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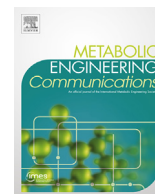
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Engineering of a new *Escherichia coli* strain efficiently metabolizing cellobiose with promising perspectives for plant biomass-based application design

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ABSTRACT

The necessity to decrease our fossil energy dependence requests bioprocesses based on biomass degradation. Cellobiose is the main product released by cellulases when acting on the major plant cell wall polysaccharide constituent, the cellulose. *Escherichia coli*, one of the most common model organisms for the academy and the industry, is unable to metabolize this disaccharide. In this context, the remodeling of *E. coli* to catabolize cellobiose should thus constitute an important progress for the design of such applications. Here, we developed a robust *E. coli* strain able to metabolize cellobiose by integration of a small set of modifications in its genome. Contrary to previous studies that use adaptative evolution to achieve some growth on this sugar by reactivating *E. coli* cryptic operons coding for cellobiose metabolism, we identified easily insertable modifications impacting the cellobiose import (expression of a gene coding a truncated variant of the maltoporin LamB, modification of the expression of *lacY* encoding the lactose permease) and its intracellular degradation (genomic insertion of a gene encoding either a cytosolic β -glucosidase or a cellobiose phosphorylase). Taken together, our results provide an easily transferable set of mutations that confers to *E. coli* an efficient growth phenotype on cellobiose (doubling time of 2.2 h in aerobiosis) without any prior adaptation.

1. Introduction

For several years now, the ability to degrade the inexpensive, very abundant and renewable lignocellulosic material has grown a massive interest since it constitutes a solution to overcome the problems arisen from the consumption of fossil energy. Hydrolysis of the major plant biomass component, the cellulose, by enzymatic cocktails mainly generates the disaccharide cellobiose (Cb), composed of 2 glucosyl residues (Parisutham et al., 2017; Shi and Weimer, 1996). *Escherichia coli* is unable to utilize Cb, but this microorganism is the most well-known and characterized bacterium, grows rapidly, and can easily be modified thanks to the numerous available genetic tools. This model bacterium is therefore an attractive microorganism for industrial applications including the biofuel production sector. Different strategies were thus formerly applied to enable *E. coli* to catabolize this disaccharide such as

the reactivation of the cryptic operons *cel*, *bgl*, *chb* or *asc* but only limited growth on this carbon source could be achieved (Hall and Xu, 1992; Krickler and Hall, 1984; Parisutham and Lee, 2011, 2015; Parker and Hall, 1990). The secretion or cell surface exposure of β -glucosidases, an enzyme cleaving the β -1,4 glycosidic bond in Cb (Aso et al., 2019; Gao et al., 2015; Luo et al., 2014; Muñoz-Gutiérrez et al., 2012; Soma et al., 2012), has also been used to extracellularly hydrolyze this sugar into two glucose units in sufficient quantity to support the growth (Aso et al., 2019; Ma et al., 2011; Singhania et al., 2013).

In *E. coli*, glucose is then imported in the cytoplasm via the phosphotransferase system (PTS) known to directly control the uptake and utilization of various carbohydrates (Tchieu et al., 2001). In short, the PTS triggers a sugar catabolic repression that allows *E. coli* to quickly adapt to its preferred energy and carbon source: glucose. However, this fast adaptation is a burden for the development of lignocellulolytic based

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biotechnological applications since the use of other fermentable sugars released in the medium during the plant cell wall degradation process (arabinose, galactose or xylose) is likely to be hampered (Park et al., 2012).

To alleviate this PTS induced catabolic repression, the expression of a cytosolic β -glucosidase or cellobiose phosphorylase (Cb phosphorylase) could be envisioned to generate intracellular glucose (de Groeve et al., 2011; Parisutham et al., 2017). In this respect, the Cb phosphorylase produces one glucose and one α -glucose 1-P (Fosses et al., 2017), the latter being converted into glucose 6-P by the α -phosphoglucomutase (PGM) which directly enters the glycolytic pathway, thus allowing to save one ATP molecule. The major limitation of this strategy is the import of the Cb. Previous studies related to *E. coli* strains producing either a β -glucosidase or a Cb phosphorylase reported an interaction of the inner membrane lactose permease LacY encoded by the *lac* operon with Cb and that a $\DeltalacY mutant strain producing a Cb phosphorylase was found unable to grow on this sugar (Collins et al., 1989; Sekar et al., 2012), suggesting the involvement of this permease in Cb uptake.$

In the present study, by combining genomic editing with directed evolution of *E. coli* followed by genome sequencing, we have designed a robust strain of *E. coli* engineered by markerless chromosomal modifications to metabolize Cb by 1/genomic integration of genes coding for a β -glucosidase or a Cb phosphorylase allowing the intracellular use of Cb, 2/insertion of specific mutations favorizing the uptake of Cb (either by production of a modified maltoporin LamB and/or by tuning the expression level of the *lacY* permease). We have thus defined a set of modifications that could be genetically transferred in any *E. coli* strain to provide an optimized Cb utilization phenotype without prior adaptation. We also propose a model of Cb catabolism of the engineered *E. coli* strains synthesizing either the β -glucosidase or the Cb phosphorylase.

2. Materials and methods

2.1. Bacterial strains and plasmids

The strains *Ruminococcus albus* F40, *Zymomonas mobilis* 4 (ATCC 31821), *Ruminiclostridium cellulosum* H10 (ATCC 35319) and *Escherichia coli* MG1655 were used in this study. All the primers used in this study are listed in Supplemental Table 1. All the engineered *E. coli* strains and plasmids used are reported in Supplemental Table 2.

2.2. Construction of pET28-bglA and pET28-bglA_{A781S} expression vectors

The sequence of the β -glucosidase encoding gene *bglA* of *R. albus* was amplified by PCR using the genomic DNA of *R. albus* as the template and the primers rbgA F and rbgA R (Suppl. Table 1). The amplicon was cloned between the *Nco*I and *Xho*I restriction sites into the pET28a(+) generating the pET28-bglA vector coding for the recombinant protein rBglA bearing 6 histidine residues at its C-terminus. The resulting plasmid was introduced in *E. coli* NEB 5- α competent cells (NEB, Ipswich, MA, Suppl. Table 2). Recombinant clones were identified by PCR and the insert subsequently verified by sequencing.

The pET28-bglA_{A781S} vector coding for the variant rBglA_{A781S} in which the residue Ala781 is replaced by Ser was constructed by the one-step PCR method (Ansaldi et al., 1996) using plasmid pET28-bglA as a template and the two overlapping divergent primers: bglA A781S F and bglA A781S R (Suppl. Table 1). The DpnI treated PCR product was transferred to *E. coli* NEB 5- α competent (NEB, Suppl. Table 2). Clones were analyzed as described above.

2.3. Strains construction

2.3.1. Construction of the integrative vectors containing bglA or cbpA expression cassette

A multi-cloning site (MCS) containing the restriction sites: BamHI, BglII, *Xho*I and *Sph*I, was generated by the annealing of the

oligonucleotides pKD3-MCS F and pKD3-MCS R (Mounts et al., 1989; Suppl. Table 1). This double strand DNA was then cloned in the *Sph*I-linearized close to the *cat* selection marker gene (coding for the chloramphenicol resistance) in pKD3 vector (Datsenko and Wanner, 2000). The resulting pKD3-MCS vector was subsequently introduced in competent *E. coli* BW25141 (pir+) cells (Suppl. Table 2). Transformants were selected on LB solid medium containing 34 μ g/mL of chloramphenicol at 37 °C. The sequence of plasmids extracted from liquid cultures of clones was verified with the primer seq-MCS F (Suppl. Table 1).

The cassette “Ppdc_{Zm}-bglA-Tgfor_{Zm}” containing the coding sequence of the gene *bglA* from *R. albus* fused to the promoter *Ppdc* from *Z. mobilis* (*Ppdc*_{Zm}) and the terminator *Tgfor* from *Z. mobilis* (*Tgfor*_{Zm}), was amplified by PCR using the primers BamHI bglA F and *Xho*I bglA R (Suppl. Table 1) and the vector pJIR300-RA as template (courtesy of L. Dedieu, Ohmiya et al., 1991). The “Ppdc_{Zm}-bglA-Tgfor_{Zm}” cassette was cloned between the BamHI and *Xho*I sites of the pKD3-MCS vector to generate the pKD3-bglA plasmid. After transformation of competent *E. coli* BW25141 cells (Suppl. Table 2), the corresponding plasmid was extracted and the sequence verified as described above.

The coding sequence of the gene *cbpA* was amplified using the primers NdeI cbpA F and *Xba*I cbpA R (Suppl. Table 1) and the genomic DNA of *R. cellulosum* H10 as template. The internal NdeI restriction sequence of the *cbpA* amplicon was then submitted to site directed mutagenesis by overlapping PCR using the primers cbpA mut NdeI F and cbpA mut NdeI R (Suppl. Table 1). The corresponding PCR fragment was subcloned between the NdeI and *Xba*I in pKD3-bglA to create the pKD3-cbpA vector containing the cassette “Ppdc_{Zm}-cbpA-Tgfor_{Zm}”. The corresponding plasmid was extracted and its sequence verified as described above.

2.3.2. Genomic integration of bglA and cbpA between the ybfl and ybfD loci in E. coli MG1655 strain

“Ppdc_{Zm}-bglA-Tgfor_{Zm} cat” or the “Ppdc_{Zm}-cbpA-Tgfor_{Zm} cat” cassettes were integrated in the intergenic region between the loci *ybfl* and *ybfD*, located at 34 min in *E. coli* MG1655 (Suppl. Table 2) genome, using the method described by Datsenko and Wanner (2000). PCR fragments containing the “Ppdc_{Zm}-bglA-Tgfor_{Zm} cat” or the “Ppdc_{Zm}-cbpA-Tgfor_{Zm} cat” cassettes were produced respectively using the pKD3-bglA or pKD3-cbpA as templates and the primers pKD3-ybfl F and pKD3-ybfl R (Suppl. Table 1). Electrotransformation of *E. coli* MG1655 pKD46 (Suppl. Table 2) by the purified PCR product was then carried out using an Eporator device (Eppendorf, Hamburg, Germany) to generate the chromosomal mutations *zde::bglA* or *zde::cbpA* according to the genetic nomenclature for an insertion in a bacterial genome at the non-coding position located at 34 min (Maloy et al., 1994). Integrants were analyzed by PCR using external primers *verif ybfl* F/*verif ybfl* R (Suppl. Table 1). After curing of the pKD46 plasmid at 42 °C, pCP20 electrotransformed clones were thermally selected to generate markerless mutations and obtain the strains CbB (Cellobiose using BglA) clones 2 and 3 (CbB2 and CbB3, Suppl. Table 2), harboring the mutation *zde::bglA*, and CbCzero (Cellobiose using CbpA) clones 1 and 2 (CbCzero1 and CbCzero2, Suppl. Table 2), harboring the mutation *zde::cbpA*, respectively.

2.3.3. Construction of the E. coli strain CbB3 Δ lacI

The strain MG1655 Δ lacI (Suppl. Table 2) was generated by integration of a *cat* cassette in *lacI* using the method of Datsenko and Wanner (2000) as described above. The *cat* cassette was synthesized using the pKD3 vector as the template and the primers *lacI*-cat F/*lacI*-cat R (Suppl. Table 1). Integrants were selected on LB solid medium containing 17 μ g/mL of chloramphenicol at 30 °C and analyzed by PCR using the pair of external primers seq *lacI* F/seq *Plac* R (Suppl. Table 1) and pKD46 plasmid was cured at 42 °C. Following the method of Baba and coworkers (Baba et al., 2006), the resulting Δ lacI::cat deletion was transduced in the strain CbB3 (Suppl. Table 2). Transductants were selected on LB solid medium containing 17 μ g/mL of chloramphenicol at 37 °C and analyzed

by PCR using the pair of external primers seq lacI F/seq Plac R (Suppl. Table 1).

2.3.4. Construction of the strain CbC

The *zde::Ppdc_{Zm}-cbpA-Tgfor_{Zm} cat* mutation was transduced from CbCzero1 strain (Suppl. Table 2; Baba et al., 2006) to the evolved strain CbB3-evo1 (Suppl. Table 2, see below “Directed evolution of *E. coli* strains on Cb and genome sequencing”). The resulting strains CbC clones 2 and 3 (CbC2 and CbC3, Suppl. Table 2) displaying the mutation $\Delta bglA::cbpA$, in which the gene *cbpA* was transduced in place of the *bglA* gene, was verified by PCR using external primers *verif ybfD* F/*verif ybfD* R (Suppl. Table 1).

2.3.5. Replacements of lac operon promoter and Ppdc_{Zm} in *E. coli* CbB and CbC genomes

The Plac inducible promoter controlling the *lacZYA* operon was replaced by the *PacpP* or *PtonB* promoters. Similarly, the *Ppdc_{Zm}* promoter controlling the expression of the genome-integrated *bglA* or *cbpA* genes was replaced by the *PgmK* promoter from *E. coli*. First, *PacpP*, *PtonB* and *PgmK* promoters' sequences were amplified from *E. coli* MG1655 (Suppl. Table 2) genomic DNA using the pairs of primers *cat-PacpP* F/*PacpP-lacZ* R, *cat-PtonB* F/*PtonB-lacZ* R and *cat-PgmK* F/*bglA PgmK* R or *cat-PgmK* F/*cbpA PgmK* R, respectively (Suppl. Table 1). Second, “*FRT-cat-FRT*” cassettes were amplified using the pKD3 template and the pairs of primers *Plac-cat* F/*cat-PacpP* R, *Plac-cat* F/*cat-PtonB* R or *cat-ybfD* F/*cat-PgmK* R (Suppl. Table 1). Finally, the corresponding purified PCR fragments were combined by overlapping PCR using the pairs of primers *Plac-cat* F/*PacpP-lacZ* R or *Plac-cat* F/*PtonB-lacZ* R, and *cat-ybfD* F/*bglA PgmK* R or *cat-ybfD* F/*cbpA PgmK* R, respectively (Suppl. Table 1). “*FRT-cat-FRT* – promoter” amplicons were then integrated into the genome of *E. coli* MG1655 (Suppl. Table 2) as described above (Datsenko and Wanner, 2000) to generate the strains CbB3 *PacpP::lac*, CbB3 *PtonB::lac*, CbB3-evo1-*PgmK*, and CbB3-evo1-*PgmK*, respectively (Suppl. Table 2). Integrants were verified by PCR using the pairs of external primers *seq lacI* F/*seq Plac* R or *verif ybfD* F/*verif bglA* R or *verif ybfD* F/*verif cbpA* R (Suppl. Table 1). The pKD46 plasmid was then cured at 42 °C, before markerless mutations were obtained as described above using the pCP20 vector (Datsenko and Wanner, 2000).

2.3.6. Construction and transduction of marked loci *lamB_{Δ363-446}::cat* and *yfeN_{S121P}::kan*

PCR fragments were synthesized using the vectors pKD3 (Cm^r) and pKD4 (Km^r) as templates with primers *lamB* mut-Cter F/*lamB* mut-Cter R or *yfeN-kan* F/*yfeN-kan* R respectively (Suppl. Table 1) to generate specific resistance cassettes flanked by the intergenic region downstream *lamB_{Δ363-446}* (named *lamB** mutation) or *yfeN_{S121P}* loci. The strain CbC2-evo1 (Suppl. Table 2) previously transformed by pKD46 vector was separately electrotransformed with these PCR fragments to mark the mutated loci using the method of Datsenko and Wanner (2000). Integrants were selected on LB solid medium containing 17 µg/mL of chloramphenicol or 35 µg/mL of kanamycin at 37 °C and analyzed by PCR using the external primers *seq lamB* F/*seq lamB* R or *seq yfeN* F/*seq yfeN* R. Then, pKD46 plasmid was cured at 42 °C. The resulting mutations *lamB_{Δ363-446}::cat* or *yfeN_{S121P}::kan* were transduced into CbC2 and CbB3-evo1 strains (Suppl. Table 2; Baba et al., 2006).

2.3.7. Construction of the strain CbB3-evo1 $\Delta lamB$

Following the method developed by Baba and coworkers (Baba et al., 2006), the mutation $\Delta lamB::kan$ was transduced from the strain of the Keio collection (Baba et al., 2006) into CbB3-evo1 strain (Suppl. Table 2). Transductants were selected on LB solid medium containing 35 µg/mL of kanamycin at 37 °C and analyzed by PCR with the pair of external primers *seq lamB* F/*verif lamB* R (Suppl. Table 1).

2.3.8. Genetic engineering of the *E. coli* strain able to metabolize the Cb

Mutation *lamB_{Δ363-446}::cat* was transduced into the engineered strain

CbB3 *PacpP::lac* (Suppl. Table 2; Baba et al., 2006), leading to CbB3 *PacpP::lac lamB**. The strain with markerless mutation was obtained using the pCP20 vector as described above (Datsenko and Wanner, 2000).

2.4. Construction of the reporter plasmid pUA-Ppdc

The sequence of the *Ppdc_{Zm}* promoter from *Z. mobilis* was amplified by PCR using pKD3-bglA plasmid as a template and the primers *Ppdc_{Zm}* XhoI F and *Ppdc_{Zm}* BamHI R (Suppl. Table 1). The amplicon was cloned between the XhoI and BamHI restriction sites of the pUA-66 (Zaslaver et al., 2006) generating the pUA-Ppdc reporter vector coding for the transcriptional fusion *Ppdc_{Zm}-gfp*.

The resulting plasmid was introduced in *E. coli* NEB 5-α competent cells (Suppl. Table 2). Transformants were analyzed by PCR and positive clones were subsequently verified by sequencing.

2.5. Growth in M9 minimum medium

Overnight cultures of *E. coli* strains were performed under shaking (150 rpm) at 37 °C in LB medium supplemented with the appropriate antibiotics. 200 µL of preculture were then used to inoculate aerobically 20 mL of M9 liquid medium supplemented with 0.5% w/v Cb (M9 Cb) or 0.2% w/v glucose (M9 Glu) under shaking (150 rpm) at 37 °C, and growth was monitored at 600 nm. For anoxic cultures, M9 medium was boiled under argon flow, and aliquoted in sealed 50 mL serum bottle, prior to sterilization. Other compounds (MgSO₄, CaCl₂, Thiamine, FeSO₄) including glucose (5 g/L) or cellobiose (5 g/L) were subsequently added anaerobically. Cultures were inoculated anaerobically using 200 µL of overnight cultures in LB medium supplemented with the appropriate antibiotics.

2.6. Directed evolution of *E. coli* strains on Cb and genome sequencing

Cb utilization directed evolution of strains CbB2 and CbB3 (*zde::bglA*, Suppl. Table 2), CbCzero1 and CbCzero2 (*zde::cbpA*, Suppl. Table 2) and CbC2 and CbC3 (CbB3-evo1 $\Delta bglA::cbpA$, Suppl. Table 2) were performed in M9 Cb liquid medium at 37 °C as described above. When the OD_{600nm} reached a value of 1, subcultures were performed in fresh M9 Cb medium. Clones from the corresponding subcultures grown until the stationary phase were first isolated on M9 Cb solid medium at 37 °C, and secondly on LB plate at 37 °C. PCR were performed on the evolved largest colonies CbB2-evo1, CbB2-evo2, CbB3-evo1 and CbB3-evo2 (Suppl. Table 2) to analyze the sequence of specific gene targets as: *lacI*, *Plac*, *Ppdc_{Zm}*, *lacY*, *ompF*, *ompC*, *micF*. Amplicons from PCR using respectively the primer pairs *verif micF* F/*verif ompC* R, *verif ompF* F/*verif ompF* R, *seq lacI* F/*seq Plac* R, *seq lacY* F/*seq lacY* R (Suppl. Table 1), were sequenced. Isolated clones from strains CbB3-evo1 and CbC2-evo1 were also subcultured in LB medium at 37 °C under shaking (150 rpm) for genomic DNA extraction using Wizard genomic DNA preparation kit (Promega, Madison, WI) to perform whole genome sequencing (GATC-Eurofins, Luxembourg city, Luxembourg).

2.7. Promoter activity assay

Cultures of MG1655 transformed with pUA66, pUA-Plac, pUA-PacpP, pUA-PtonB, pUA-PgmK, pUA-PmsC and pUA-PrnB from the plasmid GFP reporter collection of Zaslaver and coworkers (Zaslaver et al., 2006) and pUA-Ppdc were performed in triplicate on microreader plate in 200 µL M9 medium supplemented with 100 µg/mL of ampicillin, 0.2% casamino acids and 0.2% glucose at 37 °C. OD_{600nm} and GFP fluorescence (excitation at 488 nm and emission at 521 nm) were measured every 10 min with a Spark 10 M device (Tecan, Männedorf, Switzerland). Alternatively, the growth and the GFP fluorescence of CbB3-evo1 transformed with the same vectors and cultured in 50 mL M9 supplemented with 100 µg/mL of ampicillin, 0.2% casamino acids and either 0.2% glucose or 0.5% cellobiose, at 37 °C were monitored similarly.

2.8. Production and purification of rBglA and rBglA_{A781S}

E. coli BL21(DE3) (NEB, Suppl. Table 2) transformed with the vectors pET28-bglA or pET28-bglA_{A781S}, were grown at 37 °C under shaking (150 rpm) in 2 x 1 L of LB liquid medium supplemented with 50 µg/mL of kanamycin. 200 µM of isopropyl-thio-β-D-galactoside (Euromedex, France) were added at OD_{600nm} 1 and the growth temperature was switched to 16 °C overnight. Cells were harvested by centrifugation (10 min at 4 °C and 3,000 g) and resuspended in 50 mL of 30 mM Tris-HCl pH 8.0, 5 mM Imidazole, containing few mg of DNaseI (Roche, Mannheim, Germany). Cells were broken using a French Press (Stansted Fluid Power Ltd, Harlow, UK) and the crude extract was subsequently centrifuged (15,000 g, 10 min, 4 °C) to remove cell debris, prior to loading on 3 mL of Ni-NTA resin (Qiagen, Hilden, Germany) equilibrated in 30 mM Tris-HCl, 5 mM Imidazole pH 8.0. The proteins of interest were eluted using 30 mM Tris-HCl, 100 mM Imidazole pH 8.0, buffer. Finally, the purified enzymes were buffer exchanged against 10 mM Tris-HCl pH 8.0 and concentrated by ultrafiltration using Vivaspin20 (cut off 30 kDa, Sartorius, Göttingen, Germany), and stored at -80 °C. The concentration of the proteins was estimated using the absorbance at 280 nm and the absorption coefficient calculated with the program ProtParam tool (www.expasy.org/tools/protparam.html).

2.9. Determination of the kinetic parameters of rBglA and rBglA_{A781S}

The kinetic parameters of rBglA and rBglA_{A781S} were determined on Cb as follows. Enzymes (1 µM) were incubated at 37 °C with Cb at concentrations of 0.8, 1, 1.25, 1.67, 2.5, 5, 10, 20, 50, and 250 mM in 20 mM Hepes pH 7.0 and 5 mM CaCl₂. After 5 and 10 min of incubation, aliquots (1–10 µL) were pipetted and diluted in a final volume of 250 µL in 0.1 M NaOH. Samples were analyzed by High Pressure Anion Exchange Chromatography coupled with Pulsed Amperometric Detection (HPAEC-PAD) (ThermoFisher, Waltham, MA). Quantification of glucose and Cb was performed by applying 25 µL of diluted samples to a Dionex CarboPac PA1 column (4 x 250 mm) (ThermoFisher) and the corresponding guard column (4 x 50 mm) at 30 °C. Sugars were eluted with the buffers 0.1 M NaOH and 0.5 M sodium acetate +0.1 M NaOH as the eluents A and B, respectively. The following multi-step procedure was used: isocratic separation (5 min, 95% A + 5% B), separation gradient (8 min, 10–37% B), column wash (2 min, 99% B) and subsequent column equilibration (2.5 min, 95% A + 5% B). The flow rate was kept at 1 mL/min in all cases. Injection of samples containing glucose and Cb (Sigma-Aldrich, Saint-Louis, MI) at known concentrations ranging from 4 to 100 µM were used for quantification.

2.10. Determination of CbpA and BglA activities in crude extracts

Fifty-mL overnight cultures of CbB3-evo1, CbB3-evo1-PgmK, CbC2-evo1, CbC2-evo1-PgmK and MG1655 (Suppl. Table 2) in lysogenic broth were centrifuged at 7,000 g for 10 min at 4 °C. The cell-containing pellet was resuspended in 8 mL of 50 mM KHPO₄ buffer (pH 7.0) supplemented with few mg of DNase I, and broken in a French press. The crude extract was centrifuged at 15,000 g for 10 min at 4 °C, and the supernatant was concentrated by ultrafiltration on Vivaspin20 (cut off 30 kDa) to 1 mL. The activity of BglA was monitored by incubating 10 µL of 20-fold diluted sample of CbB3-evo1, CbB3-evo1-PgmK concentrated crude extracts, or 10 µL of MG1655 non-diluted concentrated crude extract with 990 µL of *p*-nitrophenyl-β-D-glucoside (pNP-Glu) at 1 g/L in 50 mM KHPO₄ buffer (pH 7.0) at 37 °C. The activity was determined by monitoring the release of *p*-nitrophenol at 400 nm as previously described (Ravachol et al., 2016). The Cb phosphorylase activity was determined by incubating 20 µL of CbC2-evo1, CbC2-evo1-PgmK or MG1655 crude extract with 980 µL of 10 mM Cb in 50 mM KHPO₄ buffer (pH 6.2) containing 0.01% NaN₃ (w/v) at 37 °C. At specific time points,

200 µL were pipetted and mixed with 50 µL of 0.025 M H₂SO₄. Twenty-five µL of samples were applied on an (300 x 7.8 mm) Aminex HPX87H column (Bio-rad, Hercules, CA) preceded by the corresponding (30 x 4.6 mm) guard column at 65 °C. Sugars were eluted using the eluent 5 mM H₂SO₄ (isocratic separation for 15 min), and detection was performed using a refractive index detector (Iota, Marseille, France). Injections of glucose and Cb at known concentrations (ranging from 0.5 to 10 mM) were used to determine the amount of consumed Cb and released glucose.

2.11. Sugar consumption by CbB3 PacpP::lac lamB* and MG1655 strains, and release of fermentation products in anaerobiosis

The consumption of glucose and Cb by CbB3 PacpP::lac lamB* (Suppl. Table 2) and MG1655 strains under aerobic conditions was followed by pipetting 1-mL culture samples at specific time points. Samples were centrifuged 10 min at 4 °C at 15,000 g, and 200 µL of supernatant were mixed with 50 µL of 0.025 M H₂SO₄ prior to analyses on Aminex HPX87H as described above.

For anaerobic condition, the same procedure was followed except that the isocratic separation was extended to 25 min, and in addition to glucose and Cb, injection of succinate, lactate, formate, acetate and ethanol at known concentration (0.5–30 mM) were performed to quantify both the sugar consumption and the release of fermentation products.

3. Results and discussion

3.1. Selection of the Cb degrading enzymes and determination of the catalytic properties of the β-glucosidase (BglA) of *Ruminococcus albus*

In this study, we aimed to create an *E. coli* strain able to intracellularly catabolize Cb. Two major types of enzymes can cleave the stable β-1,4 glycosidic bond linking the two glucosyl residues in Cb: the β-glucosidases and the Cb phosphorylases, that release two glucoses or one glucose and one α-glucose 1-phosphate, respectively (de Groeve et al., 2011; Fosses et al., 2017; Kim et al., 2018; Parisutham et al., 2017; Wilson, 2009). We selected 1/the β-glucosidase BglA from *R. albus* F40 (Ohmiya et al., 1991) belonging to the Glycoside Hydrolase 3 family (Lombard et al., 2014) and whose plasmid-based overproduction in *E. coli* allowed its growth in M9 Cb (L. Dedieu, personal communication), and 2/the efficient Cb phosphorylase CbpA from *R. cellulosyticum* H10 belonging to the Glycoside Hydrolase family 74 (Lombard et al., 2014) that was previously characterized (Fosses et al., 2017), and known to be the most active enzyme of *R. cellulosyticum* on Cb (Liu et al., 2010, 2019). In order to precise the enzymatic properties of BglA, its encoding gene was therefore cloned in a pET28 vector which was subsequently used to transform the strain BL21(DE3). Recombinant protein rBglA was purified to homogeneity and *k*_{cat} and *K*_M values of 1045.3 ± 22.6 min⁻¹ and 49.32 ± 2.81 mM were obtained on Cb (at pH 7.0 and 37 °C), respectively. The Cb phosphorylase CbpA was formerly purified and characterized, providing *k*_{cat} and *K*_M values of 1458.4 ± 108.0 min⁻¹ and 2.85 ± 0.47 mM, respectively (Fosses et al., 2017). Both Cb degrading enzymes thus exhibit catalytic velocities in the same range, but rBglA displays a 17-fold higher *K*_M for the disaccharide, thus leading to a 24-fold lower catalytic efficiency (*k*_{cat}/*K*_M) compared with CbpA (Suppl. Table 3).

Both enzymes were assayed without *a priori* to generate a Cb-utilization phenotype to *E. coli* since no preliminary information were available concerning the Cb intracellular concentration and fluxes. It is however worth considering that rBglA hydrolyses Cb into two glucose residues whereas CbpA generates one glucose and one α-glucose 1-P, thereby allowing the saving of one ATP molecule when the α-glucose 1-P is converted into glucose 6-P by the α-phosphoglucosyltransferase (PGM) of *E. coli* (Lu and Kleckner, 1994) and directly enters the glycolytic pathway.

3.2. Effect of the genomic integration of *bglA* or *cbpA* genes in *E. coli*: metabolism of the Cb and growth

As stated above, the expression of the *bglA* gene of *R. albus* cloned in the pJRD300Ra expression vector sustained a limited growth of the *E. coli* recipient strain on M9 minimal medium supplemented with 0.5% Cb. To overcome any problems due to vector instability (Hershberger and Ros-teck, 1991; Jakupciak and Wells, 1999; Patient and Summers, 1993; Standley et al., 2019), we chose to integrate the expression “*Ppdc_{Zm}-bglA-Tgfor_{Zm}*” cassette in the *E. coli* MG1655 chromosome. In this cassette *bglA* expression is under the control of the strong promoter *Ppdc* from *Z. mobilis* strain 4 and the terminator *Tgfor* from *Z. mobilis* strain 4 (Reynen et al., 1990; Seo et al., 2005). We successfully integrated this cassette between the loci *ybfL* and *ybfD* in the intergenic region *zde* (Maloy et al., 1994), a position allowing foreign DNA integration up to 4 kb (Espeli et al., 2008; Lesterlin et al., 2012). The generated strain harboring the mutation *zde::bglA* is hereafter named CbB (Cellobiose using BglA). Corresponding isolated clones identified as *E. coli* CbB2 and CbB3 were selected. Similarly, *E. coli* strain harboring the mutation *zde::cbpA* was obtained by the integration of the “*Ppdc_{Zm}-cbpA-Tgfor_{Zm}*” cassette between the same loci, and named CbCzero (Cellobiose using CbpA). Corresponding isolated clones CbCzero1 and CbCzero2 strains were selected.

Upon sequencing of all integrated regions, one mutation was found in the *bglA* gene leading to the replacement of the Ala 781 by a Ser residue. The mutated gene was cloned in a pET28 vector, and the corresponding modified rBglA_{A781S} was produced and purified using the same procedure as the wild type (WT) rBglA. The kinetic parameters of rBglA_{A781S} were established on Cb as formerly described and both k_{cat} ($1231.0 \pm 18.3 \text{ min}^{-1}$) and K_M ($53.99 \pm 2.09 \text{ mM}$) were similar to that of the WT rBglA (Suppl. Table 3). Therefore, this mutation leading to a “neutral” aminoacid substitution in rBglA in *E. coli* CbB2 and CbB3 strains was kept in this following strain engineering experiments.

The ability of each strain to use the disaccharide as the sole carbon source was then evaluated on M9 minimum medium supplemented with 0.5% Cb. Despite the presence of *cbpA*, no growth was observed for *E. coli* CbCzero1 and CbCzero2 upon 15 days of cultivation (data not shown) whereas *E. coli* CbB2 and CbB3 displayed a growth after a 5-day lag phase (Fig. 1a) but no lag phase was observed upon direct subculture in the same fresh minimum medium supplemented with 0.5% Cb (Fig. 1b) suggesting that an adaptation event occurred. These results indicate that the integration of *bglA* or *cbpA* genes in *E. coli* genome is not sufficient alone to directly induce a growth on Cb, and that an *in vivo* evolution step conferred the capacity to grow on Cb-based medium to *E. coli* CbB2 and

CbB3. Colonies of evolved clones were isolated by plating dilutions of each liquid cultures on M9-Cb solid medium. The 4 largest colonies named *E. coli* CbB2-evo1, CbB2-evo2 and CbB3-evo1, CbB3-evo2 were stored for further studies. Among them, the clone CbB3-evo1 which generated the largest colonies on M9-Cb plates, was therefore selected for subsequent analyses and engineering.

3.3. Identification of the mutation in *lacI* responsible of the Cb growth phenotype of the CbB3-evo1 strain

To identify the beneficial traits implicated in the adaptation of the CbB2-evo1, CbB2-evo2, CbB3-evo1 and CbB3-evo2 clones to grow on Cb, we firstly performed conventional PCR-based sequencing on specific loci. We targeted 1/the *Ppdc_{Zm}* promoter sequence that we introduced to control the expression of *bglA*, 2/various regions of the *E. coli lac* operon coding for genes previously proposed to be involved in Cb uptake in adapted strain (Collins et al., 1989; Sekar et al., 2012; Vilar et al., 2003) such as the *PlacZ* promoter of the *lac* operon (Donovan et al., 1996), *lacI* coding the repressor of the expression of *lac* operon (Lewis, 2005), *lacY* coding the lactose permease proposed to import the Cb (Guan and Kaback, 2006; Kaback, 2005), and 3/*ompC* and *ompF*, coding porins of the outer membrane known to leave sugars enter into the periplasm (Prilipov et al., 1998).

Only *lacI* was found to be affected by different types of mutation in the 4 clones we tested, and this exclusive event was confirmed by the complete genome resequencing of *E. coli* CbB2-evo1 and CbB3-evo1. The various modifications identified on LacI structure are mapped in Sup-plemental Fig. 1. One single-nucleotide polymorphism (SNP) impacts the helix-turn-helix motif implicated in the interaction with the *lacO* operator (Val 23 changed into Met in the clone CbB3-evo2). We also detected modifications potentially altering the structure of the tetramerization region of LacI: two SNPs changing the Glu 181 into Tyr and Asp 247 into Ala for clones CbB2-evo2 and CbB2-evo1 respectively, and 4-base pair insertion generating a premature stop codon and leading to the production of the truncated LacI_{Δ198-331} (hereafter called LacI*) for the clone CbB3-evo1. None of these mutations were ever reported in the literature concerning LacI variants (Barker et al., 1998; Bell et al., 2001; Miura-Onai et al., 1995; Tang et al., 2006; Xu and Matthews, 2009).

All these mutations suggest that the Cb growth phenotype upon adaptation results from a modification of the *lac* operon expression engendered by LacI variants presumably exhibiting decreased affinity for the *lacO* operators or altered tetramerization properties, thereby hampering the regulation activity of these modified repressors (Goodson et al., 2013). We hypothesized that the impaired binding of LacI

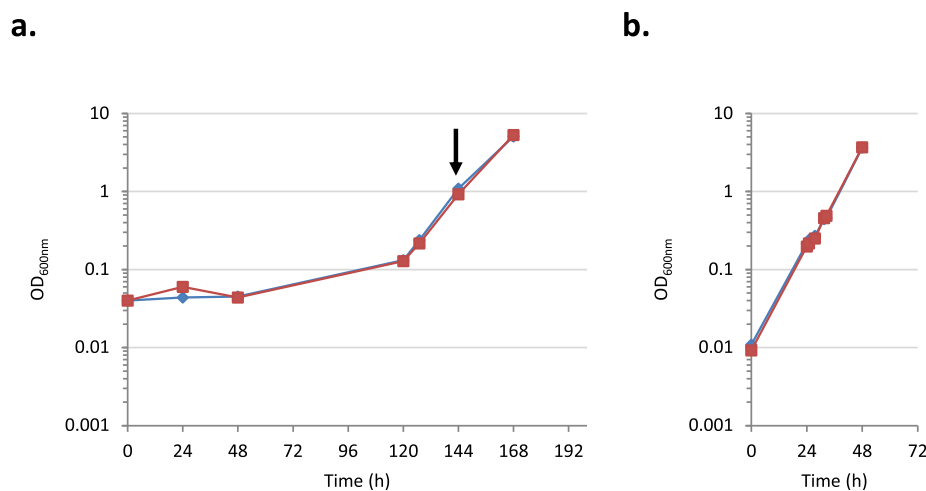


Fig. 1. Growth of CbB strains in Cb-based M9 medium. **a.** CbB2 (blue curve) and CbB3 (red curve) were cultured in M9 medium containing 0.5% Cb at 37 °C. They were sub-cultured when OD_{600nm} reached 1 (black arrow). **b.** Sub-cultures of CbB2 (blue curve) and CbB3 (red curve) grown in M9 medium containing 0.5% Cb at 37 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

drastically modifies the expression of the *lac* operon leading to an increased synthesis of the encoded proteins, as the inner membrane permease LacY previously proposed to prone to transport Cb into the cytosol (Collins et al., 1989; Sekar et al., 2012). In former studies (Beckwith, 1967; Desai et al., 2014; Donovan et al., 1996; Ibrahim et al., 2017; Mueller-Hill et al., 1964; Yang et al., 2015), the role of LacY in the Cb import was not investigated as IPTG was used as an inducer for the expression of Cb catabolic enzymes, which indeed also triggered the expression of the *lac* operon.

3.4. Tuning the expression of the *lac* operon impacts the Cb metabolism and the growth of the resulting modified *E. coli*

To verify that the mutations in *lacI* increased the expression of the *lac* operon, we firstly used pUA-*Plac* from the plasmid reporter library of Zaslaver et al. (2006) containing the *Plac-gfp* transcriptional fusion to investigate the repression activity of the *LacI*^{*} variant we identified. *E. coli* CbB3 expressing *lacI* WT, CbB3-evo1 expressing *lacI*^{*} and a newly constructed strain CbB3 Δ *lacI*, in which the gene *lacI* has been fully deleted, were transformed with the pUA66 (carrying for the *gfp* gene without any promoter) or the pUA-*Plac*. Fluorescence images of the corresponding transformed clones on LB plates are shown in Fig. 2.

As expected, all pUA66 harboring clones were not fluorescent. Unsurprisingly, a high fluorescence signal was observed for the non-evolved *E. coli* CbB3 Δ *lacI* pUA-*Plac* clones because of the non-repression of the *gfp* gene in the absence of *LacI* repressor. The same pattern of fluorescence was observed for the evolved *E. coli* CbB3-evo1 pUA-*Plac* clones indicating that the *LacI*^{*} is less active than the WT variant. The non-evolved *E. coli* CbB3 pUA-*Plac* did not display any GFP emission since the expression of the *gfp* gene is not induced due to the presence of the *LacI* WT repressor. Consequently, these data suggest that the repression of the *lac* operon in the *E. coli* CbB3-evo1 strain is significantly reduced, allowing the strain to produce the inner membrane LacY permease in a suitable quantity to favor the efficient uptake of Cb. Nevertheless, the CbB3 Δ *lacI*, strain was found unable to grow on both Cb and lactose (Suppl. Fig. 2b and c), suggesting that a total de-repression of the *lac* operon induces an overproduction and an aggregation of LacY, as formerly reported (Geerstma et al., 2008), thereby decreasing the amount of functional permease in the inner membrane. This observation also suggests that in CbB3-evo1 strain, the C-terminal truncation of *LacI* does not trigger a full de-repression of the *lac* operon.

However, to secure the import of Cb through the inner membrane, these results prompted us to replace the *Plac* promoter by another promoter of *E. coli*. We selected a set of constitutive promoters displaying various promoter activity: *Pgmk* < *PtonB* < *PmscS* < *PacpP* << *PrrnB* (rRNA promoter

known as one of the strongest promoters of *E. coli*) according to Zaslaver and coworkers (Zaslaver et al., 2006, 2009). We searched among these constitutive promoters which could mimic the strength of the *Plac* promoter like in the CbB3-evo1 strain harboring the *LacI*^{*} variant. *E. coli* CbB3 and CbB3-evo1 were then transformed with the plasmids: pUA66 (no promoter -*gfp*) as a negative control, pUA-p*Plac* (*Plac-gfp*) as a comparative reference, pUA-p*PacpP* (*PacpP-gfp*), pUA-p*Pgmk* (*Pgmk-gfp*), pUA-p*PmscS* (*PmscS-gfp*), pUA-p*PtonB* (*PtonB-gfp*), and pUA-*PrrnB* (*PrrnB-gfp*) from the *E. coli* *gfp* reporter plasmids library (Zaslaver et al., 2006, 2009). The corresponding strains were tested for their capacity to grow in M9 containing 0.2% casamino acids and 0.2% glucose at 37 °C and no growth defect was observed for any of the tested strains (Fig. 3a). As expected, a significant difference in the fluorescence profiles was observed for the strains *E. coli* CbB3 and CbB3-evo1 both bearing the pUA-*Plac* (Fig. 3b, red curves) due to the de-repression effect of the *LacI*^{*} in the evolved strain. The selected promoters did not display altered activity in presence of *LacI* WT or its variant *LacI*^{*} (Fig. 3b, dotted and filled curves, respectively). The strongest *PrrnB* promoter (Fig. 3b, dark green curves) exhibits a much higher constitutive activity than the *LacI*^{*} de-repressed *Plac* one. In contrast, both *PacpP* (blue curves) and *PmscS* (green curves) promoters displayed an activity similar to the *LacI*^{*} de-repressed *Plac* promoter (filled red curve) whereas *Pgmk* (orange curves) and *PtonB* (purple curves) promoters exhibit a weaker activity.

Based on these observations, we replaced the chromosomal *Plac* promoter by an equivalent (*PacpP*) or a weaker (*PtonB*) promoter in the *E. coli* CbB3 strain to test the impact of the promoter strength on the cell capacity to use Cb for its growth. Growth curves on Cb-based medium of the corresponding strains CbB3 *PacpP*::*lac* and CbB3 *PtonB*::*lac* are reported in Fig. 3c. Only the *PacpP* promoter in CbB3 *PacpP*::*lac* strain was able to restore a growth phenotype similar to that of the evolved *E. coli* CbB3-evo1 in M9-Cb (Fig. 3c, blue and green curves, respectively) with a comparable doubling time (Table 1).

Altogether, these data confirm that the control of the *lac* operon expression is the keystone of the evolution of the *E. coli* CbB3 strain toward the *E. coli* CbB3-evo1 strain able to use Cb. They also demonstrate that the replacement of *Plac* by *PacpP* is sufficient to establish a Cb growth phenotype in a non-evolved strain.

3.5. Identification of other complementary mutations that improve the Cb growth phenotype of the modified *E. coli*

Considering that CbpA displays higher catalytic efficiency than BglA and allows to save one ATP molecule per metabolized Cb, that should thus provide a benefit in terms of metabolism, we replaced the integrated gene *bglA* by *cbpA* in the *E. coli* CbB3-evo1 strain. The cassette “*PpdcZ_m*-*cbpA*-*Tgfor_m* cat” was transduced into CbB3-evo1 to generate the strain CbC, displaying the mutation Δ *bglA*::*cbpA*, and clones CbC2 and CbC3 were studied. In contrast to CbCzero1 or 2 (see above), these genetically reengineered strains started to grow after 5 days of culture in M9-Cb medium (Fig. 4a, orange and green curves) and subsequent subcultures did not display any lag phase (Fig. 4b, red curve) suggesting that another evolution event occurred. From CbC2 adapted culture, several clones called CbC2-evo1 to CbC2-evo6 were isolated. They all exhibited similar growths in M9-Glucose (Fig. 4c) and -Cb (Fig. 4d) minimum media, with a doubling time ranging from 2.39 to 2.58 h (Table 1). CbC2-evo1 displays a more than two-fold reduced generation time compared to CbB3-evo1 strains (Table 1) demonstrating that the growth of CbC2-evo1 is significantly improved compared to the initial CbB3-evo1 strain (Suppl. Fig. 3, 6a and b).

Genome sequencing of CbC2-evo1 revealed the presence of two additional mutations in loci *yfeN* and *lamB*. Both mutations were confirmed by Sanger sequencing of the target loci. *YfeN* is a putative outer membrane protein belonging to the Tsx nucleoside transporter family (Ye and Van der Berg, 2004). The SNP mutation leads to the replacement of the Serine residue at position 121 by a Proline that could modify the tertiary structure of this porin. *LamB*, also known as Lambda

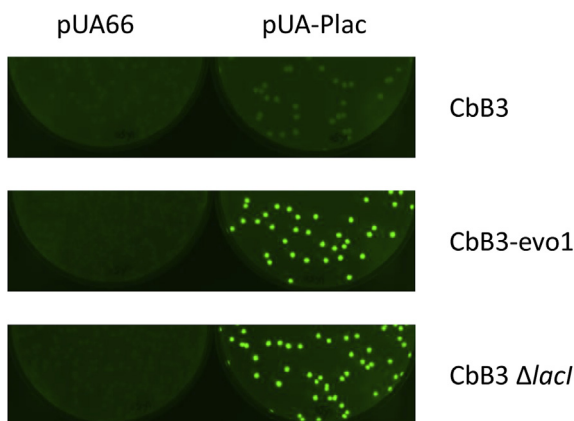


Fig. 2. Expression of the *lac* operon in CbB and CbB evo strains revealed by *Plac-gfp* reporter. Fluorescence signal of the colonies grown on LB plate read Typhoon FLA9500 imager for CbB3, CbB3-evo1 and CbB3- Δ *lacI*, transformed with vectors pUA66 (*gfp* without any promoter) or pUA-*Plac* (*Plac-gfp*).

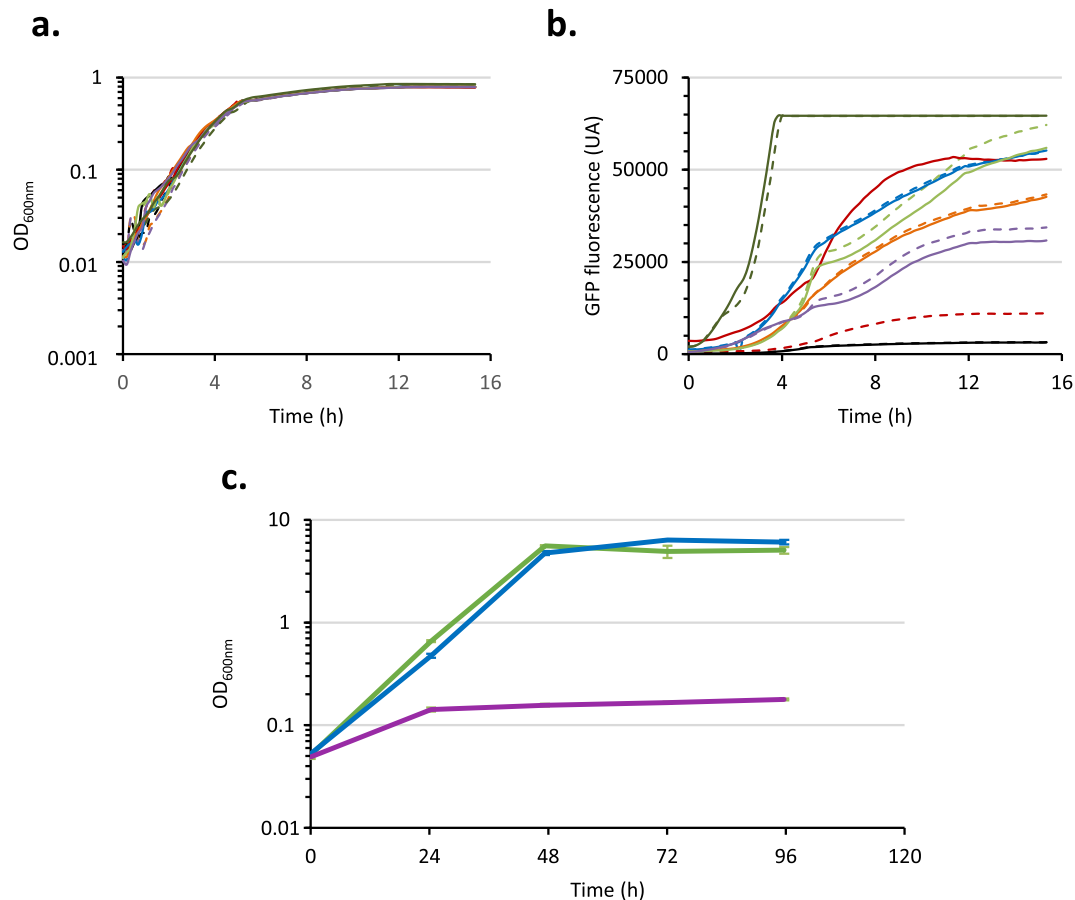


Fig. 3. Promoter activities in CbB3 and CbB3-evo1 strains grown on glucose (a and b) and growth of CbB3 *PacpP::lac* and CbB3 *PtonB::lac* strains in Cb-based M9 medium (c). Cells (a and b) were cultured in M9 0.2% CASA 0.2% glucose at 37 °C, and growth and fluorescence signal were monitored in a Tecan Spark plate reader. a. Optical density at 600 nm. b. Fluorescence level of GFP (excitation 488 nm / emission 521 nm). CbB3 (dotted line) or CbB3-evo1 (filled line) transformed by pUA66 (black), pUA-Plac (red), pUA-PacpP (blue), pUA-PgmK (orange), pUA-PmscS (light green), pUA-PtonB (purple), or pUA-PrrnB (dark green). The mean of the data obtained on three culture replicates is shown. c. CbB3-evo1 (green), CbB3 *PacpP::lac* (blue), and CbB3 *PtonB::lac* (purple) strains were cultured in M9 medium containing 0.5% Cb at 37 °C. The mean of the data obtained on three culture replicates is shown. Bars indicate the standard deviations. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Generation time of CbB and CbC strains in M9 based Cb medium.

Strain	Cb catabolic enzyme	Doubling time (h)		
CbB3	BglA	ND		
CbB3-evo1	BglA	7.03	+/-	0.61
CbCzero1	CbpA	ND		
CbC2	CbpA	ND		
CbC2-evo1	CbpA	2.58	+/-	0.06
CbC2-evo2	CbpA	2.39	+/-	0.07
CbC2-evo3	CbpA	2.57	+/-	0.05
CbC2-evo4	CbpA	2.49	+/-	0.09
CbC2-evo5	CbpA	2.52	+/-	0.03
CbC2-evo6	CbpA	2.58	+/-	0.02
CbC2 <i>lamB</i> *	CbpA	2.62	+/-	0.11
CbC2 <i>yfeN</i> _{S121P}	CbpA	ND		
CbB3-evo1 <i>lamB</i> *	BglA	2.48	+/-	0.03
CbB3-evo1 <i>yfeN</i> _{S121P}	BglA	7.20	+/-	0.79
CbB3-evo1 Δ <i>lamB</i>	BglA	6.94	+/-	0.38
CbB3-evo1-PgmK	BglA	19.59	+/-	2.30
CbC2-evo1-PgmK	CbpA	49.19	+/-	4.07
CbB3 <i>PacpP::lac</i>	BglA	6.94	+/-	0.53
CbB3 <i>PtonB::lac</i>	BglA	ND		
CbB3 <i>PacpP::lac lamB</i> *	BglA	2.20	+/-	0.05

The mean of the data obtained on three culture replicates is shown.
ND: not determined.

phage receptor (Berkane et al., 2006; Chatterjee and Rothenberg, 2012), is the maltose outer membrane porin involved in the import of malto-dextrins in the periplasm (Charbit, 2003; Klebba, 2002; Mulvihill et al., 2019). Mutation observed in *lamB* (hereafter named *lamB**) resulted from the insertion of the natural transposable element IS2D (Gonçalves et al., 2014; Saedler and Heiss, 1973). The modification of the ORF sequence at *lamB* locus led to the addition after the 362nd residue of four codons (Arg-Leu-Ala-Pro) and a premature termination codon thus resulting in the production of a truncated LamB (Δ 363–446) whose 83 C-terminal residues are missing.

3.6. A mutation in *lamB* gene modifying the structure of the porin allows the strain to grow faster on Cb

To assess the impact of these mutations, *yfeN*_{S121P} and *lamB** were marked by integration of *kan* and *cat* resistance genes respectively, and were subsequently individually transduced into non-evolved CbC2 strain and CbB3-evo1 strain. Growth was followed on Cb-based medium (Fig. 5a). The transduction of *lamB** mutation was found sufficient to allow the strain CbC2 *lamB** to grow on M9 Cb without any adaptation step (orange curve). In contrast, the mutation *yfeN*_{S121P} does not provide any benefit to both strains (yellow and blue curves). Surprisingly, CbB3-evo1 carrying *lamB** mutation is able to grow as fast as CbC2-evo1, exhibiting a doubling time reduced to 2.5 h (Table 1).

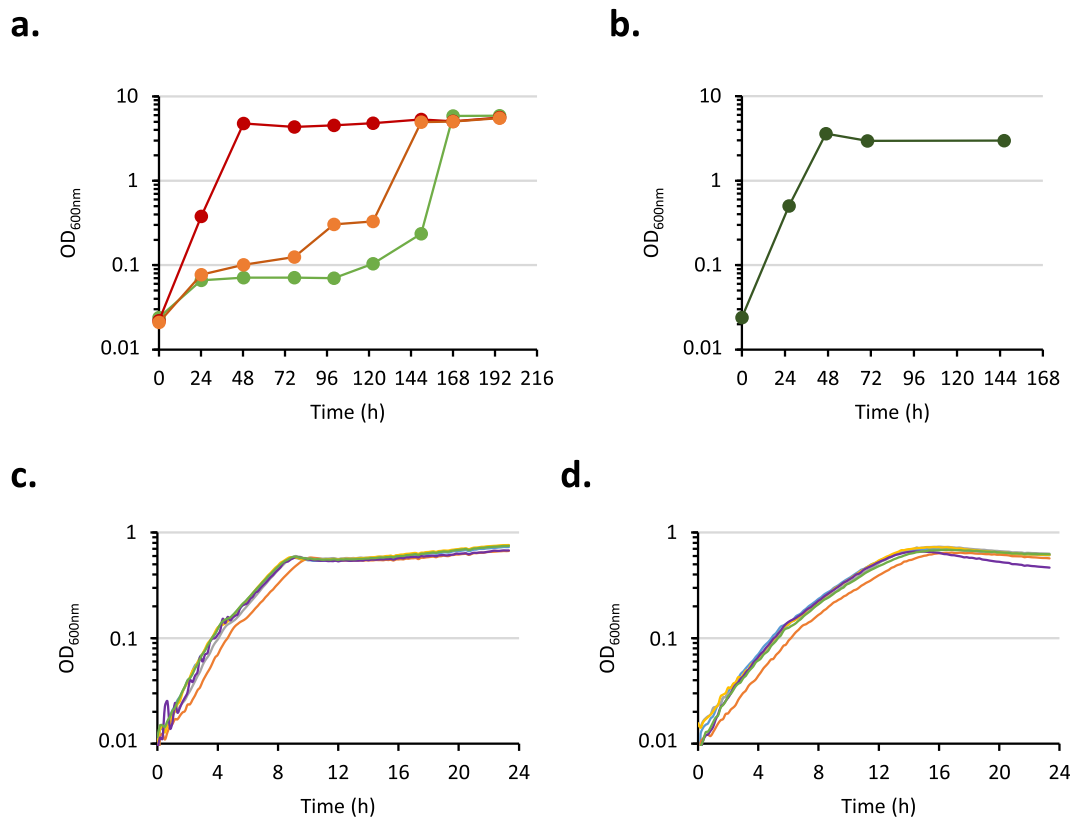


Fig. 4. Growth of CbC strains in Cb-based M9 medium. **a.** CbC2 (orange curve), CbC3 (green curve), and CbB3-evo1 (red curve) strains were cultured in M9 medium supplemented with 0.5% Cb at 37 °C. **b.** CbC2 was sub-cultured when initial culture reached the stationary phase. Sub-culture (dark green curve) was performed in M9 containing 0.5% Cb at 37 °C. **c.** Culture of isolated colonies CbC2-evo1 (blue), CbC2-evo2 (orange), CbC2-evo3 (grey), CbC2-evo4 (yellow), CbC2-evo5 (purple), and CbC2-evo6 (green) in M9 0.2% glucose at 37 °C and **d.** in M9 containing 0.5% Cb at 37 °C. Growth was monitored in Tecan Spark plate reader, of isolated colonies CbC2-evo1 (blue), CbC2-evo2 (orange), CbC2-evo3 (grey), CbC2-evo4 (yellow), CbC2-evo5 (purple), or CbC2-evo6 (green). The mean of the data obtained on three culture replicates (c. and d.) is shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

LamB barrels are assembled into trimers in the outer membrane (Baldwin et al., 2011; Luckey et al., 1991; Vos-Scheperkeuter and Witholt, 1984). The mutation engenders a deletion of 83 residues encompassing the three last β -sheets (Δ 363–446) at the C-terminal extremity (Suppl. Fig. 4a) which contains the key residues Trp383 and Trp445 (Trp358 and Trp420 in the mature LamB) involved in the direct interaction with maltose, and allowing the specific transport of the disaccharide through the membrane (Nikaido, 2003). Indeed, it is well known that LamB porin allows the diffusion of several other disaccharides displaying α -linkage (maltose, trehalose, melibiose, Suppl. Fig. 4b) but restricts the diffusion of disaccharides harboring β -linkage (cellobiose, sucrose, lactose) (Luckey and Nikaido, 1980). This Δ 363–446 truncation should lead to a reshaping of the internal channel (Suppl. Fig. 4b and c) of LamB as illustrated by the predicted structure of the truncated LamB* based on the Omp-Pst2 from *Providencia stuartii* (Suppl. Fig. 4c), another outer membrane protein belonging to the porin superfamily as LamB. Omp-Pst2 is the closest porin, sharing 30.8% similarity with the truncated LamB* in the same range of size (Suppl. Fig. 5). Overall, the in-depth modification of the inner channel and the missing recognition residues might explain the ability of Cb to diffuse through LamB*.

We then compared the capacity of the various Cb-metabolizing strains to grow on limited available Cb. The strains CbB3-evo1, CbC2-evo1, and CbB3-evo1 *lamB** were grown on M9 medium containing a range of Cb concentrations from 0.025% to 1% (Suppl. Fig. 6). The CbB3-evo1 strain is still able to grow at a minimal concentration of 0.1% Cb, whereas the CbC2-evo1 strain displays a significant growth at only 0.05% Cb. Nevertheless, the CbB3-evo1 *lamB** strain is the most efficient as it can

grow, thanks to the *lamB** mutation at only 0.025% Cb. These results indicate that the production of the truncated LamB favors the growth of the strains (CbC2-evo1 and CbB3-evo1 *lamB**), especially at low Cb concentration, compared to the strain displaying the full-length LamB porin (CbB3-evo1).

Since LamB* variant as found in CbC2-evo1 strain provides a better growth phenotype on Cb to both CbC2 and CbB3-evo1 *lamB** strains compared to CbB3-evo1, we investigated if in the initial strain CbB3-evo1, the native porin LamB is involved in the transport of Cb through the outer membrane. For this study, we constructed the strain CbB3-evo1 Δ *lamB* which does not produce LamB and its growth was compared to that of the strain CbB3-evo1 on Cb-based medium (Fig. 5b). Both strains exhibit similar growth, suggesting that Cb diffused through the outer membrane in the periplasm by another transport system in the strain CbB3-evo1. Thus, truncation of LamB improves the growth in the strain CbB3-evo1 *lamB** by providing an additional Cb-transport system to the periplasm.

3.7. A stronger expression of *bglA* or *cbpA* is deleterious for the growth on Cb

To improve the Cb based growth capacity of the engineered strains, we first investigated the strength of *PpdcZm* promoter in *E. coli* and the effect of the replacement of this promoter controlling the expression of *bglA* and *cbpA* by stronger ones. The CbB3-evo1 *lamB** strain was selected to monitor the promoter activity during the growth on Cb. This strain was transformed with pUA vectors containing *PacpP*, *Plac*, *PgmK* (Zaslavsky et al., 2006) or *PpdcZm* promoters, the latter being cloned in a pUA66

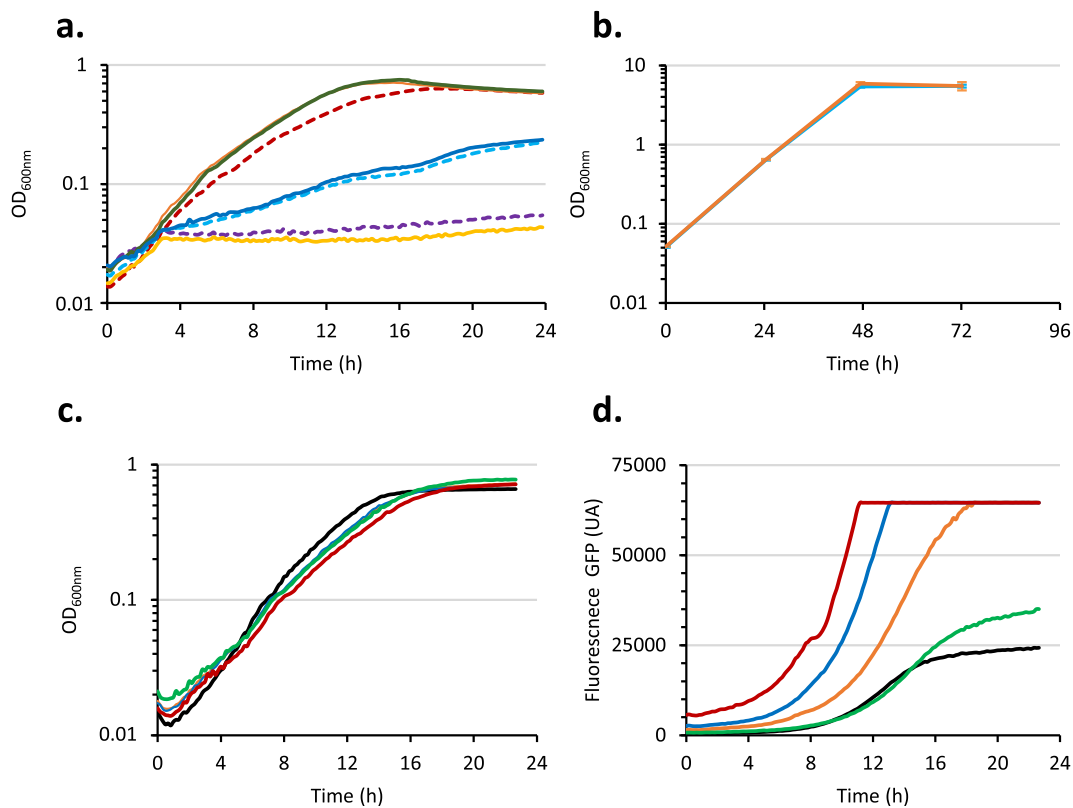


Fig. 5. Growth of strains carrying *lamB* mutations in Cb-based M9 medium (a and b) and promoter activities in CbB3-evo1 *lamB** strain grown on Cb (c and d). **a.** Culture in M9 containing 0.5% Cb at 37 °C of the strains CbB3evo1 (blue, dotted line), CbB3evo1 *yfeN*_{5121P} (blue), CbB3evo1 *lamB** (green), CbC2 (purple, dotted line), CbC2evo1 (red, dotted line), CbC2 *yfeN*_{5121P} (yellow), and CbC2 *lamB** (orange). The growth was monitored in Tecan Spark plate reader, and the data show the mean of three culture replicates. **b.** Culture in M9 containing 0.5% Cb at 37 °C of the strains CbB3-evo1 (blue) and CbB3-evo1 *DlamB* (orange). The mean of the data obtained on three culture replicates is shown. **c.** and **d.** The strains CbB3-evo1 *lamB** transformed with pUA66 (black), pUA-Plac (red), pUA-PacpP (blue), pUA-PgmK (orange), pUA-Ppdc (green) were grown in M9 containing 0.5% Cb at 37 °C. Growth and fluorescence signal were monitored in Tecan Spark plate reader. The mean of the data obtained on three culture replicates is shown. **c.** Optical density at 600 nm. **d.** Fluorescence level of GFP (excitation 488 nm / emission 521 nm). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

upstream of the gene encoding the GFP. The activity of the promoters was then measured on cells grown in M9 medium containing Cb (Fig. 5c and d). Compared to *PacpP* (blue curve), *Plac* (red curve), or *PgmK* (orange curve), the fluorescence level reached with the *Ppdc_{Zm}* promoter is very low depicting a weaker strength of this promoter.

Based on these observations, the *Ppdc_{Zm}* promoter regulating the expression of *bglA* and *cbpA* was replaced by *PgmK* in the strains CbB3-evo1 and CbC2-evo1, respectively. The growth of the resulting strains was afterwards examined on Cb-based medium (Fig. 6). CbB3-evo1-*PgmK* (in yellow) and CbC2-evo1-*PgmK* (in green) exhibited a very limited growth compared to CbB3-evo1 (blue curve) and CbC2-evo1 (red curve). In the case of CbC2-evo1-*PgmK*, the doubling time was increased up to 33 h. Clearly, the replacement of *Ppdc_{Zm}* promoter by the stronger promoter *PgmK* severely affected the growth of both strains. This observation indicates that the use of a stronger promoter is detrimental and that *Ppdc_{Zm}* induces a suitable expression of the genes encoding the heterologous Cb-degrading enzymes. Analyses of the activity of crude extracts of strains CbB3-evo1, CbB3-evo1-*PgmK*, CbC2-evo1 and CbC2-evo1-*PgmK*, revealed that the *BglA* activity on pNP-Glu in CbB3-evo1-*PgmK* extract ($2,445 \pm 148$ iu/mL) was reduced by 70% compared with CbB3-evo1 extract ($7,907 \pm 317$ iu/mL), whereas the *CbpA* activity on Cb measured in CbC2-evo1-*PgmK* extract (8.5 ± 0.4 iu/mL) was more than 100-fold lower than that observed for CbC2-evo1 extract ($1,240 \pm 32$ iu/mL). These drastic diminutions observed for both CbB3-evo1-*PgmK* and CbC2-evo1-*PgmK* are consistent with their impaired growth on Cb-based medium (Fig. 6), and may suggest that a large fraction of the heterologous Cb-degrading enzymes aggregates in these modified strains.

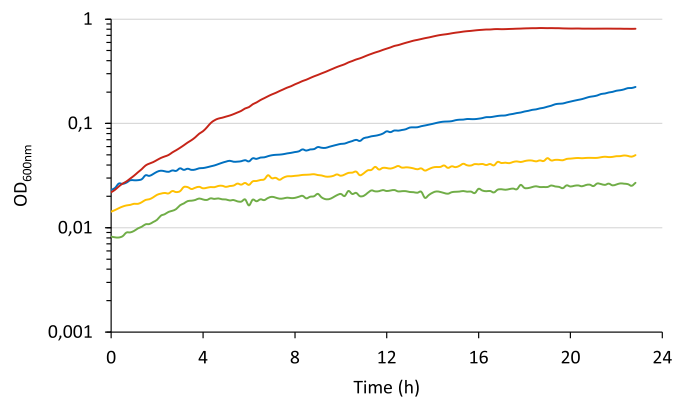


Fig. 6. Growth of CbB3-evo1-*PgmK* and CbC2-evo1-*PgmK* strains in Cb-based M9 medium. CbB3-evo1 (blue curve) CbC2-evo1 (red curve), CbB3-evo1-*PgmK* (yellow curve), and CbC2-evo1-*PgmK* (green curve) strains were cultured in M9 supplemented with 0.5% Cb at 37°C. OD at 600 nm was monitored in a Tecan Spark plate reader. The mean of the data obtained on three culture replicates is shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.8. *Ppdc_{Zm}* exhibits a promoter activity in the range of the majority of promoters of *E. coli*

Since the *Ppdc_{Zm}* promoter provides an appropriate expression of *bglA* or *cbpA* genes to sustain the growth on Cb-medium, we investigated its

promoter strength among *E. coli* promoters. First, based on the culture of CbB3-*evol1 lamB** pUA-PacpP, pUA-PgmK or pUA-Ppdc grown on M9 Cb 0.5% (Fig. 5c and d), the promoter activity (PA) of *Ppdc_{Zm}*, *PgmK* and *PacpP* was calculated using the formula of Zaslaver and coworkers ($PA = \frac{dFluorescence/dt}{OD_{600nm}}$) (Zaslaver et al., 2006) and their maximum of activity (maxPA) was extracted from the PA profiles for all the promoters (Suppl. Fig. 7a). Since PA were measured from a fluorescence quantification that strongly depends on the sensitivity of the device used, maxPA values were arbitrarily normalized to the *PacpP* maxPA to facilitate the comparison with the previously reported data (Zaslaver et al., 2006, 2009). *PgmK* displays here a maxPA corresponding to 51% of the *PacpP* maxPA whereas the maxPA of *Ppdc_{Zm}* drops to 21% (Suppl. Fig. 7a). These results are consistent with already published data from the *E. coli* promoter database, in which *PgmK* displays a maxPA of 50% compared to *PacpP* (Suppl. Table 4). The maxPA of the 1916 promoters from the complete *E. coli* promoter database were also normalized with respect to *PacpP* maxPA (Suppl. Fig. 7b). It appears that 2/3 of the promoters (1208 promoters) have maxPA lower or similar to that of *Ppdc_{Zm}* (21% of *PacpP* maxPA) whereas only 124 promoters exhibit similar or higher maxPA compared to *PgmK* (50% of *PacpP* maxPA). Thus, *Ppdc_{Zm}* promoter seems to exhibit an activity in *E. coli* in the range of the majority of promoters found in this bacterium.

3.9. Genome editing of *E. coli* MG1655 can provide an efficient Cb metabolism

Altogether our data provide a list of genetic modifications enabling to convert the *E. coli* strain MG1655 into a bacterium able to efficiently catabolize and use Cb as the sole carbon source. As summarized in Supplemental Fig. 3 and in the Cb metabolism model depicted in the Fig. 7, the following mutations were thus inserted in the wild type MG1655 genome by P1 phage transduction: *zde::bglA* to provide a metabolic enzyme cleaving Cb into glucose (CbB3 strain, Suppl. Table 2); $\Delta Plac::PacpP$ to allow a constitutive expression of the *lac* operon and to provide a sufficient amount of LacY permease in the inner membrane (CbB3 *PacpP::lac* strain, Suppl. Table 2); *lamB** to generate a truncated

form of the outer membrane LamB porin that efficiently uptakes Cb (CbB3 *PacpP::lac lamB** strain, Suppl. Table 2).

The resulting strain CbB3 *PacpP::lac lamB** was able to grow aerobically without any lag phase on M9 Cb medium, displayed a doubling time of 2.2 h and reached a final OD_{600nm} of 5 (Fig. 8b). This strain is also able to ferment cellobiose anaerobically (Fig. 8 d, and Suppl. Fig. 8) and exhibits a doubling time of 12 h on Cb under anoxic condition.

4. Conclusions

E. coli is not able to grow directly on Cb-based medium but previous studies showed that the strain can evolve to metabolize Cb by reactivating the cryptic operons *chb* (Chitinibiose utilization loci, cryptic for Cb utilization) and *asc* (encoding a β -glucosidase and a PTS transporter for Cb) followed by 30 days of Cb-based adaptative evolution (Parisutham and Lee, 2011, 2015). Nevertheless, the formerly engineered strain had a limited growth on Cb, only exhibiting a maximal OD_{600nm} = 1.6 and a generation time around 4 h when grown on minimum medium at a concentration of 0.4% Cb. In the present study, we have developed robust *E. coli* strains to provide an efficient organism able to grow on Cb as single carbon source. The best strain we obtained (CbB3 *PacpP::lac lamB**) reached on M9 0.5% Cb at 37 °C a final OD_{600nm} = 5 in aerobiosis and 0.6 in anaerobiosis (Fig. 8b) and displayed respectively a doubling time of 2.2 h in aerobiosis (Table 1) and around 12 h in anaerobiosis (Fig. 8d). This capacity to efficiently catabolize Cb was attained by chromosomal insertion of a β -glucosidase that cleaves β -1,4 linkage to produce two glucose molecules as well as of a Cb phosphorylase that produces one glucose and one α -glucose 1-P by phosphorolysis. We have also unraveled mutations in two distinct genes improving the uptake of Cb: a *lamB* mutant gene encoding a truncated outer membrane maltoporin (LamB*) and a *lacI* mutant gene encoding a dysfunctional LacI repressor leading to an upregulation of the *lac* operon and an appropriated production of the LacY permease. Furthermore, we engineered a constitutive *lac* operon expressing LacY under the control of *PacpP* promoter, without prior adaptation or involvement of LacI, a key repressor whose activity is used to control the expression of genes in many biotechnological applications

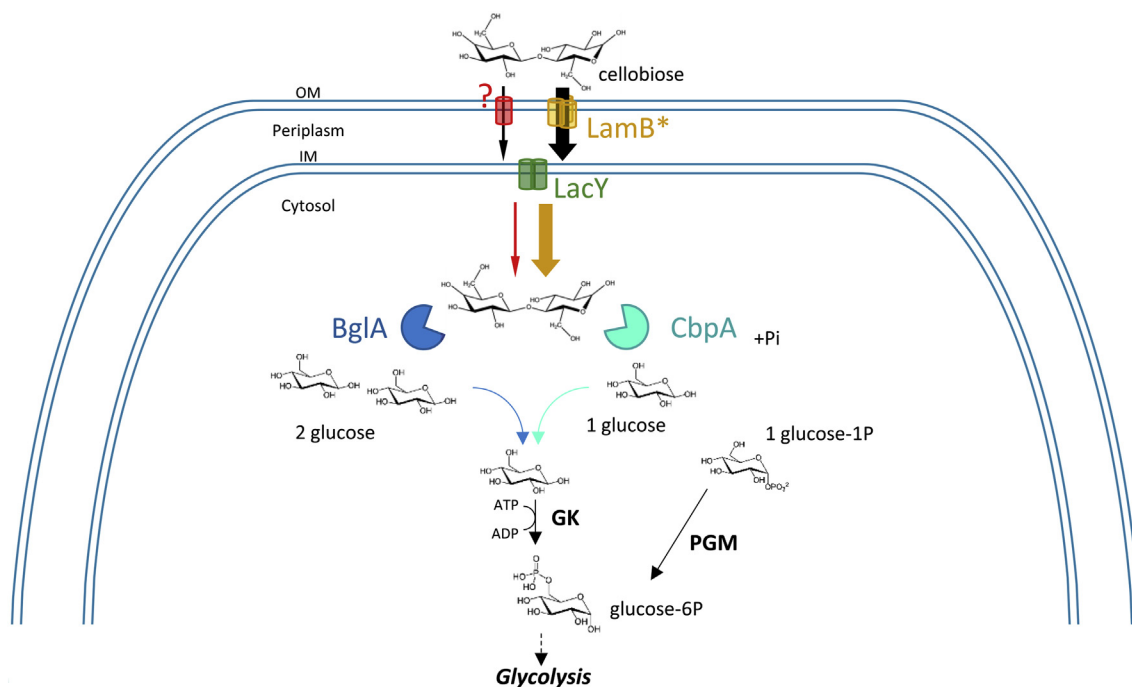


Fig. 7. Model of Cb uptake and metabolism by engineered *E. coli* strains. LamB*, truncated *E. coli* LamB ($\Delta 363$ -446) porin; LacY, *E. coli* lactose permease; BglA, *Ruminococcus albus* β -glucosidase A; CbpA, *Ruminiclostridium cellulolyticum* Cb phosphorylase A; GK, *E. coli* glucokinase; PGM, *E. coli* phosphoglucomutase; IM, inner membrane; OM, outer membrane.

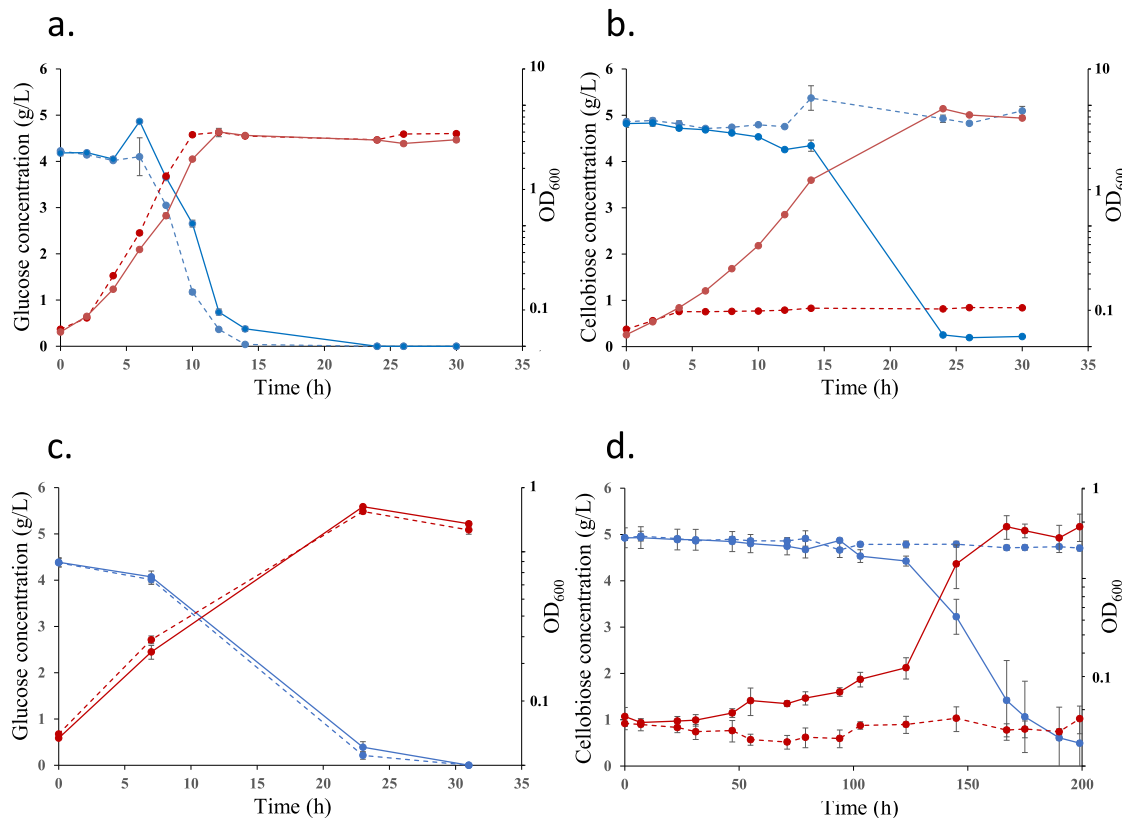


Fig. 8. Growth of CbB3 *PacpP::lac lamb** and MG1655 strains in aerobic and anaerobic conditions on glucose and cellobiose. The growth and sugar consumption under aerobic conditions on glucose and cellobiose are shown in a. and b., respectively. The growth and sugar consumption under anaerobic conditions on glucose and Cb are shown in c. and d., respectively. The growth (right axes) was monitored at 600 nm (red curves), and the sugar concentration (left axes) was determined by HPLC using an Aminex HPX87H column (blue curves). CbB3 *PacpP::lac lamb** and MG1655 strains are represented by solid and dotted lines, respectively. The data show the mean of three biological replicates, and bars represent the standard deviations. Fermentations products related to figures c. and d., are reported in supplemental Fig. 8a and b, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Ahmad et al., 2018; Gopal and Kumar, 2013; Gupta and Shukla, 2017; Hayat et al., 2018). Such a robust strain able to metabolize cellobiose should be valuable for future bioprocess engineering such as fermentation of saccharified cellulosic wastes into biomolecules of interest (bio-fuels, commodity chemicals...).

Authors contribution

RB: Conceptualization, Investigation, Formal analysis, Visualization, Writing - Original Draft **NV:** Conceptualization, Investigation, Formal analysis, Writing - Original Draft - Review & Editing **NF:** Writing - Review & Editing **CT:** Writing - Review & Editing **SP:** Writing - Review & Editing **HPF:** Conceptualization, Investigation, HPAEC-PAD and HPLC-RI analyses, Funding acquisition, Supervision, Resources, Writing - Review & Editing.

Declaration of competing interest

None.

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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.2020.e00157>.

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