Alone at last! – Heterologous expression of a single gene is sufficient for establishing the five-step Weimberg pathway in *Corynebacterium glutamicum*

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**ABSTRACT**

*Corynebacterium glutamicum* can grow on α-xylose as sole carbon and energy source via the five-step Weimberg pathway when the pentacistronic *xylXABCD* operon from *Caulobacter crescentus* is heterologously expressed. More recently, it could be demonstrated that the *C. glutamicum* wild type accumulates the Weimberg pathway intermediate α-xy-lonate when cultivated in the presence of α-xylose. Reason for this is the activity of the endogenous dehydrogenase IolG, which can also oxidize α-xylose. This raised the question whether additional endogenous enzymes in *C. glutamicum* contribute to the catabolization of α-xylose via the Weimberg pathway. In this study, analysis of the *C. glutamicum* genome in combination with systematic reduction of the heterologous *xylXABCD* operon revealed that the hitherto unknown and endogenous dehydrogenase Ksd (Cg0535) can also oxidize α-ketoglutarate semialdehyde to the tricarboxylic acid cycle intermediate α-ketoglutarate, the final enzymatic step of the Weimberg pathway. Furthermore, heterologous expression of either *xylX* or *xylD*, encoding for the two dehydratases of the Weimberg pathway in *C. crescentus*, is sufficient for enabling *C. glutamicum* to grow on α-xylose as sole carbon and energy source. Finally, several variants for the carbon-efficient microbial production of α-ketoglutarate from α-xylose were constructed. In comparison to cultivation solely on α-glucose, the best strain accumulated up to 1.5-fold more α-ketoglutarate in α-xylose/α-glucose mixtures.

1. Introduction

The Gram-positive bacterium *Corynebacterium glutamicum* has a long history in the industrial production of proteinogenic amino acids. In particular l-glutamate and l-lysine are produced at million ton-scale with this microorganism (Eggeling and Bott, 2015; Lee and Wendisch, 2017). Furthermore, *C. glutamicum* strains for more than 70 biotechnologically interesting compounds such as alcohols, organic acids or polyphenols have been engineered over the last years (Becker et al., 2018; Kallscheuer et al., 2016, 2017; Vogt et al., 2016; Wieschalka et al., 2013). However, all large-scale applications for amino acid production with *C. glutamicum* use α-glucose from starch hydrolysates or α-fructose (and sucrose) from molasses and the substrate spectrum of *C. glutamicum* variants engineered for other small molecules is also for the most part limited to these hexoses (Blombach and Seibold, 2010).

More recent studies focus on engineering *C. glutamicum* for the utilization of lignocellulose-derived pentoses α-xylose and l-arabinose as *C. glutamicum* cannot naturally catabolize these sugars (Kawaguchi et al., 2006, 2008). In case of α-xylose, two different metabolic routes have been individually added to the catabolic repertoire of *C. glutamicum*. In the Isomerase pathway, α-xylose is first converted to α-xy-lulose by a heterologous α-xylose isomerase (encoded by *xylA* from either *Escherichia coli* or *Xanthomonas campestris*) and subsequently phosphorylated by an endogenous α-xy-lulokinase (encoded by *xylB*) yielding α-xy-lulose-5-phosphate, which can be rapidly metabolized (Kawaguchi et al., 2006; Meiswinkel et al., 2013). Several *C. glutamicum* strains, capable of utilizing α-xylose via the Isomerase pathway have been engineered for the production of succinate, ethanol, lysine, glutamate, ornithine, putrescine and 1,5-diaminopentane (Buschke et al., 2011; Jo et al., 2017; Meiswinkel et al., 2013). In contrast, functional introduction of the *xylXABCD* operon from *Caulobacter crescentus* enabled *C. glutamicum* to grow on α-xylose as sole carbon and energy source via the five-step Weimberg pathway (Radek et al., 2014). In this pathway, α-xylose is initially oxidized to 1,4-α-xylo-nolactone via a xylose dehydrogenase (XylB) and subsequently hydrolyzed by a α-xylo-nolactonase (XylC) yielding α-xy-lonate (Fig. 1). Two subsequent dehydration reactions, catalyzed by a α-xylo-nolate dehydratase (XylD) and a 2-keto-3-deoxyxylonate dehydratase (XylX), lead to...
α-ketoglutarate semialdehyde, which is finally oxidized by an α-ketoglutarate semialdehyde dehydrogenase (XylA) to the tricarboxylic acid (TCA)-cycle intermediate α-ketoglutarate. However, C. glutamicum WMB1 as the first engineered strain having the Weimberg pathway allowed only for a growth rate of \( \mu = 0.07 \, \text{h}^{-1} \) on D-xylose containing defined medium. Adaptive laboratory evolution improved D-xylose utilization by 260 % yielding the strain C. glutamicum WMB2evo \( (\mu_{\text{max}} = 0.26 \, \text{h}^{-1}) \) (Radek et al., 2017). Genome sequencing of this strain revealed a functional loss of the transcriptional regulator IolR, which controls the expression of 22 genes for the most part believed to be involved in myo-inositol metabolism (Klafl et al., 2013). Among these genes is iolT1 encoding for the myo-inositol/proton symporter IolT1, which turned out to also contribute to α-xylose uptake in C. glutamicum (Brüsseler et al., 2018). By rationally introducing two point mutations into the IolR-binding site of the iolT1-promoter yielding C. glutamicum P\text{DG} iolT1, this effect could be successfully mimicked. Furthermore, an endogenously encoded α-xylose dehydrogenase (IolG) contributing to the oxidation of α-xylose in C. glutamicum could be identified, which was subsequently employed for the carbon efficient production of α-xylose with C. glutamicum (Tenhau et al., 2018).

These studies show that the C. glutamicum wild type, although not capable of α-xylose utilization via the Weimberg pathway or any other catabolic strategy by nature, does already possess individual Weimberg pathway components enabling α-xylose transport and initial α-xylose oxidation. This causes one to wonder whether there are additional endogenous enzymatic activities contributing to α-xylose utilization, which would help to reduce the number of heterologous genes required for establishing the Weimberg pathway in this bacterium.

In this study, we performed an analysis of the C. glutamicum genome in combination with systematic reduction of the xylXABCD operon to identify such enzymes. Furthermore, we exploited the Weimberg pathway for the direct conversion of α-xylose to α-ketoglutarate and could show that this represents a promising strategy for the microbial production of α-ketoglutarate with C. glutamicum.

2. Materials and methods

2.1. Bacterial strains, plasmids, media and growth conditions

All used bacterial strains and plasmids including their characteristics and sources are listed in Table 1. Escherichia coli DH5α, used for cloning purposes only, was routinely cultivated on a rotary shaker (170 rpm, 37 °C) in reaction tubes with 5 mL Lysogeny Broth (LB) medium (Bertani, 1951) or on LB agar plates (LB medium with 1.8 % [wt/vol] agar). All C. glutamicum strains are derived from C. glutamicum ATCC 13032 (Abe et al., 1967) and were aerobically cultivated on a rotary shaker either in reaction tubes (170 rpm, 30 °C) or in baffled shake flasks (130 rpm, 30 °C). As cultivation medium, brain heart infusion (BHI) medium (Difco Laboratories, Detroit, USA) or defined CGXII medium (Keilhauer et al., 1993) supplemented with different D-glucose/D-xylose mixtures were used. For plasmid propagation, kanamycin was added to final concentrations of 25 μg mL\(^{-1}\) (C. glutamicum) or 50 μg mL\(^{-1}\) (E. coli). Where appropriate, the antibiotic spectinomycin was added to a final concentration of 100 μg mL\(^{-1}\). Induction of gene expression was achieved by isopropyl β-D-thiogalactoside (IPTG) supplementation to a final concentration of 1 mM. In general, growth of bacterial strains, cultivated in baffled shake flasks, was followed over time by measuring the optical density at 600 nm (OD\text{600}). Cultivations in the microtiter plate format were performed in Flower Plates with optodes using the microbioreactor BioLector (m2plabs, Baesweiler, Germany), enabling online determination of backscatter, pH and dissolved oxygen. BioLector cultivations were routinely inoculated to an OD\text{600} of 1 and incubated at 30 °C, 1300 rpm and 80 % humidity. The total culture volume was always 1 mL and the backscatter gain was set to 15.

2.2. Plasmid and strain construction

All enzymes were purchased from Thermo Scientific (Schwerte, Germany) whereas codon-optimized synthetic genes for expression in
Table 1
Strains and plasmids used in this study.

| Strain or plasmid | Relevant characteristics | Source or reference |
|-------------------|--------------------------|---------------------|
| **C. glutamicum strains** | | |
| ATCC 13002 (WT) | biotin auxotroph wild-type strain | Abe et al. (1967) |
| *p*OstT1 | Derivative of C. glutamicum ATCC 13032 with two point mutations in the promoter of *ostT1*, relative to the start codon at position –113 (A→G) and –112 (C→G) respectively | (Brüsseler et al., 2018) |
| *p*OstT1 Δcg0535 | Derivative of C. glutamicum *p*OstT1 with in-frame deletion of cg0535 (kanA) | This study |
| *p*OstT1 ΔodhA | Derivative of C. glutamicum *p*OstT1 with in-frame deletion of odhA (cg1280) | This study |
| **E. coli strains** | | |
| DH5α | F− ΔθlacZΔM15 ΔlacZYA-argF]/U169 recA1 endA1 hsdR17 (rK− mK−) proA-supE44 Δ(λ−i) gyr96 relA1 | Invitrogen (Karlsruhe, Germany) |
| BL21 (DE3) | F− ompT hsdR (rK− mK−) dcm | Invitrogen (Karlsruhe, Germany) |
| **C. glutamicum Plasmids** | | |
| pEKEx3 | SpeI; C. glutamicum/E. coli shuttle vector for regulated gene expression; (pBR322) | (Gande et al., 2007) |
| pEKEx3-xylXADCcr-opt | SpeI; pEKEx3 derivative for the regulated expression of xylXADCcr of C. crescentus | Radek et al. (2017) |
| pHK52 | SpeI; pEKEx3-xylXADCcr-opt | This study |
| pHX104K | SpeI; pEKEx3 derivative for the regulated expression of xylXADCcr of C. crescentus | This study |
| pEKEx3-xylXDDcr-opt | SpeI; pEKEx3 derivative for the regulated expression of xylXDDcr of C. crescentus | This study |
| pEKEx3-xylEXDcr-opt | SpeI; pEKEx3 derivative for the regulated expression of xylEXDcr of C. crescentus | This study |
| pEKEx3-xylEXDcr-opt | SpeI; pEKEx3 derivative for the regulated expression of xylEXDcr of C. crescentus | This study |
| pK19mobbacB-DΔcg0535 | Kanβ; plasmid for in-frame deletion of cg0535 (kanA) | This study |
| pK19mobbacB-DΔodhA | Kanβ; plasmid for in-frame deletion of odhA (cg1280) | This study |
| **E. coli Plasmids** | | |
| pET-28b(+) | Kanβ; Vector for overexpression of genes in E. coli, adding an N-terminal hexahistidine affinity tag to the synthesized protein (pBR322 orVhcr, Pcyt-lacI) | Novagen (Darmstadt, vector, Germany) |
| pET-28b(+) cg0535 | Kanβ; pET-28b(+) derivative for the regulated expression of cg0535 (kanA) of C. glutamicum | This study |

*a Kanβ; Kanamycin resistance, SpeI; Spectinomycin resistance.

C. glutamicum were obtained from Life Technologies (Darmstadt, Germany). Oligonucleotides were synthesized by Eurofins genomics (Ebersfeld, Germany) and are listed in Table 2. For molecular cloning work, standard protocols, e.g. PCR and Gibson were used (Gibson et al., 2009; Sambrook and Russell, 2001). Verification of the constructed plasmids was performed either by restriction analysis or colony PCR. DNA sequencing was conducted at Eurofins Genomics (Ebersfeld, Germany). E. coli DH5α was routinely transformed using the RbCl-method, whereas C. glutamicum was always transformed by electroporation followed by an additional heat shock at 46 °C for 6 min (Riegeling and Bott, 2005; Hanahan, 1983). In-frame deletion of odhA and cg0535 (kanA) was performed by two-step homologues recombination using the plasmids pK19mobbacB-ΔodhA and pK19mobbacB-Δcg0535 as previously described (Schäfer et al., 1994).

2.3. Microbial production of α-ketoglutarate

For initial biomass formation, all constructed C. glutamicum strains were cultivated in 50 mL BHI medium with 10 g/L d-glucose in 500 mL baffled shake flasks at 130 rpm and 30 °C on a rotary shaker. Cells were harvested by centrifugation at 4000 rpm for 10 min, resuspended in defined CGXII medium with either 4 % d-glucose or a 1 % d-glucose/3 % α-xylene mixture and then further cultivated for 40 h at 130 rpm and 30 °C on a rotary shaker. For α-ketoglutarate production, defined CGXII medium with either 4 % d-glucose or a 1 % d-glucose/3 % α-xylene mixture was inoculated to an OD600 of 4. If appropriate, gene expression was induced by adding IPTG to a final concentration of 1 mM.

2.4. Heterologous expression of Cg0535 in E. coli and protein purification

The plasmid pET-28b(+) cg0535 was transformed into E. coli BL21 for heterologous gene expression of cg0535. Cultivations for this purpose were performed in 10 mL 2xYT medium in baffled shake flasks for 15 h at 37 °C and 130 rpm on a rotary shaker. 1 mL of this culture was used to inoculate an expression culture in 100 mL 2xYT medium with 50 mg L−1 kanamycin and cultivated at 37 °C and 130 rpm. At an optical density of OD600 ≈ 1.5, gene expression was induced by the addition of 0.5 mM IPTG and then further incubated at 18 °C and 130 rpm for 18 h. Cells were harvested by centrifugation for 30 min at 6000 rpm and the cell-free supernatant was discarded. Cell pellets were routinely stored at −80 °C if not further processed the same day. In order to avoid protein degradation, all subsequent steps for protein isolation were performed at 4 °C. Frozen cell pellets were first thawed on an ice-water mixture and resuspended in 15 mL lysis buffer (50 mM Tris-HCl pH 7.6, 100 mM NaCl, 10 mM Imidazol, 5 % Glycerin and 1 mM DTT). Crude cell extracts were obtained by using a Branson Sonifier 250 (intensity, 7; duty cycle, 40 %, 6 min; Branson Ultrasonics, Danbury, USA). After removal of the cellular debris by two centrifugation steps (30 min at 6000 rpm and 45 min at 50,000 rpm) Cg0535 was purified from the protein fraction by affinity chromatography using a GE ÄktA pure chromatography system (GE Healthcare Life Sciences, Chicago, USA).

2.5. Kinetic characterization of KsaD (Cg0535)

In all dehydrogenase assays performed, the initial NAD(P)H generation due to KsaD-mediated α-ketoglutarate semialdehyde oxidation was monitored at 340 nm and 30 °C using an Shimadzu UV-1601 Spectrophotometer (Kyoto, Japan). The enzyme assays contained 0.5–5 mM α-ketoglutarate semialdehyde (FCH Group, Chernigiv, Ukraine, supplied by AKos Consulting & Solutions Deutschland GmbH, Steinen, Germany), 5 mM NAD(P)−, 100 mM Potassium phosphate, pH 7.5. Assays were linear over time and proportional to the protein concentration used.

2.6. Quantification of α-xylene

For quantification of α-xylene, a commercial enzyme assay kit was used according to the manufacturer's instructions (Xylose Assay Kit, Megazymes, Wickow, Ireland). A set of different α-xylene concentrations served as external standards.

2.7. HPLC analysis

Identification and quantification of metabolites was performed using a High Performance Liquid Chromatography (HPLC) 1260 Infinity system (Agilent, Waldbronn, Germany). Separation was achieved by using an Organic acid H+ column (8 µm, 300 mm by 7.80 mm; Phenomenex, Torrance, CA, USA) at 80 °C with an isocratic elution program using 5 mM sulfuric acid. For detection of organic acids and d-glucose, a diode array detector (DAD) at 210 nm or a refraction index (RI) detector was used, respectively. Data acquisition and analysis was performed using the Agilent OpenLAB Data Analysis - Build 2.200.0.528 software (Agilent,
Growth of the resulting strain plasmid was then transferred to nase (encoded by xylC respectively), indicating that heterologous expression of the xylose dehydrogenase activity of the endogenous dehydrogenase IolG oxidizing D-xylose to \( \mu_{\text{max}} = 0.26 \) \( /C_6 \) (Fig. 2A). Surprisingly, growth of both strains was indistinguishable (\( \mu_{\text{max}} = 0.26 \pm 0.006 \) h \(^{-1} \)), indicating that heterologous expression of the xylose dehydrogenase (encoded by xylB) and the xylonolactonase (encoded by xylC) is neither necessary nor beneficial for growth of C. crescentus.

Motivated by these results, we performed a genome-wide search based on sequence similarity to identify genes potentially encoding for enzymes with XylX-, XylD- or XylA-activity in C. glutamicum. For a better assessment, secondary structures of Cg0535 and XylA were calculated and aligned using PROMALS3D (PROfile Multiple Alignment with predicted Local Structures and three-dimensional constraints) (Supplementary Fig. S1) (Pei et al., 2008). This in silico analysis revealed a striking resemblance between both proteins with regard to their secondary structure triggering further investigations. To the best of our knowledge, nothing about regulation and expression of cg0535 in C. glutamicum is known. Nonetheless, C. glutamicum Po6 iolT1 pEKE3-xylXADc-opt with a further reduced operon was constructed to find out whether heterologous expression of XylA from C. crescentus is required for establishing the Weimberg pathway in C. glutamicum. Comparative cultivation of C. glutamicum Po6 iolT1 pEKE3-xylXADc-opt and C. glutamicum Po6 iolT1 pEKE3-xylXADc-opt revealed that C. glutamicum indeed does have an endogenous \( \alpha \)-ketoglutarate semialdehyde dehydrogenase activity as both strains exhibited the same growth rate (\( \mu_{\text{max}} = 0.26 \pm 0.006 \) h \(^{-1} \)), \( \mu_{\text{max}} = 0.26 \pm 0.006 \) h \(^{-1} \), respectively) (Fig. 2A). Subsequently, cg0535 was deleted in the genome of C. glutamicum Po6 iolT1 yielding C. glutamicum Po6 iolT1 \( \Delta \)cg0535. After transformation of this strain with pEKE3-xylXADc-opt, the resulting strain C. glutamicum Po6 iolT1 \( \Delta \)cg0535 pEKE3-xylXADc-opt was compared to that of C. glutamicum Po6 iolT1 pEKE3-xylXADc-opt bearing the complete xylA-ABC operon (Fig. 2A). Surprisingly, growth of both strains was indistinguishable (\( \mu_{\text{max}} = 0.26 \pm 0.006 \) h \(^{-1} \)), \( \mu_{\text{max}} = 0.26 \pm 0.004 \) h \(^{-1} \), respectively), indicating that heterologous expression of the xylonolactonase (encoded by xylB) and the xylonolactonase (encoded by xylC) is neither necessary nor beneficial for growth of C. glutamicum Po6 iolT1 \( \Delta \)cg0535 pEKE3-xylXADc-opt confirming that Cg0535 indeed has \( \alpha \)-ketoglutarate semialdehyde dehydrogenase activity (in silico) of an endogenous \( \alpha \)-ketoglutarate semialdehyde dehydrogenase activity

### Table 2

Oligonucleotides used in this study.

| Name | DNA Sequence (5’-3’) |
|------|----------------------|
| pET16b-fw | GATCCCGCGAAATTAATACG |
| pET16b-rv | CAAGACCCGTTAGAGGCC |
| pET16b-rv_rev | TTGTAAAACGACGGCCAGTGTCCATTATCGTAGGTGATGG |
| pET16b-rv_rev_rev | TTGTAAAACGACGGCCAGTGCGCTAGATTTAGGCCTTG |
| pe3_xylXD_xylD_rev | CTGTAAAACGACGGCCAGTGTTATTAGTGGTTGTGGCGTG |
| pe3_xylXD_xylD_fw | GTCCTAATAAGCTAGTATAAGGAGATATAGATATGAC |
| pe3_xylXD_xylX_rev | CGGAGCGCATATCTATATCTCCTTATACTAGCTTATTACAGCAG |
| pe3_xylXD_xylX-fw | GCCAAGCGCAATATAGTACGGTCTAGCCCCAGAGGTTATCG |
| pe3_check rev | GATATGACCATGATTACGCCAAGC |
| pe3_check fw | CGGCGTTTCACTTGGTTGTGGTGTCGG |
| pe3_xylXAD_xylD_rev | CTGTAAAACGACGGCCAGTGTTATTAGTGGTTGTGGCGT |
| pe3_xylXAD_xylD_fw | GTCCTAATAAGCTAGTATAAGGAGATATAGATATGAC |
| pe3_xylXAD_xylA-fw | GCCAAGCGCAATATAGTACGGTCTAGCCCCAGAGGTTATCG |
| pe3_xylXAD_xylX_rev | CGGAGCGCATATCTATATCTCCTTATACTAGCTTATTACAGCAG |
| pe3_xylXAD_xylX-fw | GCCAAGCGCAATATAGTACGGTCTAGCCCCAGAGGTTATCG |
| pe3_check rev | GATATGACCATGATTACGCCAAGC |
| pe3_check fw | CGGCGTTTCACTTGGTTGTGGTGTCGG |

**Waldbronn, Germany.**

### 3. Results

#### 3.1. Identification of an endogenous \( \alpha \)-ketoglutarate semialdehyde dehydrogenase activity

Presence of the endogenous dehydrogenase IolG oxidizing \( \alpha \)-xylose to 1,4-\( \alpha \)-xyloolactonolactone and the observation that hydrolyzation of this lactone can occur spontaneously, indicates that heterologous expression of the xylose dehydrogenase (encoded by xylB) and the \( \alpha \)-xyloolactonolactonase (encoded by xylC) from C. crescentus might not be required for establishing the Weimberg pathway in C. glutamicum. Since reduction of the Weimberg pathway encoding operon has not been tried yet, a synthetic operon comprised of codon-optimized genes for 2-keto-3-deoxy-xyloolactonolactonase (\( \alpha \)X), xylonolactonolactonase (\( \alpha \)D) and the \( \alpha \)-ketoglutarate semialdehyde dehydrogenase (\( \alpha \)A), all originating from C. crescentus, was constructed. The resulting plasmid pEKE3-xylXADc-opt plasmid was then transferred to C. glutamicum Po6 iolT1, which is characterized by deregulation of the myo-inositol/proton symporter gene iolT1. Growth of the resulting strain C. glutamicum Po6 iolT1 pEKE3-xylXADc-opt was compared to that of C. glutamicum Po6 iolT1 pEKE3-xylXADc-opt bearing the complete xylA-ABC operon (C. crescentus) (Fig. 2A). Surprisingly, growth of both strains was indistinguishable (\( \mu_{\text{max}} = 0.26 \pm 0.006 \) h \(^{-1} \)), \( \mu_{\text{max}} = 0.26 \pm 0.004 \) h \(^{-1} \), respectively), indicating that heterologous expression of the xylose dehydrogenase (encoded by xylB) and the xylonolactonolactonase (encoded by xylC) is neither necessary nor beneficial for growth of C. crescentus.

**Motivated by these results, we performed a genome-wide search based on sequence similarity to identify genes potentially encoding for enzymes with XylX-, XylD- or XylA-activity in** C. glutamicum ATCC 13032. These analyses suggested that the gene cg0535 could encode for an enzyme having a \( \alpha \)-ketoglutarate semialdehyde dehydrogenase activity. However, the predicted protein Cg0535 shares only 25 % sequence identity with XylA of C. crescentus. For a better assessment, secondary structures of Cg0535 and XylA were calculated and aligned using PROMALS3D (PROfile Multiple Alignment with predicted Local Structures and three-dimensional constraints) (Supplementary Fig. S1) (Pei et al., 2008). This in silico analysis revealed a striking resemblance between both proteins with regard to their secondary structure triggering further investigations. To the best of our knowledge, nothing about regulation and expression of cg0535 in C. glutamicum is known. Nonetheless, C. glutamicum Po6 iolT1 pEKE3-xylXADc-opt with a further reduced operon was constructed to find out whether heterologous expression of XylA from C. crescentus is required for establishing the Weimberg pathway in C. glutamicum. Comparative cultivation of C. glutamicum Po6 iolT1 pEKE3-xylXADc-opt and C. glutamicum Po6 iolT1 pEKE3-xylXADc-opt revealed that C. glutamicum indeed does have an endogenous \( \alpha \)-ketoglutarate semialdehyde dehydrogenase as both strains exhibited the same growth rate (\( \mu_{\text{max}} = 0.26 \pm 0.008 \) h \(^{-1} \), \( \mu_{\text{max}} = 0.26 \pm 0.006 \) h \(^{-1} \), respectively) (Fig. 2A). Subsequently, cg0535 was deleted in the genome of C. glutamicum Po6 iolT1, yielding C. glutamicum Po6 iolT1 \( \Delta \)cg0535. After transformation of this strain with pEKE3-xylXADc-opt, the resulting strain C. glutamicum Po6 iolT1 \( \Delta \)cg0535 pEKE3-xylXADc-opt was compared to its parent strain C. glutamicum Po6 iolT1 pEKE3-xylXADc-opt. These experiments showed that deletion of cg0535 completely abolished growth of C. glutamicum Po6 iolT1 \( \Delta \)cg0535 pEKE3-xylXADc-opt confirming that Cg0535 indeed has \( \alpha \)-ketoglutarate semialdehyde dehydrogenase activity
conducted with the dehydratases XylX and XylD of C. crescentus showed that both dehydratases accept α-xylene as substrate (Dahms and Donald, 1982). Since both dehydratase substrates α-xylene and 2-keto-3-deoxy-xylene of the Weimberg pathway, are chemically quite similar, it makes one wonder why two separate enzymes appear to be necessary (Fig. 1). Unfortunately, no experimental data shedding more light on this interesting aspect are available for the enzymes of C. crescentus. However, a conducted comparison of both enzymes as part of this study revealed only a low sequence identity (18 %) and an analysis using PROMALS3D suggested two very different secondary structures (data not shown). Nevertheless, driven by curiosity, the plasmids pEKEx3-xylXABCDCc-opt and pEKEx3-xylIDc-opt were constructed and individually introduced into C. glutamicum P06 iolT1. The resulting strains C. glutamicum P06 iolT1 pEKEx3-xylXABCDCc-opt and C. glutamicum P06 iolT1 pEKEx3-xylIDc-opt were compared with regard to growth to C. glutamicum P06 iolT1 pEKEx3 (Fig. 2B). As a result, both strains expressing either xylX or xylD could grow on this defined medium with α-xylene as sole carbon and energy source, whereas C. glutamicum P06 iolT1 could not. The growth rates of C. glutamicum P06 iolT1 pEKEx3-xylXABCDCc-opt and C. glutamicum P06 iolT1 pEKEx3-xylIDc-opt were identical (vmax = 0.25 ± 0.006 h⁻¹, klin = 0.006 ± 0.004 h⁻¹, respectively). Apparently, both dehydratases can complement for each other in C. glutamicum and heterologous expression of either xylX or xylD from C. crescentus is sufficient for enabling α-xylene utilization via the Weimberg pathway in C. glutamicum P06 iolT1.

3.2. Expression of xylD or xylX enables growth on α-xylene

Analysis of the C. glutamicum genome did not identify any hitherto unknown dehydratases potentially catalyzing the two subsequent dehydration reactions of the Weimberg pathway. Noteworthy, enzyme assays

Fig. 2. Microbioreactor cultivations of C. glutamicum strains engineered for α-xylene utilization via the Weimberg pathway. (A) C. glutamicum P06 iolT1 pEKEx3-xylXABCDCc-opt (black), C. glutamicum P06 iolT1 pEKEx3-xylXADc-opt (brown) and C. glutamicum P06 iolT1 pEKEx3-xylDCc-opt (red); (B) C. glutamicum P06 iolT1 pEKEx3-xylXABCDCc-opt (cyan), C. glutamicum P06 iolT1 pEKEx3-xylXADc-opt (green) and C. glutamicum P06 iolT1 pEKEx3 (orange). All strains were cultivated in a BioLector microbioreactor system using defined CGXII medium with 40 g L⁻¹ α-xylene as sole carbon and energy source. All data represent mean values from three biological replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
much less α-ketoglutarate in the supernatant compared to the strain with the full xylXABCD-operon (1.27 ± 0.1 g L\(^{-1}\) (8.71 ± 0.7 mM) and 1.26 ± 0.0 g L\(^{-1}\) (8.62 ± 0.0 mM), respectively). This was somewhat surprising, as these results hint towards a limitation of the flux through the Weimberg pathway during product formation, which was not observable during growth experiments with \(C. glutamicum\) strains without \(\alpha\)dhA-deletion. Subsequent construction and characterization of \(C. glutamicum\) \(\textit{P}_{\text{o6}}^{\textit{ioiT1 \alpha\text{dhA}}}\) \(\textit{PEKEK3-xylXABCD-opt}\) (black, 10 g L\(^{-1}\) \(\alpha\)-glucose and 30 g L\(^{-1}\) \(\beta\)-xylose), \(C. glutamicum\) \(\textit{P}_{\text{o6}}^{\textit{ioiT1 \Delta\alpha\text{dhA} \textit{PEKEK3-xylXABCD-opt}}}\) (red, 10 g L\(^{-1}\) \(\alpha\)-glucose and 30 g L\(^{-1}\) \(\beta\)-xylose), \(C. glutamicum\) \(\textit{P}_{\text{o6}}^{\textit{ioiT1 \Delta\alpha\text{dhA} \textit{PEKEK3-xylXABCD-opt}}}\) (cyan, 10 g L\(^{-1}\) \(\alpha\)-glucose and 30 g L\(^{-1}\) \(\beta\)-xylose), \(C. glutamicum\) \(\textit{P}_{\text{o6}}^{\textit{ioiT1 \Delta\alpha\text{dhA} \textit{PEKEK3-xylXABCD-opt}}}\) (green, 10 g L\(^{-1}\) \(\alpha\)-glucose and 30 g L\(^{-1}\) \(\beta\)-xylose). The data represent mean values and standard deviations obtained from three independent cultures. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

Functional introduction of a pathway from another organism or implementation of a novel synthetic pathway usually means addition of new enzymatic activities to the catalytic repertoire of the respective host organism. However, sometimes the “new” enzymes have overlapping substrate specificities with endogenous enzymes rendering their introduction unnecessary. This could be already shown for \(C. glutamicum\) \(R\) and \(C. glutamicum\) ATCC 13032 when establishing the two-step isomerase pathway for \(\alpha\)-xylose utilization as both strains already have a xylulokinase (XylB) and thus only require a heterologous gene encoding for a xylose isomerase (Johnsen et al., 2009).

In our experiments, microbial synthesis of α-ketoglutarate from \(\alpha\)-glucose/\(\beta\)-xylose mixture with engineered \(C. glutamicum\) strains having the Weimberg pathway turned out to be more beneficial for product formation compared to cultivations using \(\alpha\)-glucose as only substrate. This could be a direct consequence of the carbon efficiency of the Weimberg pathway offering a theoretical product yield of 100 %. In contrast, \(\alpha\)-ketoglutarate synthesis from \(\alpha\)-glucose is always accompanied by loss of carbon as \(\text{CO}_2\) during isocitrate oxidation in the TCA-cycle, which eventually only allows for a maximum theoretical yield of 83 %. However, we could observe that reduction of the \(\text{xylXABCD-opt}\) operon also reduced final product concentrations in the constructed \(\alpha\)-dhA-deletion strains. At this stage, we can only speculate that deletion of \(\alpha\)-dhA, necessary for the accumulation of significant amounts of α-ketoglutarate, causes this effect as this is the only genetic difference to the other \(\alpha\)-xylose consuming \(C. glutamicum\) strains evaluated in the context of the \(\text{xylXABCD-opt}\) operon reduction. However, this indicates that heterologous expression of the whole pentasteric \(\text{xylXABCD-opt}\) operon might not be necessary for growth of \(C. glutamicum\) in \(\alpha\)-xylose containing defined medium, but is beneficial for product formation via the Weimberg pathway, especially in more engineered strains.
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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.mec.2019.e00090.

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