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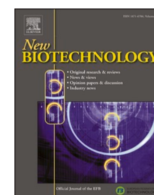

Full length Article

In vivo evolution of lactic acid hyper-tolerant *Clostridium thermocellum*

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A B S T R A C T

Lactic acid (LA) has several applications in the food, cosmetics and pharmaceutical industries, as well as in the production of biodegradable plastic polymers, namely polylactides. Industrial production of LA is essentially based on microbial fermentation. Recent reports have shown the potential of the cellulolytic bacterium *Clostridium thermocellum* for direct LA production from inexpensive lignocellulosic biomass. However, *C. thermocellum* is highly sensitive to acids and does not grow at pH < 6.0. Improvement of LA tolerance of this microorganism is pivotal for its application in cost-efficient production of LA. In the present study, the LA tolerance of *C. thermocellum* strains LL345 (wild-type fermentation profile) and LL1111 (high LA yield) was increased by adaptive laboratory evolution. At large inoculum size (10 %), the maximum tolerated LA concentration of strain LL1111 was more than doubled, from 15 g/L to 35 g/L, while subcultures evolved from LL345 showed 50–85 % faster growth in medium containing 45 g/L LA. Gene mutations (pyruvate phosphate dikinase, histidine protein kinase/phosphorylase) possibly affecting carbohydrate and/or phosphate metabolism have been detected in most LA-adapted populations. Although improvement of LA tolerance may sometimes also enable higher LA production in microorganisms, *C. thermocellum* LA-adapted cultures showed a yield of LA, and generally of other organic acids, similar to or lower than parental strains. Based on its improved LA tolerance and LA titer similar to its parent strain (LL1111), mixed adapted culture LL1630 showed the highest performing phenotype and could serve as a framework for improving LA production by further metabolic engineering.

Introduction

The global market for lactic acid (LA) is rapidly expanding [1]. Apart from traditional applications in the food industry (e.g. as preservative or acidifier) and the production of cosmetics and pharmaceuticals, LA is used for the synthesis of biodegradable plastic polyesters, namely polylactides (PLAs). Contamination of almost every ecosystem by traditional (i.e. non-biodegradable) plastics is among the main current environmental threats [2]. Potentially, PLAs could replace oil-derived non-biodegradable polymers as general purpose plastics, but their current cost is too high and mainly depends on LA price [3]. Industrial production of LA is largely based on fermentation of expensive food crops, such as corn [3,4]. Both ethical and economic motivations have stimulated research on alternative feedstocks for LA fermentation, with significant attention on lignocellulose [5,6]. Since microorganisms that naturally produce high amounts of LA, such as lactic acid bacteria (LAB), several bacilli and fungi belonging to *Rhizopus sp.*, cannot directly ferment lignocellulose, metabolic engineering has been used to develop strains combining both lignocellulose fermentation and efficient LA production. Almost all the studies reported so far have attempted to express minimal cellulase systems in LAB with only moderate success owing to the high complexity of these enzyme systems [5]. However, improving LA production in native cellulolytic microorganisms is a promising alternative strategy because (i) gene tools are now available for several microbial models such as *Clostridium thermocellum*, *C. cellulolyticum*, *C. cellulovorans* and *Caldicellulosiruptor bescii,* and (ii) these strategies should not face hurdles linked to the expression of heterologous cellulases [6].

Recently, recombinant strains of *C. thermocellum,* one of the most efficient cellulose degraders isolated so far, showing improved

Abbreviations: F1,6BP, fructose 1,6-bisphosphate; HPrK/P, histidine protein kinase/phosphorylase; LA, lactic acid; LAB, lactic acid bacteria; LDH, lactate dehydrogenase; PEP, phosphoenolpyruvate; PLA, polylactide; PPDK, pyruvate phosphate dikinase.

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production of LA have been obtained through either deletion of *adhE* encoding its main alcohol/aldehyde dehydrogenase [7] or overexpression of the gene encoding the native lactate dehydrogenase (LDH, Clo1313_1160) [8]. In particular, the Δ*adhE* strain showed the highest LA yield reported in *C. thermocellum* so far, namely 0.78 mol/mol glucose equivalent. In this strain, LA is the main fermentation product, with a yield of about 40 % of the theoretical maximum [7]. Although further improvement of LA production is necessary to meet industrial process requirements, these results confirm the potential of *C. thermocellum* towards production of cellulosic LA. However, improving LA production efficiency may not be sufficient for industrial application of anaerobic cellulolytic bacteria, since they generally are very acid-sensitive [9,10]. A recent study reported that *C. thermocellum* growth is severely limited at pH values around 6.0 [10]. To date, there have been no reports describing LA tolerance in cellulolytic clostridia. Although some strong LA producers, such as LAB, can tolerate acidic pH as low as 3.2, growth inhibition by acidic pH and organic acid accumulation is also common in these strains and causes decreased LA productivity [1,11]. Acidic extracellular pH causes dissipation of the proton gradient across the cytoplasmic membrane and protonation of weak acids, such as LA, thus increasing their passive diffusion into the cell. Since the cytoplasmic pH is more alkaline, weak acids can then dissociate and further collapse the ΔpH [10]. The decrease of intracellular pH leads to several types of cell damage such as enzyme denaturation, alteration of nutrient uptake, cytoplasmic membrane damage, depurination and depyrimidination of DNA, and dissipation of amino acid pools [12].

Limited tolerance of microbial strains to acidic pH and/or LA has traditionally been circumvented by process engineering strategies, such as the use of neutralizing agents, or systems for continuous removal of LA, e.g. electrodialysis, solvent extraction, adsorption and membrane bioreactors [11,13]. However, these strategies increase the complexity and cost of the whole process [13,14]. Hence, developing microbial strains with improved acidic pH/LA tolerance is among the key strategies for reducing the cost of fermentative LA production. Improvement of acid tolerance of microorganisms has been pursued through different strategies that include evolutionary engineering and rational metabolic engineering [10,15]. Studies aimed at improving LA tolerance have mainly been performed on strong natural LA producers such as LAB and were based on rational metabolic engineering [16–18]. These studies focused on overexpression of genes which are up-regulated upon acid exposure such as molecular chaperones [19] and DNA repair proteins [19]. An adaptive evolution approach has been recently used to increase LA tolerance of *Leuconostoc mesenteroides* [12]. Evolved *L. mesenteroides* strains showed increased growth rate in media containing LA concentrations ranging between 30−70 g/L. Furthermore, improved LA tolerance also corresponded to 2-fold higher LA titer of up to 76.8 g/L. A combination of random chemical mutagenesis and *in vivo* evolution has recently allowed for increased acidic pH tolerance of the anaerobic cellulolytic bacterium *Fibrobacter succinogenes*, although to a limited extent in which the pH limit for growth was lowered only from 6.10 to 5.65 [15]. Similar enhancement of acid tolerance of *C. cellulovorans* was obtained by adaptive evolution together with rational metabolic engineering [20].

In the present study, tolerance of *C. thermocelllum* DSM1313 to two main organic acids, LA and acetic acid, was determined. Both the parent strain LL345 and LL1111 (Δ*adhE*, LA overproducing strain) were investigated and an evolutionary engineering approach was used to improve their LA tolerance. *C. thermocellum* strains were progressively adapted to increasing LA concentration up to 37.5 g/L for LL1111 and 47.5 g/L for LL345. Resequencing of the genomes of LA adapted strains revealed interesting mutations which are the result of possible convergent evolution and that may affect carbohydrate and/or phosphate metabolism.

Materials and methods

Bacterial strains and culture conditions

All reagents used in this study were of molecular grade, and obtained either from Sigma Aldrich (St. Louis, MO, USA) or Thermo-Fisher Scientific (Waltham, MA, USA), unless otherwise stated. The strains employed in this study are listed in Table 1. *C. thermocellum* DSM1313 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). Strain LL345 has a deletion at the *hpt* locus to allow for counter-selection with 8-azahypoxanthine (8AZH), but exhibits a wild type fermentation phenotype. LL345 is the ancestor of all other strains described in this work (including LL1111). The construction of LL345 is described elsewhere [21]. LL1111 was previously engineered by deleting the native *adhE* gene, that encodes the main bifunctional alcohol/-aldehyde dehydrogenase [7]. In addition to eliminating ethanol production, this strain exhibits a LA hyperproducing phenotype. The LA hyperproduction phenotype is due, in part, to a mutation that eliminates fructose 1,6-bisphosphate (F1,6BP) regulation of LDH activity [7].

Strains were grown in chemically defined MTC-5 medium at an initial pH of 7.4 [22] supplemented with 5 g/L cellobiose as the main carbon source. Cultures were incubated at 55 °C under anaerobic conditions either in conical tubes in an anaerobic chamber (Coy Laboratory Products, Grass Lakes, MI, USA) or in 125 mL (50 mL working volume) butyl stoppered vials. Bacterial growth was monitored by measuring absorbance at 600 nm (OD_{600}). When required, culture samples (1 mL) were harvested for measurement of substrate consumption and fermentation products.

For measurement of growth parameters, strains were grown in a 96-well plate in 200 μL of MTC-5 medium and OD_{600} was determined every 3 min for 72 h in a Powerwave XS plate reader (Agilent Bio Tek, Santa Clara, CA, USA) as previously described [23]. Either 1 or 10 % (v/v) of inoculum were used, as specified below.

Data for substrate consumption/fermentation products and growth parameters are averages from biological triplicate experiments.

Adaptation of C. thermocellum to LA

Adaptation procedure was used for both *C. thermocellum* strains LL345 and LL1111. Quadruplicate serial transfers in 10 mL tubes containing 6 mL of MTC-5 medium supplemented with 5 g/L cellobiose were performed in anaerobic conditions (anaerobic chamber, Coy Laboratory Products). Sodium lactate was added to each tube to a final concentration in the range 0–47.5 g/L. Sodium lactate was used instead of LA to avoid medium acidification. Each transfer was 10 % volume (0.6 mL). Serial transfer to obtain LA tolerant mutants consisted of inoculation into medium with increasing lactate concentration alternated with medium with lower (10−15 g/L added lactate) or no selective pressure, according to [24]. At the end of the adaptation procedure, each whole final subculture, consisting of a mixed population of strains with

Table 1
C. thermocellum strains and LA-adapted mixed cultures used in this study. *, mixed adapted cultures.

Strain/ subculture	Description	SRA accession	Reference
LL345	LL1004 Δ*hpt*	SRP053786	[21]
LL1004	DSM 1313 *C. thermocellum*, wild type	SRP077312	DSMZ
LL1111	LL345 Δ*adhE*	SRP049310	[7]
LL1628	LL1111 grown with added LA*	SRP192458	this work
LL1629	LL345 grown with added LA*	SRP192462	this work
LL1630	LL1111 grown with added LA*	SRP192463	this work
LL1631	LL1111 grown with added LA*	SRP192464	this work
LL1632	LL345 grown with added LA*	SRP192460	this work
LL1633	LL345 grown with added LA*	SRP192459	this work
LL1634	LL345 grown with added LA*	SRP192461	this work

improved LA tolerance, was saved and further characterized. For each mixed population, genomic DNA was re-sequenced, and growth rate, LA tolerance and fermentation profile were determined.

Analytical techniques

Cellobiose, glucose, acetate, citrate, formate, ethanol, lactate, malate, pyruvate and succinate were measured by HPLC using an Aminex HPX-87H column (BioRad, CA, USA) equipped with both refractive index and UV detector as previously reported [25].

Genome re-sequencing

Whole-genome resequencing of LA adapted subcultures was performed to detect possible mutations. Analyses were performed by the Department of Energy Joint Genome Institute. Raw genome resequencing data are available from the NCBI Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/Traces/sra) (Table 1). Data were analyzed with the CLC Genomic Workbench version 11.0.1, (Qiagen Inc., Hilden, Germany), as previously described [25].

Protein sequence alignments

Alignment of protein sequences was performed by Geneious version 8.1 (Biomatters) (http://www.geneious.com).

Results and discussion

LA titer and productivity of C. thermocellum strain LL1111 with increasing amounts of cellobiose

C. thermocellum DSM1313 (LL345) naturally produces a very low yield of LA of 0.01 mol/mol hexose equivalent, while its main catabolites are acetate, ethanol and a mixture of other compounds, i.e. formic acid, H_2 and CO_2 [7]. However, deletion of the bifunctional alcohol/-aldehyde dehydrogenase AdhE in strain LL1111 eliminated ethanol production and increased LA yield to 0.78 mol/mol hexose equivalent, or 40 % of the maximum theoretical value [7]. LL1111 is the *C. thermocellum* strain with the highest LA yield obtained to date. The second most abundant product of LL1111 is acetate. Small scale (50 mL working volume, in butyl stoppered vials) fermentation assays were performed on LL1111 to determine the maximum LA titer and productivity. It was grown in chemically defined medium (MTC-5) supplemented with different initial concentrations of cellobiose (5, 10, 20, 50 g/L) (Fig. 1, Supplementary Fig. 1). Cultures supplemented with 10 g/L cellobiose displayed the highest growth efficiency, both as maximum specific growth rate and maximum biomass, and the highest final titers of both LA (6.45 g/L) and acetic acid (1.87 g/L). Maximum volumetric productivities of LA (0.20−0.24 g/L/h) and acetic acid (0.07−0.10 g/L/h) were similar in all the conditions tested (Fig. 1, Supplementary Fig. 1). The pH of the medium (initial pH 7.3) was progressively acidified while the bacteria were growing and accumulating organic acids, then its value slightly increased as growth and metabolism stopped (Supplementary Fig. 1). The highest acidification was observed in cultures with 10 g/L cellobiose substrate, which also correlated with the highest levels of LA and acetic acid production. An initial concentration of cellobiose >10 g/L resulted in less efficient growth and metabolism (Fig. 1). In cultures supplemented with 5 or 10 g/L cellobiose, the substrate was depleted at the end of growth, while about 20 % and 75 % residual substrate was measured in cultures containing 20 and 50 g/L of cellobiose, respectively. Accordingly, increasing amounts of glucose were accumulated in the latter conditions.

It can be concluded that *C. thermocellum* growth was likely stopped by cellobiose exhaustion in cultures supplemented with 5 or 10 g/L cellobiose, while other factors limited growth in media supplemented with 20 or 50 g/L cellobiose. It is worth noting that minimum pH values measured in cultures supplemented with 10, 20 and 50 g/L cellobiose are very similar (5.9–6.0). Recently, it was reported that growth of *C. thermocellum* DSM1313 is limited at pH < 6.24 [10] and this is consistent with the acid-sensitivity of other cellulolytic clostridia [26]. Growth assays were performed to measure *C. thermocellum* LL1111 tolerance to acetic acid and LA and test if accumulation of these products may have contributed to growth arrest.

C. thermocellum strain LL1111 tolerance to acetic acid and LA

LL1111 was inoculated (1% v/v) into MTC-5 medium containing 5 g/L cellobiose and increasing concentrations of acetic acid (up to 20 g/L) or LA (up to 30 g/L) (Fig. 2a, b). For acetate, no decrease in maximum biomass and only a slight reduction of maximum specific growth rate of up to about 20 % was observed, up to a concentration of 10 g/L (Fig. 2b). Even at 20 g/L acetate, maximum specific growth rate and maximum biomass were about 60 % and 75 % of those measured in control conditions. LA had a stronger inhibitory effect since no growth was observed for initial LA concentrations of 20 g/L or higher. At 15 g/L LA, the

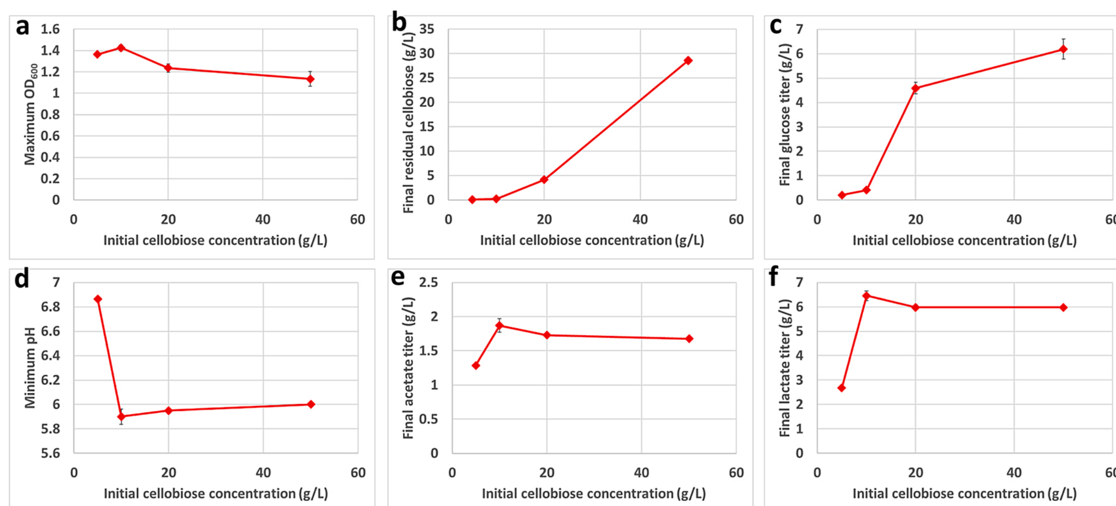


Fig. 1. Fermentation parameters of *C. thermocellum* LL1111 grown in chemically defined MTC-5 medium supplemented with different amounts of cellobiose as the main carbon source: a) maximum OD_{600}; b) final residual cellobiose; c) final glucose titer; d) minimum pH of the growth medium; e) final acetate titer; f) final LA titer. Data are the averages from biological triplicate experiments. Error bars represent one standard deviation.

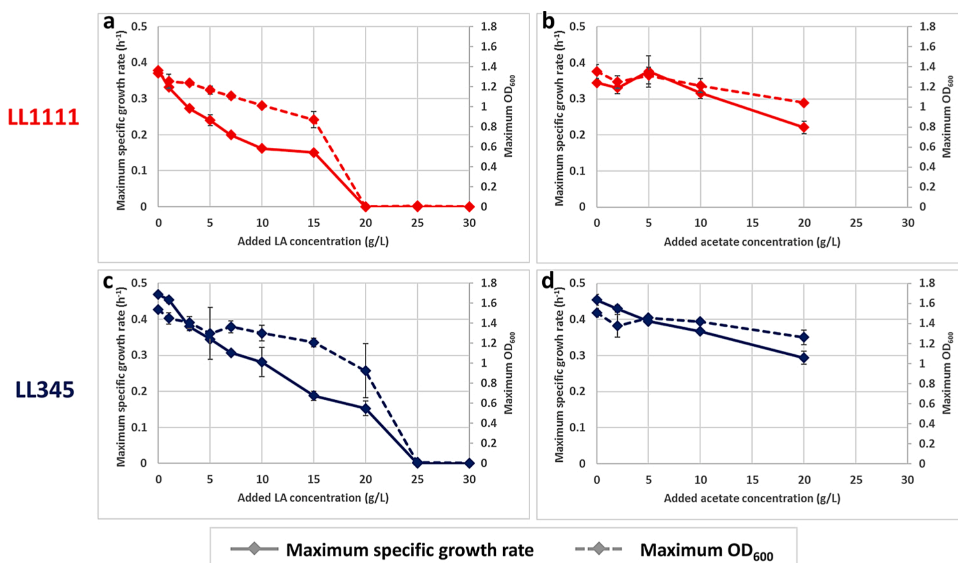


Fig. 2. Maximum specific growth rate (μ_{max}) (solid lines) and maximum absorbance at 600 nm (OD_{600}) (dashed lines) of *C. thermocellum* LL1111 (red, a, b) and LL345 (blue, c, d) grown in MTC-5 medium supplemented with increasing amounts of sodium lactate (LA) (a, c) or sodium acetate (b, d). Addition of sodium salts instead of acids was used in order to avoid medium acidification. For each culture, 1% (v/v) inoculum was used. Data are the averages from three biological replicates. Error bars represent one standard deviation.

maximum growth rate and biomass were about 38 % and 57 % respectively of those measured in control conditions. Based on these results, final titers of acetic acid and LA produced in *C. thermocellum* cultures supplemented with 20 or 50 g/L cellobiose (Fig. 1) should not be able *per se* to stop *C. thermocellum* LL1111 growth. However, simultaneous production of acetic acid and LA in these cultures might have had additive effects which were most probably further enhanced by the parallel decrease of the growth medium pH [10].

Organic acid tolerance of native cellulolytic microorganisms has not been well studied. If compared with the most efficient producers of LA, such as some LAB, *C. thermocellum* shows significantly lower tolerance to acidic pH (LAB able to tolerate pH 3.2 have been reported) and LA [1]. Natural LAB able to produce up to 192 g/L LA in batch fermentation have been described [27–29]. Low LA tolerance limits both LA titer and productivity [11]. It is therefore evident that application of *C. thermocellum* to plant biomass fermentation for LA requires significant improvement of its acid tolerance for this process to be competitive with more established microorganisms. In the present study, adaptive evolution was used to increase *C. thermocellum* tolerance to LA.

In vivo evolution of C. thermocellum LA hyper-tolerant strains

To explore the adaptive landscape more broadly, adaptive evolution was applied to both LL1111 (LA hyperproduction phenotype) and LL345 (wild-type fermentation phenotype) strains. LL345 showed a slightly higher tolerance to both LA and acetic acid than LL1111 (Fig. 2c, d). The maximum added lactate concentration that still allowed LL345 to grow was 20 g/L, and 20 g/L acetic acid caused only a 16 % reduction of maximum biomass. Each strain was inoculated into four independent cultures, which were sequentially transferred into growth media containing increasing amounts of sodium lactate, alternated with transfers into fresh medium without or with lower lactate concentration (Fig. 3) as previously described [24]. At the end of the LA-adaptation procedure, whole final subcultures, each consisting of a mixed population, were saved and characterized. An advantage in working with mixed populations is that genome sequencing enhances the signal from mutations that result from selective pressure (random mutations that are present in only a subset of the population are filtered out). When single colonies are isolated from a population, mutations arising from genetic drift become fixed and are difficult to distinguish from selective mutations, unless a large number of colonies are isolated and re-sequenced. On the other hand, working with mixed populations implies that resulting phenotypes (LA tolerance and fermentation products) cannot necessarily be attributed to a single mutation. This can be mitigated by using relatively large inocula (1%) for phenotype assays, and choosing a high cutoff threshold of >95 % for calling mutations. Through 32–33 transfers, or approximately 116 generations, subcultures derived from LL345 (LL1629, LL1632, LL1633, and LL1634) were adapted to grow with up to 40–47.5 g/L of LA. LL1111 grows slower that LL354, and through 29–32 transfers, or approximately 108 generations, mixed populations derived from it (LL1628, LL1630, and LL1631) were adapted to grow with 35–37.5 g/L of LA. After *in vivo* evolution, all the adapted subcultures were able to grow with at least two-fold higher concentration of LA than their parent strains. Genome re-sequencing indicated that one of the subcultures derived from LL1111 (LL1635) was contaminated. The latter population was then excluded from further characterization.

Growth characteristics of LA adapted C. thermocellum subcultures

To confirm adaptation of *C. thermocellum* subcultures to increased concentrations of LA, their tolerance to LA was tested as described above for parent strains LL1111 and LL345. Fig. 4 shows the maximum specific growth rate and maximum OD_{600} of cultures grown in MTC-5 medium containing LA amounts ranging from 0 to 50 g/L. LA adaptation mainly improved the growth rate of evolved populations: all showed significantly (*p-value* < 0.05) higher maximum specific growth rates than their parent strain, under all the conditions in which growth was observed, i.e. 0−25 g/L LA for LL1111 derived strains and 0−30 g/L LA for LL345 derived strains. Improvement of biomass production in LA-evolved populations was also observed in media containing LA, i.e. 15−25 g/L LA for LL1111 derived cultures and 20−30 g/L LA for LL345 derived cultures.

Although, during adaptive evolution, strains evolved from LL1111 were able to tolerate 35 g/L LA and those evolved from LL345 were able to tolerate 45 g/L LA, plate reader-based growth assays showed lower maximum LA tolerance of 25 g/L and 35 g/L respectively. It was suspected that this was due to differences in fermentation conditions. Repeating the analysis with a larger inoculum size, 10 % vs. 1%, resolved the discrepancy, confirming that strains derived from LL1111 and LL345 could grow with 35 g/L and 45 g/L LA, respectively (Fig. 5). As previously determined, LL1111 could not grow with 35 g/L LA but, unexpectedly, LL345 was able to grow with 45 g/L using this procedure (Fig. 5e) These results could depend both on reduction of lag phase time, owing to higher number of inoculated cells, and increased dilution of LA in the medium, owing to the larger volume of inoculum. However,

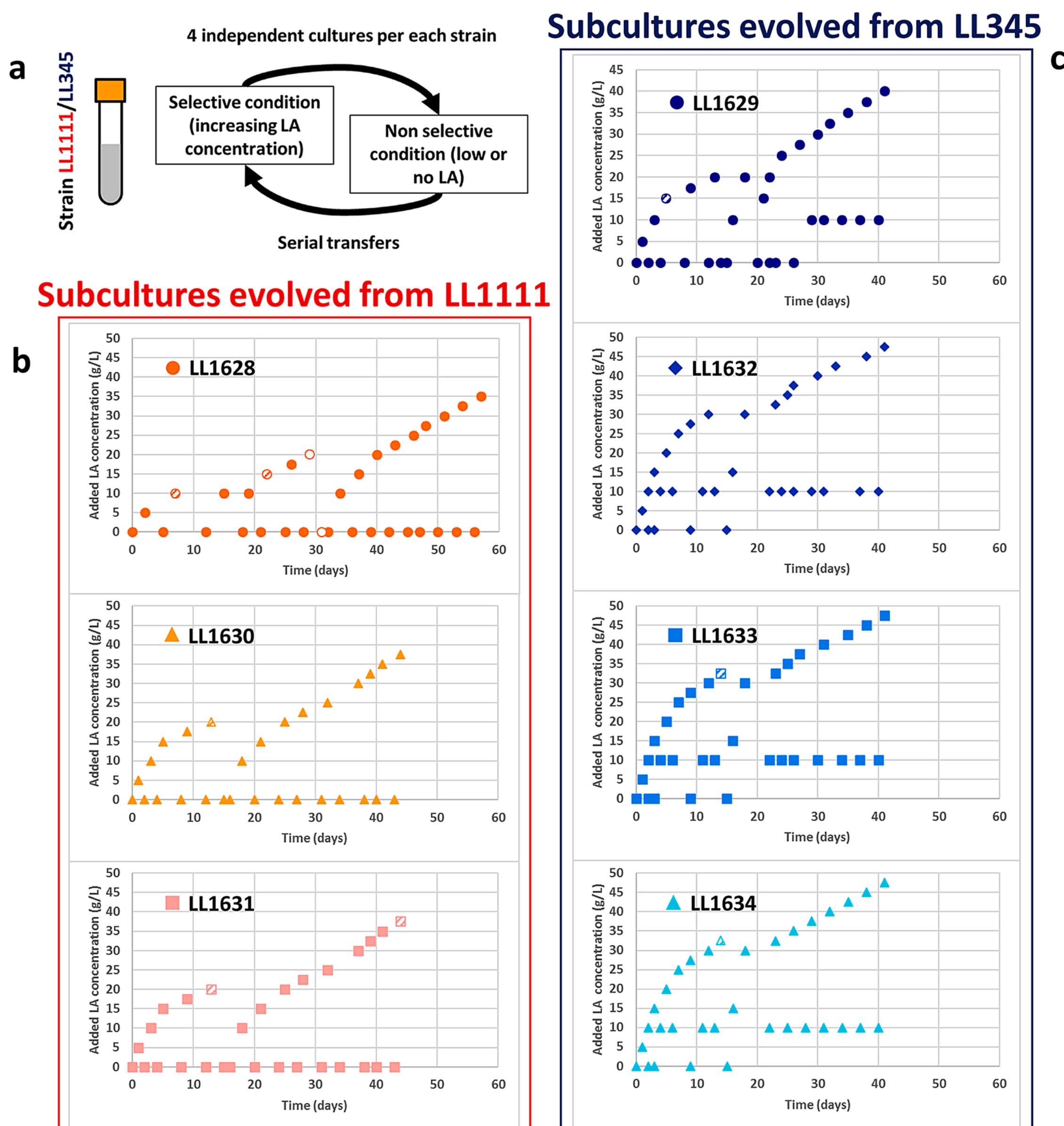


Fig. 3. Schemes representing the serial transfer strategy used (a) to adapt *C. thermocellum* strain LL1111 (b) and LL345 (c) to increasing concentration of sodium lactate (LA). Sodium lactate was used instead of LA in order to avoid medium acidification. As non selective condition, cultures with no added LA (LL1111) or supplemented with low (10-15 g/L) LA were used. In each transfer, 10 % (v/v) was used. As indicator of growth efficiency, the ratio (R) of final optical density at 600 nm (OD_{600}) over initial OD_{600} was indicated ($R > 4$, solid symbols; $2 < R < 4$, dashed symbols; $R < 2$, empty symbols). After this procedure, each whole final subculture was saved. These mixed populations were considered as mostly single derivatives. Subcultures LL1628, LL1630 and LL1631 were derived from LL1111, while subcultures LL1629, LL1632, LL1633 and LL1634 were derived from LL345.

LL345 growth in 45 g/L LA-supplemented medium was significantly slower compared to its LA adapted derivatives (Fig. 5e, f). The effects of LA adaptation on maximum biomass production were more heterogeneous. Subcultures LL1629 and LL1632 actually showed higher biomass production than LL345 in 45 g/L LA supplemented medium, whereas LL1632 and LL1633 showed lower biomass production than LL345 in control medium (no LA).

For mixed populations evolved from LL1111, their growth rate in 35 g/L LA supplemented medium was about 30–40 % of that shown by these populations in control medium (Fig. 5a, c). LA supplementation seems to have had less dramatic effects on biomass production of these derivative populations, at least for subculture LL1628 (Fig. 5b).

The adaptive evolution experiment was designed to select for strains with faster growth in the presence of added LA, and it was confirmed that this selection succeeded as intended. LA adaptation mainly increased the growth rate of *C. thermocellum* strains in LA supplemented media. A similar observation was reported also by another recent LA adaptation study on *L. mesenteroides* [12].

Re-sequencing of LA adapted strain genome

Genome sequencing of LA adapted *C. thermocellum* strains detected several mutations in each subpopulation (Supplementary Fig. 2). Attention was mainly focused on different mutations occurring in the same gene locus in different subcultures, which most probably resulted from convergent evolution under LA selective pressure (Fig. 6). Among these, the Clo1313_0949 locus encoding pyruvate phosphate dikinase (PPDK) shows a single nucleotide deletion in three different locations in three subcultures (LL1630, LL1632 and LL1633). In the population LL1630, a single base deletion is present just upstream of the *ppdk* start

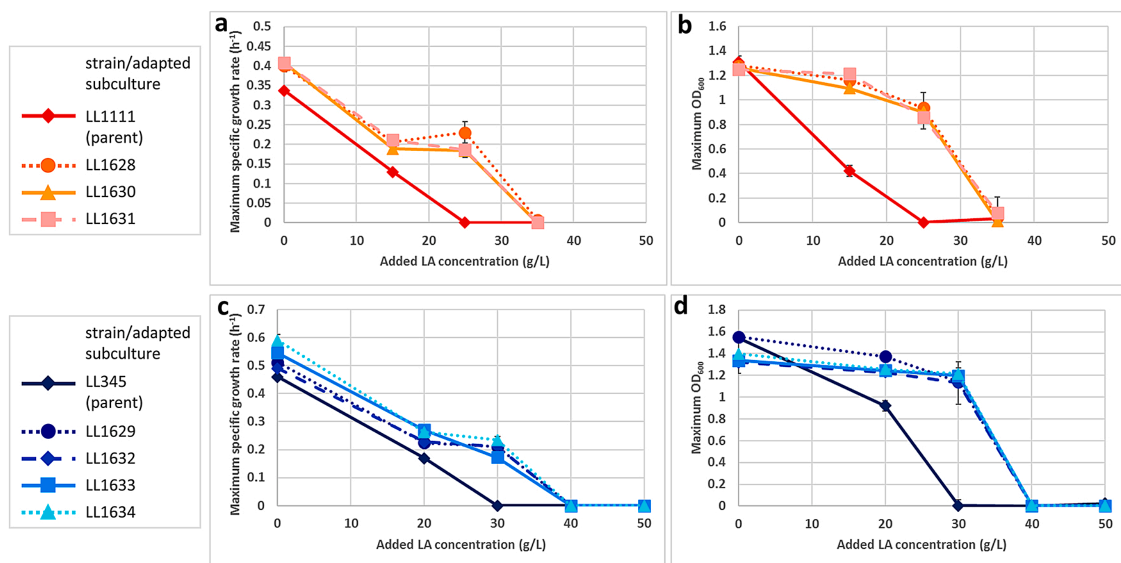
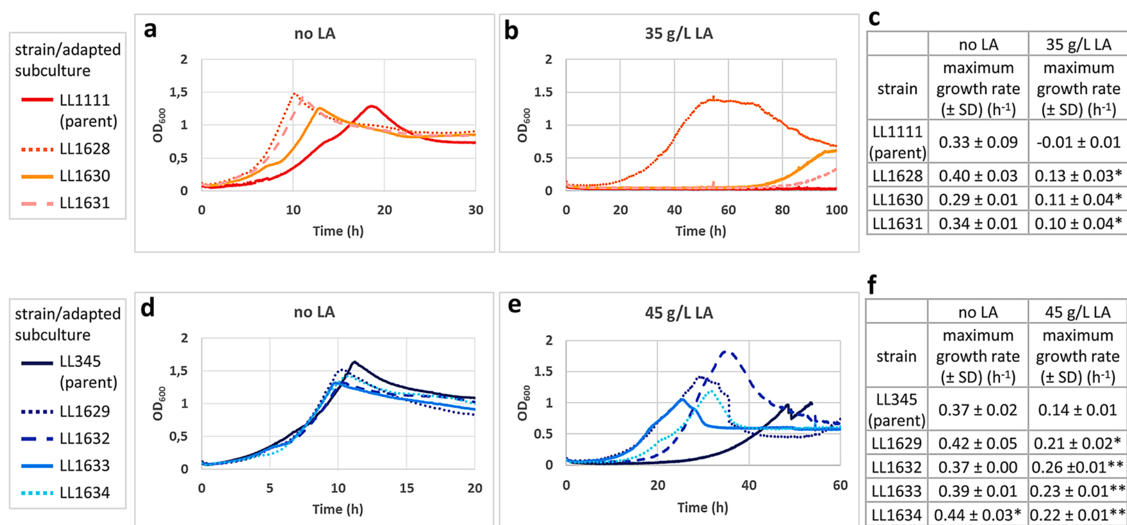


Fig. 4. Growth parameters of *C. thermocellum* LL1111 and mixed populations evolved from LL1111 (a, b) and LL345 and subcultures evolved from LL345 (c, d) grown in MTC-5 medium supplemented with increasing amounts of sodium lactate (LA). Sodium lactate was used instead of LA in order to avoid medium acidification. For each culture, 1 % (v/v) inoculum was used. Data are the averages from biological triplicate experiments. Error bars represent one standard deviation.



strain	no LA maximum growth rate (± SD) (h-1)	35 g/L LA maximum growth rate (± SD) (h-1)
LL1111 (parent)	0.33 ± 0.09	-0.01 ± 0.01
LL1628	0.40 ± 0.03	0.13 ± 0.03*
LL1630	0.29 ± 0.01	0.11 ± 0.04*
LL1631	0.34 ± 0.01	0.10 ± 0.04*

strain	no LA maximum growth rate (± SD) (h-1)	45 g/L LA maximum growth rate (± SD) (h-1)
LL345 (parent)	0.37 ± 0.02	0.14 ± 0.01
LL1629	0.42 ± 0.05	0.21 ± 0.02*
LL1632	0.37 ± 0.00	0.26 ±0.01**
LL1633	0.39 ± 0.01	0.23 ± 0.01**
LL1634	0.44 ± 0.03*	0.22 ± 0.01**

Fig. 5. Growth kinetics and maximum specific growth rate of *C. thermocellum* LL1111 and mixed populations evolved from LL1111 (a, b, c) and of strain LL345 and mixed populations evolved from LL345 (d, e, f) grown in MTC-5 medium supplemented with different amounts of sodium lactate (LA). Sodium lactate was used instead of LA in order to avoid medium acidification. For each culture, 10 % (v/v) inoculum was used. Data are the averages from biological triplicate experiments. Maximum growth rates labeled with asterisks (c, f) indicate values that are significantly higher than those measured on the parent strain grown in the same condition. * p-value < 0.05; ** p-value < 0.005.

codon, probably affecting the promoter region and *ppdk* transcription. In subcultures LL1632 and LL1633, deletions are present in the coding region which cause frameshift mutations leading to production of truncated forms of PPDK (Supplementary Fig. 3). PPDK catalyzes reversible reaction of phosphoenolpyruvate (PEP), AMP and PP_i to pyruvate, ATP and P_i [30]. *C. thermocellum* PPDK shows high amino acid sequence identity (68 %) with *C. symbiosum* PPDK, for which the crystal structure has been determined [30]. PPDK structure consists of three main domains, the N-terminal nucleotide-binding module, the C-terminal PEP/pyruvate-binding domain and a central domain containing a conserved phospho-carrier histidine residue that shuttles phosphoryl groups between C- and N-terminal domains (Supplementary Fig. 3). Based on protein sequence alignment, the deletions present in the *ppdk* locus of subcultures LL1632 and LL1633 should result in truncation of significant portions of the PEP/pyruvate-binding domain (including key amino acids for catalysis), probably eliminating function (Supplementary Fig. 3).

In most organisms, pyruvate kinase performs the conversion of PEP to pyruvate. *C. thermocellum* does not have this enzyme, and instead uses either PPDK or the "malate shunt", a 3-enzyme pathway with oxaloacetate and malate as intermediates [31]. Under some conditions, PPDK carries almost 70 % of glycolytic flux [31]. Although deletion of *ppdk* does not result in a large shift in the distribution of fermentation products, a slight increase of glucose accumulation and decrease of LA production have been reported [32]. In addition, large differences in ^{13}C labeling patterns were observed in Δ*ppdk C. thermocellum*, not all of which are fully understood, but that may affect intracellular NADH/NADPH pools [31].

Another gene locus that shows different mutations in two mixed evolved populations is Clo1313_2121, encoding histidine protein

Start Region[1]	Description	Type	Annotation name	Locus Tag	Locus description	Read fraction[2] LL345 parent				Read fraction[2] LL1111 parent		
						LL1629	LL1632	LL1633	LL1634	LL1628	LL1630	LL1631
401583	C --> T, Pro46Leu	SNV	Clo1313_0365	Clo1313_0365	cytidylyltransferase	0.0	1.0	1.0	1.0	0.0	0.0	0.0
1057976	T --> C, Leu791Pro	SNV	Clo1313_0907	Clo1313_0907	phosphoesterase RecJ domain protein	0.0	1.0	1.0	1.0	0.0	0.0	0.0
1104730	T --> -	Deletion	6 bp upstream of ppdk	Clo1313_0949	PEP-utilizing protein	0.0	0.0	0.0	0.0	0.0	1.0	0.0
1106452	A --> -, Ile573fs	Deletion	ppdk			0.0	0.0	0.7	0.0	0.0	0.0	0.0
1106841	A --> -, Ile703fs	Deletion	ppdk			0.0	0.9	0.0	0.0	0.0	0.0	0.0
1197201	C --> T	SNV	Clo1313_1021	Clo1313_1021	PKD domain containing protein	0.0	0.4	0.5	0.0	0.0	0.0	0.0
1198434	C --> T	SNV	Clo1313_1021			0.0	0.0	0.0	0.4	0.0	0.4	0.4
1206344	C --> A, Pro3718Gln	SNV	Clo1313_1021			0.0	0.0	0.0	0.0	0.5	0.0	0.0
1212030	A --> G	SNV	Clo1313_1021			0.0	0.0	0.0	1.0	0.0	0.0	0.0
2172285	A --> T, Tyr44*	SNV	Clo1313_1857	Clo1313_1857	helix-turn-helix domain protein	0.0	1.0	1.0	1.0	0.0	0.0	0.0
2493163	- --> TC, Ile307fs	Insertion	Clo1313_2121	Clo1313_2121	HPr serine kinase domain-containing protein	0.0	0.0	0.0	1.0	0.0	0.0	0.0
2493249	T --> C, Asn278Ser	SNV	Clo1313_2121			1.0	0.0	0.0	0.0	0.0	0.0	0.0
3047483	G --> A	SNV	148 bp upstream of Clo1313_2590	Clo1313_2590	response regulator receiver	0.0	0.0	0.0	0.0	0.6	1.0	1.0
3132643	C --> T	SNV	Clo1313_2666	Clo1313_2666	rca:Rcas_1719 hypothetical protein	1.0	1.0	1.0	1.0	0.0	0.0	0.0

Fig. 6. Most significant mutations found through genome sequencing of the LA-adapted *C. thermocellum* subcultures obtained in this study (the complete list of mutations found in LA adapted populations is reported in Supplementary Fig. 2). SNV, single nucleotide variation. [1]Start region is based on the coordinates from the *C. thermocellum* genome. Genbank accession number NC_017304.1. [2]Read fraction indicates the fraction of reads which support the presence of a given mutation. Read fractions > 0.95 indicate a mutation was called with high confidence. Lower read fraction values can result from a variety of causes, including duplicated genome regions, population heterogeneity, low read counts, and sequencing instrument noise.

kinase/phosphorylase (HPrK/P). HPrK/P is a sensor enzyme involved in the regulation of sugar uptake and carbon metabolism in several bacteria [33]. HPrK/P catalyzes phosphorylation/dephosphorylation of Ser46 of HPr, a protein of the PEP-dependent sugar phosphotransferase system (PTS) [34]. Conditions leading to an increase of F1,6BP concentration activate kinase activity of HPrK/P, which improves the level of P-Ser46-HPr [33]. P-Ser46-HPr acts as a co-regulator, mainly a co-repressor, of gene transcription, e.g. of genes involved in carbohydrate transport and catabolism, including glycolysis. [35]. On the other hand, increase in intracellular levels of P_i inhibits kinase activity of HPrK/P and stimulates its phosphorylase activity [34]. *C. thermocellum* HPrK/P shows significant (42.3 %) sequence identity with that from *Lactobacillus casei* [34]. The crystal structure of the C-terminal domain (aa 128–325) of *L. casei* HPrK/P has been resolved [34]. Based on sequence homology, the single nucleotide variation observed in LL1629 causing N278S replacement is adjacent to the conserved PXXXGR motif between β strand K and α helix 3, while frameshift insertion observed in LL1634 causes truncation of α helix 4 (Supplementary Fig. 4). Both the PXXXGR motif and α helix 4 are important for stabilizing the quaternary structure (homoexamer) of *L. casei* HPrK/P, which seems essential for its enzymatic activity [34]. Additionally, α helix 4 is important for binding HPr [36]. It is therefore possible to speculate that mutations observed in HPrK/P of LA evolved *C. thermocellum* strains influence the structure and activity of this enzyme.

Both mutations in PPDK and HPrK/P suggest modifications in the carbohydrate metabolism in *C. thermocellum* LA adapted populations. Curiously, no subculture has both these mutations, but either one or the other (Fig. 6). Apart from the effect on intracellular pH acidification, growth with high LA concentration may have other metabolic effects, for instance end-product inhibition of pyruvate reduction to LA by LDH. It has been predicted that even at LA concentrations in the order of magnitude of 100 mM, LDH reaction should be thermodynamically favorable in the direction of lactate production in *C. thermocellum* [37]. Furthermore, it was reported that *C. thermocellum* LDH is not inhibited by up to 160 mM LA [38]. However, LA adapted *C. thermocellum* populations have been exposed to higher LA concentrations ranging between 310−420 mM. Although the *C. thermocellum* LDH has a K_m for lactate of 59.5 mM, about 200-fold higher than that for pyruvate of 0.3 mM, it is possible that the rate of pyruvate reduction could be decreased in these extreme conditions, thus causing possible accumulation of glycolytic intermediates, such as F1,6BP. In fact, HPrK/P is among the enzymes which are allosterically regulated by F1,6BP [33]. On the other hand, diversion of PEP flux from PPDK to PEP carboxykinase and malate shunt may involve modification of intracellular levels of important regulators or cofactors.

In addition to affecting carbohydrate metabolism, mutations in PPDK or HPrK/P may affect phosphate metabolism. When PEP is converted to pyruvate by PPDK, AMP and PP_i are consumed producing ATP and P_i. In contrast, if flux proceeds via the malate shunt, *C. thermocellum* PEPCK preferentially uses GDP and produces GTP [32]. Using the malate shunt instead of PPDK may therefore increase PP_i and GTP levels and reduce P_i concentration, thus possibly affecting other reactions of the *C. thermocellum* carbohydrate metabolism, e.g. GTP and PP_i are the cofactors of glucokinase and phosphofructokinase, respectively, while P_i is a repressor of HPrK/P kinase activity [39]. It is tempting to hypothesize that mutations in genes encoding PPDK and HPrK/P could be strategies to compensate possible imbalances in glycolytic flux.

A number of other gene loci (Clo1313_0365, Clo1313_0907, Clo1313_1021, Clo1313_1857, Clo1313_2590, Clo1313_2666) are mutated in several LA evolved populations (Fig. 6). However, in these cases it is more difficult to predict the phenotypic consequence of these mutations, either because they are silent or because the structure and/or the function of the protein they encode is not known. Interestingly, a single nucleotide variation affecting the amino acid sequence (L791P) of the gene product was found in Clo1313_0907 of subcultures LL1632, LL1633, LL1634. This gene encodes the single-stranded-DNA-specific exonuclease RecJ, an important enzyme involved in DNA repair and recombination [40]. However, the effect of the observed mutations on RecJ structure/function cannot be hypothesized, since it occurs in a region which is absent in other RecJ proteins for which the crystal structure has been determined [40,41]. It is worth remembering that, because of DNA damage possibly caused by acidic conditions, overexpression of proteins involved in DNA repair such as RecA, RecO and UvrABCD has been detected in strains undergoing acid stress [18,42].

The strategies most frequently used by microorganisms to face acid stress involve mechanisms for intracellular pH homeostasis (such as proton pumping F_0F_1 ATPases and PP_i-ases, decarboxylation and deamination), modification of cell membrane and overexpression of protein chaperones and DNA repair proteins [10,12,42]. These systems are not well represented in the present investigation, since apart from RecJ mentioned above, only one other mutated protein, namely the AAA ATPase central domain protein (Clo1313_2950) which is a type of molecular chaperone [43], was affected by the LA evolution procedure (strain LL1630).

Fermentation profiles of LA adapted subpopulations

Since genome re-sequencing suggested possible modifications of carbohydrate metabolic flux in most of the LA adapted subcultures, the fermentation profile of these mixed populations in MTC-5 chemically-defined medium, supplemented with 5 or 50 g/L cellobiose, was compared with that of their parent strains (Figs. 7,8). As regards strains

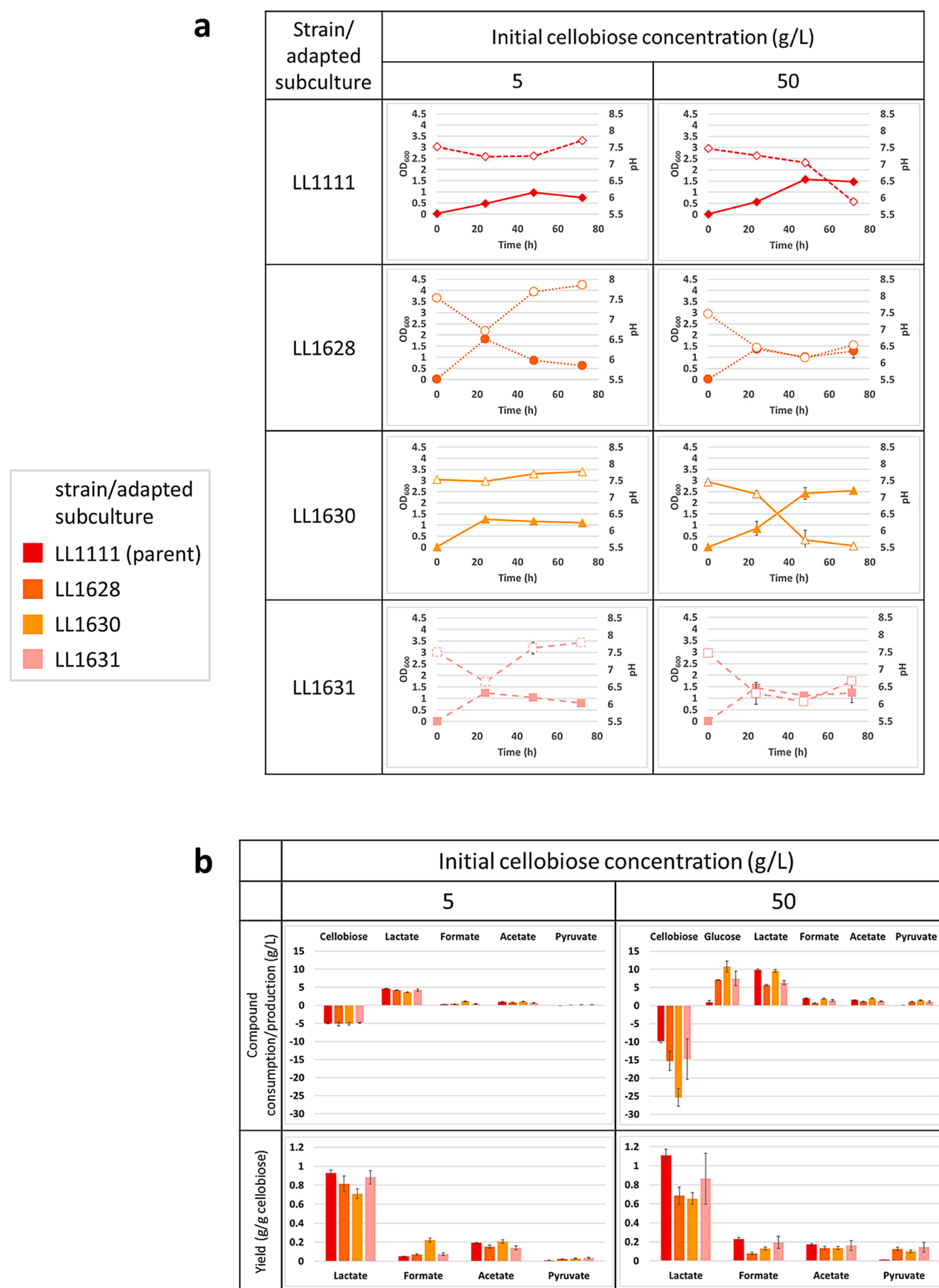


Fig. 7. Growth, pH kinetics (a) and fermentation profile (b) of strain LL1111 and subcultures evolved from it in MTC-5 medium supplemented with 5 or 50 g/L cellobiose. a) Solid symbols, OD_{600}; open symbols, pH. b) Product yield is expressed as [g product / (g cellobiose consumed – g glucose accumulated)]. Data are the averages from biological triplicate experiments. Error bars represent one standard deviation.

evolved from *C. thermocellum* LL345, no major differences in product yield among evolved and parent strains were detected in cultures supplemented with 50 g/L cellobiose, while about 3-fold higher formate yield was produced by LA-adapted populations in 5 g/L cellobiose-supplemented cultures (Fig. 8b). About 20–40 % reduction, depending on the derivative population, of LA yield and to a similar extent in formate and acetate yield, was observed in populations evolved from *C. thermocellum* LL1111 with respect to their parent strain in 50 g/L cellobiose-supplemented cultures (Fig. 7b). In addition, these evolved populations showed important increases in glucose and pyruvate accumulation (Fig. 7b). As a consequence, the LA titer of LL1111-derived strains was similar to, or lower than, that of the parent strain. Interestingly, the maximum LA titer of 9.88 g/L, measured in these cultures performed in conical tubes, was higher than that determined in butyl-stoppered vials (6.45 g/L) and may reflect these different growth conditions. Fermentation profiles of these populations seem more similar to each other in 5 g/L cellobiose-supplemented cultures (Fig. 7b). In this growth condition, subculture LL1630, showing mutation at the *ppdk* locus, had the most divergent profile, characterized by increased formate yield and titer. Curiously, this metabolic feature was also common to subpopulations evolved from LL345 that show mutation at the loci encoding PPDK or HPrK/P. However, reduced organic acid (except pyruvate) yield in medium supplemented with 50 g/L cellobiose applied to all the populations evolved from LL1111 and was not associated with a specific genotype, such as mutations at the *ppdk* locus.

Improvement of LA tolerance of microorganisms has sometimes also been reported to enable higher LA production, such as in *L. mesenteroides* [12]. However, the present results on *C. thermocellum* LA-adapted strains

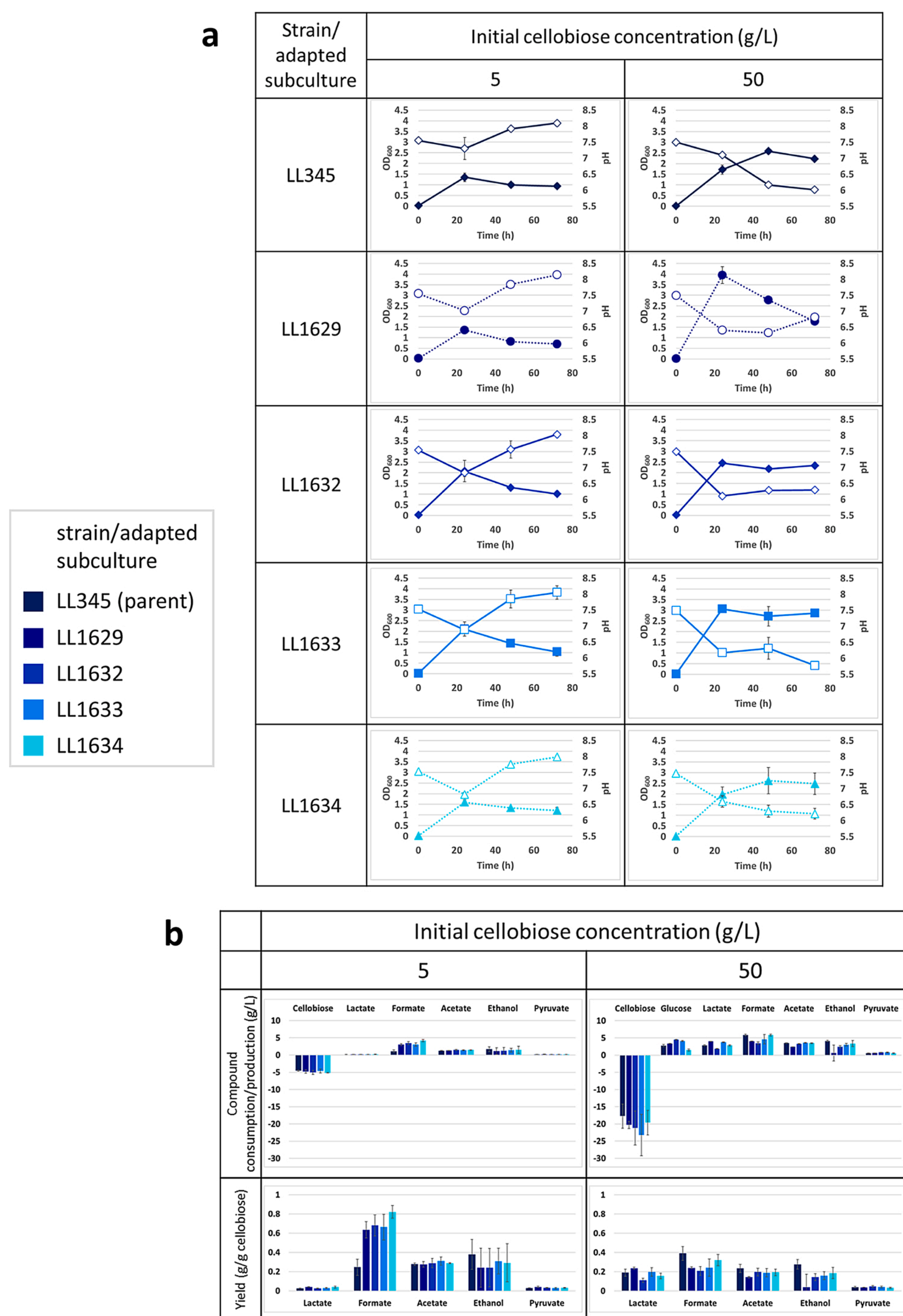


Fig. 8. Growth, pH kinetics (a) and fermentation profile (b) of strain LL345 and subcultures evolved from it in MTC-5 medium supplemented with 5 or 50 g/L cellobiose. a) Solid symbols, OD_{600}; open symbols, pH. b) Product yield is expressed as [g product / (g cellobiose consumed – g glucose accumulated)]. Data are the averages from biological triplicate experiments. Error bars represent one standard deviation.

do not confirm association between enhanced LA tolerance and production. In the growth conditions used, pH acidification seems the main factor limiting *C. thermocellum* growth and LA titer. From an application standpoint, subculture LL1630 showed the best phenotype among the LA-evolved *C. thermocellum* populations since, despite reduced LA yield, it maintained an LA titer similar to that of parent strain LL1111.

Conclusions

C. thermocellum is among the best cellulose degraders isolated so far and has shown interesting potential as a producer of LA through direct fermentation of cellulose. In a previous study, the *C. thermocellum* strain LL1111 was engineered by disrupting *adhE* encoding its main alcohol/aldehyde dehydrogenase, and in which LA was the main fermentation product with a yield of 40 % of the maximum theoretical value [7]. Improved LA production of LL1111 was also due to a spontaneous mutation that eliminated F1,6BP regulation of LDH activity. However, industrial fermentation processes require robust microbial strains able to produce LA with at least 80 % yield, 2.5 g/L/h productivity and 100 g/L titer [44]. Apart from insufficient yield, the present study indicated that LA productivity (max about 0.2 g/L/h) and titer (max9.9 g/L) of LL1111 also require significant further improvement to satisfy industrial

requirements. The present investigation showed that exogenously added LA caused *C. thermocellum* growth to stop at concentrations of 15−20 g/L, depending on the strain. Adaptive evolution was able to improve *C. thermocellum* tolerance to LA. The maximum tolerated LA concentration was more than doubled in LA hyperproducing strain, LL1111, from 15 g/L to 35 g/L. Mixed populations evolved from LL345, the wild type fermentation profile, showed a 50–80 % increase of maximum growth rate in medium containing 45 g/L LA. Mutations in the gene loci encoding PPDK and HPr K/P suggest that some modification of the carbohydrate and or phosphate metabolism may have occurred in five out of seven evolved subcultures. Curiously, these mixed populations showed altered formate production. More direct investigation of the effect of the observed mutations on LA tolerance and fermentation profile of the evolved *C. thermocellum* populations could be obtained by re-introducing these mutations into the parent strain(s). Besides improved understanding, this approach could provide gene targets for rational engineering of LA tolerant *C. thermocellum* strains and will be considered for future studies. Apart from this, all the LA-adapted strains show LA yield and titer similar to or lower than their parent strains, especially those derived from LL1111. From an application standpoint of industrial production of LA, subculture LL1630 shows the highest performing phenotype among the evolved populations, since the reduced LA yield is compensated by higher biomass production leading to an LA titer similar to that of LL1111. Based on its improved LA tolerance, subculture LL1630 could serve as a framework for improving LA production by further metabolic engineering modifications, such as improvement of LDH activity and elimination of acetate and/or formate production [7,8].

Author contributions

R.M., D.G.O., E.K.H. and L.R.L. conceived the experimental design. R.M. and A.M.C. performed the experiments. D.G.O. analyzed resequencing results. All the Authors contributed to discussing the results and writing the manuscript.

Declaration of Competing Interest

L.R.L. is a founder of the Enchi Corporation, which has a financial interest in *Clostridium thermocellum*.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.nbt.2021.12.003.

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