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Engineering of *Corynebacterium glutamicum* for growth and production of L-ornithine, L-lysine, and lycopene from hexuronic acids

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Abstract

Background: Second-generation feedstocks such as lignocellulosic hydrolysates are more and more in the focus of sustainable biotechnological processes. *Corynebacterium glutamicum*, which is used in industrial amino acid production at a million-ton scale, has been engineered towards utilization of alternative carbon sources. As for other microorganisms, the focus has been set on the pentose sugars present in lignocellulosic hydrolysates. Utilization of the hexuronic acids D-galacturonic acid (abundant in pectin-rich waste streams such as peels and pulps) and D-glucuronic acid (a component of the side-chains of plant xylans) for growth and production with *C. glutamicum* has not yet been studied.

Results: Neither aldohexuronic acid supported growth of *C. glutamicum* as sole or combined carbon source, although its genome encodes a putative uronate isomerase sharing 28% identical amino acids with UxaC from *Escherichia coli*. Heterologous expression of the genes for both uptake and catabolism of D-galacturonic acid and D-glucuronic acid was required to enable growth of *C. glutamicum* with either aldohexuronic acid as the sole carbon source. When present in mixtures with glucose, the recombinant *C. glutamicum* strains co-utilized D-galacturonate with glucose and D-glucuronate with glucose, respectively. When transformed with the plasmid for uptake and catabolism of the aldohexuronates, model producer strains were able to grow with and produce from D-galacturonate or D-glucuronate as sole carbon source.

Conclusions: An easily transferable metabolic engineering strategy for access of *C. glutamicum* to aldohexuronates was developed and applied to growth and production of the amino acids L-lysine and L-ornithine as well as the terpene lycopene from D-galacturonate or D-glucuronate.

Background

Corynebacterium glutamicum is a rod-shaped Gram-positive aerobic bacterium, which can be found in soil, sewages, vegetables, and fruits [1]. This bacterium is capable of utilizing various sugars as well as organic acids [2]. Among others, *C. glutamicum* has the ability to metabolize glucose, fructose, and sucrose as well as lactate, pyruvate, and acetate [2-4]. Characteristic of *C. glutamicum* is the capability of growing on mixtures of different carbon sources with a monoauxic growth [5,6] as opposed to diauxic growth observed for many other microorganisms such as *Escherichia coli* and *Bacillus subtilis* [7]. Only a few exceptions have been reported as in the case of glucose-ethanol or acetate-ethanol mixtures, where preferential substrate utilization was observed [8].

Since its discovery, *C. glutamicum* has become an indispensable microorganism for the biotechnological industry [9]. From its initial use as a natural L-glutamate producer [10], it is currently used for production of other amino acids such as L-lysine, L-ornithine, L-methionine, and L-aspartate [11-14]. However, its importance has further increased as it was for production of of non-natural products [9] such as isobutanol [15], ethanol [16], putrescine [14,17,18], cadaverine [19], carotenoids and terpenoids [20-25], and xylitol [26].

Recently, efforts with *C. glutamicum* have shifted from optimizing production processes to also include access to alternative carbon sources. As yet, feed in the industry

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relies mainly on glucose and fructose [8], which also have competing uses in the food industry. So far, alternative carbon source utilization in *C. glutamicum* has been successfully established, among others, for xylose [27,28], galactose [29], arabinose [14,30], glucosamine [31], N-acetyl-glucosamine [32], and glycerol [33]. Plant cell wall materials such as lignocellulose and pectin are promising alternatives as carbon source. These materials are readily and abundantly available as agricultural waste or forestry residues [34]. Among the sugar constituents of plant cell wall are the hexuronic acids D-galacturonate and D-glucuronate found in pectin. These acidic sugars are naturally consumed by most plant pathogenic bacteria such as *Erwinia carotovora*, *Pseudomonas syringae*, and *Agrobacterium tumefaciens*, but also by *E. coli*.

There are three pathways for utilization of hexuronic acids, namely via isomerization, oxidation, and reduction [35]. The isomerization pathway in *E. coli* consists of seven reactions, which yield the central intermediates D-glyceraldehyde-3-phosphate and pyruvate (Figure 1). Both *uxaC-uxaA* and *uxuA-uxuB* are located within operons. However, these operons and the remaining genes

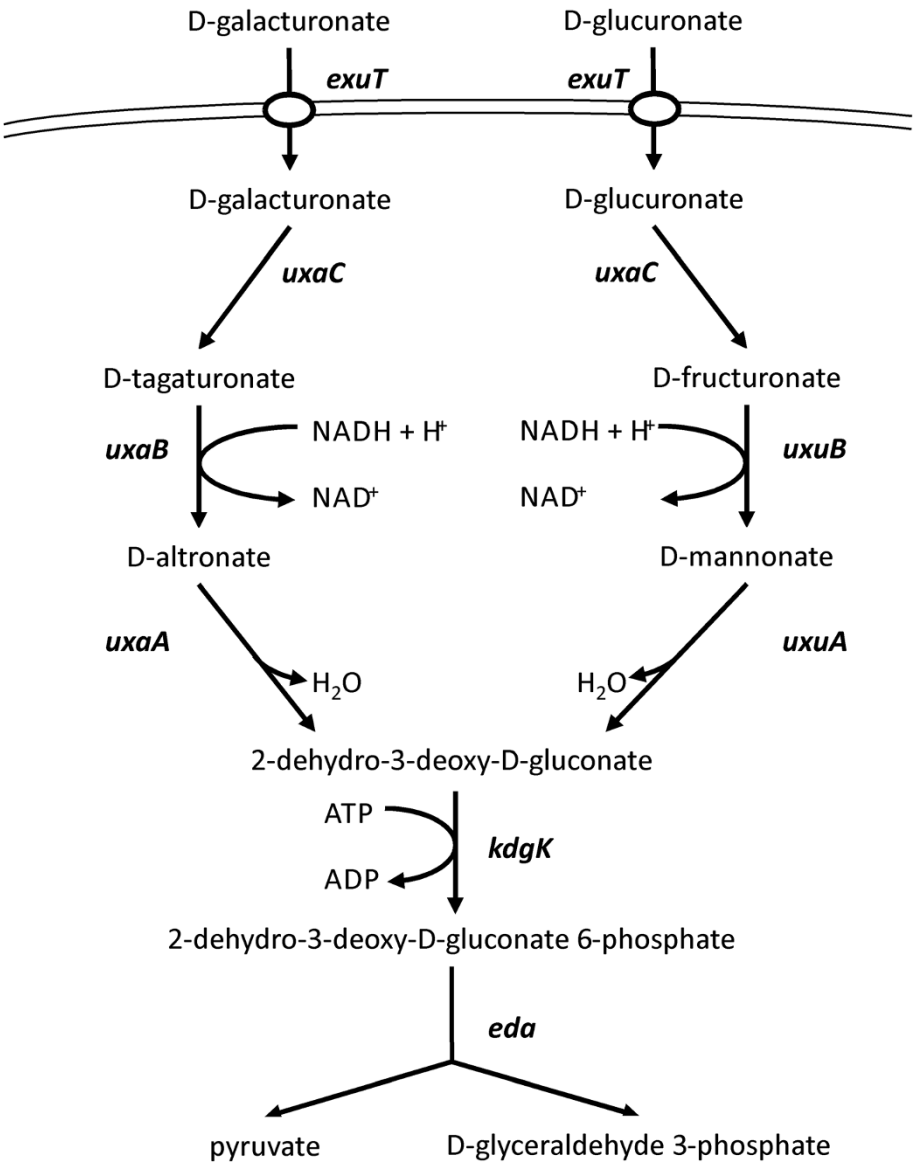

Figure 1 Schematic representation of the D-galacturonate and D-glucuronate catabolic pathways of *E. coli*. Gene names are given adjacent to the reactions: *eda*, 2-keto-3-deoxygluconate-6-phosphate aldolase; *exuT*, uptake system for hexuronic acids; *kdgK*, 2-keto-3-deoxygluconokinase; *uxaA*, altronate dehydratase; *uxaB*, altronate oxidoreductase; *uxaC*, uronate isomerase; *uxuA*, mannonate dehydratase; *uxuB*, mannonate oxidoreductase.

are not clustered but spread across the genome of *E. coli*. The genes *kdgK* and *eda* belong to the modified Entner-Doudoroff pathway, which is present in many Gram-negative bacteria, pseudomonads, and Archaea [36]. Bioinformatic analysis revealed an *uxaC* ortholog in the genome of *C. glutamicum* with 28% protein similarity to that of *E. coli*. However, homologs of *uxaB*, *uxuA*, *uxuB*, *kdgK*, and *eda* appear to be absent. Therefore, in this work, *C. glutamicum* was engineered for the uptake and utilization of D-galacturonate and D-glucuronate as alternative carbon sources. The potential of this synthetic pathway was then analyzed with respect to the production of the amino acids L-lysine and L-ornithine as well as the carotenoid lycopene.

Methods

Microorganisms, plasmids, and cultivation conditions

The wild-type strain *C. glutamicum* ATCC 13032 used in this study was obtained from the American Type Culture Collection (ATCC). Other strains include *C. glutamicum* ORN1 [14], *C. glutamicum* DM1933 [37], and *C. glutamicum* Δ*crtYEB* [23] that are derived from the wild-type strain (Table 1). The hexuronic acid utilization and transporter genes originated from *E. coli* MG1655, whereas the strain *E. coli* DH5α [38] was used for plasmid construction. Both *E. coli* strains were obtained from the Coli Genetic Stock Center (CGSC). For cultivations, the Luria broth (LB) complex medium and CGXII minimal medium [39] were used and contained glucose,

Table 1 Strains, plasmids, and oligonucleotides used in this study

Name	Relevant genotype/information	Reference
***E. coli* strain**		
DH5α	*(ϕ80lacZΔM15) Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK–, mK–) supE44 thi1 gyrA96 relA1*	[38]
***C. glutamicum* strains**		
WT	Wild-type strain ATCC 13032, auxotrophic for biotin	[40]
ORN1	In-frame deletion of *argR* and *argF*, L-ornithine overproducing strain derived from *C. glutamicum* WT ATCC 13032, auxotrophic for L-arginine	[14]
DM1933	Δ*pck* deletion mutant of *C. glutamicum* WT also carrying the chromosomal changes *pyc*(*P458S*), *hom*(*V59A*), two copies of *lysC*(*T311I*), two copies of *asd*, two copies of *dapA*, two copies of *dapB*, two copies of *ddh*, two copies of *lysA*, two copies of *lysE*	[37]
Δ*crtYEb*	$crtY_eY_fEb$ deletion mutant of *C. glutamicum* MB001	[23]
Plasmids		
pEKEx3	$Spec^R$; *E. coli/C. glutamicum* shuttle vector for regulated gene expression (P_{tac}, $lacI^q$, pBL1 $oriV_{Cg}$)	[41]
pHexA	pEKEx3 derivative for IPTG-inducible expression of *uxaCAB*, *uxuAB*, *kdgK*, and *eda* from *E. coli* containing artificial ribosome binding sites each	This work
pVWEx1	Km^R; *E. coli/C. glutamicum* shuttle vector for regulated gene expression (P_{tac}, $lacI^q$, pCG1 $oriV_{Cg}$)	[42]
pVWEx1-*exuT*	pVWEx1 derivative for IPTG-inducible expression of *exuT* from *E. coli* containing artificial ribosome binding site	This work
Primers		
G1a	GCAGGTCGACTCTAGAGGATCCCCGAAAGGAGGCCCTTCAGATGACTCCGTTTATGACTGAAGATTTC	
G1b	GTACTAGCTAATGCAATCAGTGATGTTATAGCGTTACGCCGCTTTTG	
G2d	CATCACTGATTGCATTAGCTAGTACGAAAGGAGGCCCTTCAGATGAAAACACTAAATCGTCGCGAT	
G2c	GCTAATGGTGCTATCTGGTACGATCTTAGCACAACGGACGTACAG	
G3d	GATCGTACCAGATAGCACCATTAGCGAAAGGAGGCCCTTCAGATGGAACAGACCTGGCGC	
G3f	GCAGGTCGACTCTAGAGGATCCCCATGGAACAGACCTGGCGCTGGTACGGCC	
G3c	CGTTCTAGTTACTTTGGAACGTACCTTACAGCGCAGCCACACA	
G4d	GGTACGTTCCAAAGTAACTAGAACGGAAAGGAGGCCCTTCAGATGTCCAAAAAGATTGCCGTGAT	
G4c	GTCAATCCATGGCATTCTAGCCAAGTTACGCTGGCATCGCCTC	
G5d	CTTGGCTAGAATGCCATGGATTGACGAAAGGAGGCCCTTCAGATGAAAAACTGGAAAACAAGTGCAG	
G5c	GCCAGTGAATTCGAGCTCGGTACCCTTACAGCTTAGCGCCTTCTACAG	
ExuT-fw	CTGCAGGTCGACTCTAGAGGAAAGGAGGCCCTTCAGA	
ExuT-rv	CGGTACCCGGGGATCTTAATGTTGCGGTGCGGGATC	

D-galacturonate, or D-glucuronate in concentrations as indicated in the 'Results' section. Kanamycin (25 μg ml^{-1}), spectinomycin (100 μg ml^{-1}), and/or isopropyl-β-D-thiogalactopyranoside (IPTG; 20 μM) were added to the medium when necessary. Cultivations were carried out in 50-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 120 rpm and 30°C for *C. glutamicum* or 37°C for *E. coli*. In both cases, the growth in liquid cultures was followed by measuring the optical density at 600 nm (OD_{600}).

DNA preparation, modification, and transformation

Standard procedures were used for plasmid and chromosomal DNA isolation, molecular cloning, and transformation of *E. coli*. Plasmid isolation for *C. glutamicum* was carried out as described previously [43]. Transformation of *C. glutamicum* by electroporation was carried out as described [39]. PCR experiments were carried out in a thermocycler (Analytik Jena AG, Jena, Germany) with KOD Hot Start DNA Polymerase (Novagen, Merck KGaA, Darmstadt, Germany) and with oligonucleotides obtained from Eurofins MWG Operon (Eurofins Genomics, Ebersberg, Germany) as listed in Table 2. Restriction enzymes, T4 DNA Ligase, and alkaline phosphatase were obtained from New England BioLabs, Inc. (Ipswich, MA, USA) and used according to the manufacturer's protocol.

Construction of plasmids and strains

For the construction of pHexA, the *uxaCA* and *uxuAB* operons as well as genes *uxaB*, *kdgK*, and *eda* were amplified by PCR from *E. coli* MG1655. The *uxaCA* operon was amplified with primers G1a + G1b resulting in a 2,981-bp product, whereas the *uxuAB* operon was amplified with primers G3f + G3c resulting in a 2,775-bp product. Gene *uxaB* was amplified with primers G2d + G2c, gene *kdgK* with primers G4d + G4c, and *eda* was amplified with primers G5d + G5c resulting in 1,519-, 997-, and 709-bp PCR products, respectively. Through these primers, appropriate linker sequences and a ribosomal binding site (RBS) sequence were attached to each gene or operon to facilitate the Gibson assembly [44]. The genetic load was first divided due to the insert size. Therefore, genes *uxuAB*, *kdgK*, and *eda* were cloned into the *SmaI*-digested pEKEx3 resulting in pEKEx3-*uxuAB-kdgK-eda*, designated as pAB5. The insert of pAB5 was amplified via PCR with primers G3d and G5d with a 4481-bp PCR product. The pAB5 amplicon, *uxaB*, and *uxaCA* were then used for Gibson assembly into the *SmaI*-digested pEKEx3 and yielded the final vector pHexA. The aldohexuronate transporter gene *exuT* was amplified via PCR with primers ExuT-fw and ExuT-rv from *E. coli* MG1655 and used for Gibson assembly into the BamHI-digested pVWEx1. The plasmid inserts were verified by sequencing (Sequencing Core Facility CeBiTec, Bielefeld, Germany), and the plasmids were used to transform *C. glutamicum* ATCC 13032.

DNA microarray analysis

C. glutamicum ATCC 13032 was cultivated in CGXII medium with 50 mM D-galacturonate plus 50 mM glucose, 50 mM D-glucuronate plus 50 mM glucose, or 50 mM glucose as carbon source. Exponentially growing cells were harvested after 4 h. RNA isolation, cDNA synthesis, and microarray hybridization were performed according to previous protocols [45]. Microarray images were analyzed with ImaGene software (BioDiscovery, Inc., Hawthorne, CA, USA), whereas the EMMA platform was used for data evaluation.

Quantification of amino acids and carbohydrates

To evaluate the amino acid and carbohydrate production, culture samples were taken and centrifuged (13.000×*g*, 10 min), and the supernatant analyzed by high-pressure liquid chromatography (HPLC, 1200 series, Agilent Technologies Inc., Santa Clara, CA, USA) as described previously [14,21].

Computational analysis

Protein alignments were carried out via the BLASTP algorithm [46] of NCBI (Bethesda, MD, USA). The GenBank accession number for the annotated genome sequence of *C. glutamicum* ATCC 13032 and *E. coli* MG1655 is NC_006958 [47] and NC_000913 [48], respectively.

Results

Response of *C. glutamicum* WT to D-galacturonate and D-glucuronate

D-galacturonate and D-glucuronate were tested as potential carbon sources of *C. glutamicum*. However, although its genome encodes a putative uronate isomerase sharing 28% identical amino acids with UxaC from *E. coli*, both compounds did not support growth as sole carbon sources at 50 mM (data not shown). When present in addition to 50 mM glucose, *C. glutamicum* wild type (WT) grew to comparable maximal OD_{600} values of about 18 irrespective of the presence or absence of D-galacturonate or D-glucuronate (data not shown). Surprisingly, the presence of 50 mM D-galacturonate in minimal medium with 50 mM glucose accelerated growth slightly (0.24 h^{-1} as compared to 0.17 h^{-1}), while the addition of 50 mM D-glucuronate to glucose minimal medium slowed growth of WT (0.13 h^{-1}). These observations prompted us to carry out DNA microarray experiments to study global gene expression under these conditions. Genes differentially expressed in cells growing exponentially with 50 mM D-galacturonate plus 50 mM glucose, 50 mM D-glucuronate plus 50 mM glucose, and 50 mM glucose alone are listed in Table 2. As expected, the presence of

Table 2 Gene expression analysis of *C. glutamicum* WT in CGXII minimal medium with 50 mM glucose[a]

	Gene ID	Description	*M*
Differentially expressed genes in the presence of D-glucuronate	*cg3219*	*ldh*, L-lactate dehydrogenase	1.7
	cg3303	Transcriptional regulator PadR family	1.6
	cg0580	Hypothetical protein	1.5
	cg2789	*nrdH*, glutaredoxin-like protein NrdH	−1.5
	cg2182	ABC-type peptide transport system, permease component	−1.5
	cg3300	Cation transport ATPase	−1.5
	cg2477	Hypothetical protein	−1.6
	cg1809	DNA-directed RNA polymerase subunit omega	−1.7
	cg0935	Hypothetical protein	−1.8
	cg1286	Hypothetical protein	−1.8
Differentially expressed genes in the presence of D-galacturonate	*cg2313*	*idhA3*, myo-inositol 2-dehydrogenase	2.0
	cg1118	Pyrimidine reductase, riboflavin biosynthesis	1.9
	cg0687	*gcp*, putative O-sialoglycoprotein endopeptidase	1.9
	cg1116	*tdcB*, threonine dehydratase	1.9
	cg1784	*ocd*, putative ornithine cyclodeaminase	1.9
	cg3096	*ald*, aldehyde dehydrogenase	1.9
	cg0792	Thioredoxin domain-containing protein	1.8
	cg0682	ATPase or kinase	1.7
	cg1003	*fthC*, 5-formyltetrahydrofolate cycloligase	1.7
	cg1134	*pabAB*, para-aminobenzoate synthase components I and II	1.7
	cg1438	ABC-type transport system, ATPase component (C-terminal fragment)	1.7
	cg2430	Hypothetical protein	1.7
	cg1560	*uvrA*, excinuclease ATPase subunit	1.7
	cg1014	*pmt*, protein O-mannosyltransferase	1.6
	cg1668	Putative membrane protein	1.6
	cg2625	*pcaF*, β-ketoadipyl CoA thiolase	1.6
	cg2094	Hypothetical protein	1.6
	cg1241	Hypothetical protein	1.5
	cg1876	Glycosyltransferase	1.5
	cg2417	Short-chain-type oxidoreductase	1.5
	cg3118	*cysI*, ferredoxin-sulfite reductase	−1.5
	cg0504	*qsuD*, shikimate 5-dehydrogenase	−1.5
	cg1740	Putative nucleoside-diphosphate-sugar epimerase	−1.5
	cg3225	Putative serine/threonine-specific protein phosphatase	−1.5
	cg2945	*ispD*, 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	−1.5
	cg1614	Hypothetical protein	−1.5
	cg3427	*parA1*, ATPase involved in chromosome partitioning	−1.5
	cg1252	*fdxC*, ferredoxin	−1.5
	cg0518	*hemL*, glutamate-1-semialdehyde 2,1-aminomutase	−1.5
	cg2587	Phosphoglycerate dehydrogenase or related dehydrogenase	−1.6
	cg1551	*uspA1*, universal stress protein UspA	−1.6
	cg0059	*pknA*, serine/threonine protein kinase	−1.7
	cg0156	*cysR*, transcriptional activator of assimilatory sulfate reduction	−1.7
	cg0966	*thyA*, thymidylate synthase	−1.7
	cg0060	*pbpA*, D-alanyl-D-alanine carboxypeptidase	−1.7
	cg1045	Hypothetical protein	−1.7
	cg3119	*fpr2*, probable sulfite reductase (flavoprotein)	−1.8
	cg1253	Succinyldiaminopimelate aminotransferase	−1.8
	cg0045	Putative ABC-type transporter membrane protein	−1.8
	cg3117	*cysX*, ferredoxin-like protein	−1.8
	cg3430	Hypothetical protein	−1.9
	cg3115	*cysD*, sulfate adenylyltransferase subunit 2	−1.9
	cg1037	*rfp2*, RPF2 precursor	−1.9

[a]Statistical evaluation was carried out with the *t*-test, where $p \leq 0.05$, log expression ratio $M \geq 1.5$ or ≤ -1.5, and signal intensity $A \geq 10$. Values are averages of three independent cultivations.

D-galacturonate elicited different gene expression changes than D-glucuronate. Since neither D-galacturonate nor D-glucuronate was metabolized, these gene expression changes are likely due to regulatory or secondary effects. To elicit such regulatory changes, transport of minute

amounts of D-galacturonic or D-glucuronic acid might be sufficient.

Increased expression of fermentative lactate dehydrogenase gene *ldhA* in the presence of D-glucuronate might have slowed growth with glucose since lactate is known to accumulate transiently and since high *ldhA* levels have been implied to slow growth of *C. glutamicum* with sugars [49]. Furthermore, expression of the gene for subunit omega of RNA polymerase was reduced, thus transcription might have been negatively affected more in general. The gene expression changes due to the presence of D-galacturonate did not allow deriving a potential explanation for faster growth with glucose. However, decreased expression of *cg1551* encoding putative universal stress protein UspA is in line with faster growth of *C. glutamicum* in the presence of D-galacturonate.

Expression of genes for catabolism of D-galacturonate and D-glucuronate in *C. glutamicum* WT

Plasmid pHexA was constructed for heterologous expression of the *E. coli* genes for degradation of D-galacturonate and D-glucuronate to the glycolytic intermediates pyruvate and glyceraldehyde-3-phosphate. To this end, the operons *uxaCA* and *uxuAB* as well as genes *uxaB*, *kdgK*, and *eda* were cloned with attached RBS sequences as synthetic operon into IPTG-inducible gene expression vector pEKEx3. The resulting plasmid pHexA was used to transform *C. glutamicum* WT. However, the transformants were unable to grow with either D-galacturonate or D-glucuronate as sole carbon sources (data not shown).

Co-expression of *exuT* from *E. coli* was required for uptake of hexuronic acids

Since endowing *C. glutamicum* with D-galacturonate or D-glucuronate catabolism proved insufficient for utilization of these substrates, the gene for the respective uptake system *exuT* was co-expressed from a compatible plasmid. In preliminary experiments, *C. glutamicum* WT (pHexA)(pVWEx1-*exuT*) did indeed grow with either D-galacturonate or D-glucuronate as sole carbon source, however, very slowly. Based on the assumption that overproduction of transmembrane protein ExuT perturbed growth, the concentration of the inducer IPTG was titrated. Moreover, it was required to pre-cultivate the strain in minimal medium with a mixture of 50 mM glucose and 50 mM of either D-galacturonate or D-glucuronate as carbon source. In the main culture with 50 mM of either D-galacturonate or D-glucuronate, no growth was observed for *C. glutamicum* WT(pHexA)(pVWEx1). By contrast, *C. glutamicum* WT(pHexA)(pVWEx1-*exuT*) was able to grow with D-galacturonate and D-glucuronate, respectively, with growth rates of 0.06 ± 0.01 and 0.05 ± 0.01 h^{-1}, respectively (data not shown). Thus, these results revealed that *C. glutamicum* WT lacks the ability for uptake and catabolism of hexuronic acids and that heterologous expression of the genes from *E. coli* for uptake and catabolism of hexuronic acids enabled access of *C. glutamicum* to D-galacturonate and D-glucuronate as growth substrates.

Co-utilization of hexuronic acids with glucose expression by *C. glutamicum* WT(pHexA)(pVWEx1)

A hallmark of *C. glutamicum* is its ability to co-utilize various carbon sources when these are added as carbon source mixtures. To assay if hexuronic acids are utilized simultaneously with glucose, the preferred carbon source of *C. glutamicum*, the growth and substrate consumption of *C. glutamicum* WT(pHexA)(pVWEx1) in minimal medium containing either 100 mM D-galacturonate plus 100 mM glucose or 100 mM D-glucuronate plus 100 mM glucose were determined (Figure 2). In minimal medium with the mixture of D-galacturonate plus glucose, *C. glutamicum* WT(pHexA)(pVWEx1) grew with a growth rate of 0.25 ± 0.02 h^{-1} and co-utilized glucose with D-galacturonate (Figure 2). Specific uptake rates of 28 ± 3 and 39 ± 4 nmol (mg cell dry weight (CDW))$^{-1}$ min^{-1} were derived for utilization of D-galacturonate and glucose, respectively. In minimal medium with a blend of 100 mM D-glucuronate plus 100 mM glucose, both carbon sources were utilized simultaneously and support a growth rate of 0.25 ± 0.02 h^{-1} for *C. glutamicum* WT(pHexA)(pVWEx1) (Figure 2). The specific uptake rates were 21 ± 2 nmol (mg CDW)$^{-1}$ min^{-1} for glucose and 18 ± 2 nmol (mg CDW)$^{-1}$ min^{-1} for D-glucuronate.

Production of L-lysine, L-ornithine, and lycopene by recombinant *C. glutamicum* strains from D-galacturonate and D-glucuronate

The natural substrate spectrum of *C. glutamicum* has been broadened to realize a flexible feedstock concept for production processes using this bacterium [27-33]. To test if recombinant *C. glutamicum* strains engineered to accept D-galacturonate and D-glucuronate as growth substrates are able to produce, e.g., amino acids from these substrates, model L-lysine, L-ornithine, and lycopene producer strains were transformed with plasmids pHexA and pVWEx1-*exuT*. These strains were cultivated in CGXII minimal medium with 20 μM IPTG and either 100 mM D-galacturonate or 100 mM D-glucuronate as sole carbon source.

The lysine-producing strain *C. glutamicum* DM1933 carries a number of chromosomal changes known to be beneficial for L-lysine production [37]. DM1933(pHexA) (pVWEX1-*exuT*) hardly grew with either D-galacturonate or D-glucuronate (Table 3). However, DM1933(pHexA) (pVWEX1-*exuT*) produced 6.5 ± 0.2 mM L-lysine from 100 mM D-galacturonate and 9.3 ± 1.1 mM L-lysine from 100 mM D-glucuronate (Table 3).

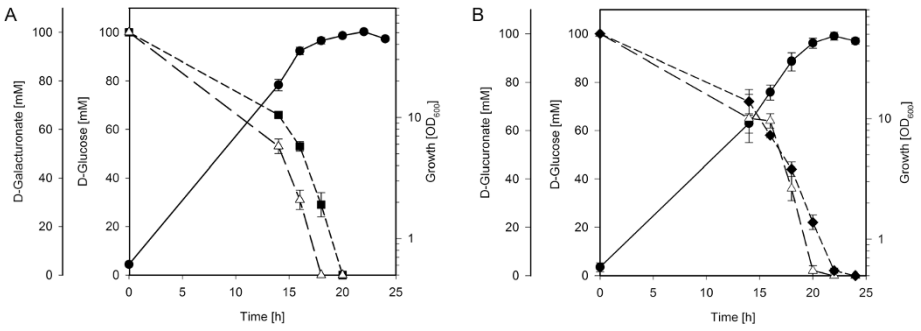

Figure 2 Growth of *C. glutamicum* strains WT(pHexA)(pVWEx1-*exuT*) with blends of D-glucose and D-galacturonate (A) or D-glucose and D-glucuronate (B). Residual concentrations of D-galacturonate (closed squares), D-glucuronate (closed diamonds), and D-glucose (open triangles) and OD600 values (closed circles) are given as means and standard deviations of triplicate cultivations in CGXII minimal medium, pH 6.3, with 20 μM IPTG, and either a mixture of 100 mM D-galacturonate and 100 mM D-glucose **(A)** or a mixture of 100 mM D-glucuronate plus 100 mM D-glucose **(B)** as carbon sources.

Lycopene accumulates in *C. glutamicum* Δ*crtYEb* due to disruption of the pathway for biosynthesis of the endogenous carotenoid decaprenoxanthin [20]. *C. glutamicum* Δ*crtYEb*(pHexA)(pVWEX1-*exuT*) grew with D-galacturonate (0.02 ± 0.01 h^{-1}) and D-glucuronate (0.04 ± 0.01 h^{-1}), respectively, as sole carbon source (Table 3). The strain produced 0.7 ± 0.1 mg (g CDW)$^{-1}$ lycopene in minimal medium with D-galacturonate and 0.8 ± 0.3 mg (g CDW)$^{-1}$ lycopene with D-glucuronate (Table 3).

Table 3 Batch fermentations of L-lysine-, L-ornithine-, and lycopene-producing strains in minimal medium with D-galacturonate or D-glucuronate

		D-galacturonate	D-glucuronate
L-lysine by DM1933(pHexA) (pVWEx1-*exuT*)			
	C_{Lys} (mM)	6.5 ± 0.2	9.3 ± 1.1
	μ (h^{-1})	0.01 ± 0.01	0.02 ± 0.01
	$Y_{p/s}$ (g_{Lys} $g_{substrate}^{-1}$)	0.04 ± 0.01	0.07 ± 0.01
Lycopene by Δ*crtYEb*(pHexA) (pVWEx1-*exuT*)			
	C_{Lyc} (mg/g)	0.7 ± 0.1	0.8 ± 0.3
	μ (h^{-1})	0.02 ± 0.01	0.04 ± 0.01
	$Y_{p/s}$ (g_{Lyc} $g_{substrate}^{-1}$)	0.09 ± 0.01	0.08 ± 0.02
L-ornithine by ORN1(pHexA) (pVWEx1-*exuT*)			
With 0.75 mM L-arginine	C_{Orn} (mM)	2.4 ± 0.2	<0.5
	μ (h^{-1})	0.03 ± 0.01	0.03 ± 0.01
	$Y_{p/s}$ (g_{Orn} $g_{substrate}^{-1}$)	0.01 ± 0.01	<0.01
With 0.125 mM L-arginine	C_{Orn} (mM)	1.7 ± 0.1	0.6 ± 0.2
	μ (h^{-1})	0.04 ± 0.01	0.03 ± 0.01
	$Y_{p/s}$ (g_{Orn} $g_{substrate}^{-1}$)	0.01 ± 0.01	<0.01

CGXII minimal medium was used with 100 mM of the indicated carbon source.

C. glutamicum ORN1 is an L-arginine auxotrophic derivative of *C. glutamicum* WT that secretes L-ornithine due to deletions of the L-ornithine carbamoyltransferase gene *argF* and the L-arginine biosynthesis repressor gene *argR* [14]. When supplemented with either 0.75 mM or 0.125 mM L-arginine, *C. glutamicum* ORN1(pHexA)(pVWEX1-*exuT*) grew in D-galacturonate and D-glucuronate minimal medium with growth rates of about 0.04 ± 0.01 h^{-1} (Table 3). However, L-ornithine accumulated only to low concentrations corresponding to yields of about 1 to 2 mol% (Table 3).

Discussion

C. glutamicum WT is not capable of utilizing hexuronic acids. Heterologous expression of gene for catabolism and uptake of the hexuronic acid pathway from *E. coli* in *C. glutamicum* enabled utilization of both D-galacturonate and D-glucuronate as sole carbon sources in minimal medium. Moreover, both hexuronates were co-utilized with glucose by the recombinant *C. glutamicum* strains developed here. Simultaneous utilization of several carbon sources as required for efficient utilization of substrate mixtures such as in lignocellulosic hydrolysates is a hallmark of *C. glutamicum* [6,8] and also pertains to co-utilization of non-native substrates by the respective recombinants [14,27,28,30-33].

Notably, the aldohexuronate transporter ExuT was strictly required, indicating that *C. glutamicum* lacks the capacity to import sugar acids. ExuT belongs to the major facilitator superfamily (MFS) class of transporters, more specifically to the anion:cation symporter (ACS) family [50]. This class of symporters transfers organic/inorganic anions while simultaneously co-transporting H^+/Na^+, respectively. ExuT has not been reported to transport other

substrates than the aldohexuronic acids. Inspection of the genome of *C. glutamicum* WT showed only one protein with 22% similarity to ExuT, namely putative lincomycin resistance protein LMRB (YP 226924.1). Engineering *C. glutamicum* for growth with other non-native carbon sources does not necessarily require heterologous expression of a gene encoding a transport system. Introduction of catabolic genes for conversion of glycerol, arabinose, and xylose was sufficient to enable utilization of these carbon sources by these recombinant *C. glutamicum* strains, while additional introduction of the respective uptake system accelerated carbon source utilization [27,28,33,51,52]. Transport engineering was not required for the amino sugar glucosamine, which is a substrate of the endogenous glucose-specific PTS [31], whereas the amino sugar N-acetylglucosamine could only be utilized if NagE from the related *Corynebacterium glycinophilum* was introduced [32]. In the latter case as well as in the present study, it was necessary to adjust the concentration of the inducer IPTG. It is often observed that too high levels of a transmembrane protein such as a transport protein results in growth perturbation [53,54]. In addition, expression levels of several genes of a pathway may need to be tuned to avoid accumulation of potentially inhibitory intermediates as demonstrated for *C. glutamicum* engineered for decaprenoxanthin overproduction [23].

It is not known if the recombinant *C. glutamicum* strain WT(pHexA)(pVWEX1-*exuT*) would be able to grow with sugar acids related to the aldohexuronates D-galacturonate and D-glucuronate since ExuT is specific for aldohexuronate uptake. In *E. coli*, the intermediate of aldohexuronate catabolism D-fructuronate serves as carbon source and its utilization requires uptake by GntP [55]. *C. glutamicum* possesses GntP for gluconate uptake [56], but it is unknown whether GntP from *C. glutamicum* accepts D-fructuronate or the related intermediates of aldohexuronate catabolism D-tagaturonate, D-altronate, or D-mannonate as substrates. Recently, it was shown that *E. coli* may grow with L-galactonate and L-gulonate as sole carbon sources with L-galactonate-5-dehydrogenase YjjN being required for their conversion to D-tagaturonate and D-fructuronate, respectively [57]. Under osmotic stress conditions, *E. coli* may use a different pathway, i.e., 5-keto 4-deoxyuronate isomerase KduI and 2-deoxy-D gluconate 3-dehydrogenase KduD may compensate for reduced levels of UxaC, UxaB, and UxuB under osmotic stress conditions [58]. Since ExuT from *E. coli* was required for aldohexuronate utilization by recombinant *C. glutamicum*, it is likely that introduction of the respective uptake systems for the related sugar acids described above is a prerequisite for their use as carbon sources.

Degradation of aldohexuronate to pyruvate yields one mole of ATP per mole of aldohexuronate by substrate-level phosphorylation as compared to two moles of ATP per glucose in the Embden-Meyerhof-Parnas (EMP) pathway of glycolysis as present in *C. glutamicum*. In the EMP pathway of glycolysis, two moles of nicotinamide adenine dinucleotide (NADH) are generated from glucose, while no net formation of NADH occurs in aldohexuronate conversion to pyruvate (Figure 1). The maximal OD_{600} values reflecting the maximal biomass concentration with 50 mM D-galacturonate consequently were lower (OD_{600} of about 6.5) than with 50 mM glucose (OD_{600} of about 18). The maximal OD_{600} (about 4.5) with 50 mM D-glucuronate was even lower (data not shown). The reduced biomass yields for aerobic growth with the aldohexuronates can be explained at least in part by their lower ATP yields as compared to glucose catabolism. The growth rates in minimal medium with D-galacturonate (0.06 h^{-1}) and D-glucuronate (0.05 h^{-1}), respectively, obtained with *C. glutamicum* WT(pHexA)(pVWEx1-*exuT*) are five to six times lower than with glucose. Aldohexuronate utilization may be accelerated, e.g., by improving heterologous gene expression or by using catabolic enzymes of other microorganisms as in the case of xylose [28]. Instability of the plasmids pHexA and pVWEX1-*exuT* were not observed in the experiments described here; however, it might be possible that plasmid instability poses a challenge when using these strains in large fermentation vessels.

The low biomass yields and slow growth rates observed with *C. glutamicum* WT(pHexA)(pVWEx1-*exuT*) were also found when the respective plasmids were transformed into model producer strains. The product yields observed were low, e.g., about 6 to 9 mol% for L-lysine (Table 3). Thus, the aldohexuronates are not good substrates for growth and production by *C. glutamicum*. However, endowing *C. glutamicum* strains with aldohexuronate catabolism may be a requirement for complete and efficient utilization of second-generation feedstocks ensuring that not only the major sugar fractions are converted to value-added products.

Conclusions

Access of *C. glutamicum* to the aldohexuronates D-galacturonate and D-glucuronate was established by heterologous expression of genes for catabolism and uptake of the aldohexuronates from *E. coli* in *C. glutamicum*. This metabolic engineering strategy could be applied to D-galacturonate- or D-glucuronate-based growth and production of the amino acids L-lysine and L-ornithine as well as the terpene lycopene.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AH planned and performed the experiments, analyzed the data, and drafted the paper. IK performed the experiments and analyzed the data. SNL planned the experiments and analyzed the data. VFW designed and

coordinated the study, analyzed the data, and wrote the paper. All authors read and approved the final manuscript.

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