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Adaptive evolution and metabolic engineering of a cellobiose- and xylose-negative *Corynebacterium glutamicum* that co-utilizes cellobiose and xylose

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Abstract

Background: An efficient microbial cell factory requires a microorganism that can utilize a broad range of substrates to economically produce value-added chemicals and fuels. The industrially important bacterium *Corynebacterium glutamicum* has been studied to broaden substrate utilizations for lignocellulose-derived sugars. However, *C. glutamicum* ATCC 13032 is incapable of PTS-dependent utilization of cellobiose because it has missing genes annotated to β-glucosidases (bG) and cellobiose-specific PTS permease.

Results: We have engineered and evolved a cellobiose-negative and xylose-negative *C. glutamicum* that utilizes cellobiose as sole carbon and co-ferments cellobiose and xylose. NGS-genomic and DNA microarray-transcriptomic analysis revealed the multiple genetic mutations for the evolved cellobiose-utilizing strains. As a result, a consortium of mutated transporters and metabolic and auxiliary proteins was responsible for the efficient cellobiose uptake. Evolved and engineered strains expressing an intracellular bG showed a better rate of growth rate on cellobiose as sole carbon source than did other bG-secreting or bG-displaying *C. glutamicum* strains under aerobic culture. Our strain was also capable of co-fermenting cellobiose and xylose without a biphasic growth, although additional pentose transporter expression did not enhance the xylose uptake rate. We subsequently assessed the strains for simultaneous saccharification and fermentation of cellulosic substrates derived from Canadian Ponderosa Pine.

Conclusions: The combinatorial strategies of metabolic engineering and adaptive evolution enabled to construct *C. glutamicum* strains that were able to co-ferment cellobiose and xylose. This work could be useful in development of recombinant *C. glutamicum* strains for efficient lignocellulosic-biomass conversion to produce value-added chemicals and fuels.

Keywords: *Corynebacterium glutamicum*, Cellobiose and xylose, Cofermentation, Intracellular β-glucosidase, Adaptive evolution

Background

Advances in metabolic engineering and synthetic biology have opened-up opportunities for us to engineer microbial hosts to produce a range of industrially-relevant chemicals and fuels [1, 2]. In addition, oligonucleotide-mediated

or CRISPR-CAS9-mediated genome editing technologies have been used to accelerated genomic evolution and enhance development of new strains [3, 4]. More efficient utilization of hexose and pentose derived sugars from lignocellulosic biomass (cellulose: ~48 %, xylan: ~22 %, lignin: ~25 %) [5] is advantageous to achieve economically-attractive bioprocesses for improving titers, productivities, and yields of value-added chemicals.

An industrial amino acid producer, *Corynebacterium glutamicum* [6] showed a broad range of sugar utilization

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such as hexose (i.g. glucose and gluconate) and disaccharide (i.g. maltose and sucrose) but some pentose (arabinose but xylose) [7]. Since there are great potentials of *C. glutamicum* as a microbial cell factory to produce other commercially relevant chemicals and fuels [8–10] from renewable lignocellulosic biomass, efficient utilization of cellulosic sugars is an inevitable goal. *C. glutamicum* has been successfully engineered for cell growth and production of biochemical using pentose sugars via either the heterologous xylose-isomerase pathway [11, 12] or Weimberg pathway [13]. For cellobiose utilization in *C. glutamicum*, recent genome sequencing of *C. glutamicum* R strain showed possible gene clusters that encode for functional EII permeases of PTS (BglF1 and BglF2), and for functional phospho- β -glucosidases (BglG1 and BglG2) [14]. Thus, an adaptive strain of *C. glutamicum* R strain grown in minimal medium with 0.2 % cellobiose and glucose has revealed a single-substitution mutation BglF(V217A or V317 M) for cellobiose utilization [14, 15]. The *C. glutamicum* R-CEL strain has been shown to utilize cellobiose, glucose and xylose simultaneously, but only possible under anaerobic conditions [16].

However, *C. glutamicum* ATCC 13032 is incapable of PTS-dependent utilization of methyl β -D-glucoside and cellobiose because it does not have any genes annotated to β -glucosidases (bG). In addition, no genes encoding for cellobiose-specific PTS permease were annotated [17]. To metabolize cellobiose, *C. glutamicum* ATCC 13032 must have an enzyme that cleaves the β -(1 → 4)-glycosidic linkage of cellobiose. Thus, bG-displaying or secreting *C. glutamicum* strains have been developed and exhibited the complete consumption of 20 g/L cellobiose in 4 days with L-lysine production [18]. The cellobiose utilization was quite slow, compared to the glucose consumption of current *C. glutamicum* strains. Thus, optimizing gene expression and maximizing the activity of bG was necessary for better production of L-lysine and other chemicals. Recently, *Saccharomyces cerevisiae* (a cellobiose- and xylose-negative yeast strain) was engineered for cellobiose utilization by expressing cellobextrin transporter (CDT-1) and intracellular bG along with a xylose-consuming pathway in order to resolve carbon catabolite repression by glucose in hydrolysates [19]. This engineering has enabled simultaneous utilization of cellobiose and xylose as a model hydrolysate, and increased the productivity of ethanol.

Like *S. cerevisiae*, *C. glutamicum* ATCC 13032 is not able to utilize either cellobiose or xylose as sole carbon source. First, we performed metabolic engineering of *C. glutamicum* for cellobiose utilization by expressing a cellobextrin transporter and an intracellular bG (Fig. 1) and evolved the strains for the efficient cellobiose utilization. Subsequently, NGS-genomic and DNA microarray

transcriptomic analysis were performed to characterize the evolved strains. Next, we have introduced the xylose-isomerase pathway [11, 12, 20] to the cellobiose-utilizing *C. glutamicum* strain for co-utilization of cellobiose and xylose. Our cellobiose-utilizing engineered strains were used to ferment the cellobiose and glucose derived from Canadian Ponderosa Pine in simultaneous saccharification and fermentation (SSF). Canadian Ponderosa Pine was used as a model lignocellulosic biomass.

Results and discussion

Utilization of cellobiose in *C. glutamicum* using metabolic engineering and adaptive evolution

To test whether heterologous expressions of either CDT-1 transporter and bG or bG alone allow utilization of cellobiose in *C. glutamicum*, the *N. crassa* *cdt-1* and *gh1-1* gene were codon-optimized and introduced into a CoryneBrick vector [11], pBbEB1c (Table 1). We attempted to grow *Cg-Cello01* strain containing both the *cdt-1* and *gh1-1* genes, and *Cg-Cello02* containing the *gh1-1* gene in CgXII minimal medium containing 2 % (w/v) cellobiose as sole carbon source. No growth of *Cg-Cello01* was observed for 4 d. Surprisingly, the cultures of *Cg-Cello01* strain exhibited the growth only after day 16 (Fig. 2). As soon as we observed the maximal cell growth of each strain (corresponding to the growth in presence of 2 % glucose), the adapted strain was transferred to fresh CgXII minimal medium containing 2 % (w/v) cellobiose for 48 h. After the first transfer, the growth and residual sugars in the culture medium were determined for each cell culture. However, no cell growth was observed for *Cg-pBbEB1c* as a control. During the adaptive evolution of *Cg-Cello01* strain by three serial transfers, cellobiose was gradually consumed during cell cultures (Fig. 2b). Interestingly, glucose in the medium was detected up to 12 g/L during the evolution and then glucose derived from cellobiose was consumed after cellobiose was completely depleted. In the last evolutionary round (third serial transfer), *Cg-Cello01(evo)* showed the complete cellobiose consumption in 12 h and glucose (5 g/L) was minimally secreted.

On the other hand, fewer adaptations of *Cg-Cello02* were performed to obtain *Cg-Cello02(evo)*. *Cg-Cello02* strains exhibited the growth after day 11. Only twice serial transfers were performed to obtain the desired phenotype, of which the strain completely consumed its cellobiose in 12 h. No glucose derived from cellobiose was secreted during the adaptive evolution rounds (Fig. 2c). Finally, we confirmed no phenotypic changes (cell growth and cellobiose consumption) shown over more than twenty's serial transfers into fresh medium (not shown) after *Cg-Cello01(evo)* and *Cg-Cello02(evo)* were obtained. As a result, the patterns of cell growth and

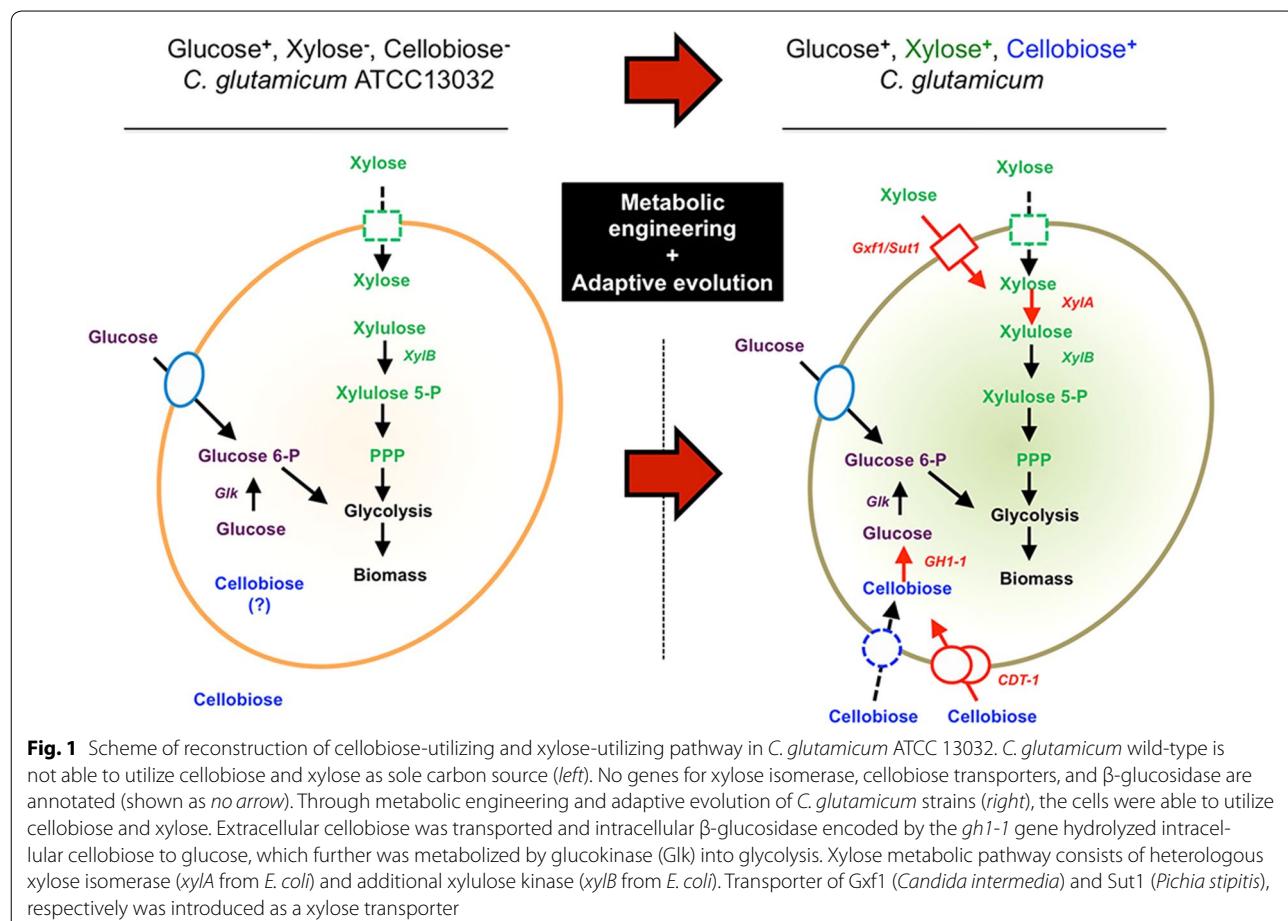


Fig. 1 Scheme of reconstruction of cellobiose-utilizing and xylose-utilizing pathway in *C. glutamicum* ATCC 13032. *C. glutamicum* wild-type is not able to utilize cellobiose and xylose as sole carbon source (left). No genes for xylose isomerase, cellobiose transporters, and β -glucosidase are annotated (shown as no arrow). Through metabolic engineering and adaptive evolution of *C. glutamicum* strains (right), the cells were able to utilize cellobiose and xylose. Extracellular cellobiose was transported and intracellular β -glucosidase encoded by the *gh1-1* gene hydrolyzed intracellular cellobiose to glucose, which further was metabolized by glucokinase (*Glk*) into glycolysis. Xylose metabolic pathway consists of heterologous xylose isomerase (*xylA* from *E. coli*) and additional xylulose kinase (*xylB* from *E. coli*). Transporter of *Gxf1* (*Candida intermedia*) and *Sut1* (*Pichia stipitis*), respectively was introduced as a xylose transporter

2 % (w/v) sugar consumption of *Cg-Cello01(evo)* and *Cg-Cello01(evo)* were almost identical to those of the wild-type [11], regardless of cellobiose or glucose. Finally, we obtained *Cg-Cello01(evo)* and *Cg-Cello02(evo)* strains as cellobiose-utilizing *C. glutamicum* ATCC 13032 derivative strains. *Cg-Cello01(evo)* strain is the fastest cellobiose-utilizing strain known under aerobic conditions.

Cellobiose-utilizing *C. glutamicum* requires activities of bG and glucokinase (*Glk*) for the catabolism of cellobiose in the cytosol. Thus, we checked whether the bG and *Glk* activities of the *Cg-Cello01(evo)* and *Cg-Cello02(evo)* strains were altered (Fig. 3). As a result, the control (*Cg-pBbEB1c*; the wild-type carrying the empty vector) showed no bG activity (both cell extract and supernatant). On the other hand, levels of the bG activities in the cell extracts of *Cg-Cello01(evo)* and *Cg-Cello02(evo)* grown on 2 % cellobiose were measured at 0.2 ± 0.01 and 0.17 ± 0.002 U/mg, respectively (Fig. 3). Significantly low or none of the bG activities were seen in the cell-free supernatants from *Cg-Cello01(evo)* or *Cg-Cello02(evo)* cultures. Also, the bG activity levels were quite similar

to a bG activity from wild-type expressing GH-1-1 alone (initial *Cg-Cello02*) was grown on 2 % glucose, the bG activity (0.17 ± 0.01). Thus, these results indicated that the adaptive evolution did not alter the intracellular expression of heterologous bG. Also, no exogenous expression of bG was occurred due to possible genetic mutations. In addition, we measured *Glk* activity over the culture interval until the carbon sources were depleted. The *Glk* activity of *Cg-Cello01(evo)* and *Cg-Cello02(evo)* was not significantly different the *Glk* activity from *Cg-pBbEB1c*. Therefore, having bG activity in the cytosol of *C. glutamicum* is one of key steps to utilization of cellobiose, but increasing or high bG activity is not necessary for better cellobiose utilization.

Characterization of the evolved cellobiose-positive *C. glutamicum* strains

Through metabolic engineering and adaptive evolution of *C. glutamicum*, we obtained the cellobiose-utilizing strains, *Cg-Cello01(evo)* and *Cg-Cello02(evo)*. Since *C. glutamicum* wild-type does not have any genes annotated

Table 1 Bacteria strains and plasmids used in this study

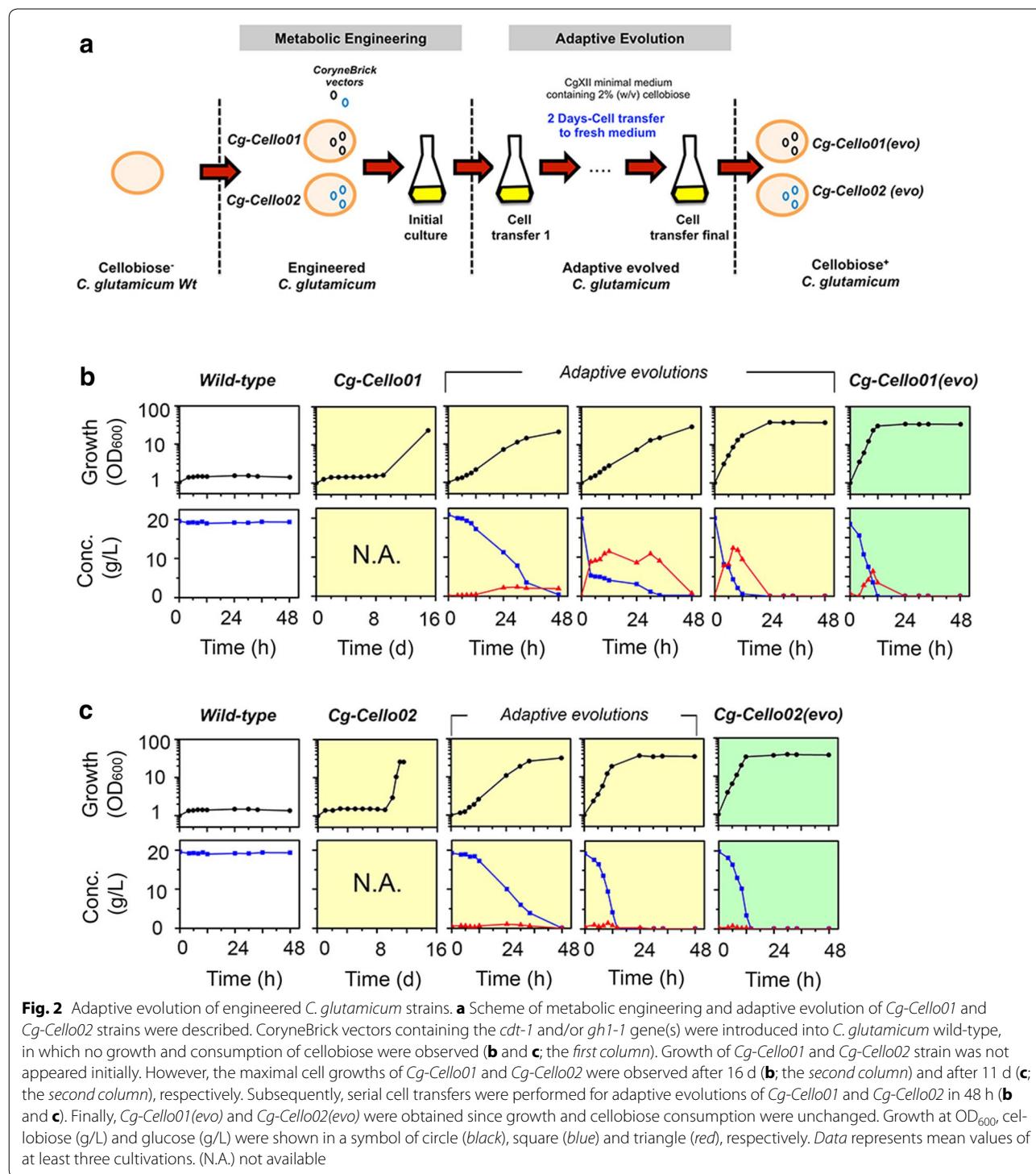
Strain or plasmid	Relevant characteristics	Source or reference
Strains		
<i>E. coli</i> HIT-DH5a	F ⁻ (80d <i>lacZ</i> M15) (<i>lacZYA-argF</i>) U169 <i>hsdR17(r-</i> m ⁺) <i>recA1 endA1 relA1 deoR96</i>	[38]
<i>C. glutamicum</i> ATCC 13032	<i>C. glutamicum</i> wild-type strain (ATCC 13032)	ATCC
<i>Cg-pBbEB1c</i>	<i>C. glutamicum</i> wild-type harboring pBbEB1c, Cm ^r CoryneBrick empty vector	[39]
<i>Cg-EcXylAB</i>	<i>C. glutamicum</i> wild-type harboring pBbEB1c- <i>EcXylA</i> (cg.co)- <i>EcXylB</i> (cg.co), Cm ^r	[11]
<i>Cg-Cello01</i>	<i>C. glutamicum</i> wild-type harboring pBbEB1c-CT-bG, Cm ^r	This study
<i>Cg-Cello02</i>	<i>C. glutamicum</i> wild-type harboring pBbEB1c-bG, Cm ^r	This study
<i>Cg-Cello01(evo)</i>	Celllobiose-adapted <i>Cg-Cello01</i> strain, Cm ^r	This study
<i>Cg-Cello02(evo)</i>	Celllobiose-adapted <i>Cg-Cello02</i> strain, Cm ^r	This study
<i>Cg-Evo1</i>	Celllobiose-adapted <i>Cg-Cello01</i> strain after plasmid curing (plasmid-free), celllobiose ⁻ , Cm ^s	This study
<i>Cg-Evo2</i>	Celllobiose-adapted <i>Cg-Cello02</i> strain after plasmid curing (no plasmids), celllobiose ⁻ , Cm ^s	This study
<i>Cg-Cello03</i>	<i>Cg-Evo1</i> harboring pBbEB1c-bG, Cm ^r	This study
<i>Cg-Cello04</i>	<i>Cg-Evo2</i> harboring pBbEB1c-bG, Cm ^r	This study
<i>Cg-Cello03-Xyl01</i>	<i>Cg-Cello03</i> harboring pBbEB1c-bG-XIK, Cm ^r	This study
<i>Cg-Cello03-Xyl02</i>	<i>Cg-Cello03</i> harboring pBbEB1c-bG-XIK-XTg, Cm ^r	This study
<i>Cg-Cello03-Xyl03</i>	<i>Cg-Cello03</i> harboring pBbEB1c-bG-XIK-XTs, Cm ^r	This study
<i>Cg-Cello04-Xyl01</i>	<i>Cg-Cello04</i> harboring pBbEB1c-bG-XIK, Cm ^r	This study
<i>Cg-Cello04-Xyl02</i>	<i>Cg-Cello04</i> harboring pBbEB1c-bG-XIK-XTg, Cm ^r	This study
<i>Cg-Cello04-Xyl03</i>	<i>Cg-Cello04</i> harboring pBbEB1c-bG-XIK-XTs, Cm ^r	This study
Plasmids		
pBbEB1c	Cole1 (Ec), pBL1 (Cg), Cm ^r , P _{trc} BglBrick sites, CoryneBrick vector	[39]
pBbEB1c-CT-bG	pBbEB1c derivative containing each codon-optimized <i>N. crassa</i> <i>cdt1</i> and <i>gh1-1</i> genes	This study
pBbEB1c-bG	pBbEB1c derivative containing each codon-optimized <i>N. crassa</i> <i>gh1-1</i> gene	This study
pBbEB1c-bG-XIK	pBbEB1c derivative containing each codon-optimized <i>N. crassa</i> <i>gh1-1</i> gene and <i>E. coli</i> <i>xylA</i> and <i>xylB</i> genes	This study
pBbEB1c-bG-XIK-XTg	pBbEB1c derivative containing each codon-optimized <i>N. crassa</i> <i>gh1-1</i> gene and <i>E. coli</i> <i>xylA</i> and <i>xylB</i> genes and <i>C. intermedia</i> <i>gxfl</i> gene	This study
pBbEB1c-bG-XIK-XTs	pBbEB1c derivative containing each codon-optimized <i>N. crassa</i> <i>gh1-1</i> gene and <i>E. coli</i> <i>xylA</i> and <i>xylB</i> genes and <i>P. stipitis</i> <i>sut1</i> gene	This study

celllobiose transporter, we investigated if the evolved cells have either functional CDT-1 transporters or altered transporters for the uptake of celllobiose.

First, plasmids used for metabolic engineering were isolated to characterize genetic mutations occurred during the adaptive evolution. When we compared the original sequence of plasmids, pBbEB1c-CT-bG and pBbEB1c-bG, in-frame deletion and a point mutation were found in the region of replication of origins (Additional file 1: Figure S1), but no mutations were found on the sequence of the *gh1-1* gene. Interestingly, the *cdt-1* gene that was present on the pBbEB1c-CT-bG in the *Cg-Cello01* strain was missing, which was confirmed by DNA sequencing. This could be due to intra-molecular recombination that occurs during the adaptive evolution at the identical and ribosomal binding synthetic DNA sequences of the *cdt-1* and *gh1-1* genes. Also, the gel images of the *cdt-1* gene by colony PCR were shown for the loss of *cdt-1* gene during the evolutionary process of the *Cg-Cello01* to *Cg-Cello01(evo)* strains (Additional

file 1: Figure S2). This result indicated that heterologous expression of CDT-1 was not suitable for the celllobiose uptake in *C. glutamicum*. Also, we found that there were no mutations found in the sequence of the *gh1-1* gene encoding for bG on the plasmids although a substitution mutation of BglF (V217A or V317 M) was found on the adaptive *C. glutamicum* R strain [15]. Thus, the plasmid sequencing results confirmed that the intracellular expression of heterologous bG was sufficient to utilize celllobiose as sole carbon source in *Cg-Cello01(evo)* and *Cg-Cello02(evo)* strains (Fig. 3).

To characterize the genetic basis of celllobiose-utilizing *C. glutamicum*, next-generation sequencing (NGS) analysis was applied to fully-evolved *Cg-Cello01(evo)* and *Cg-Cello02(evo)* strains, compared with the reference genome sequence of *C. glutamicum* ATCC 13032 (Additional file 1: Table S1 and Table S2). As a result, in the genome sequence of *Cg-Cello01(evo)* strain thirty-six genes were mutated with thirty-one single-nucleotide variants including missense (15 variants) and



silent mutations (five variants) in the coding regions, two multi-nucleotide variants, one insert and two deletions (Additional file 1: Table S1). *Cg-Cello02(evo)* strain exhibiting shorter adaption were shown for more mutations occurred. Three hundred single-nucleotide variants

including missense (123 variants), nonsense (six variants), silent mutations (98 variants) in the coding regions were identified along with 41 insertions and 28 deletions in the nucleotide sequence. Yet, the reasons for the high number of mutations for the evolved strains were unclear

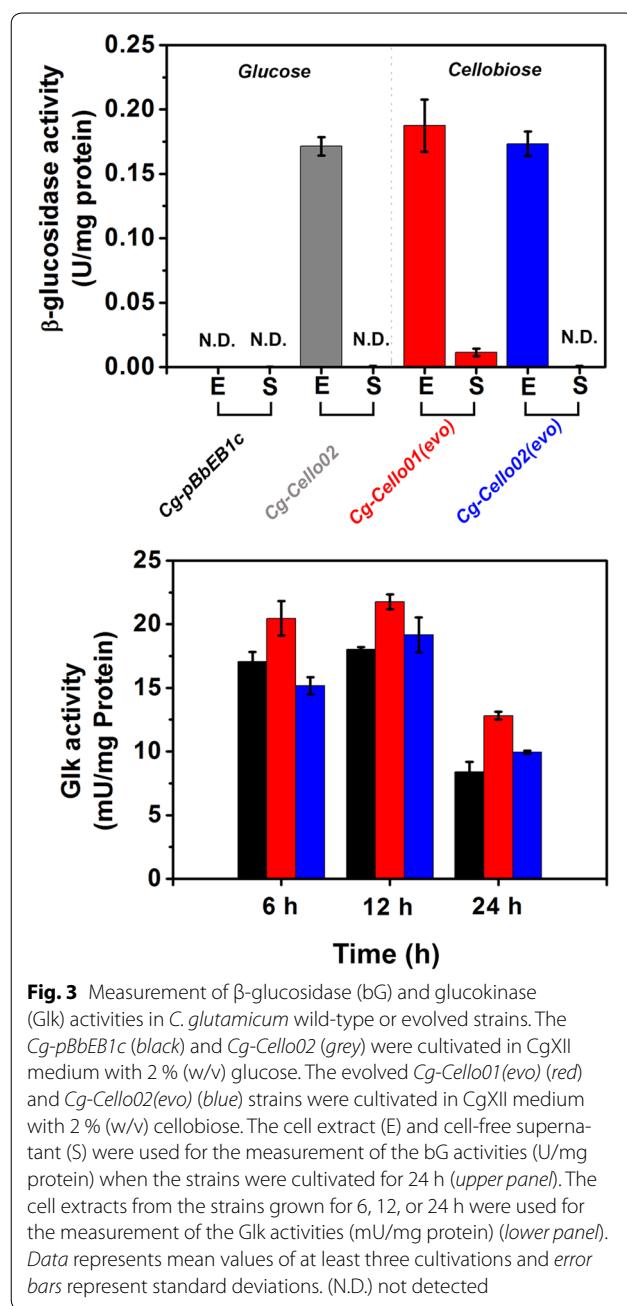


Fig. 3 Measurement of β -glucosidase (bG) and glucokinase (GK) activities in *C. glutamicum* wild-type or evolved strains. The *Cg-pBbEB1c* (black) and *Cg-Cello02* (grey) were cultivated in CgXII medium with 2 % (w/v) glucose. The evolved *Cg-Cello01(evo)* (red) and *Cg-Cello02(evo)* (blue) strains were cultivated in CgXII medium with 2 % (w/v) cellobiose. The cell extract (E) and cell-free supernatant (S) were used for the measurement of the bG activities (U/mg protein) when the strains were cultivated for 24 h (upper panel). The cell extracts from the strains grown for 6, 12, or 24 h were used for the measurement of the GK activities (mU/mg protein) (lower panel). Data represents mean values of at least three cultivations and error bars represent standard deviations. (N.D.) not detected

since the complete genome sequences of cellobiose-utilizing *C. glutamicum* R-CEL and CEL2 strains were not available [14, 15]. Specifically, multiple genetic variants were found in genes encoding for putative proteins, phage integrase, ATPase component of ABC-type transport system (*Cg2184*), GTPase, transposase (*Cg2461*), and intergenic regions. Thus, we searched the identical genetic variants in between *Cg-Cello01(evo)* and *Cg-Cello02(evo)* strains. Finally, 10 identical genetic variants were identified (Table 2). Three genes encoding for

membrane proteins (ABC-type transporter, RibX, LysE-type transloactor) were shown for the missense mutations, which could be responsible membrane proteins for the cellobiose uptake. In addition, two genes (*wzz* and *fruR*) involved in sugar metabolism were mutated, changing its amino acid sequences (Glu363Asp and Gly75Val), respectively. *MiaB* (tRNA methylthiotransferase (*MiaB*)), Maltose-binding protein (*AmyE*), and Benoate 1,2-dioxogenase (*BenA*) were also confirmed to be mutated. Unlike evolved *C. glutamicum* R strains, *C. glutamicum* ATCC1304 required multiple mutations for the cellobiose uptake in a concert of altered membrane proteins, metabolic and regulatory proteins, translational processing, and auxiliary proteins. The mechanism of those mutations for cellobiose utilization remains unclear.

Next, to characterize the genome-wide gene expression of the *Cg-Cello01(evo)* and *Cg-Cello02(evo)* strains utilizing cellobiose, we performed a DNA microarray-transcriptomic analysis to investigate whether the gene-expression levels of sugar transporters, or other membrane proteins, were differentially altered in the evolved strains during cellobiose consumption. We analyzed three groups: (1) gene expression of the evolved strains grown on glucose with a control (*Cg-pBbEB1c*) on glucose, (2) gene expression of the evolved strains grown on cellobiose with a control (*Cg-pBbEB1c*) on glucose, and (3) gene expression of the evolved strains grown on cellobiose with evolved strains on glucose. Those with gene expression that was 2-fold up- and 0.5-fold down-regulated in Group 2 and Group 3 were selected for further analysis (Fig. 4 and Additional file 1: Figure S3 and Table S3). Among 32 and 66 differentially altered genes for *Cg-Cello01(evo)* and *Cg-Cello02(evo)*, respectively, the gene expressions of four and fourteen membrane proteins (respectively) were changed significantly. Most of them are hypothetically annotated, according to the National Center for Biotechnology Information (NCBI) (accession no. NC003450).

In a comparison of transcriptomic analysis with next-generation sequencing-analysis, the mRNA expression of the regulon of *FruR* (the *ptsH*, *ptsF*, *pfkB1*, *ptsI* gene encoding for EII components of the PTS system) were not altered although the transcriptional *FruR* has been known to attenuate the induction of EII components of the PTS system in *C. glutamicum* R [21]. Thus, mutated *FurR* in the evolved strain may be not functional as a transcriptional regulator. However, the mRNA expression of a *DeoR*-type transcriptional regulator (*FruR*, *Cg2118*) was highly up-regulated (5-fold) in the *Cg-Cello02(evo)* strain in presence of cellobiose than glucose (Group 3). Also, the mRNA expression of benoate 1,2-dioxogenase (*BenA*) was only down-regulated (0.3-fold) in the *Cg-Cello02(evo)* strain in presence of cellobiose than glucose

Table 2 List of common mutations of *C. glutamicum* Cg-Cello01(evo) and Cg-Cello02(evo) strains

Reference position	Gene name	Type	Reference nucleotide	Allele nucleotide	Coding region change	Amino acid change	Annotation
32227	cg0045	MISSENSE	A	T	551A > T	Asn184Ile	Probable ABC transport protein, membrane component
364912	cg0414	MISSENSE	A	C	1089A > C	Glu363Asp	Wzz, cell surface polysaccharide biosynthesis/chain length determinant protein
1689677	cg1796	MISSENSE	C	T	103G > A	Glu35Lys	RibX, putative membrane protein-C. ammoniagenes RibX homolog
2041951	cg2118 ^a	MISSENSE	G	T	224G > T	Gly75Val	FruR, transcriptional regulator of sugar metabolism, DeoR family
2058943	cg2135	MISSENSE	A	G	490T > C	Ser164Pro	MiaB, tRNA methylthiotransferase
2296630	cg2380	Deletion	C	-	270delG	Gly90 fs	Hypothetical protein Cg2380
2331324	cg2412	MISSENSE	C	T	154C > T	Pro52Ser	Hypothetical protein Cg2412
2545730	cg2637 ^b	MISSENSE	G	A	436G > A	Asp146Asn	BenA, benzoate 1,2-dioxygenase alpha subunit (aromatic ring hydroxylation dioxygenase A)
2607484	cg2705	MISSENSE	G	A	407C > T	Ala136Val	AmyE, maltose-binding protein precursor
2826260	cg2941	MISSENSE	T	C	577A > G	Ile193Val	LysE type translocator

Full list of all mutations of *C. glutamicum* Cg-Cello01(evo) and Cg-Cello02(evo) strains were described in the Additional file 1: Table S1 and S2

^a The mRNA expression of the cg2118 gene was highly up-regulated

^b The mRNA expression of the cg2637 gene was highly down-regulated. See the details in the text

(Group 3), in which mRNA expression is repressed by global transcriptional regulator GlxR in sugar metabolism [22].

Since we observed the overexpressed hypothetical membrane proteins from the gene expression profiling of the cellobiose-utilizing strains (Fig. 4), we looked into the fatty acid profiles of cellular membranes of the wild-type strain, Cg-Cello01(evo), and Cg-Cello02(evo). Compared to the fatty acid profiles of the wild-type, lower palmitic acids ($C_{16:0}$) and higher unsaturated steric acids ($C_{18:1w9c}$) were measured in the lipids of the cellobiose-utilizing strains (Cg-Cello01(evo) and Cg-Cello02(evo)) (Table 3). This altered lipid profile of *C. glutamicum* was also shown for the wild-type growing on sodium oleate, and for mutants [23]. Moreover, cellobiose utilization influenced the fatty acid profiles of a recombinant

Rhodococcus opacus PD630 expressing bG, which accumulated fatty acids from cellobiose [24]. Multiple mutations metabolic and regulatory proteins, translational processing, and auxiliary proteins, intergenic regions in evolved *C. glutamicum* could be responsible for altered lipid profiles.

Based on the NSG- and transcriptomic analysis, altered ABC-type transporters/hypothetical membrane proteins and sugar metabolism were responsible for efficient cellobiose utilization in *C. glutamicum*. However, it was difficult to pinpoint which single transporter is mainly designated for the cellobiose uptake. Rather, multiple gene mutations could be required for the efficient cellobiose uptake in *C. glutamicum*. Those mutated genes in common could be good targets for further engineering of *C. glutamicum* wild-type to explore cellobiose uptake

Fig. 4 Heat map of altered gene expressions of *C. glutamicum* strains with cellobiose or glucose. Evolved *C. glutamicum* strains [*Cg-Cello01(evo)* and *Cg-Cello02(evo)*] grown on 2 % (w/v) cellobiose were tested with either a control (*Cg-pBbEB1c*) or *Cg-Cello01(evo)* and *Cg-Cello02(evo)* grown 2 % (w/v) glucose. The mRNA expression changed with 2-fold up- and 0.5-fold down-regulated were selected in the evolved strains with cellobiose over glucose (the third columns) as well as a control (the second columns). The mRNA ratios are averages from at least duplicated experiments. Heat maps generated by MeV (MultiExperiment Viewer ver. 4.8) showed differential gene expression of significantly changed genes. Up-regulated signals relative to the mean were colored in red. Down-regulated were colored in green (scale bar, log 2 of mRNA ratio). The criterion used for selection of RNA ratios was a signal-to-noise ratio of >3 for either Cy5 fluorescence. For the significantly changed genes, P < 0.05 as determined by a one-way ANOVA. The ID numbers of *C. glutamicum* were given at the last column, of which data were described in the Additional file 1: Table S1

and corresponding sugar metabolism or protein structures. Thus, comprehensive next-generation sequencing-analysis could be required to analyze the evolving and evolved strains to investigate the most critical mutations for the cellobiose utilization.

Reconstruction of cellobiose-positive chassis of the adaptive evolved *C. glutamicum* strains

Inverse engineering the *C. glutamicum* wild-type is necessary to construct rational microbial cells for cellobiose utilization. However, lack of multiple genome editing technology such as RNA-guided CRISPR-CAS9 [4] or MAGE [3] system of *C. glutamicum* has led to limitation of inverse engineering of *C. glutamicum* in this study. Thus, we decided to re-construct a cellobiose-positive chassis in which all multiple genetic changes were already reflected, for further engineering. We obtained plasmid-free strains by curing of plasmids in *Cg-Cello01(evo)* and *Cg-Cello02(evo)*, resulting in *Cg-Evo1* and *Cg-Evo2* (Table 1).

After the cultivation of *Cg-Evo1* and *Cg-Evo2* using 2 % cellobiose as sole carbon source, we confirmed that *Cg-Evo1* and *Cg-Evo2* did not grow at all. Thus, pBbEB1c-bG plasmid was introduced to *Cg-Evo1* and *Cg-Evo2*, yielding *Cg-Cello03* and *Cg-Cello04* strains (Fig. 5). *Cg-Cello03* and *Cg-Cello04* strains showed complete growth and consumption of cellobiose in the CgXII medium containing 2 % (w/v) cellobiose, without any adaptations or pre-culture with cellobiose (Fig. 5). This result supports that the *Cg-Evo1* and *Cg-Evo2* have already multiple genetic changes from its parental strain for the efficient cellobiose uptake.

When we compared the profiles of growth and cellobiose consumption, the *Cg-Cello03* strain showed almost identical patterns of growth and cellobiose consumption

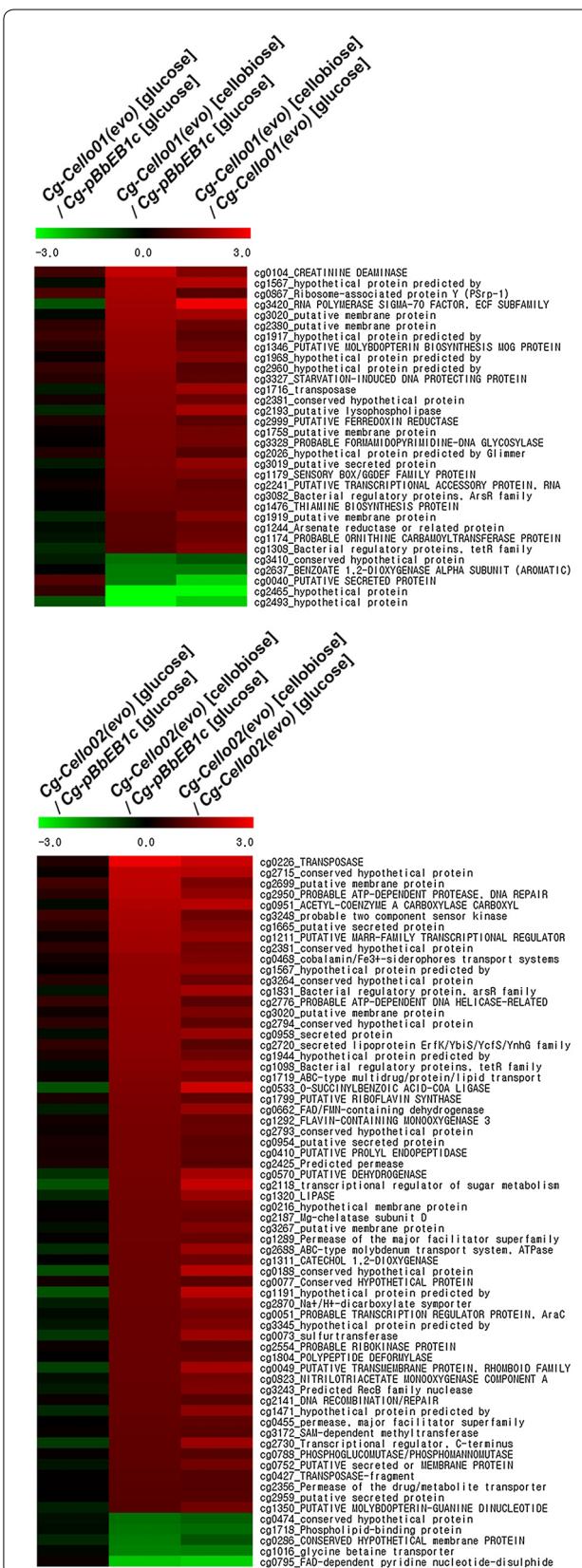


Table 3 Fatty acid profiles of the cellobiose-utilizing *C. glutamicum* strains and the wild-type

Fatty acid composition (%)	<i>Cg-pBbEB1c</i>	<i>Cg-Cello01(evo)</i>	<i>Cg-Cello02(evo)</i>
C _{10:0}	0.15	0.08	0.08
C _{12:0}	0.25	–	–
C _{12:0} 3OH	–	0.06	0.05
C _{14:0}	1.33	0.43	0.39
C _{16:1} w9c	0.84	0.51	0.46
C _{16:0}	41.04	35.89	33.28
C _{16:0} 3OH	0.31	0.69	0.34
C _{18:1} w9c	54.24	59.97	63.4
C _{18:0}	0.47	0.44	0.41
C _{18:0} 10-methyl	1.27	1.54	1.26
C _{19:1} iso I	0.09	0.33	0.33
C _{19:1} w6c/unknown fatty acids*	–	0.06	–

The *Cg-Cello01(evo)* and *Cg-Cello02(evo)* strains were cultivated with 2 % cellobiose as sole carbon source. The *Cg-pBbEB1c* strain was cultivated with 2 % glucose as sole carbon source. The fatty acid profiles of the *Cg-pBbEB1c* were almost identical to the profiles of the *C. glutamicum* wild-type [23]. Analysis of fatty acid and fatty acid methyl ester were followed by the standard protocol of Sherlock® Microbial Identification System (MIS) of Microbial IDentification Inc. (MIDI)

Data represents mean values of duplicated cultivations

* Equivalent chain lengths (ECL) value = 18.846

as its parental strain, *Cg-Cello01(evo)*. Glucose derived from cellobiose was secreted into the medium while both *Cg-Cello01(evo)* and *Cg-Cello03* strains consumed the cellobiose. In the case of *Cg-Cello04* strain, of which the parental strain is the *Cg-Cello02(evo)* strain, the rates of cell growth and cellobiose consumption were slightly retarded but no glucose was detected as for the parental strain. The reason for slower cellobiose consumption remains unclear.

Instead of inverse engineering, we successfully constructed cellobiose-positive *C. glutamicum* chassis strain that utilize cellobiose as sole carbon source under the conditions of aerobic culture by expressing intracellular bG alone. Moreover, the strains expressing intracellular bG exhibited better cellobiose utilization than any other strain either secreting bG into the medium, or displaying bG on the cell surface [18] in terms of the cellobiose consumption rate under aerobic conditions. Therefore, we obtained cellobiose-positive *C. glutamicum* chassis strains to perform metabolic engineering with cellobiose as sole carbon source.

Co-utilization of cellobiose and xylose in *C. glutamicum* through metabolic engineering

Using the cellobiose-positive *C. glutamicum* chassis strains, we focused on co-utilization of xylose and

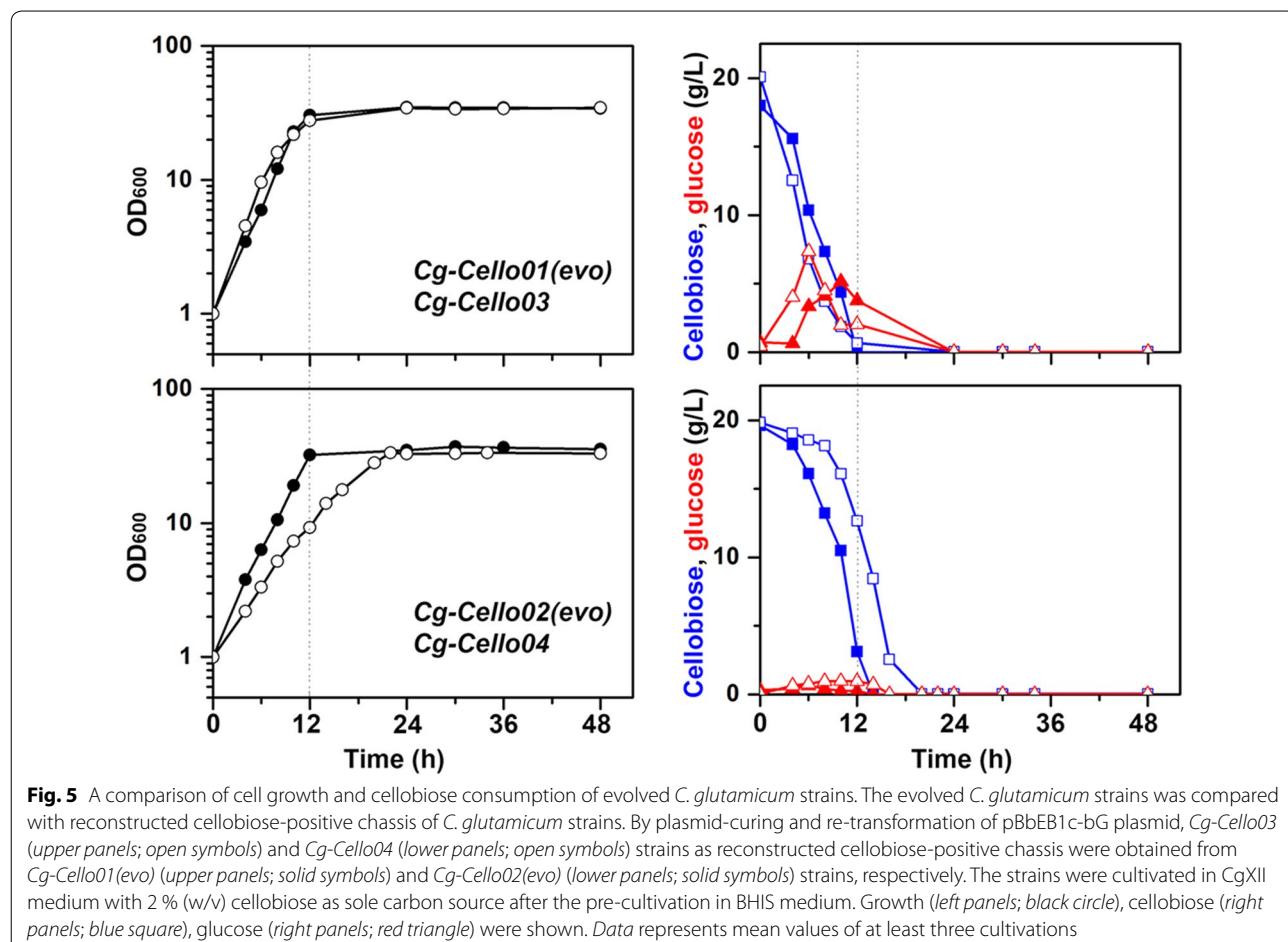
cellobiose in *C. glutamicum* via direct cellobiose uptake and intracellular hydrolysis of cellobiose. As a result, *Cg-Cello03-Xyl01* and *Cg-Cello04-Xyl01* strains were able to co-utilize cellobiose and xylose under aerobic conditions (Fig. 6). Compared to the xylose consumption by *Cg-EcXylAB* in the presence of glucose, the xylose consumption rates of the engineered strains were improved in the presence of cellobiose, instead of glucose.

As shown in the culture of *Cg-EcXylAB* strain with glucose and xylose, a biphasic growth of *Cg-Cello03-Xyl01* strain was observed after 12 h when cellobiose was completely consumed. However, a biphasic growth behavior was not shown by *Cg-Cello04-Xyl01* strain because the cellobiose was slowly consumed before 12 h, and cellobiose and xylose were almost simultaneously utilized and depleted in the culture between 12 and 24 h (Fig. 6). Moreover, *Cg-Cello04-Xyl01* strain reached higher optical density at first, compared to the *Cg-Cello03-Xyl01* strain. To further improve the engineered cellobiose- and xylose-utilizing strains, we introduced genes coding for sugar transporters (*Gxf1* and *Sut1*) into *Cg-Cello03-Xyl01* and *Cg-Cello04-Xyl01* strains, respectively. But, the engineered strains with additional heterologous transporters did not show significant improvement of xylose utilization or co-utilization under the conditions of aerobic culture (Fig. 7).

Simultaneous consumption of cellobiose and xylose in engineered *S. cerevisiae* has resolved carbon catabolite repression and significantly increased ethanol productivity through co-fermentation [19]. Engineering of cellobiose-positive *C. glutamicum* also enabled the co-consumption of cellobiose and xylose (Figs. 6 and 7). However, compared to the cellobiose-utilizing *S. cerevisiae*, our cellobiose-positive *C. glutamicum* strains showed much faster cellobiose-consumption rates during aerobic culture. On the other hand, their xylose consumption rate was not much increased during co-fermentation. Expression of a pentose-specific transporter did not increase the rate, either. Therefore, we concluded that inefficient xylose utilization by *C. glutamicum* was another bottleneck for the co-fermentation of cellobiose and xylose although we did optimize the codon-usage of the corresponding *Escherichia coli* gene sequence [11]. Exploring an alternative xylose utilization pathway in *C. glutamicum* is necessary to provide faster xylose uptake [13, 20]. This strategy could then be further applied for co-fermentation of xylose and cellobiose.

Hydrolysis of Canadian biomass and efficient SSF by engineered *C. glutamicum*

Taking advantage of the capability for cellobiose utilization by two engineered *C. glutamicum* strains (*Cg-Cello03* and *Cg-Cello04*), we applied the strains for



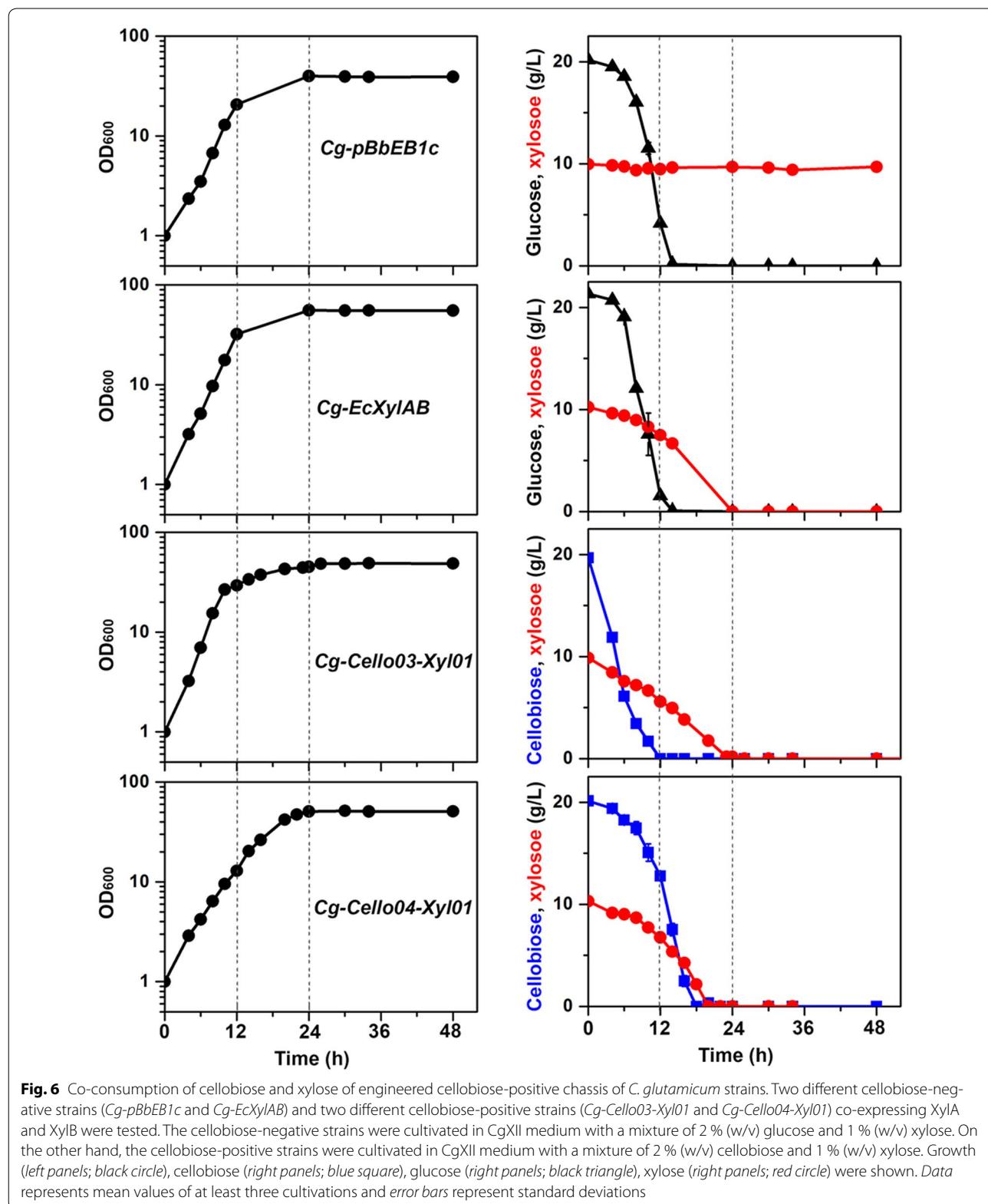
efficient SSF of Canadian cellulosic hydrolysates. Before fermentation by *C. glutamicum*, we hydrolyzed either 1 % (w/v) Avicel® PH-101, or 1 % (w/v) dissolving pulp (DP), with cellulase (Celluclast 1.5 L) under the same culture conditions (except for the cell type). As a result, equal amounts of cellobiose and glucose were detected for both cellulosic substrates (Fig. 8; upper panels), and similar conversion yields of total sugar (16.7 % and 16.15 %) were obtained for Avicel and DP, respectively. Also, enzymatic hydrolysis terminated after 12 h. There were not many differences between Avicel and DP as a cellulosic substrate for enzymatic hydrolysis with Celluclast 1.5 L.

Based on the enzymatic hydrolysis, we investigated whether the engineered strains (Cg-Cello03 and in Cg-Cello04) were able to utilize cellulosic substrates during SSF. Thus, we cultivated 1 % (w/v) cellulosic substrate of either Avicel or DP as sole carbon source for the evolved *C. glutamicum* strains with cellulase. No lag phase was shown by either culture (Fig. 8; lower panels). Compared to a control (Cg-pBbEB1c; the wild-type with empty vector), the Cello03 and Cg-Cello04 strains showed faster growth, and reached nearly double growth at the end. When total

amount of sugars was quantified independently, the total amount of sugars consumed in the Cg-Cello03 and Cg-Cello04 cultures were higher than that of a control. Moreover, we measured cellobiose and glucose in the supernatant, with the result that no cellobiose and glucose were detected during the culture of Cello03 and Cg-Cello04. Therefore, the engineered Cg-Cello03 and in Cg-Cello04 strains were able to grow by simultaneously utilizing cellobiose and glucose from cellulosic hydrolysates although low conversion yields of cellulosic substrates limit further cell growth during SSF. Along with improvements of the enzymatic hydrolysis, the simultaneous saccharification and co-fermentation (SSCF) of pretreated plant biomass (hexose and pentose) could be accomplished using engineered *C. glutamicum* strains (Cg-Cello03-Xyl01 and Cg-Cello04-Xyl01).

Conclusions

Adaptive evolution of the microbial host to acquire desired environmental phenotypes was quite difficult unless growth-associated evolutions [25, 26]. In this study, integrated metabolic engineering and adaptive evolution allowed us to develop a cellobiose- and



xylose-negative *C. glutamicum* strain that co-utilize cellobiose and xylose using. For further studies, we envision development of recombinant *C. glutamicum* strains

based on the chassis strain, for efficient lignocellulosic-biomass conversion to create valuable products such as L-glutamate or L-lysine.

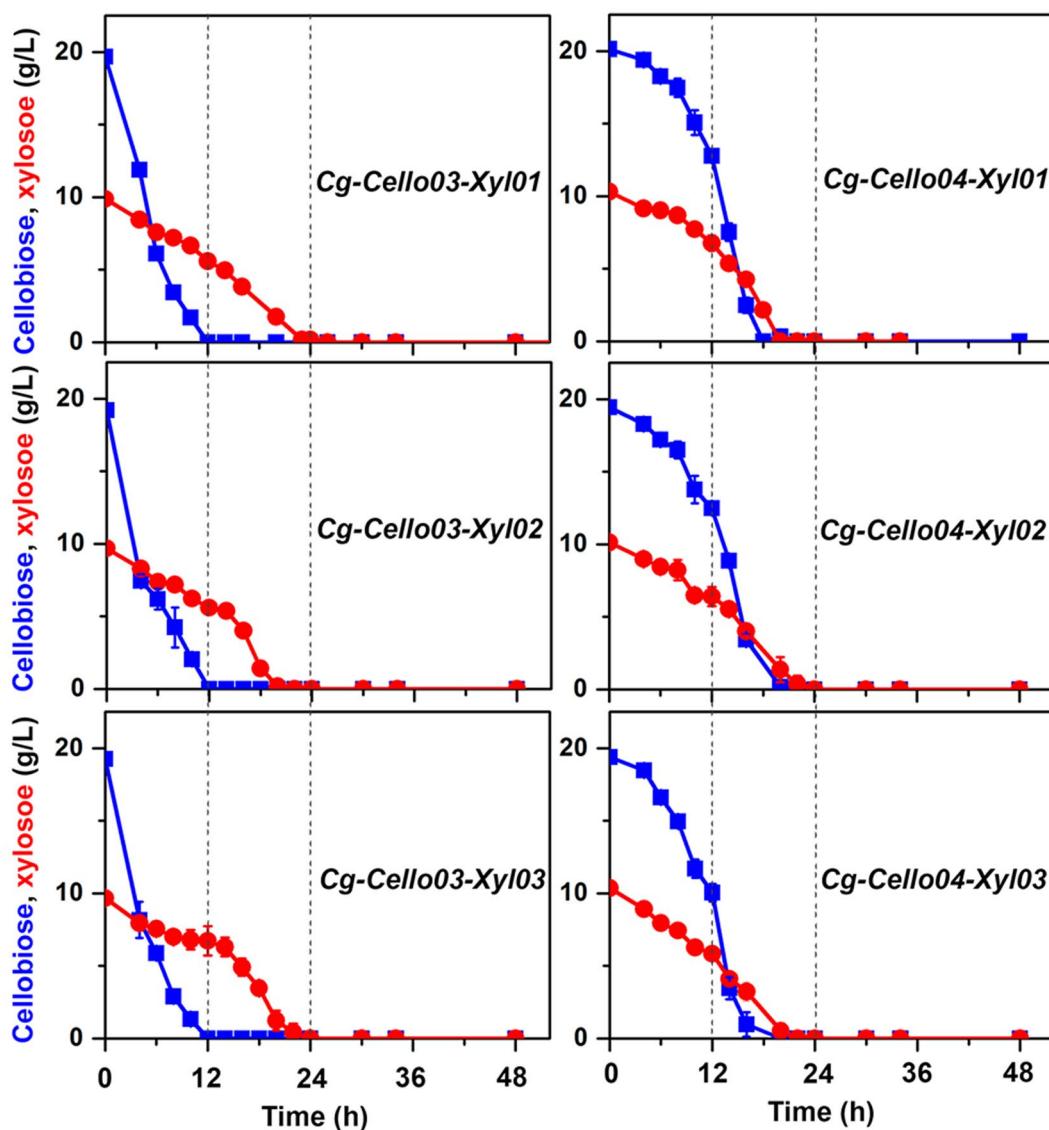


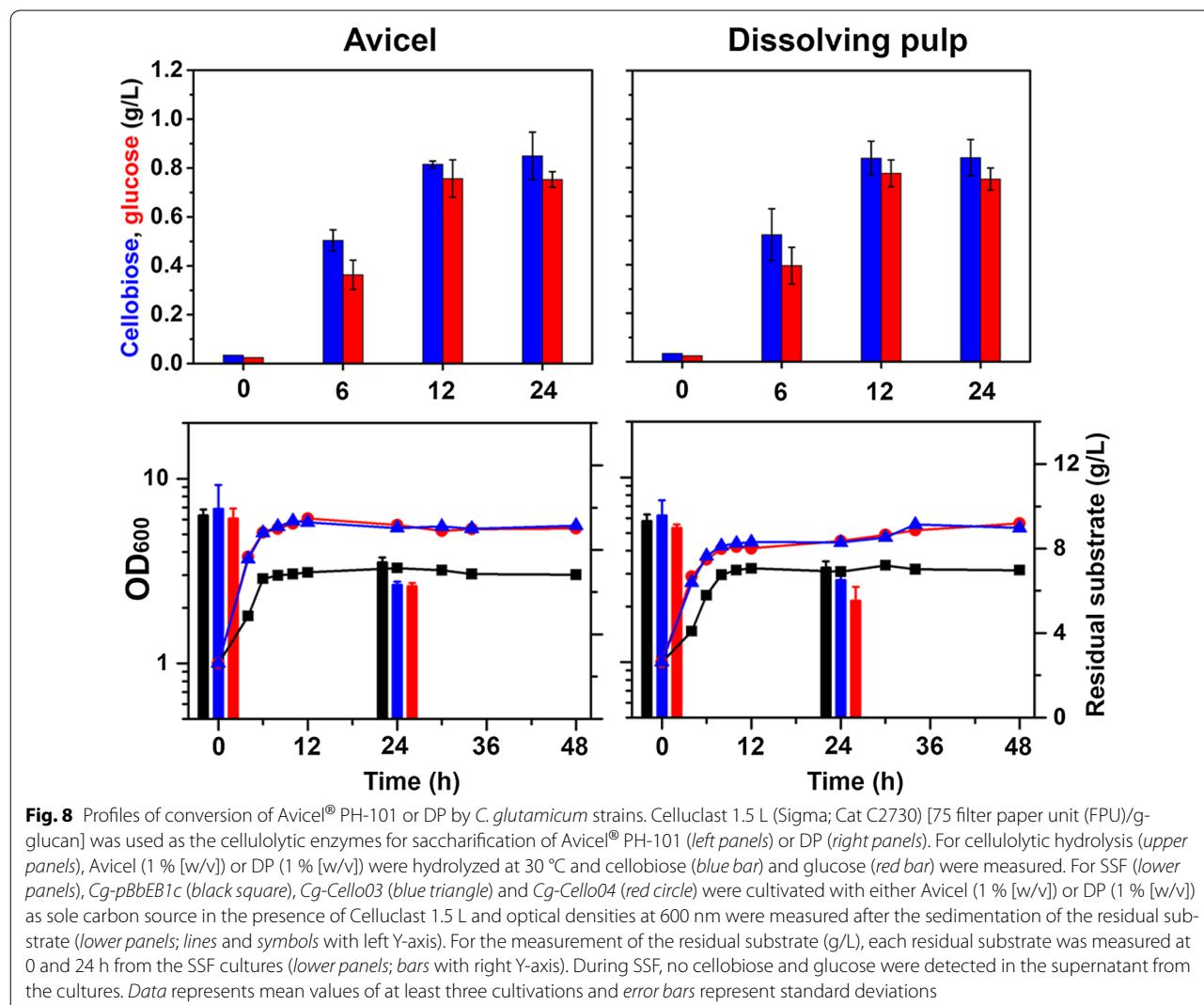
Fig. 7 Additional sugar transporters for co-consumption of cellobiose and xylose. Two different cellobiose-positive strains [Cg-Cello03-Xyl01 (top left panel) and Cg-Cello04-Xyl01 (top right panel)] co-expressing XylA and XylB were further engineered with additional sugar transporters such as a Gxf1 (*Candida intermedia*) [29] and Sut1 (*Pichia stipites*) [30], yielding Cg-Cello03-Xyl02 and Cg-Cello03-Xyl03 (left panels) and Cg-Cello04-Xyl02 and Cg-Cello04-Xyl03 (right panels), respectively. The cellobiose-positive and xylose-positive strains were cultivated in CgXII medium with a mixture of 2 % (w/v) cellobiose and 1 % (w/v) xylose. Cellobiose (blue square), xylose (red circle) were shown. Data represents mean values of at least three cultivations and error bars represent standard deviations

Methods

Bacterial strains, plasmids and culture conditions

All bacterial strains and plasmids used or constructed in this work are listed in Table 1. *E. coli* strains were grown in LB medium (containing per liter: 10 g tryptone, 5 g yeast extract, and 5 g NaCl) at 37 °C and 200 rpm. *C. glutamicum* ATCC 13032 and its derivatives were cultivated in BHIS medium (containing per liter: 37 g brain heart infusion, 91 g sorbitol) [27] at 30 °C and 200 rpm

overnight and then incubated aerobically in CgXII medium (50 in 250 mL baffled Erlenmeyer flasks) [27] containing 2 % (w/v) cellobiose or a mixture of 2 % (w/v) cellobiose and 1 % (w/v) xylose supplemented with 25 µg/mL chloramphenicol at 30 °C on a rotary shaker at 200 rpm. All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, Mo). 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added for induction.



Construction of plasmids and recombinant *C. glutamicum* strains

The *cdt-1* (NCU00801) and *gh1-1* (NCU00130) genes from *Neurospora crassa* [19] encoding for a cellobextrin transporter and a bG, respectively, were chosen for constructing a synthetic pathway in *C. glutamicum*. Each target gene was synthesized (Genscript, USA) with codon-optimization (Gene Designer 2.0 software [28]) for *C. glutamicum* and was assembled using a standard Bgl-Brick cloning method, where the target gene is inserted at the *Eco*RI and *Xba*I sites of the CoryneBrick plasmid pBbEB1c [11]. Thus, the synthesized *gh1-1* gene was inserted, resulting pBbEB1c-bG. Subsequently, the synthesized *cdt-1* gene was placed in front of the *gh1-1* gene, resulting pBbEB1c-CT-bG. For utilization of xylose, the codon-optimized *xylA* and *xylB* genes from *E. coli* [11] were, subsequently, inserted to pBbEB1c-bG, resulting

pBbEB1c-bG-XIK. In addition, the *gxfl* (*Candida intermedia*) [29] and *sut1* (*Pichia stipites*) [30] gene encoding for sugar-transporter were codon-optimized and inserted to pBbEB1c-bG-XIK, yielding pBbEB1c-bG-XIK-XTg and pBbEB1c-bG-XIK-XTs, respectively. Cloned DNA fragments were correctly verified by DNA sequencing.

The resulting plasmids were introduced into *C. glutamicum* by electroporation, and strain validation was performed by colony PCR [27]. The resulting strains are listed in Table 1.

Adaptive Evolution of recombinant *C. glutamicum* strains

Cg-Cello01 and *Cg-Cello02* strains were cultivated in CgXII minimal medium containing 2 % (w/v) cellobiose as sole carbon source. After the maximal cell growth of *Cg-Cello01* and *Cg-Cello02* were observed in 16 and 11 days, respectively, the cells were transferred to the

fresh CgXII minimal medium containing 2 % (w/v) cellobiose, starting OD₆₀₀ of 1 (Fig. 2a). Subsequently, the cells were transferred to the same fresh medium after every 48 h. Each culture of the cell was analyzed using HPLC to investigate the changes of the profiles of cellobiose utilization. The cell transfers were conducted until the rates of growth and cellobiose consumption were not changed, yielding *Cg-Cello01(evo)* and *Cg-Cello02(evo)* strains. *Cg-Cello01(evo)* and *Cg-Cello02(evo)* strains were further analyzed using DNA microarray. Plasmids from *Cg-Cello01(evo)* and *Cg-Cello02(evo)* strains were isolated and their DNA sequences were identified using partial overlapping primer-walking. In addition, plasmid-free strains were obtained by curing of plasmids in *C. glutamicum* as follows: after the electroporation of *Cg-Cello01(evo)* and *Cg-Cello02(evo)* strains, nonselective BHIS medium was inoculated at 30 °C. Each single colony was streaked onto BHIS agar plates either with or without chloramphenicol (25 µg/mL), yielding plasmid-free (Cm^s) *Cg-Evo1* and *Cg-Evo2* strains, respectively. For co-utilization of cellobiose and xylose, the *xylA* (encoding for xylose isomerase) and *xylB* (encoding for xylulose kinase) genes were introduced into cellobiose-utilizing *Cg-Cello03* and *Cg-Cello04* strains, yielding *Cg-Cello03-Xyl01* and *Cg-Cello04-Xyl01* strains.

HPLC analysis for glucose, xylose, and cellobiose quantification

For the measurement of the concentrations of glucose, xylose and cellobiose, culture supernatant was passed through a syringe filter (pore size of 0.2 µm) after centrifugation at 10,000g for 10 min. The concentrations of glucose and xylose were determined by high-performance liquid chromatography (HPLC system Agilent 1260, Waldbronn, Germany) equipped with a refractive index detector (RID) and an Aminex HPX-87 H Ion Exclusion Column (300 mm by 7.8 mm, Bio-Rad, Hercules, CA, USA) under the following conditions: sample volume of 20 µL, mobile phase of 5 mM H₂SO₄, flow rate of 0.6 mL/min, and column temperature of 65 °C.

Enzymatic measurement of β-glucosidase and glucokinase activity

Recombinant strains were cultivated in CgXII medium containing 2 % (w/v) cellobiose but 2 % (w/v) glucose was used for the control (*Cg-pBbEB1c*). After incubation at 30 °C for 24 h, bG activities in the cell-free extracts or in the culture supernatants, respectively, were quantitatively measured in a 1 mL mixture containing 590 µL 500 mM potassium phosphate buffer (pH 7.0), 10 µL 500 mM MgCl₂, 200 µL sample, 200 µL *p*-nitrophenyl-β-d-glucopyranoside (*p*NPG) as a substrate at 410 nm [16] (U; µmol of *p*NPG reduced min⁻¹). For the determination

of Glk activity (U/L) [31], the Glk activity in cell-free extracts was determined at 25 °C by measuring of the formation of NADPH at 340 nm in a coupled reaction containing 100 mM potassium phosphate buffer (pH 7.0), 20 mM glucose, 2 mM ATP, 25 mM MgCl₂, 2 mM NADP and 2 U glucose-6-phosphate dehydrogenase (U; µmol of NADP reduced min⁻¹).

NGS-based genomic DNA sequencing analysis

Genomic DNAs of *Cg-Cello01(evo)* and *Cg-Cello02(evo)* were isolated from a single colony's culture and purified using Wizard Genomic DNA purification kit (Promega, Cat.No. A1125). The genomes of *Cg-Cello01(evo)* and *Cg-Cello02(evo)* strains were sequenced using the Illumina MiSeq 300 bp paired-end system (Illumina, Inc, San Diego, CA, USA) at ChunLab, Inc. (Seoul, South Korea). We obtained 5,753,368 reads of the genome to reach a 428.63-fold depth of coverage on *Cg-Cello01(evo)* and *Cg-Cello02(evo)*. The re-sequencing data were annotated by RNAmmer 1.2, tRNA scan-SE 1.21, Rapid Annotation using Subsystem Technology (RAST), Pipeline, and CLgenomics program (ChunLab, Inc). The detail procedures were described in the previous study [32].

Transcriptomic analysis

Total RNA from *Cg-Cello01(evo)* and *Cg-Cello02(evo)* were sampled in the exponential phase. For Transcriptome analysis, extraction of total RNA and preparation of cDNA was followed by previous methods [33]. The cDNA probes were cleaned up using Microcon YM-30 column (Millipore, Bedford, MA) and then followed by Cy5-dye (Amersham Pharmacia, Uppsala, Sweden). The Cy5-labelled cDNA probes were cleaned up using the QIAquick PCR Purification Kit (Qiagen). Dried Cy5-labelled cDNA probes were re-suspended in hybridization buffer. Hybridization to a microarray slide (*Corynebacterium_glutamicum* 3 × 20 K) (Mycroarray.com, Ann Arbor, MI), staining, and scanning of the probe array were performed according to the manufacturer's instructions. Hybridization image on the slide was scanned by Axon 4000B (Axon Instrument, Union City, CA). The analysis of the microarray data was performed using GenePix Pro 6.0 (Axon Instruments). The averages of the normalized ratios were calculated by dividing the average normalized signal channel intensity by the average normalized control channel intensity. All measurements were performed on duplicated technical replicates. Two independent samples were analyzed; their measurements were averaged for further analysis. The microarray data were deposited at the NCBI Gene Expression Omnibus, GEO under accession no. GSE65076 and at <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE65076>.

Fatty acids and lipid analysis

Fatty acid methyl esters were prepared as described previously [23, 34], and identified by gas chromatography with model 5898A microbial identification system (Microbial ID). Trimethylsilylated derivatives of fatty acids and methyl esters were analyzed by high-temperature gas chromatography on an HP 5790A gas chromatograph (Hewlett Packard), equipped with a flame-ionization detector on a 12 m high throughput screening (HTS) column with hydrogen gas as the carrier. Derivatives were identified by comparing their retention times to those of standards and by gas chromatography mass spectrometry analysis on a KRATOS MS50 spectrometer (ion source temperature set to 200 °C and ionization energy to 70 eV), respectively. For the analysis, colonies of *Cg-Cello01(evo)* and *Cg-Cello02(evo)* strains were obtained on CgXII agar plate containing 2 % cellobiose and colonies of *Cg-pBbEB1c* stain was obtained on CgXII agar plate containing 2 % glucose as.

Cellulolytic hydrolysis of Avicel® PH-101 and Canadian biomass and SSF by *C. glutamicum*

Avicel® PH-101 (Sigma), microcrystalline cellulose and dissolving pulp (DP, pure cellulosic substrate less than 0.5 % lignin, less than 3 % xylan) [35] from Canadian Ponderosa Pine were used as substrate for cellulolytic hydrolysis and SSF by the cellobiose-utilizing *C. glutamicum* strains. Each cellulolytic hydrolysis and SSF was carried out in CgXII medium (pH 7.0) with 1 % (w/v) Avicel® PH-101 or 1 % (w/v) dissolving pulp at 30 °C and 200 rpm. Celluclast 1.5 L (Sigma; Cat C2730) [75 filter paper unit (FPU)/g-glucan] was used as the cellulolytic enzymes for saccharification of Avicel® PH-101 or DP. Cellulase activity of the Celluclast 1.5 L was determined by the standard filter paper assay with the 3,5-dinitrosalicylic acid (DNS) method [36]. One unit of cellulose activity is defined as the amount of enzyme required to release 1 μmol of reducing sugar per min at pH 4.8 and 50 °C. The enzyme activity of Celluclast 1.5 L was measured to be 28 FPU/mL. A colorimetric method based on the phenol–sulfuric acid reaction was used to determine the amount of residual substrate during SSF by quantifying total sugars [37].

Availability of supporting data

The data set supporting the results of this article is available at NCBI GEO repository, [GSE65076 and <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE65076>].

Additional file

Additional file 1. Additional data and analysis for the evolved *C. glutamicum* strains.

Abbreviations

bG: β-glucosidase; SSF: simultaneous saccharification and fermentation; HPLC: high-performance liquid chromatography; RID: refractive index detector; NGS: next-generation sequencing; GEO: gene expression omnibus; Glk: glucokinase; DP: dissolving pulp; SSCF: simultaneous saccharification and co-fermentation.

Authors' contributions

JL performed and analyzed all experiments and helped in drafting the manuscript. JNS and YU assisted in analysis and helped in drafting the manuscript. HMW analyzed all experiments and coordinated the study and drafted the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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