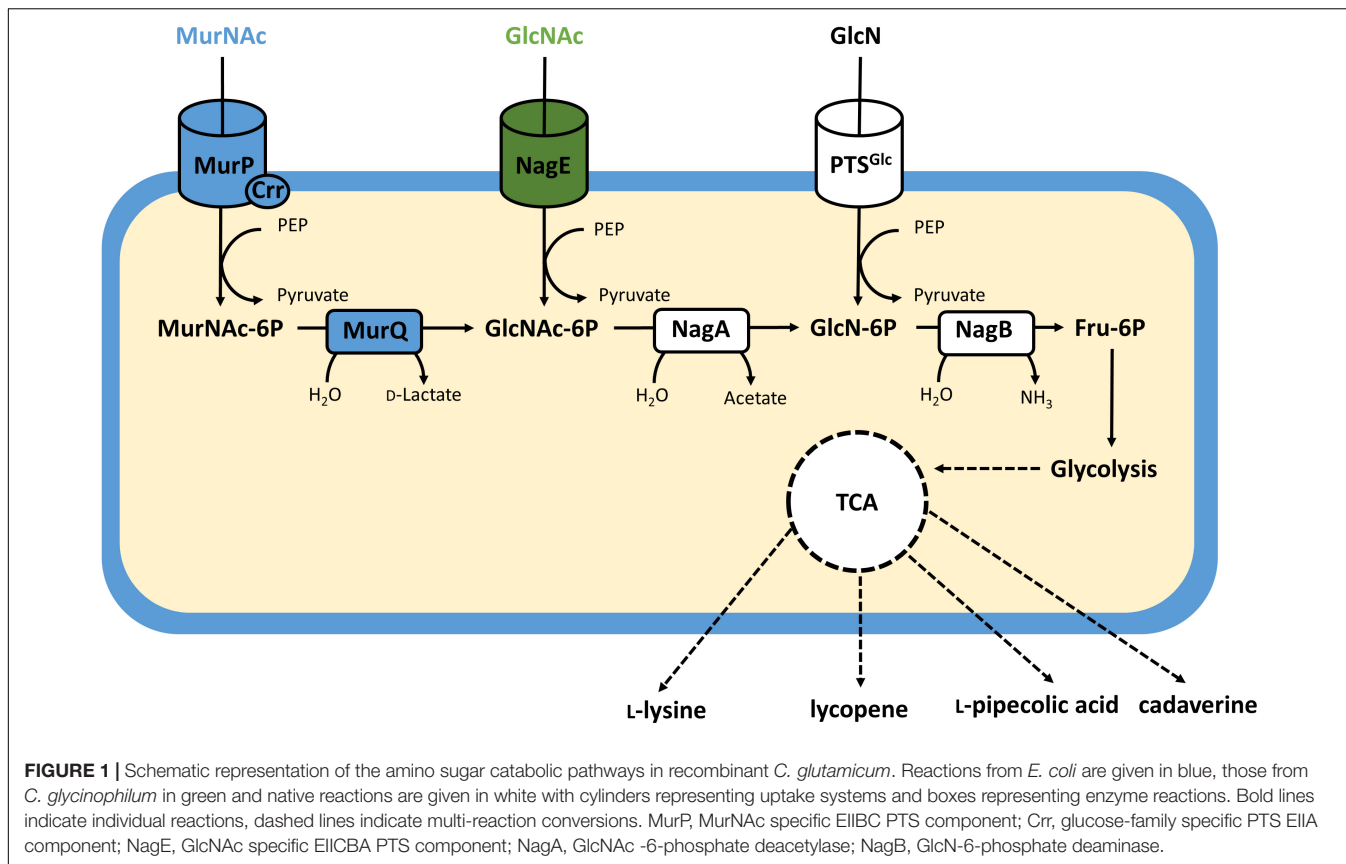
Corynebacterium glutamicum is able to take up GlcN (Figure 1) by using its glucose specific PTS PtsG (Arndt and Eikmanns, 2008; Uhde et al., 2013). Efficient growth with GlcN required high levels of the endogenous NagB, e.g., in the absence of the repressor protein NanR (Matano et al., 2016). By contrast, high levels of NagA and NagB were not sufficient to support growth with GlcNAc unless *nagE* from *C. glycinophilum* was expressed heterologously (Matano et al., 2014). Recombinant *C. glutamicum* strains carrying a *nanR* deletion and expressing *nagE* from *C. glycinophilum* produced several value-added products from GlcN or GlcNAc.

Here, *C. glutamicum* strains were developed for utilization of MurNAc as carbon, nitrogen and energy source and for MurNAc-based production of the food amino acid L-glutamate, the feed amino acid L-lysine, L-lysine-derived chemicals as well as the carotenoid lycopene.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The strains and plasmids used in this work are listed in Table 1. Pre-cultivation of *C. glutamicum* strains was carried out at 30°C in baffled shake flasks using BHI supplemented with 45.5 g/L D-sorbitol. *E. coli* was grown at 37°C in LB (Lysogeny Broth) medium. Kanamycin (50 µg/mL), chloramphenicol (4.5 and 25 µg/mL for *C. glutamicum* and *E. coli*, respectively) or tetracycline (5 µg/mL) were added, if necessary. To adjust both cultures to growth conditions in the Biolector® system (m2pLabs,



Baesweiler), precultures were washed after 24 h with TN-buffer and transferred to CGXII medium (Eggeling and Bott, 2005) with 100 mM GlcN and antibiotics, if necessary. After 24 h, these were transferred to 1 mL cultures in the Biolector® system (1100 rpm) with CGXII medium containing and, if not otherwise stated, 25 mM MurNAc (BACHEM, Bubendorf, Switzerland) as sole carbon source or a combination of 25 mM MurNAc and 25 mM GlcNAc. To trigger glutamate production, penicillin G (10 μM) was added in the main culture. The initial OD₆₀₀ was 1 and gene expression from plasmids pVWEx1 and pEC-XT99A was induced by addition of 25 μM IPTG, if not otherwise stated. Correlation factors for light scattering in the Biolector system, OD₆₀₀ and biomass concentrations were determined.

Construction of Expression Vectors

Escherichia coli DH5α was used for cloning. Codon usage of *murP*, *crr* and *murQ* from *E. coli* for *C. glutamicum* was examined using the graphical codon usage analyzer¹. The analysis showed, that the codon ATA occurred twice in the sequence of *murP*. This codon is rarely used in *C. glutamicum* and was changed to the more frequently used codon of ATC via site directed mutagenesis (SDM). The mutated variation of *murP* was called *murP*^{opt}. The genes of *murP*, *crr* and *murQ* were amplified via PCR from genomic DNA of *E. coli* K-12, while *nagE* from *C. glycinophilum*, was amplified from

pVWEx1_ *nagE* (Matano et al., 2016). The primers used in this study (see **Supplementary Table S1**) were obtained from Metabion international AG, Planegg. Using Gibson assembly (Gibson, 2011), the vectors pVWEx1_ *murP*, pVWEx1_ *murP*^{opt}, pVWEx1_ *murP**crr*, pVWEx1_ *murP*^{opt}*crr*, pCXE50_ *murQ* and pEC-XT99A_ *nagE* were constructed. The vectors pVWEx1 and pEC-XT99A are IPTG inducible while pCXE50 has a constitutive EF_{tu} promoter. *E. coli* was transformed by the CaCl₂ method while transformation through electroporation was applied for *C. glutamicum* at 2500 V, 25 μF and 200 Ω.

Carotenoid Extraction

Lycopene was extracted as described before (Heider et al., 2012). In short, 5 wells each containing 1 ml cell suspension were combined and pelleted in safe lock micro reaction tubes by centrifugation at 10,000 g for 15 min and resuspended in 800 μL of a 7:3 methanol/acetone mixture and incubated for 15 min at 60°C and 750 rpm in a thermomixer (Eppendorf). The cell debris was removed by centrifugation and the supernatant used for HPLC analysis. The procedure was repeated to ensure complete extraction until white pellets were obtained.

Quantitation of Fermentation Products

The quantification of MurNAc, GlcNAc, lycopene, L-glutamate, and L-lysine was conducted by HPLC analysis (1200 series

¹<http://gcua.schoedl.de/>

TABLE 1 | Plasmids and strains used in this study.

Strain ID	Characteristics	Reference
<i>E. coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>dlacZ</i> Δ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Hanahan, 1983
JW2421-1	F-, Δ (<i>araD-araB</i>)567, Δ <i>lacZ4787</i> (<i>rrmB-3</i>), λ -, Δ <i>murQ757::kan</i> , <i>rph-1</i> , Δ (<i>rhaD-rhaB</i>)568, <i>hsdR514</i> ; Kan ^R	Baba et al., 2006
JW2421-1 (pCXE50_ <i>murQ</i>)	JW2421-1 carrying pCXE50_ <i>murQ</i>	this work
<i>C. glutamicum</i>		
Δ <i>nanR</i>	ATCC 13032 Δ <i>nanR</i>	(Matano et al., 2016)
Δ <i>nanR</i> PQ	Δ <i>nanR</i> carrying pVWEx1_ <i>murP</i> and pCXE50_ <i>murQ</i>	This work
Δ <i>nanR</i> POQ	Δ <i>nanR</i> carrying pVWEx1_ <i>murP</i> ^{opt} and pCXE50_ <i>murQ</i>	This work
Δ <i>nanR</i> PCQ	Δ <i>nanR</i> carrying pVWEx1_ <i>murP_crr</i> and pCXE50_ <i>murQ</i>	This work
Δ <i>nanR</i> POCQ	Δ <i>nanR</i> carrying pVWEx1_ <i>murP</i> ^{opt} _ <i>crr</i> and pCXE50_ <i>murQ</i>	This work
Δ <i>nanR</i> PCQnE	Δ <i>nanR</i> carrying pVWEx1_ <i>murP_crr</i> , pCXE50_ <i>murQ</i> and pEC-XT99A_ <i>nagE</i>	This work
DM1729 Δ <i>nanR</i> PCQ	DM1729 Δ <i>nanR</i> carrying pVWEx1_ <i>murP_crr</i> and pCXE50_ <i>murQ</i>	This work
DM1729 Δ <i>nanR</i>	ATCC 13032 <i>pycP</i> ^{458S} <i>hom</i> ^{V59A} <i>lysC</i> ^{T311I} Δ <i>nanR</i> , L-lysine overproducing strain	Matano et al., 2014
DM1729 Δ <i>nanR</i> PCQnE	DM1729 Δ <i>nanR</i> carrying pVWEx1_ <i>murP_crr</i> , pCXE50_ <i>murQ</i> and pEC-XT99A_ <i>nagE</i>	This work
DM1729 Δ <i>nanR</i> PCQ <i>ldcC</i>	DM1729 Δ <i>nanR</i> carrying pVWEx1_ <i>murP_crr</i> , pCXE50_ <i>murQ</i> and pEC-XT99A_ <i>ldcC</i>	This work
DM1729 Δ <i>nanR</i> PCQ LPA	DM1729 Δ <i>nanR</i> carrying pVWEx1_ <i>murP_crr</i> , pCXE50_ <i>murQ</i> and pECXT99A_ <i>lysDH</i> _{S.prom} - <i>proC</i> _{C.g}	This work
Δ <i>crtYeb</i> Δ <i>nanR</i>	lycopene producing derivative of WT carrying in-frame Δ <i>crtYeb</i> Δ <i>nanR</i>	Matano et al., 2014
Δ <i>crtYeb</i> Δ <i>nanR</i> PCQ	Deletion of <i>crtY</i> and <i>crtEb</i> carrying pVWEx1_ <i>murP_crr</i> , pCXE50_ <i>murQ</i>	This work
Δ <i>crtYeb</i> Δ <i>nanR</i> PCQnE	Δ <i>crtYeb</i> Δ <i>nanR</i> carrying pVWEx1_ <i>murP_crr</i> , pCXE50_ <i>murQ</i> and pEC-XT99A_ <i>nagE</i>	This work
Plasmids		
pVWEx1	<i>E. coli</i> / <i>C. glutamicum</i> shuttle vector, Kan ^R	Peters-Wendisch et al., 2001
pEC-XT99A	Tet ^R , <i>C. glutamicum</i> / <i>E. coli</i> shuttle vector (Ptrc, <i>lacI</i> , pGA1 OriV _{C.g} .)	Kirchner and Tauch, 2003
pCXE50_ <i>porB</i>	Constitutive pE _{f_{lu}} promoter, Cm ^R	Lee, 2014
pVWEx1_ <i>murP</i>	pVWEx1-derivative for IPTG inducible expression of <i>murP</i> from <i>E. coli</i> , Kan ^R	This work
pVWEx1_ <i>murP</i> ^{opt}	pVWEx1-derivative for IPTG inducible expression of the for <i>C. glutamicum</i> codon optimized <i>murP</i> from <i>E. coli</i> , Kan ^R	This work
pVWEx1_ <i>murP_crr</i>	pVWEx1-derivative for IPTG inducible expression of <i>murP</i> and <i>crr</i> from <i>E. coli</i> , Kan ^R	This work
pVWEx1_ <i>murP</i> ^{opt} _ <i>crr</i>	pVWEx1-derivative for IPTG inducible expression of the for <i>C. glutamicum</i> codon optimized <i>murP</i> and <i>crr</i> from <i>E. coli</i> , Kan ^R	This work
pCXE50_ <i>murQ</i>	Cm ^R , expressing <i>murQ</i> from <i>E. coli</i> K12	This work
pEC-XT99A_ <i>nagE</i>	Tet ^R , expressing <i>nagE</i> from <i>Corynebacterium glycinophilum</i> DSM45794	This work
pEC-XT99A_ <i>ldcC</i>	Tet ^R , expressing <i>ldcC</i> from <i>E. coli</i> K12	This work
pECXT99A_ <i>lysDH</i> _{S.prom} - <i>proC</i> _{C.g}	Tet ^R , expressing <i>lysDH</i> from <i>Silicibacter pomeroyi</i> and <i>proC</i> from <i>C. glutamicum</i>	This work

Kan^R, kanamycin resistance; Tet^R, tetracycline resistance; Cm^R, chloramphenicol resistance.

HPLC system, Agilent Technologies Sales & Services GmbH & Co. KG, Waldbronn). The supernatant of 1 ml pelleted cell suspension was diluted and analyzed. For quantification of organic acids, the carbo column (300 × 8 mm, 10 μ m particle size, 25 Å pore diameter, CS Chromatographie Service GmbH) and a refractive index detector (RID G1362A, 1200 series, Agilent Technologies) was used for quantification of MurNAc and GlcNAc with 5 mM H₂SO₄ as buffer.

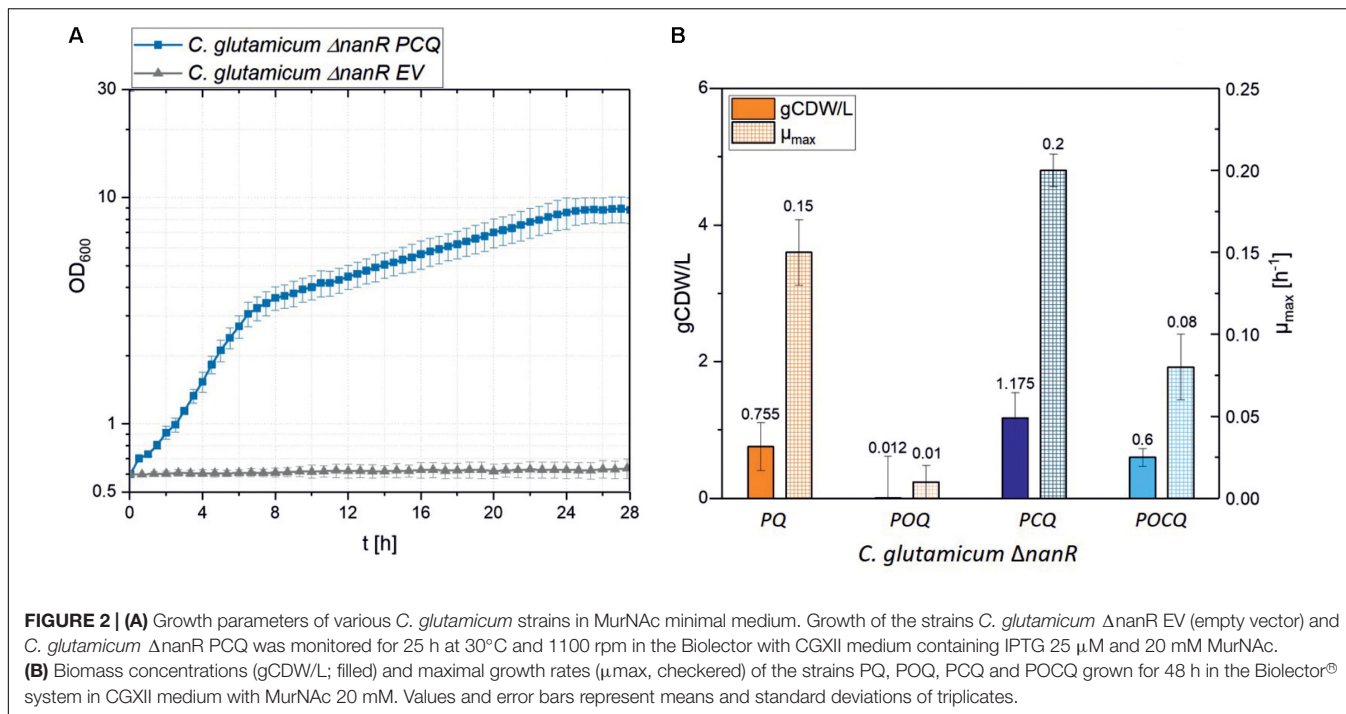
Applying OPA derivatisation, L-lysine and L-glutamate were analyzed using an RP8 column with a sodium acetate (0.25 v/v %) buffer at pH 6 and a 1:50 dilution with an internal L-asparagine

standard. Using the RP18 column with a Methanol-Milli-Q-water mixture (9:1), lycopene was quantified (Heider et al., 2012).

RESULTS

Metabolic Engineering of *C. glutamicum* for Growth With MurNAc as Carbon Source

Corynebacterium glutamicum, which has been engineered to utilize GlcN and GlcNAc (Matano et al., 2014, 2016), cannot



utilize MurNac since no growth was observed in minimal medium with 25 mM MurNac and 25 ± 0.1 mM MurNac remained in the growth medium after 25 h of incubation (Figure 2A). As expected, the *C. glutamicum* genome lacks genes encoding a MurNac PTS and MurNac-6-phosphate etherase for uptake and conversion of MurNac to GlcNac-6-phosphate, an endogenous intermediate of *C. glutamicum* metabolism. As described in Section “Materials and Methods,” the genes for the MurNac PTS *murP* from *E. coli* or codon optimized allele *murP^{opt}* (Nuoffer et al., 1988; Tchieu et al., 2001; Dahl et al., 2004) were cloned into the IPTG-inducible plasmid pVWex1 alone or as operon with *crr*. The gene for the MurNac-6-phosphate etherase *murQ* from *E. coli* (Jaeger et al., 2005) was cloned into the constitutive expression vector pCXE50 (Lee, 2014). Functional expression of *murQ* from pCXE50_ *murQ* was tested by complementation of the *E. coli murQ* mutant *E. coli* JW2421-1. While *E. coli* JW2421-1(pCXE50_ *murQ*) utilized MurNac as sole carbon source (ΔOD_{600} of 3.2 ± 0.1 and μ_{max} of 0.07 ± 0.01 h⁻¹), *E. coli* JW2421-1 $\Delta murQ$ showed no growth (see Supplementary Figure S1). Only *murQ* was tested by complementation of a *E. coli* mutant. We neither tested *murP* nor *crr* because we expected perturbations due to overexpression since MurP is a membrane protein and Crr serves a regulatory function. *C. glutamicum* $\Delta nanR$ was transformed with the constructed pVWex1 plasmids and with the pCXE50_ *murQ*. The respective strains were named *C. glutamicum* $\Delta nanR$ PQ, POQ, PCQ, POCQ (Table 1).

Strains expressing *crr* from *E. coli* grew faster in minimal medium containing 25 mM MurNac as sole source of carbon and energy than strains lacking *crr* (Figure 2B). Strains with native *murP* grew better than strains expressing codon optimized *murP*

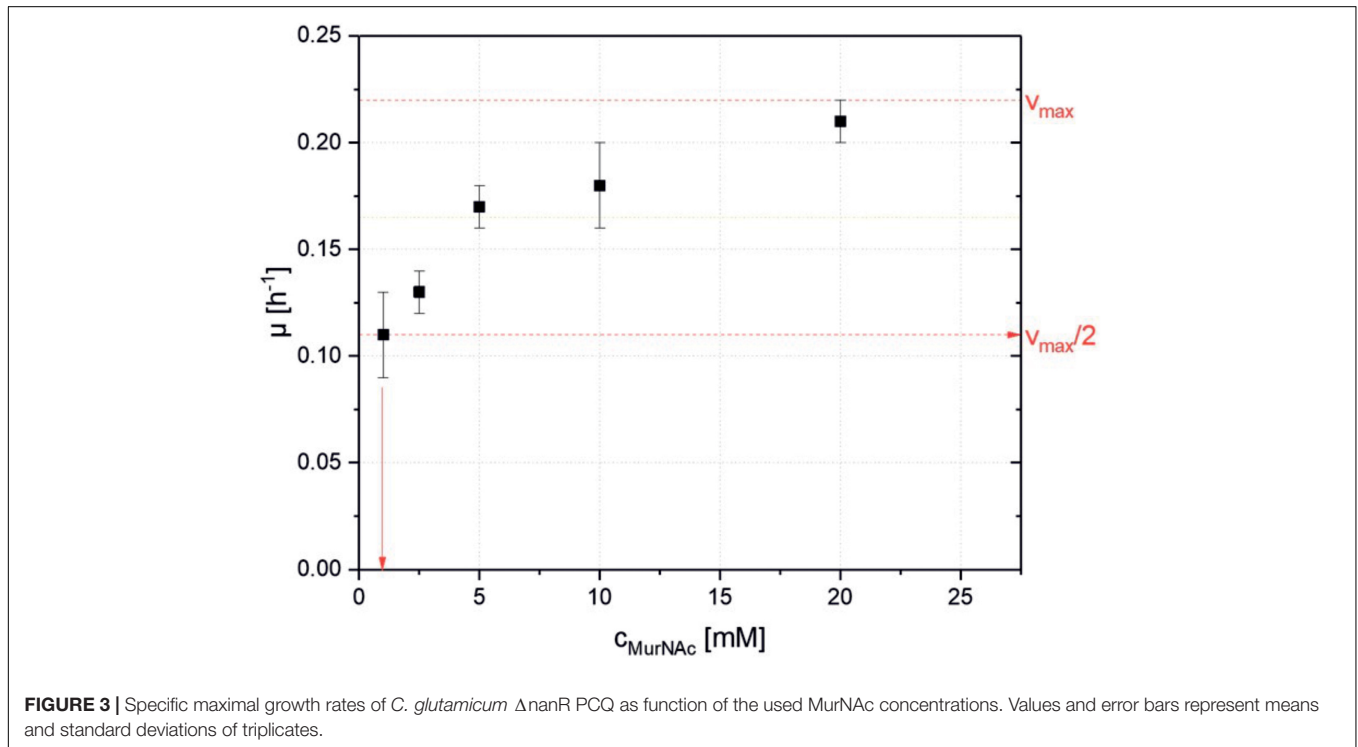
(Figure 2B). IPTG was used at a low concentration (25 μ M) to induce heterologous gene expression, since higher concentrations slowed growth (Table 2). This is not unexpected and presumably due to too high expression of transport protein genes as seen previously for *dccT* (Youn et al., 2008) and *dctA* (Youn et al., 2009), coding for dicarboxylate transporters. With 25 μ M IPTG, strain $\Delta nanR$ PCQ expressing native *murP*, *crr* and *murQ* grew in minimal medium containing 25 mM MurNac to a biomass concentration of 1.2 ± 0.3 gCDW/L and with 50 mM MurNac to a biomass concentration of 2.0 ± 0.2 gCDW/L (Table 2). Biphasic exponential growth was observed: faster growth between 0 and 6 h and slower growth between 6 and 27 h (Figure 2A). The curves appear linear as the Y axis has been logarithmized (Figure 2). During the transition from the first to the second growth phase the medium contained 3.10 ± 0.12 mM lactate. Thus, lactate released by MurQ from MurNac-6-phosphate may not have been utilized as fast as GlcNac-6-phosphate, the other product of the MurQ reaction. In consequence, lactate accumulated in the culture medium in the first exponential growth phase and presumably slowed growth in the second exponential growth phase. Transient accumulation of lactate to growth inhibitory concentrations has been observed during growth of *C. glutamicum* with various carbon sources (Engels et al., 2008).

As PTS systems typically support growth on their cognate substrates with high affinity, the dependence of the growth rate on the initial MurNac concentration in the growth medium was determined using strain $\Delta nanR$ PCQ. Different concentrations of MurNac (1, 2.5, 5, 10, and 20 mM) were used and the maximal growth rates were plotted against the MurNac concentration to derive the maximal growth rate of 0.22 h⁻¹ and the Monod constant of 0.9 ± 0.1 mM as shown in Figure 3.

TABLE 2 | Growth characteristics of *C. glutamicum* $\Delta nanR$ PCQ on different MurNAc and IPTG concentrations.

C_{MurNAc} [mM]	C_{IPTG} [μ M]	gCDW/L	μ_{max1} [h^{-1}]	μ_{max2} [h^{-1}]	$Y_{x/s}$ [$g \cdot mol^{-1}$]
20	25	1.2 \pm 0.3	0.25 \pm 0.01	0.05 \pm 0.01	58.0 \pm 0.1
	50	1.0 \pm 0.4	0.24 \pm 0.01	0.04 \pm 0.01	51.4 \pm 0.1
	100	1.0 \pm 0.1	0.26 \pm 0.02	0.05 \pm 0.01	54.5 \pm 0.1
50	25	2.0 \pm 0.2	0.25 \pm 0.04	0.04 \pm 0.01	38.4 \pm 0.1
	50	1.7 \pm 0.5	0.26 \pm 0.01	0.05 \pm 0.01	35.1 \pm 0.1
	100	1.5 \pm 0.2	0.21 \pm 0.02	0.04 \pm 0.01	30.4 \pm 0.1

The final biomass concentrations reached [given as g of cell dry weight (CDW) per L], maximal specific growth rates of the first (μ_{max1}) and second growth phase (μ_{max2}), and the biomass yield coefficients of cell dry weight formed per used substrate used ($Y_{x/s}$) are given as means with standard deviations.



A sub-millimolar Monod constant is typical for PTS mediated uptake.

Comparative Analysis of Growth With MurNAc and/or GlcNAc

Growth of recombinant *C. glutamicum* with MurNAc and/or GlcNAc as sole carbon sources was compared (Figure 4 and Table 3). With 25 mM MurNAc *C. glutamicum* $\Delta nanR$ PCQnE grew to a biomass concentration of 3.0 \pm 0.1 gCDW/L, while the maximal biomass concentration was only 2.4 \pm 0.1 gCDW/L with GlcNAc. The higher biomass concentration observed with MurNAc in comparison to GlcNAc indicated that lactate released from MurNAc by MurQ contributed to biomass formation. However, the biomass yield was higher with GlcNAc (0.44 \pm 0.01 $g \cdot g^{-1}$) than with MurNAc (0.39 \pm 0.02 $g \cdot g^{-1}$). GlcNAc catabolism was faster than MurNAc catabolism as the maximal growth rates and the specific substrate uptake rates were lower with MurNAc (0.22 \pm 0.10 h^{-1} and

1.80 \pm 0.10 $mmol \cdot g^{-1} \cdot h^{-1}$) than with GlcNAc (0.30 \pm 0.01 h^{-1} and 3.00 \pm 0.10 $mmol \cdot g^{-1} \cdot h^{-1}$) as shown in Table 3.

Unlike *E. coli* and *B. subtilis*, it is typical for *C. glutamicum* to simultaneously co-utilize carbon substrates present in blends (Blombach and Seibold, 2010). Therefore, *C. glutamicum* strain $\Delta nanR$ PCQnE was constructed by transforming strain $\Delta nanR$ PCQnE with plasmid pEC-XT99A-*nagE* for expression of the gene for the GlcNAc-specific PTS uptake system to establish whether MurNAc and GlcNAc are co-utilized or utilized sequentially. *C. glutamicum* strain $\Delta nanR$ PCQnE were grown with 25 mM MurNAc and/or 25 mM GlcNAc (Figure 4). With the blend of MurNAc and GlcNAc *C. glutamicum* $\Delta nanR$ PCQnE grew to a biomass concentration of 3.8 \pm 0.1 gCDW/L, while a biomass concentration of only 2.1 \pm 0.1 g/L was reached in the absence of *nagE*. Determination of the residual substrate concentrations revealed sequential utilization of GlcNAc before MurNAc (Figure 4C). Thus, unlike many growth substrates MurNAc and GlcNAc were not co-utilized.

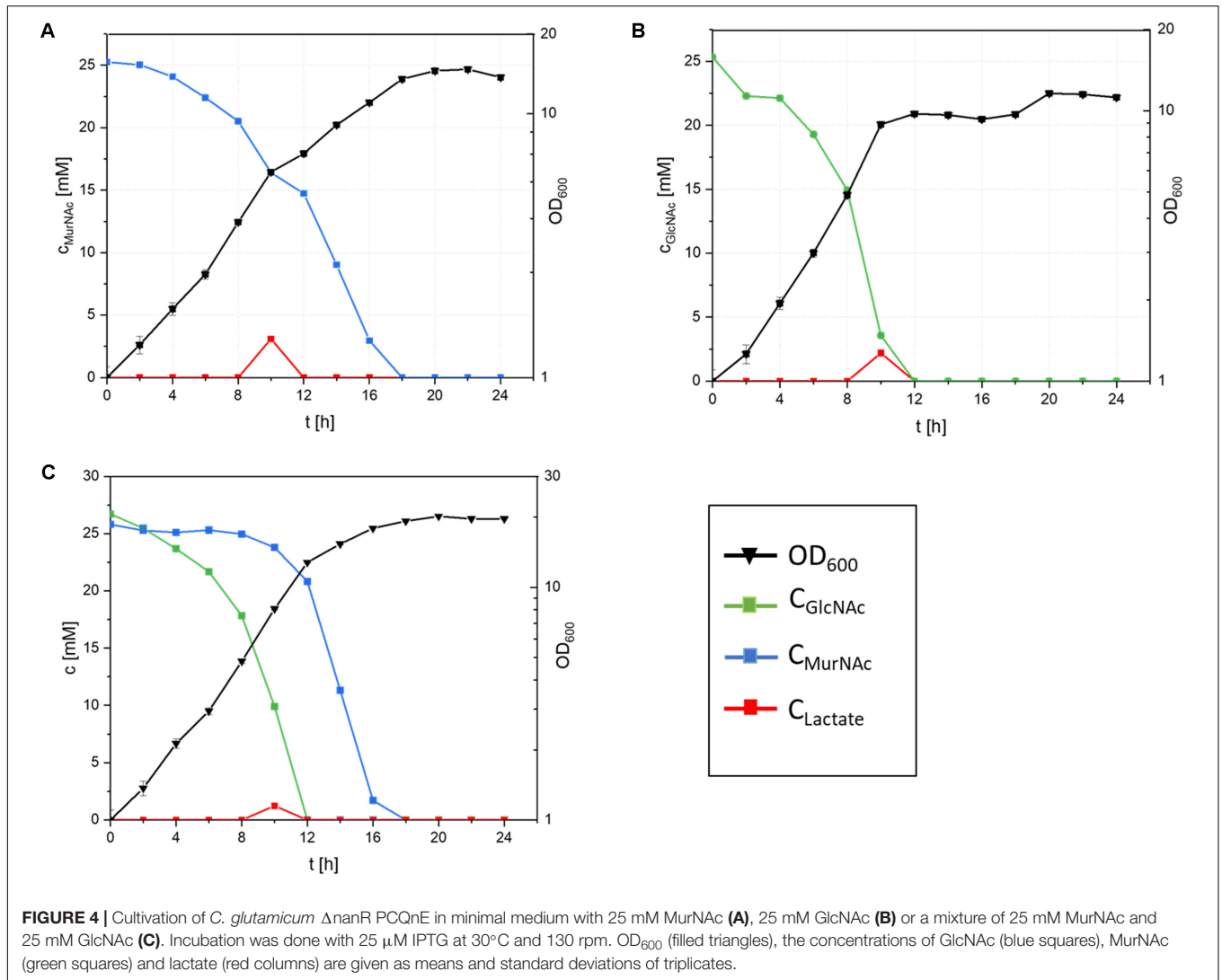


TABLE 3 | Cultivation parameters of *C. glutamicum* $\Delta nanR$ PCQnE growing on MurNAc (25 mM), GlcNAc (25 mM) or a MurNAc-GlcNAc-mixture (both 25 mM).

	MurNAc	GlcNAc	MurNAc-GlcNAc mix
ΔOD_{600} [-]	12.0 ± 0.1	9.6 ± 0.1	17.7 ± 0.1
ΔS MurNAc [mM]	25.3 ± 0.1	-	25.8 ± 0.1
ΔS GlcNAc[mM]	-	25.3 ± 0.1	26.7 ± 0.1
$Y^1_{X/S}$ [g·mmol ⁻¹]	0.14 ± 0.01	0.09 ± 0.01	0.11 ± 0.10
μ_{max}^1 [h ⁻¹]	0.18 ± 0.01	0.22 ± 0.01	0.18 ± 0.01
qS^1 [mmol·g ⁻¹ ·h ⁻¹]	1.27 ± 0.10	2.42 ± 0.10	1.68 ± 0.10
$Y^2_{X/S}$ [g·mol ⁻¹]	0.11 ± 0.01	-	0.08 ± 0.10
μ_{max}^2 [h ⁻¹]	0.10 ± 0.01	-	0.07 ± 0.20
qS^2 [mmol·g ⁻¹ ·h ⁻¹]	0.87 ± 0.10	-	0.90 ± 0.20

The parameters given are ΔOD_{600} for biomass formed and ΔS for substrate used. Monophasic growth was observed with GlcNAc. With MurNAc, two phases were observed, and biomass yield coefficients of cell dry weight formed per used substrate used ($Y_{X/S}$), maximal specific growth rates (μ_{max}) and specific substrate uptake rates (qS) are given for the phase 1 and phase 2. With MurNAc+GlcNAc, $Y_{X/S}$, μ_{max} and qS are reported for the phase where GlcNAc was utilized exclusively (phase 1; 0–8 h) as well as for the phase when MurNAc was utilized exclusively (phase 2; 12–18 h).

TABLE 4 | Parameters describing concentration in mM and production yield ($Y_{P/S}$) of L-lysine, L-PA and 1,5-diaminopentane after 72 h with either 25 mM GlcNAc, either 25 mM MurNAc or 25mM GlcNAc of indicated strains and glutamate production after 48 h from either 25 mM MurNAc either a mixture of GlcNAc and MurNAc, each 25 mM.

Product	Strain ID	C _{Substrate} [mM]	C _{product} [mM]	Y _{P/S} [mmol·mmol ⁻¹]
Lycopene	$\Delta crtYEB \Delta nanR PCQ$	25 mM MurNAc	–	0.04 ± 0.01 mg (gCDW) ⁻¹
	$\Delta crtYEB \Delta nanR PCQnE$	25mM GlcNAc + 25 mM MurNAc		0.10 ± 0.01 mg (gCDW) ⁻¹
L-lysine	DM1729 $\Delta nanR PCQnE$	25 mM GlcNAc	7.6 ± 0.3	0.30 ± 0.10
	DM1729 $\Delta nanR PCQnE$	25 mM GlcNAc + 25 mM MurNAc	10.6 ± 0.6	0.21 ± 0.10
	DM1729 $\Delta nanR$	25mM MurNAc	6.9 ± 0.6	0.27 ± 0.05
1,5-diaminopentane	DM1729 $\Delta nanR PCQ ldcC$	25 mM MurNAc	4.3 ± 0.1	0.30 ± 0.10
L-PA	DM1729 $\Delta nanR PCQ LPA$	25 mM MurNAc	4.2 ± 0.2	0.35 ± 0.10
Glutamate	$\Delta nanR PCQ$	25 mM MurNAc	1.0 ± 0.1	0.03 ± 0.00
	$\Delta nanR PCQnE$	25mM GlcNAc + 25 mM MurNAc	1.9 ± 0.1	0.04 ± 0.00

MurNAc-Based Production of Food and Feed Additives and Derived Chemicals

MurNAc was expected not only to support growth of recombinant *C. glutamicum* strains, but also production of value-added compounds. Therefore, MurNAc was tested as sole carbon source or in blends with GlcNAc for production of the amino acids L-lysine and L-glutamate, the diamine 1,5-diaminopentane, the cyclic non-proteinogenic amino acid L-pipecolic acid, and the carotenoid lycopene.

The lycopene accumulating strain *C. glutamicum* $\Delta crtYEB \Delta nanR$ (Matano et al., 2014) was transformed with the plasmids pVWEx1_{murP_crr}, pEC-XT99A_{nagE} and pCXE50_{murQ} as described above and the resulting strains were named $\Delta crtYEB \Delta nanR PCQ$ and $\Delta crtYEB \Delta nanR PCQnE$. Cells of both strains accumulated lycopene when grown in MurNAc containing minimal medium. Strain $\Delta crtYEB \Delta nanR PCQ$ showed a lycopene content of 0.04 mg ± 0.01 (g CDW)⁻¹ in MurNAc minimal medium. Growth of *C. glutamicum* $\Delta crtYEB \Delta nanR PCQnE$ with a MurNAc/GlcNAc blend led to a lycopene content of 0.10 ± 0.01 mg (g CDW)⁻¹.

The L-lysine producing strains *C. glutamicum* DM1729 $\Delta nanR PCQ$ and DM1729 $\Delta nanR PCQnE$ were constructed based on DM1729 $\Delta nanR$ as described above for lycopene accumulating strains. DM1729 $\Delta nanR PCQ$ produced 7 ± 1 mM L-lysine ($Y_{P/S}$ 0.27 ± 0.05 mmol mmol⁻¹) and DM1729 $\Delta nanR PCQnE$ produced 11 ± 1 mM L-lysine ($Y_{P/S}$ 0.21 ± 0.10 mmol mmol⁻¹) in minimal medium with either 25 mM MurNAc or a combination of 25 mM MurNAc and 25 mM GlcNAc., whereas 7.6 ± 0.3 mM L-lysine ($Y_{P/S}$ 0.30 ± 0.01 mmol mmol⁻¹) have been produced from DM1729PCQnE with 25 mM GlcNAc (Table 4).

To test if the L-lysine derived compounds 1,5-diaminopentane and L-pipecolic acid can also be produced from MurNAc, strain DM1729 $\Delta nanR PCQ$ was transformed with either pEC-XT99A_{ldcC} or pEC-XT99A_{lysDH-proC}. 1,5-Diaminopentane can be generated from L-lysine by lysine decarboxylase LdcC and L-pipecolic acid can be generated from L-lysine in a three-step pathway by L-lysine-6-dehydrogenase (encoded by

lysDH from *S. pomeroyi*), spontaneous ring formation and by pyrroline 5-carboxylate reductase (encoded by endogenous *proC*) (Pérez-García et al., 2016, 2017). Although the strains showed poor growth, *C. glutamicum* $\Delta nanR$ DM1729PCQ *ldcC* was able to produce 4.3 ± 0.1 mM of 1,5-diaminopentane ($Y_{P/S}$ 0.30 ± 0.10 mmol mmol⁻¹) and *C. glutamicum* $\Delta nanR$ DM1729PCQ *LPA* produced 4.0 ± 0.2 mM of L-pipecolic acid ($Y_{P/S}$ 0.35 ± 0.10 mmol mmol⁻¹) from MurNAc as sole carbon source as shown in Table 4.

L-Glutamate production was accomplished by *C. glutamicum* $\Delta nanR PCQ$ and *C. glutamicum* $\Delta nanR PCQnE$ using penicillin G as trigger. *C. glutamicum* $\Delta nanR PCQ$ accumulated 1 ± 0 mM of L-glutamate from 25 mM MurNAc after 48 h, whereas *C. glutamicum* $\Delta nanR PCQnE$ produced 2 ± 0 mM of L-glutamate under these conditions.

DISCUSSION

In this work, production of food and feed additives by *C. glutamicum* from MurNAc, an alternative carbon source without competing use in human and animal nutrition, has been established. The food amino acid L-glutamate, the feed amino acid L-lysine and the feed additive lycopene were produced from MurNAc, GlcNAc and blends of both hexosamines.

Metabolic engineering of *C. glutamicum* for access to MurNAc relied on *E. coli* genes. Its MurNAc PTS system was active in *C. glutamicum* and strains expressing *crr* in addition were able to grow faster and yield more biomass than the strains without heterologous Crr (Figure 2). Crr is a glucose-family specific EIIC component, but it can interact with the PTS-EIIBC components of several members of the glucose PTS family (Barabote and Saier, 2005). *C. glutamicum* has two complete PTS^{Glc} systems (Barabote and Saier, 2005). The finding that MurNAc could be utilized without heterologous Crr indicated that a PTS component of *C. glutamicum* made up for its absence. Moreover, the low Monod constant found for growth of the recombinant with MurNAc (Figure 3) indicated

that the MurNAc PTS catalyzed high-affinity MurNAc uptake in *C. glutamicum*, although the MurNAc PTS seems to have a lower affinity for its substrate than, e.g., the heterologous expressed GlcNAc specific PTS system NagE from *Corynebacterium glycinophilum* which showed a K_M value $3.8 \pm 0.6 \mu\text{M}$ (Ferenci, 1996; Matano et al., 2014). The K_M for the etherase MurQ in *E. coli* found in literature had a similar range of value (1.2 mM) (Hadi et al., 2008) with the K_M value found experimentally in this study for the MurNAc PTS ($0.9 \pm 0.1 \text{ mM}$) system, making the two enzymatic steps of up taking, phosphorylation and esterification balanced.

Growth of recombinant *C. glutamicum* with MurNAc was biphasic and lactate accumulated during the interphase (Figure 4A). MurNAc differs from GlcNAc only by one additional lactoyl group that is hydrolysed to lactate by etherase MurQ. Although *C. glutamicum* can utilize lactate as sole carbon source (Eikmanns, 2005), lactate accumulated. Utilization of D-lactate requires *dld* encoding quinone-dependent D-lactate dehydrogenase (Stansen et al., 2005; Kato et al., 2010). Utilization of L-lactate requires quinone-dependent L-lactate dehydrogenase which is encoded in the *LldR* repressed operon *cg3227-lldD* (Engels et al., 2008; Georgi et al., 2008). L-Lactate is secreted by *C. glutamicum* under certain conditions, e.g., during growth with glucose when oxygen is limiting, but is quickly re-utilized once *cg3227-lldD* is derepressed (Eggeling and Bott, 2005; Stansen et al., 2005; Georgi et al., 2008).

Corynebacterium glutamicum co-utilizes glucose simultaneously with many different carbon sources including those that required introduction of heterologous pathways, for example, xylose (requiring xylose isomerase gene from *E. coli* (Kawaguchi et al., 2006), arabinose (requiring the *araBAD* operon from *E. coli* (Kawaguchi et al., 2008; Schneider et al., 2011), cellobiose (requiring β -glucosidase) or glycerol (requiring *E. coli* glycerol kinase and glycerol-3-phosphate dehydrogenase), (Rittmann et al., 2008; Sasaki et al., 2008; Zahoor et al., 2012; Meiswinkel et al., 2013; Wendisch et al., 2016). Only rarely, glucose repression has been observed, for example, during sequential utilization of glucose before ethanol (due to catabolite repression of the alcohol dehydrogenase gene *adhA*) (Gerstmeir et al., 2004) or before glutamate (due to catabolite repression of the operon *gluABCD* encoding the glutamate uptake system) (Wendisch et al., 2000; Blombach and Seibold, 2010). The preferential utilization of GlcNAc before MurNAc by *C. glutamicum* $\Delta nanR PCQnE$ may be explained by an offset between fast uptake and hydrolysis of MurNAc yielding GlcNAc-6-P and D-lactate followed by fast utilization of GlcNAc-6-P, but accumulation of D-lactate. It is conceivable that overexpression of *dld* would accelerate D-lactate catabolism precluding transient D-lactate accumulation during growth with MurNAc.

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A proof-of-concept for MurNAc-based fermentative production of food and feed additives was reached. The engineered strain DM1729 $\Delta nanR PCQ$ and DM1729 $\Delta nanR PCQnE$ showed comparable L-lysine production as observed previously for GlcN and GlcNAc (Matano et al., 2014). Similarly, lycopene production from MurNAc by *C. glutamicum* $\Delta crtYEb\Delta nanR PCQ$ was comparable to that observed by similar strains with 100 mM glucose [$30 \pm 10 \mu\text{g}\cdot\text{g}(\text{CDW})^{-1}$] and 100 mM GlcNAc [$29.6 \pm 4.5 \mu\text{g}\cdot\text{g}(\text{CDW})^{-1}$] (Heider et al., 2012; Matano et al., 2014). To establish viable production processes with MurNAc as sole or combined carbon source, more work to increase titres, yields and volumetric productivities is needed. Conceptually, however, this work laid the foundation for recycling the cell wall fraction of bacterial biomass from large-scale production processes as substrate for fermentative production of food and feed additives.

CONCLUSION

Corynebacterium glutamicum was successfully metabolically engineered for utilization of the amino sugar MurNAc as alternative carbon source for growth and production of relevant value-added compounds, specifically L-lysine, L-glutamate and lycopene, from this carbon source lacking competing uses in human and animal nutrition.

AUTHOR CONTRIBUTIONS

VW conceived the study. VW and ES planned the experiments. ES and LB performed and analyzed the experiments. ES and LB drafted the manuscript. VW finalized the manuscript. All the authors agreed to the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

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