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Recombinant *Lactococcus lactis* for efficient conversion of cellodextrins into L-lactic acid

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Abstract

Lactic acid bacteria (LAB) are among the most interesting organisms for industrial processes with a long history of application as food starters and biocontrol agents, and an underexploited potential for biorefineries converting biomass into high-value compounds. Lactic acid (LA), their main fermentation product, is among the most requested chemicals owing to its broad range of applications. Notably, LA polymers, *i.e.*, polylactides, have high potential as biodegradable substitutes of fossil-derived plastics. However, LA production by LAB fermentation is currently too expensive for polylactide to be cost-competitive with traditional plastics. LAB have complex nutritional requirements and cannot ferment inexpensive substrates such as cellulose. Metabolic engineering could help reduce such nutritional requirements and enable LAB to directly ferment low-cost polysaccharides.

Here, we engineered a *Lactococcus lactis* which constitutively secretes a β -glucosidase and an endoglucanase. The recombinant strain can grow on cellooligosaccharides up to at least cellooctaose and efficiently metabolizes them to L-LA in single-step fermentation. This is the first report of a LAB able to directly metabolize cellooligosaccharides longer than cellohexaose and a significant step towards cost-sustainable consolidated bioprocessing of cellulose into optically pure LA.

Key words: Polylactide, metabolic engineering, recombinant cellulolytic strategy, cellulase, beta-glycosidase.

Introduction

Lactic acid (LA) is among the most requested chemicals worldwide because of broad range of applications (*e.g.*, as food preservative and flavor enhancer, emulsifier and moisturizer in the cosmetic industry, precursor of pharmaceuticals and biodegradable solvents) (Abdel-Rahman et al., 2013) and its global demand has been increasing yearly at 5-8% (Yadav et al., 2011). Its polymer polylactide (PLA) has high potential as biodegradable and biocompatible alternative to petrochemical-derived plastics (Abdel-Rahman et al., 2013). Physico-chemical features of PLA widely differ depending on proportion of D- and L-LA used for its polymerization (Auras et al., 2004). Chemical synthesis of LA is not suitable for this application, since it produces a racemic mixture of D- and L-LA. Fermentative production of LA allows biosynthesis of optically pure LA and currently accounts for almost the whole global production of LA (Abdel-Rahman et al., 2011). Notably, about 90% of the LA produced worldwide is obtained through lactic acid bacteria (LAB) fermentation (Sauer et al., 2008).

LAB have long been used in industrial processes mainly as food starters or probiotics. Several physiological (*e.g.*, high acid/alcohol tolerance and broad optimal growth temperature) and metabolic (*e.g.*, production of LA, ethanol, and polyhydroalkanoates) characteristics prompt their use also as microbial cell factories (Mazzoli et al., 2014). However, complex nutritional requirements of LAB significantly limit their use in cost sustainable biorefinery (Mazzoli et al., 2014). Generally, LAB cannot biosynthesize most amino acids, nucleotides and vitamins that must be supplemented to their growth medium thus increasing costs of fermentation and product-purification (Okano et al., 2010a). Furthermore, few natural LAB can ferment inexpensive feedstocks such as starch, and no native cellulolytic and/or hemicellulolytic LAB has been isolated so far (Okano et al., 2010a). Fermentation of enzymatically hydrolyzed lignocellulosic feedstocks by LAB has been reported (Hu et al., 2016; Shi et al., 2015). However, the high cost of commercial cellulases significantly reduces economic advantage of using cheap raw materials (Okano et al.,

2010a; Olson et al., 2012). The cost of fermentative production of LA should be at or below 0.8 \$ /kg for PLA to be economically competitive with fossil fuel-based plastics (Okano et al., 2010a).

Impressive number of studies have been aimed at engineering microorganisms producing high-value compounds (e.g., *Saccharomyces cerevisiae*, *Clostridium acetobutylicum*, *Corynebacterium glutamicum*) with heterologous cellulase systems (Fierobe et al., 2012; Ilmén et al., 2011; Kim et al., 2014) for application in single-step fermentation (i.e., consolidated bioprocessing, CBP) of cheap biomass. The moderate success of such recombinant cellulolytic strategies (RCSs) is mainly related to the high complexity of native cellulase systems. The latter generally consist of multiple glycosyl hydrolases organized according to two main paradigms, the non-complexed enzyme model of aerobic fungi and bacteria and the cellulosome complex of anaerobic microorganisms (Lynd et al., 2002). Extracellular cellulases cleave cellulose in a mixture of glucose and cellooligosaccharides (cellodextrins) (Desvaux, 2006; Lynd et al., 2002). Cellodextrins are further metabolized by two alternative pathways: i) extracellular hydrolysis to glucose; ii) direct transport into the cytoplasm followed by intracellular depolymerization (Desvaux, 2006; Taylor et al., 2006). Heterologous expression of cellulase-system components is often toxic for hosts, leading to strains which are not-viable or with severe growth deficiency (Mingardon et al., 2011; Wieczorek and Martin, 2010). Efficient biosynthesis and secretion of cellulases are major hurdles of RCSs (Mazzoli, 2012). Strategies such as codon usage optimization, engineering of signal peptides and utilization of inducible transcriptional promoters, have been used to improve translation rate, increase protein secretion or delay cellulase biosynthesis to the mid-exponential phase to reduce saturation of transmembrane transport systems, respectively (Mazzoli 2012; Mazzoli et al., 2012).

In the present study, *Lactococcus lactis* IL1403 was engineered with a mini-cellulase system derived from *Clostridium cellulovorans* 743B (Tamaru et al., 2011). Its genomic GC content (31.2 %) is similar to that of *L. lactis* (35.3 %), which suggests a similar codon usage (Bolotin et al., 2001; Tamaru et al., 2011). *C. cellulovorans* biosynthesizes a cell-surface anchored cellulosome

which acts in synergism with dozens of non-complexed glycosyl hydrolases/polysaccharide lyases (Tamaru et al., 2010). In the present research, the genes encoding two non-cellulosomal glycosyl hydrolases, *i.e.*, the β -glucan glucohydrolase BglA and the endoglucanase EngD, were cloned in *L. lactis* (Foong and Doi, 1992; Kosugi et al., 2006). Here, we demonstrate that recombinant *L. lactis* secreting both BglA and EngD efficiently ferments cellodextrins up to cellooctaose to L-LA.

Materials and methods

Bacterial strains and media

The bacterial strains used in this study are listed in Supplemental Table SI. *C. cellulovorans* 743B was purchased from DSMZ (Germany) (DSM 3052) and routinely grown in the anaerobic medium described by Sleat et al. (1984) supplemented with 0.5% (w/v) cellobiose at 37°C, without agitation. *Escherichia coli* TOP10 (Invitrogen) was routinely grown in Luria-Bertani (LB) medium at 37°C with horizontal shaking (180 rpm). *L. lactis* IL1403 was kindly provided by Dr. Muriel Cocaign-Bousquet and Dr. Pascal Loubière (INSA-Toulouse, France). *L. lactis* was routinely grown in M17 medium supplemented with 0.5 % (w/v) glucose as the sole carbohydrate source (GM17) (Terzaghi and Sandine, 1975) at 30°C without agitation, unless otherwise stated. Bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600nm}) (1 OD_{600nm} unit corresponds to 0.3 g/L dry weight).

Molecular techniques

Phusion DNA polymerase, restriction enzymes and T4 DNA ligase were from New England Biolabs (NEB). DNA samples were purified by the GeneElute PCR Clean-Up Kit (Sigma-Aldrich). All the plasmids constructed in this study were firstly transformed in chemiocompetent *E. coli* TOP10 (Invitrogen). Plasmidic DNA was purified by the GeneElute HP Plasmid Miniprep Kit (Sigma-Aldrich). Plasmids and primers used in this study are listed in Supplemental Table SI and SII, respectively.

Extraction of *C. cellulovorans* genomic DNA

Isolation of genomic DNA (gDNA) from overnight cultures of *C. cellulovorans* (3 mL) was performed as previously described (Mermelstein et al., 1992). gDNA was re-suspended in 50 µl of 10 mM Tris-HCl pH 7.5, 1 mM EDTA.

Optimization of pMG36e vector: construction of pMG36eΔ

The *E. coli-L. lactis* shuttle vector pMG36e was kindly supplied by Prof. Jan Kok (University of Groningen, The Netherlands). pMG36e is a translational fusion vector for cytoplasmic gene expression under the control of the constitutive P32 lactococcal promoter. It harbours a single selection marker, *i.e.*, an erythromycin resistance gene (Van de Guchte et al., 1989). In this study, the pMG36e vector was improved by two sequential modifications: i) the 34 bp fragment between the ribosome binding site (RBS_{P32}) and the *SacI* site of the multiple cloning site (MCS) (including the original pMG36e start codon) was removed to efficiently translate heterologous genes with their original signal sequences (or with signal sequence of choice); to this aim, the pMG36e template was amplified with the ΔATG-D/ΔATG-R primers (Supplemental Table SII), digested by *SacI* and circularized by ligation thus obtaining the pMG36eΔ vector; ii) an ampicillin resistance gene was inserted as second selection marker for easier selection of *E. coli* transformants (*E. coli* is generally naturally tolerant to erythromycin); to this aim, the 1110 bp ampicillin resistance cassette from pUC19 (NEB) was amplified by the amp-D/amp-R primers (Supplemental Table SII), and cloned into the unique *NheI* site of pMG36eΔ. The optimized plasmid was designed as pMG36eΔ.

Gene cloning

Full length *bglA* (GenBank AY268940.1) and *engD* (GenBank M37434.1) genes were amplified from the *C. cellulovorans* gDNA using the *bglA*-D/*bglA*-R and *engD*-D/*engD*-R primer pairs, respectively (see Supplemental Table SII). *bglA* and *engD* were separately cloned in the pMG36eΔ vector. *bglA* was inserted between *SacI* and *XbaI* restriction sites, while *engD* was cloned between *SacI* and *SphI* sites, thus obtaining vectors pB and pD, respectively. The pBD vector containing an artificial bicistronic operon encoding both BglA and EngD was constructed by

cloning a second RBS_{P32} followed by full length *engD* gene in the pB vector downstream of *bglA*. For this construction, *engD* was amplified from *C. cellulovorans* gDNA using the engD-RBS/engD-R primer pair (Supplemental Table SII). The engD-RBS primer was designed to contain the RBS_{P32} sequence. The PCR product was cloned between *XbaI-SphI* sites of the pB plasmid. pB, pD and pBD plasmids were transformed into chemiocompetent *E. coli* TOP10 (Invitrogen). Transformant selection was performed on LB agar plates supplemented with 100 µg/mL ampicillin.

Transformation of *L. lactis* IL1403

pMG36eaΔ, pB, pD and pBD vectors were extracted from the corresponding *E. coli* strains and transformed in electrocompetent *L. lactis* IL1403 as previously described (Gerber and Solioz, 2007). Electrocompetent *L. lactis* cells were mixed with 1 µg of plasmid DNA (re-suspended in milliQ water) in ice-cold 0.2 cm gap electroporation cuvettes (BioRad) and 2.45 kV electric field was applied by using a MicroPulser (BioRad). After electric discharge, cells were immediately supplemented with 900 µl ice-cold SM17MC medium for phenotypic expression, as previously described (Gerber and Solioz, 2007). Finally, cells were plated on SR agar (Gerber and Solioz, 2007) supplemented with 5 µg/mL erythromycin and incubated for 48 h at 30°C.

Protein expression in *L. lactis* and preparation of protein extracts

Recombinant *L. lactis* cells were grown overnight at 30°C in 100 ml GM17 medium without shaking. Biomass and culture broths were separated by centrifugation (3005 x g, 20', 4°C). Where necessary, acellular supernatants were concentrated by Vivaspin 20 ultrafiltration devices (Sartorius Stedim Biotech) with 10 or 30 KDa cut off polyethersulfone (PES) membrane. For preparation of total cellular proteins, biomass was re-suspended in 10 ml of 50 mM Tris-HCl pH 7.3, 1 mM EDTA and disrupted by ultrasonic treatment as previously described (Pessione et al., 2005). Crude extracts were clarified by centrifugation (3005 x g, 20', 4°C).

Cellulose binding assay

L. lactis acellular supernatants (100 ml) or total cellular extracts (10 ml) were incubated 1 h at 25°C with 0.8 g of crystalline cellulose (Sigmacell, Sigma), then centrifuged (3000 x g, 10',

4°C). Pellets were washed twice with 50 mM potassium phosphate buffer pH 6.0, then re-suspended in 750 µl of SDS-PAGE loading buffer (Laemmli, 1970). Samples were boiled 10 minutes, centrifuged (16000 x g, 5', 4°C) twice in order to remove any residual cellulose, and analyzed by SDS-PAGE in 10% polyacrilamide gels by using the Mini-Protean 3 Cell (Bio-Rad). Gels were stained with R-250 Coomassie Brilliant Blue. Cellulose-bound proteins were quantified by the 2D quant kit (GE Healthcare) following the manufacturer's instruction and using Bovine Serum Albumin (BSA) as the standard.

Enzyme activity assays

β-glycosidase activity was determined by using 0.9 mM p-nitrophenyl-b-D-glucopyranoside (pNPG) as the substrate and reading the absorbance of liberated p-nitrophenol at 410 nm as previously described (Kosugi et al., 2006). One unit of enzyme activity was defined as the amount of enzyme releasing 1 µmol of p-nitrophenyl/min. Endo 1-4 β glucanase activity was determined by the Azo-Carboxymethylcellulose (Azo-CMC) kit (Megazyme) following the manufacturer's instructions.

CMC-hydrolysis tests

Recombinant *L. lactis* cells were plated on GM17 medium containing 0.2% (w/v) CMC and incubated at 30°C for 72 h to enable microbial growth and enzyme expression. CMCase activity was detected by using 0.1% (w/v) Congo Red (Sigma-Aldrich) as described by Teather and Wood (1982).

Glucose and cellooligosaccharide fermentation tests

L. lactis strains were grown overnight at 30°C in GM17 medium until they reached the late exponential phase. These cultures were used to inoculate (initial OD_{600nm} = 0.1) 200 µl of fresh GM17 medium or M17 medium supplemented with: 0.5 % (w/v) cellooligosaccharide (degree of polymerization, DP, comprised between 2 and 10 as reported in Supplemental Fig. S1) mixture (OM17) or 0.1 % (w/v) glucose plus 0.4% (w/v) "pure" oligosaccharides as the sole carbohydrate sources. The DP2-10 cellooligosaccharide mixture and pure cellopentaose (97.5 % DP5, 2.5%

DP4), cellohexaose (85.3 % DP6, 14.7 % DP5), celloheptaose (80 % DP7, 20 % DP6) and a mixture enriched in cellooctaose and cellononaose (50 % DP8, 11% DP9, 35% DP7, 4% DP6) were obtained from Elicityl (France). No pure cellooctaose, cellononaose or cellodecaose was commercially available.

For each growth condition, three independent replicates were performed. Bacterial growth was monitored by measuring OD_{600nm} with a Nanovue UV-Vis microspectrophotometer (GE Healthcare). Growth rates were calculated from the logarithmic portion of each growth curve and were reported as the mean value \pm the standard deviation (SD) between the replicates. All along the bacterial growth, samples were harvested for LA and residual sugar determination in the growth medium. For this purpose, aliquots of the culture broth were harvested and centrifuged (16000 x g, 5', room temperature) and the supernatants were stored at -24°C until further analysis.

Analytic procedures

D- and L-LA quantification in the growth medium of recombinant *L. lactis* was performed by the K-D-LATE kit (Megazyme) according to the manufacturer's instructions.

For total soluble sugar quantification, acellular supernatants of *L. lactis* cultures were firstly incubated 1 h at room temperature with 1 volume of 67 % (v/v) H₂SO₄ to hydrolyze cellodextrins to glucose (Updegraff, 1969) followed by quantification by the phenol-sulfuric acid method (Dubois et al., 1956). Total sugar amount was determined by measuring absorbance at 490 nm and using glucose (0-200 μ g) as the standard.

Residual cellooligosaccharide amounts in the growth medium of *L. lactis* were determined by Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC). TLC was performed on silica gel-coated plates (10*20 cm, Merck Millipore) as previously described (Morag et al., 1993). HPLC analyses were performed by using an Agilent 1200 series instrument equipped with an Aminex HPX-42A column (Biorad), a UV-visible detector (Agilent series 1200) and a refractive index detector (Agilent series 1200) as previously described (Liu et al., 2010).

Statistical analyses

Experimental data (specific growth rate, final biomass, LA production) were analyzed by means of the Student's t-test. Data were considered as significantly different when $p < 0.05$.

Results

Cloning and expression of *bglA* and *engD* in *L. lactis*

The full length genes encoding BglA and EngD, including sequences coding for their original signal peptides, were cloned in the *E. coli-L. lactis* pMG36ea Δ shuttle vector either separately, thus obtaining the pB and pD vector, respectively, or as assembled in the artificial *bglA-engD* operon, thus obtaining the pBD vector. Plasmids pMG36ea Δ , pB, pD and pBD were transformed into *L. lactis* IL1403 thus obtaining strains *L. lactis* pMG36ea Δ , *L. lactis* pB, *L. lactis* pD and *L. lactis* pBD, respectively.

Extracellular β -glycosidase activity of strains pMG36ea Δ (empty vector) and pD was negligible (Table I). Values measured for *L. lactis* pB and pBD were about 0.2 and 1.2 U/L, respectively, indicating that BglA was secreted in a catalytically active form in these strains (Table I). Since *bglA* and *engD* were expressed under the control of the same promoter and RBS in all recombinant strains obtained in this study, we expected a similar level of β -glycosidase activity in strains pB and pBD. Two possible hypotheses could explain higher β -glycosidase activity in *L. lactis* pBD: i) a sort of synergism between BglA and EngD; ii) an effect of stabilization of *bglA* mRNA when the corresponding gene is inserted in the *bglA-engD* operon leading to higher biosynthesis of BglA. To test the first hypothesis, we measured β -glycosidase activity in a sample obtained by mixing the extracellular fractions of strains pB and pD (Table I). These analyses clearly showed that synergism between BglA and EngD is a minor phenomenon that can be quantified as 1.32 with respect to the sum of the β -glycosidase activity of the extracellular extracts containing only BglA (*i.e.*, *L. lactis* pB) or EngD (*i.e.*, *L. lactis* pD). Increased β -glycosidase activity of strain

pBD seems mostly the result of improved BglA biosynthesis probably derived by enhanced stability of *bglA* mRNA when inserted in an operonic structure (Table I).

To detect if EngD was biosynthesized by recombinant *L. lactis*, we exploited its ability to bind crystalline cellulose through its carbohydrate binding module (Bianchetti et al., 2013). Both extracellular and cellular extracts of engineered *L. lactis* strains were incubated with crystalline cellulose and the bound protein fraction was analyzed by SDS-PAGE (Fig. 1). These analyses showed that EngD is biosynthesized and efficiently secreted by both *L. lactis* pD and pBD. No EngD could be detected in cellular protein extracts. Similar EngD amounts (0.9-1 µg/mg dry weight biomass) were measured in the culture supernatants of strains pD and pBD, as also confirmed by extracellular endoglucanase activity assays (Table I). Endoglucanase activity in the strain harboring the empty vector (Table I) was negligible.

Carboxymethylcellulose (CMC) hydrolysis assay

The ability of engineered *L. lactis* strains to hydrolyze cellulose was tested by plating them on M17 medium supplemented with CMC. CMC hydrolysis halos were detected around *L. lactis* pD and pBD colonies, whereas no CMCase activity was detected for *L. lactis* pMG36eaΔ and pB (Fig. 2).

Growth parameters of recombinant *L. lactis* strains on glucose

Specific growth rate (μ) and final biomass of *L. lactis* pMG36eaΔ, pB, pD and pBD grown in GM17 medium (*i.e.*, containing glucose as the sole carbohydrate source) were determined (Fig. 3). Final biomass was the same for every strain, while a slight reduction in maximal μ was observed for *L. lactis* pD (27%) and pBD (16%) with respect to *L. lactis* pMG36eaΔ. These data indicate that expression of BglA does not significantly affect *L. lactis* growth, while EngD has very moderate negative effect on the μ only.

Cellooligosaccharide mixture fermentation

The ability of *L. lactis* pB, pD and pBD to grow on cellooligosaccharides was tested by comparison with the control strain *L. lactis* pMG36eaΔ. Cultures were performed in M17 medium

containing a cellooligosaccharide (with degree of polymerization, DP, from 2 to 10) mixture (OM17) as the sole carbohydrate source (Fig. 3). μ and final biomass of *L. lactis* pMG36ea Δ on OM17 were 44% and 40% lower than those observed for the same strain in GM17, respectively. μ of all the other recombinant *L. lactis* did not significantly differ from that of *L. lactis* pMG36ea Δ . Also final biomass of *L. lactis* pB and pD on OM17 were similar to that of *L. lactis* pMG36ea Δ in the same medium. However, *L. lactis* pBD grew at significantly higher biomass (*i.e.*, about 40% more than *L. lactis* pMG36ea Δ) which indicates that it was able to metabolize a higher amount of cellooligosaccharides of the mixture.

Cellooligosaccharide consumption in *L. lactis* pMG36ea Δ and pBD cultures was determined by TLC and HPLC (Fig. 4C, D, E, F). Resolution of oligosaccharides longer than cellopentaose was not possible by TLC, while HPLC could separate up to celloheptaose. Cellooctaose, cellononaose and cellodecaose were not separated even by HPLC, however, their relative amount in the mixture was minor (~ 5 %) (Supplemental Fig. S1 and S2). Both analyses showed that *L. lactis* pMG36ea Δ could metabolize only cellooligosaccharides up to cellotetraose (DP4) while cellodextrins with DP \geq 5 were not consumed (Fig. 4C, E). Furthermore, HPLC analyses indicated that *L. lactis* pMG36ea Δ metabolizes cellobiose faster than cellotriose and much faster than cellotetraose. Four hours after inoculum, 49% of cellobiose was consumed, while only 34% and 7% of cellotriose and cellotetraose, respectively, had been metabolized (Fig. 4E). *L. lactis* pBD metabolized almost the whole cellooligosaccharide mixture, at least up to celloheptaose (Fig. 4D, F). Interestingly, also *L. lactis* pBD seems to metabolize cellotetraose slower than the other oligosaccharides of the mixture. Four hours after inoculum, celloheptaose was depleted and 80%, 68%, 61% and 56% of cellohexaose, cellopentaose, cellobiose and cellotriose were consumed, respectively, while only 36% of DP4 was metabolized. This result indicates that DP4 was metabolized slower and/or transiently accumulated as a result of concomitant hydrolysis of longer cellodextrins by *L. lactis* pBD. The concomitant occurrence of both phenomena may be hypothesized since, as previously mentioned, slower DP4 consumption is also observed in *L. lactis* pMG36ea Δ (which does not

display any extracellular β -glucan hydrolyzing activity). However, at the end of the bacterial growth all detected cellodextrins were mostly exhausted by *L. lactis* pBD (*i.e.*, ~ 97%).

Both *L. lactis* pMG36ea Δ and pBD accumulated only L-LA (2.02 ± 0.35 g/L and 3.42 ± 0.56 g/L, respectively) in the OM17 medium, while D-LA was not detectable (Fig. 4A, B). If we take into account that about 0.3 g/L of LA was accumulated by both strains when grown in M17 medium not supplemented with any sugar (data non shown), *L. lactis* pBD produced about 81 % more LA than *L. lactis* pMG36ea Δ during growth on oligosaccharides. Based on total oligosaccharide consumption (*i.e.*, 3.50 ± 0.26 g/L), metabolized oligosaccharides were converted to L-LA with a yield of 0.89 ± 0.19 g/g by *L. lactis* pBD, that is close to the maximum theoretical yield.

“Single” cellooligosaccharide metabolism tests

Since separation of cellodextrins longer than cellopentaose was poor and those with DP > 7 were not resolved from the DP2-10 mixture even by HPLC (see Supplemental Fig. S2), to confirm the ability of *L. lactis* pBD to ferment cellooligosaccharides longer than cellotetraose cultures enriched in cellopentaose (DP5) or cellohexaose (DP6) or celloheptaose (DP7) or cellooctaose-cellononaose (DP8-9) were performed. Actually, almost pure DP5, DP6 and DP7 were used, while only a mixture containing 50 % DP8 and 11 % DP9 was commercially available (see Materials and Methods). Pure cellodecaose was not purchasable. Cultures of *L. lactis* pBD were performed in M17 medium containing 0.1 % (w/v) glucose plus 0.4 % (w/v) of single “pure” cellooligosaccharide or of the DP8-9 enriched mixture as the sole carbohydrate sources. Glucose was used to promote rapid growth of *L. lactis* pBD and increased biosynthesis of BglA and EngD. Control cultures of *L. lactis* pMG36ea Δ in the same conditions were parallelly performed (Fig. 5).

In cultures supplemented with DP6, DP7 and DP8-9, final biomass and L-LA produced by *L. lactis* pMG36ea Δ were similar to those of cultures of the same strain containing 0.1% (w/v) glucose only (data not shown) (Fig. 5). This confirmed that cellodextrins longer than DP5 were not metabolized by this strain. However, in DP5-supplemented cultures, *L. lactis* pMG36ea Δ produced

significantly higher biomass (+ 65%) and L-LA amounts (+ 65%) indicating that, in these conditions, at least a part of DP5 was fermented, contrarily to what observed in cultures on the DP2-10 mixture. In every condition tested *L. lactis* pBD grew at a higher final biomass (*i.e.*, in DP5, DP6 and DP8-9 supplemented cultures) and/or biosynthesized higher L-LA (*i.e.*, in DP6, DP7 and DP8-9 supplemented cultures) than *L. lactis* pMG36ea Δ (Fig. 5). These data confirmed that *L. lactis* pBD has improved metabolization features on cellodextrins with DP>4 with respect to the control strain, and, notably, only *L. lactis* pBD can metabolize cellodextrins with DP>5. Since DP9 was present in low amount in the DP8-9 mixture, it is not possible to definitively confirm that *L. lactis* pBD was able to consume it. However, DP6, DP7 and DP8 were at least partially metabolized by *L. lactis* pBD. It is worth noting that, in cultures supplemented with DP5, DP6, DP7 and DP8-9 mixtures biomass of *L. lactis* pBD shows a trend to progressively decrease, while L-LA production does not (Fig. 5). Previous studies have reported different carbon distribution over metabolic products/biomass formation for microorganisms growing on different oligomers/polymers of glucose (Desvaux, 2006). This may depend on the different kinetics and/or bioenergetics of uptake/metabolism of different-length oligosaccharides (Desvaux, 2006). As mentioned above, DP4 seems uptaken/metabolized slower than DP3/DP2 by *L. lactis* pBD (see Fig. 4E, F and previous section). In addition, substrate specificity of BglA and EngD may play a role in the divergent metabolism shown by *L. lactis* pBD on the different cellodextrins. Based on total sugar consumption (Supplemental Table SIII), more than 85 % of metabolized sugars were converted in L-LA by *L. lactis* pBD grown in DP5 or DP6 enriched mixtures. Dissolution of cellodextrins with DP>6 was clearly incomplete (*i.e.*, precipitates were observable), which likely led to underestimation of sugar concentration in these cultures (Supplemental Table SIII). For this reason, an estimation of sugar/L-LA conversion yield in the latter conditions was not possible.

Discussion

In the present study, the genes encoding two extracellular glycosyl hydrolases, *i.e.* the β -glucan glucohydrolase BglA and the endoglucanase D (EngD), of *C. cellulovorans* were cloned in the homofermenting *L. lactis* IL1403. BglA catalyzes hydrolysis of cellobiose, cellotriose and cellotetraose (Kosugi et al., 2006). EngD is active on both cellulose and cellooligosaccharides with a DP comprised between 4 and 6 glucose units, while cellobiose and cellotriose are not depolymerized (Bianchetti et al., 2013; Foong and Doi, 1992). Here, full length *bglA* and *engD* genes (including the sequence encoding their original N-terminal peptides) were cloned under the control of a constitutive promoter. Both gene products were secreted in a catalytically active form by *L. lactis* confirming that peptide sequences for protein export are generally conserved in different gram-positive bacteria (Cho et al., 2000). To date, several yeasts and bacteria have been engineered with heterologous proteins involved in cellulose hydrolysis, including a dozen of LAB (Mazzoli, 2012; Mazzoli et al., 2014). In several cases inducible promoters were used to delay protein biosynthesis in a suitable growth phase so as to avoid or reduce toxic effects on cells (Mingardon et al., 2011; Morais et al., 2014; Wieczorek and Martin, 2010). However, use of these inducers at the industrial fermentation scale is not economically viable. Therefore, the present study represent a step forward towards the development of strains suitable for low-cost cellulosic biomass biorefinery.

Furthermore, *L. lactis* pBD secreting a mini-cellulase system composed by both BglA and EngD was engineered in this study. Although depolymerization of most recalcitrant cellulosic substrates rely on synergistic enzyme activities, co-expression of multiple proteins in a heterologous host is extremely challenging since most cellulases are toxic (Mingardon et al., 2011; Morais et al., 2014). To circumvent this bottleneck, development of artificial syntrophic consortia (consisting of recombinant strains biosynthesizing different single cellulase-system components) has been performed at the laboratory scale (Arai et al., 2007; Morais et al., 2014; Tsai et al., 2010). However, implementation at the industrial process scale seems more complicated. Therefore, construction of

single strains secreting multiple cellulase-systems is an essential goal yet. As far as we know, this is the first study reporting the expression of multiple β -glucan hydrolases in a single LAB strain.

Efficient metabolization of cellooligosaccharides is a key element for optimal fermentation of cellulose (Galazka et al., 2010; Lane et al., 2015; Lian et al., 2014; Wei et al., 2014). In native microorganisms, cellodextrins generated by extracellular cellulases either: i) are further saccharified in the extracellular environment leading to glucose which is transported into the cell cytoplasm or; ii) enter the cells through cellodextrin transporters and are either hydrolyzed to glucose by cellodextrinases or cleaved into glucose-1-phosphate units by cellodextrin phosphorylases (Desvaux, 2006). The recombinant *L. lactis* pBD engineered in this study was able to efficiently metabolize cellooligosaccharides up to at least cellooctaose in L-LA. As far as we know, this is the first LAB that can directly ferment cellodextrins longer than cellohexaose. Natural LAB can metabolize a large spectrum of mono- and di-saccharides, including cellobiose (Mazzoli et al., 2014), while only one native *Lactobacillus plantarum* strain able to metabolize cellotriose has been described so far (Okano et al., 2010b). By traditional mutagenesis, a *Lactobacillus delbrueckii* was obtained which could convert cellotriose to L-LA with a yield of 0.85 g/g (Adsul et al., 2007). Later, a *L. plantarum* was engineered with the CelA endoglucanase from *Clostridium thermocellum* which was able to grow on cellooligosaccharides up to cellohexaose (Okano et al., 2010b). However, the main metabolic product of this strain was acetic acid while LA yield on cellohexaose was lower than 0.07 g/g. Furthermore, this strain showed lower metabolization rate on cellopentaose and was unable to grow on cellotetraose (Okano et al., 2010b). Higher LA yields through fermentation of cellohexaose by the recombinant *L. plantarum* were obtained only in anaerobic conditions (Okano et al., 2010b). As reported above, *L. lactis* pBD obtained in this study was able to metabolize all the cellooligosaccharides at least up to DP8 converting them in optically pure L-LA with a yield of 0.85-0.89 g/g of consumed sugars.

Surprisingly, our study showed that even *L. lactis* harboring pMG36ea Δ (empty vector) was able to at least partially metabolize cellooligosaccharides up to DP5 although negligible β -

glycosidase activity was detected in its extracellular extract. These data suggest that protein transporters should be present in parent *L. lactis* cells allowing the uptake of short cellodextrins into the cytoplasm. Six genes encoding potentially β -glucosidases or 6-P- β -glucosidases are annotated in the genome of *L. lactis* IL1403 which could catalyze intracellular metabolism of cellodextrins while no gene coding for cellodextrin phosphorylase is annotated in this strain (Bolotin et al., 2001). Different protein systems (*i.e.*, ATP binding cassette-transporters, phosphotransferases and cellodextrin/H⁺ symporters) are employed for cellodextrin uptake by different bacteria (Nataf et al., 2009; Tian et al., 2009). In *L. lactis* IL1403, cellobiose uptake occurs through an inducible PEP-phosphotransferase system comprising proteins CelB-PtcA-PtcB (Aleksandrak-Piekarczyk et al., 2011; Kowalczyk et al., 2008). This system displays a quite broad substrate specificity since it recognizes also β -gentiobiose, arbutin, salicilin and lactose (Aleksandrak-Piekarczyk et al., 2011). It is tempting to hypothesize that the same transporter could catalyze uptake of other cellodextrins with DP<6, as for other transporters described in the literature (Galazka et al., 2010). Direct confirmation of this hypothesis could be obtained by testing growth of *L. lactis* deleted for these genes on celloligosaccharides. However, this aspect was out the scope of the present study.

A previous research demonstrated that recombinant *S. cerevisiae* secreting a β -glucosidase (*i.e.*, Bgl1 from *Saccharomycopsis fibuligera*) and EngD from *C. cellulovorans* could directly ferment CMC and β -glucan to ethanol (Jeon et al., 2009). In the near future, the ability of *L. lactis* pBD to ferment more complex (hemi-)cellulosic substrates such as CMC, or phosphoric acid swollen cellulose will be tested to better assess its potential for CBP of cellulosic biomass.

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Figure legends

Fig. 1. SDS-PAGE of cellulose-bound proteins. Assays were performed on the total cellular (C) and extracellular (E) fractions of *L. lactis* pMG36ea Δ , *L. lactis* pD and *L. lactis* pBD. Arrows indicate protein bands corresponding to EngD ($M_R = 56$ kDa).

Fig. 2. Carboxymethylcellulase (CMCase) activity assays. Analyses performed on *L. lactis* pB (A), *L. lactis* pD (B) and *L. lactis* pBD (C) are shown. Arrows indicate clearing halos corresponding to CMCase activity.

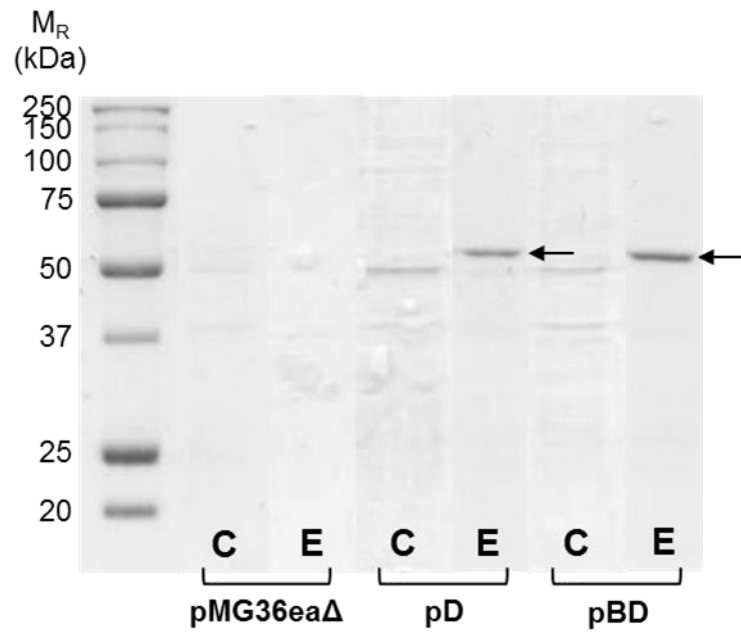
Fig. 3. Growth parameters of recombinant *L. lactis* strains obtained in this study. Strains were grown in M17 medium containing 0.5% glucose (GM17) or 0.5% celloligosaccharide (DP2-DP10) mixture (OM17) as the sole carbohydrate source. Data are expressed as the mean of three replicates \pm standard deviation (SD). Symbols *, †, and # refer, respectively to: *, specific growth rates which significantly differ from the one of *L. lactis* pMG36ea Δ grown on GM17 ($p < 0.05$); †, final biomasses which significantly differ from that observed for *L. lactis* pMG36ea Δ grown on GM17 ($p < 0.05$); #, final biomasses which significantly differ from that observed for *L. lactis* pMG36ea Δ grown on OM17 ($p < 0.05$).

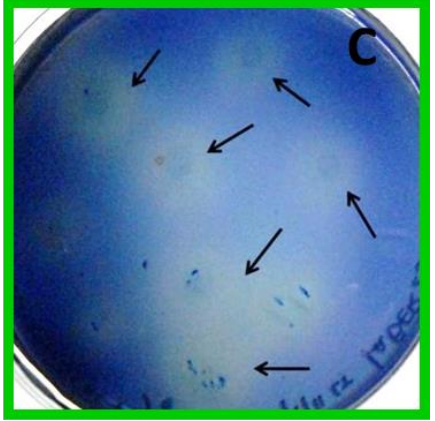
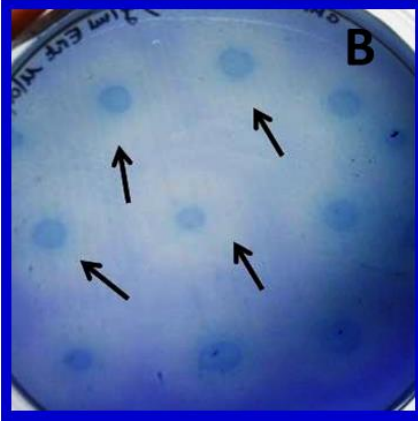
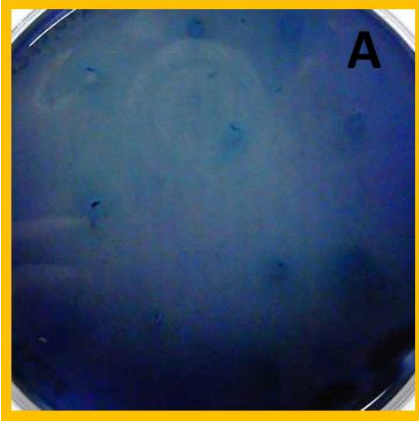
Fig. 4. Celloligosaccharide mixture fermentation tests. *L. lactis* pMG36ea Δ (A, C, E) and *L. lactis* pBD (B, D, F) were grown in M17 medium supplemented with 0.5 % w/v of a celloligosaccharide mixture (DP2-10) as the sole carbohydrate source. For each culture time-course determination of biomass (A, B, ●, solid line), extracellular LA (A, B, ▲, dashed line) and residual oligosaccharide concentration by both TLC (C, D) and HPLC (E, F) was performed. Figure resumes data from three independent cultures for each strain. L-LA (A, B) and residual oligosaccharide (E, F) concentration were expressed as the mean \pm SD.

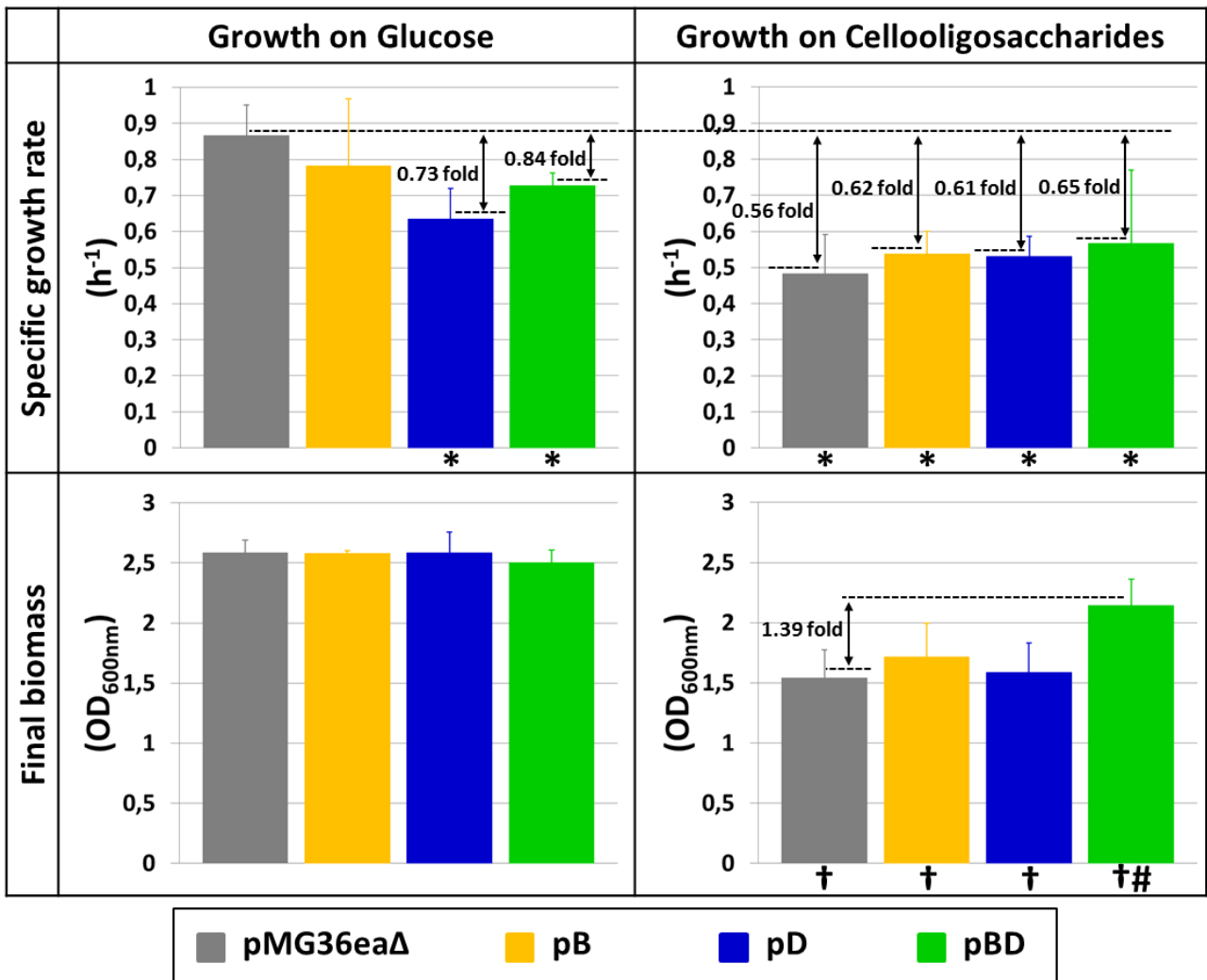
Fig. 5. Kinetics of growth (●, solid line) and extracellular LA production (▲, dashed line) of *L. lactis* pMG36eaΔ and *L. lactis* pBD in M17 medium enriched with cellopentaose (DP5), cellohexaose (DP6), cellopentaose (DP7) or a mixture containing cellooctaose and cellononaose (DP8-9). Graphs resume data from three independent cultures. L-LA concentrations are expressed as the mean ± SD.

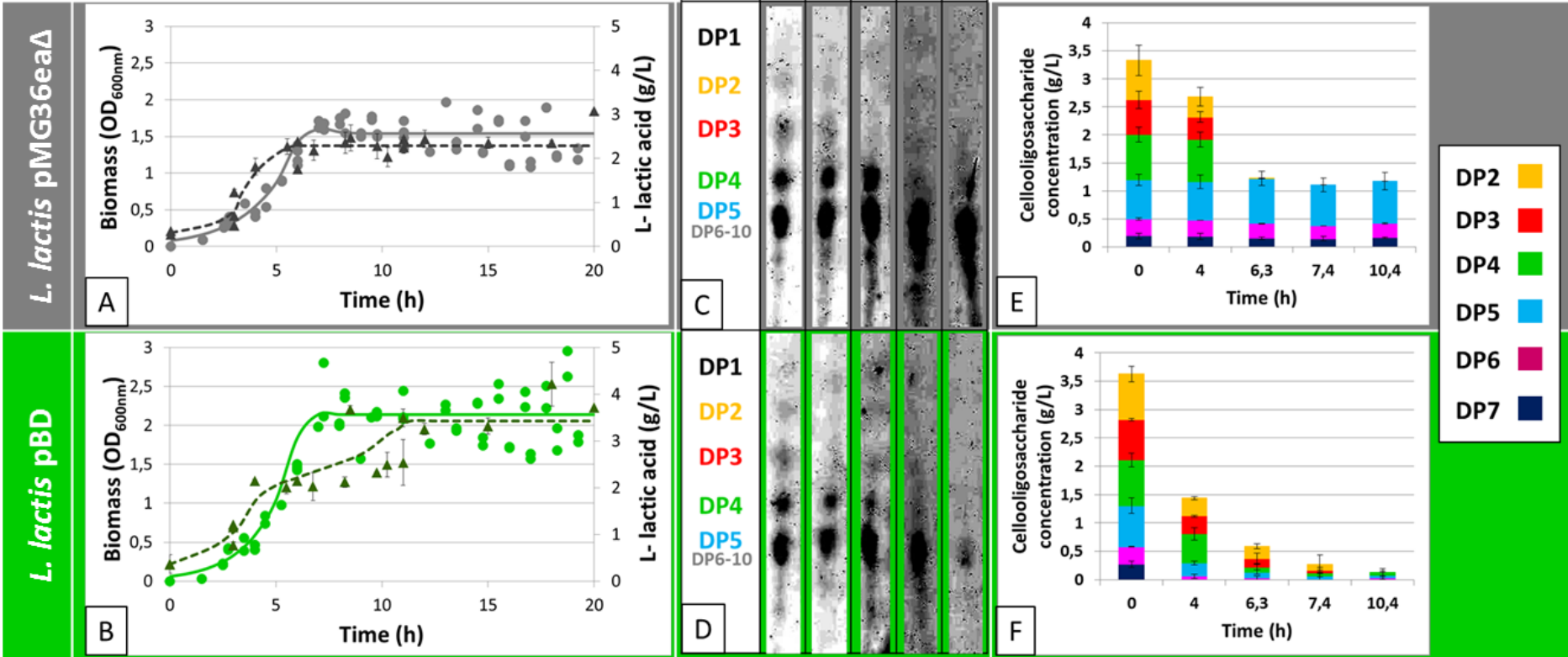
Table I: β -glycosidase and endoglucanase activity (mean of three replicates \pm standard deviation) measured in extracellular extracts of recombinant *L. lactis* strains obtained in this study. β -glycosidase and endoglucase activity were measured by using p-nitrophenyl-b-D-glucopyranoside (pNGP) and Azo-Carboxymethylcellulose (Azo-CMC) as the substrate, respectively. *L. lactis* pB-pD co-culture corresponds to a co-culture of strains *L. lactis* pB plus *L. lactis* pD. *L. lactis* pB plus pD is a sample obtained by mixing the supernatants of separate cultures of strains *L. lactis* pB and *L. lactis* pD. n.d., not determined.

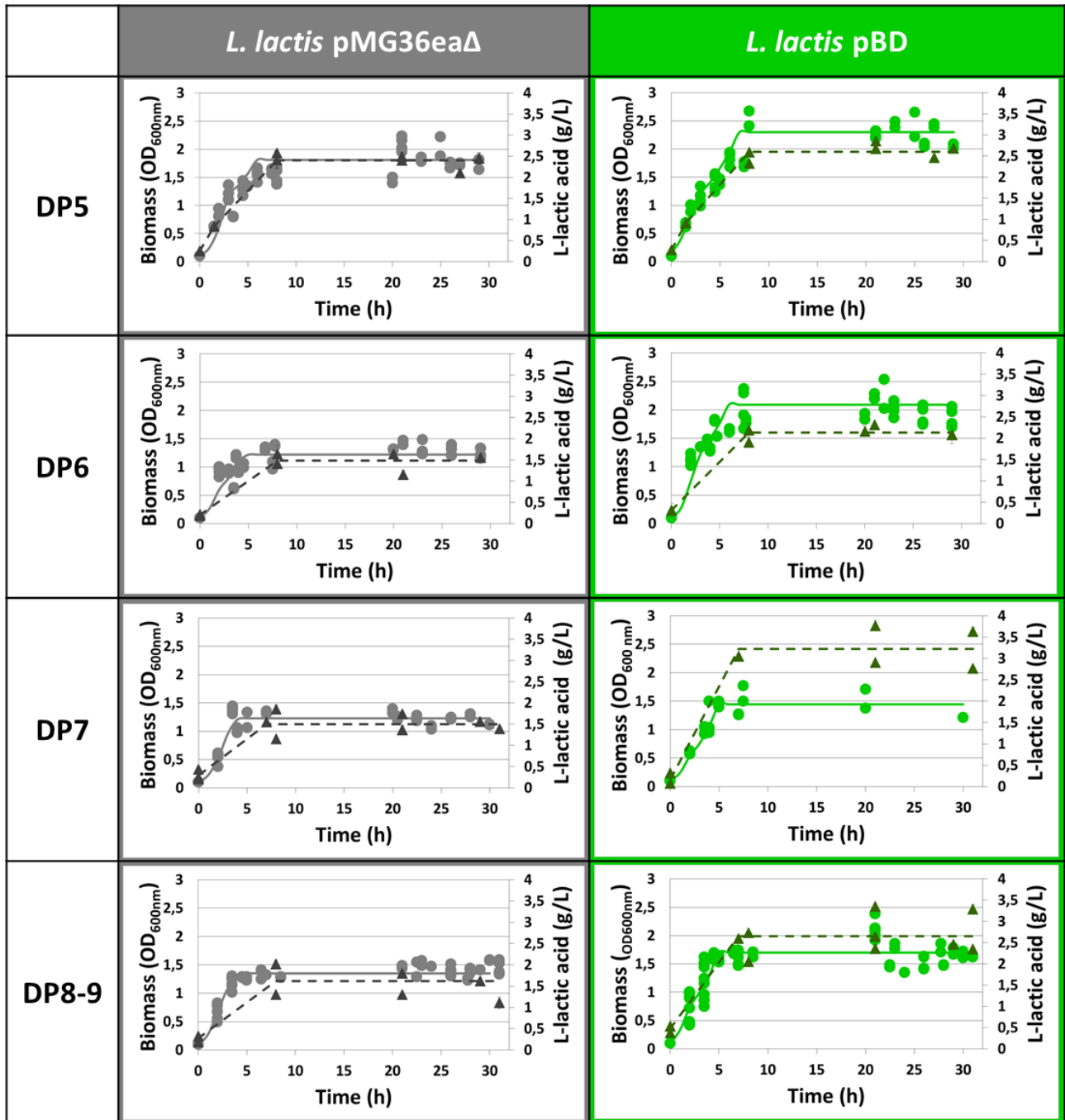
Strain	β -glycosidase activity		Endoglucanase activity	
	U/L	U/g biomass	U/L	U/g biomass
<i>L. lactis</i> pMG36ea Δ	0.035 \pm 0.032	0.045 \pm 0.041	2 \pm 3	2.5 \pm 3.8
<i>L. lactis</i> pB	0.191 \pm 0.033	0.244 \pm 0.042	n.d.	n.d.
<i>L. lactis</i> pD	0.048 \pm 0.025	0.061 \pm 0.032	155 \pm 17	197.3 \pm 25.3
<i>L. lactis</i> pBD	1.220 \pm 0.100	1.605 \pm 0.149	157 \pm 2	206.6 \pm 9.3
<i>L. lactis</i> pB plus pD	0.316 \pm 0.028	0.279 \pm 0.069	n.d.	n.d.











Supplementary online material

Table SI. Strains and plasmids used in this study.

Genotype /Description		Source
Strain		
<i>Clostridium cellulovorans</i> 743B		DSMZ
<i>Escherichia coli</i> TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara-leu</i>) 7697 <i>galU galK rpsL</i> (Str ^R) <i>endA1 nupG</i> λ-	Invitrogen
<i>Lactococcus lactis</i> IL1403		(Redon et al., 2005)
Plasmid		
pUC19	Amp ^r	Invitrogen
pMG36e	Ery ^r ; P32::RBS _{P32}	(Van de Guchte et al., 1989)
pMG36eΔ	Ery ^r ; P32::RBS _{P32} Δ1390-1423	This study
pMG36eaΔ	Ery ^r ; Amp ^r ; P32::RBS _{P32} Δ1390-1423	This study
pB	Ery ^r ; Amp ^r ; P32::RBS _{P32} Δ1390-1423:: <i>bglA</i>	This study
pD	Ery ^r ; Amp ^r ; P32::RBS _{P32} Δ1390-1423:: <i>engD</i>	This study
pBD	Ery ^r ; Amp ^r ; P32::RBS _{P32} Δ1390-1423:: <i>bglA</i> ::RBS _{P32} :: <i>engD</i>	This study

Table SII. Primers used in this study. Restriction sites are indicated in italics.

Primer name		Sequence	
ΔATG-D	5'	TTTTTT <i>GAGCTCG</i> CCCCGGGGATCGATC	3'
ΔATG-R	5'	TTTTTT <i>GAGCTCTTCAA</i> AATTCCTCCGAATATTTTTTTAC	3'
amp-D	5'	TTTTTT <i>GCTAGCAC</i> CCCTATTTGTTTATTTTTCTAAATAC	3'
amp-R	5'	TTTTTT <i>GCTAGCGTCTG</i> ACGCTCAGTGGAAACGAAAAC	3'
bglA-D	5'	TTTTTT <i>GAGCTCAT</i> GGAAAAGCTAAGATTTCCCAAAG	3'
bglA-R	5'	TTTTTT <i>TCTAGATTACT</i> TATTAGATCTTTCTATAAGCTCC	3'
engD-D	5'	TTTTTT <i>GAGCTCAT</i> GATTA AACATCTATTATCACGGGG	3'
engD-R	5'	TTTTTT <i>GCATGCTAT</i> TTTACTGTGCATTCAGTACCATTC	3'
engD-RBS	5'	TTTTTT <i>TCTAGAGGTAG</i> GTAAAAAATATTCGGAGGAATTTGAAATGATTA AACATCTATTATCACGGGG	3'

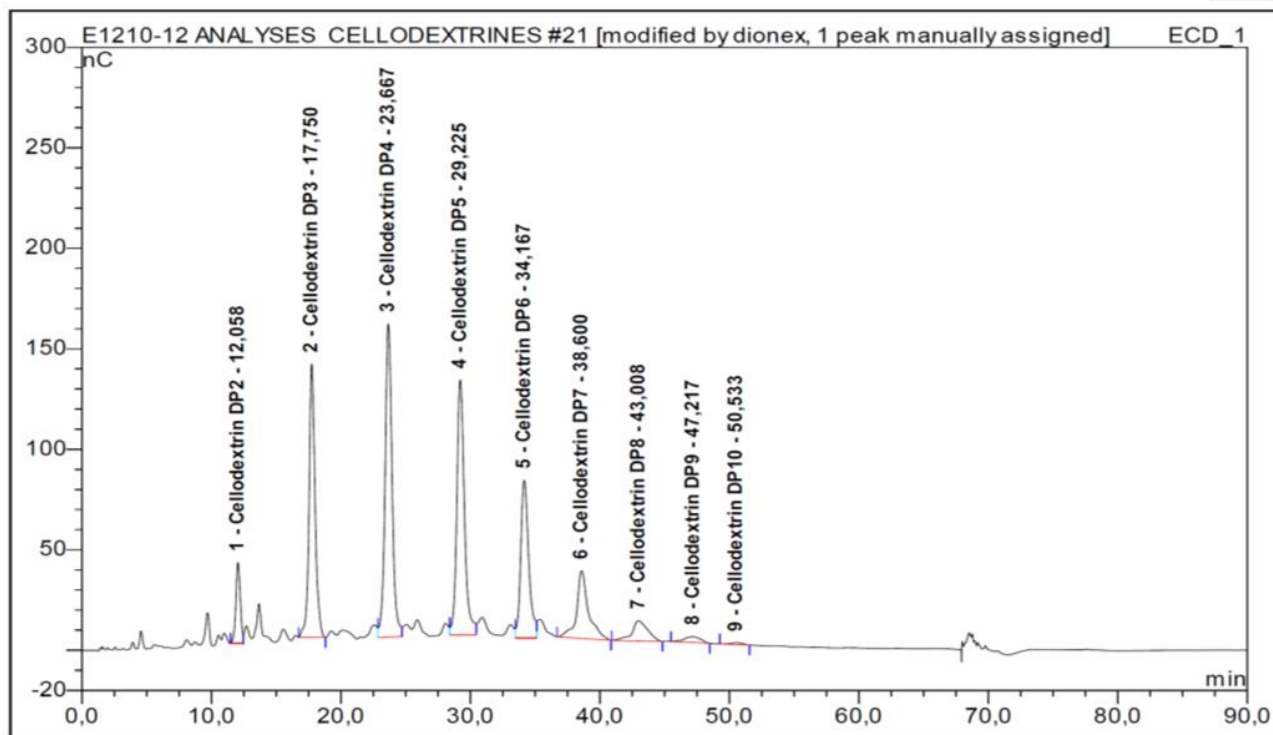
Table SIII. Sugar consumption and L-LA production of *L. lactis* pMG36ea Δ and pBD strains grown in M17 medium containing 0.1 % w/v glucose plus 0.4 % w/v of single “pure” cellopentaose (DP5), or cellohexaose (DP6) or celloheptaose (DP7) or of a cellooctaose-cellononaose (DP8-9) mixture as the sole carbohydrate sources. Total sugars were determined by the phenol-sulphuric acid method. L-LA was determined by the K-D-LATE kit (Megazyme). Values are the mean of three independent cultures \pm SD. n.d., not determined.

Cellodextrin	<i>L. lactis</i> strain	Initial total sugar concentration \pm SD (g/L)	Final total sugar concentration \pm SD (g/L)	Consumed sugar \pm SD (g/L)	Initial L-LA concentration \pm SD (g/L)	Final L-LA concentration \pm SD (g/L)	L-LA produced \pm SD (g/L)
DP5	pMG36ea Δ	4.98 \pm 0.74	2.88 \pm 0.29	2.09 \pm 0.79	0.25 \pm 0.05	2.41 \pm 0.16	2.15 \pm 0.17
	pBD	4.55 \pm 0.16	3.21 \pm 0.34	1.35 \pm 0.37	0.28 \pm 0.06	2.60 \pm 0.19	2.32 \pm 0.20
DP6	pMG36ea Δ	4.75 \pm 0.24	n.d	n.d	0.20 \pm 0.02	1.48 \pm 0.20	1.28 \pm 0.21
	pBD	4.50 \pm 0.69	2.74 \pm 0.10	1.76 \pm 0.70	0.31 \pm 0.01	2.12 \pm 0.14	1.81 \pm 0.14
DP7	pMG36ea Δ	2.71 \pm 0.33	n.d	n.d	0.30 \pm 0.08	1.50 \pm 0.22	1.20 \pm 0.24
	pBD	2.82 \pm 0.14	2.05 \pm 0.33	0.77 \pm 0.36	0.16 \pm 0.15	3.23 \pm 0.45	3.07 \pm 0.47
DP8-9	pMG36ea Δ	3.11 \pm 0.34	n.d	n.d	0.27 \pm 0.08	1.68 \pm 0.28	1.41 \pm 0.29
	pBD	3.00 \pm 0.15	2.43 \pm 0.27	0.58 \pm 0.31	0.47 \pm 0.09	2.65 \pm 0.43	2.17 \pm 0.44

Figure S1

Cromatographic Trace:

A



Integration Table:

B

No.	Ret.Time min	Peak Name	Height nC	Area nC*min	Rel.Area %	Amount mg/L	Type
1	12,06	Cellodextrin DP2	40,133	15,955	4,16	2418,332	BM *
2	17,75	Cellodextrin DP3	136,002	73,072	19,05	9810,006	BMB
3	23,67	Cellodextrin DP4	155,780	97,010	25,29	15023,853	M *
4	29,23	Cellodextrin DP5	126,928	86,362	22,51	14258,927	M *
5	34,17	Cellodextrin DP6	78,346	56,214	14,66	11641,977	M *
6	38,60	Cellodextrin DP7	33,639	35,636	9,29	15021,708	BMB
7	43,01	Cellodextrin DP8	10,038	14,108	3,68	n.a.	BMB [^]
8	47,22	Cellodextrin DP9	2,910	4,230	1,10	n.a.	BMB
9	50,53	Cellodextrin DP10	0,931	0,995	0,26	n.a.	BMB*
Total:			584,706	383,582	100,00	68174,804	

Fig. S1. HPLC analysis of the cellooligosaccharide mixture (DP2-10) used in this study as performed by the mixture supplier (Elicityl, France). A, chromatogram, B integration table. n.a., not available.

Figure S2

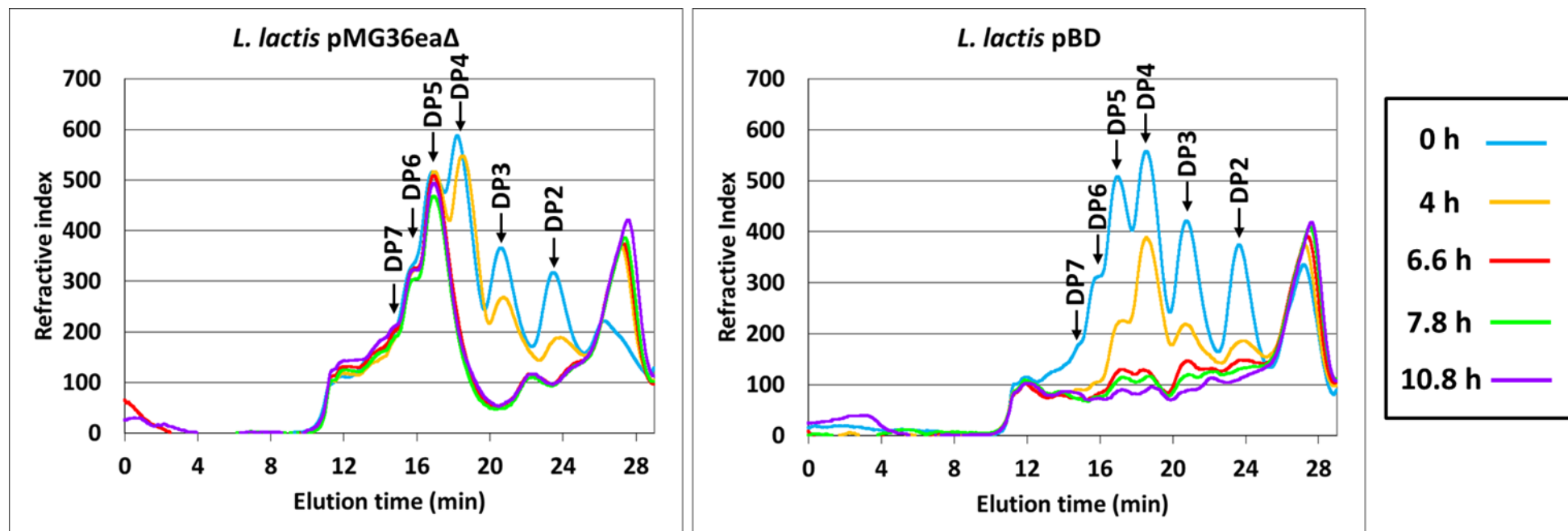


Fig. S2. HPLC analysis of residual cellooligosaccharides present in the growth medium of *L. lactis* pMG36eaΔ (left) and *L. lactis* pBD (right) grown in OM17. For each strain, chromatograms of samples harvested at 0 (light blue), 4 (yellow), 6.6 (red), 7.8 (green) and 10.8 (violet) h after inoculum were superimposed. Elution peaks corresponding to cellobiose (DP2), cellotriose (DP3), cellotetraose (DP4), cellopentaose (DP5), cellohexaose (DP6) and celloheptaose (DP7) are indicated by arrows.