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The stressostat: A novel approach in adaptive laboratory evolution to improve end-product resistance

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ABSTRACT

End-product inhibition in pH-controlled batch cultures, is the major limiting factor for bacterial biomass formation in the starter culture industry as well as in many other biotechnological processes. Adaptive laboratory evolution (ALE) has emerged over the past decades as a powerful tool for phenotype optimization, but none of the existing ALE methods could select for improved end-product resistance. Therefore, we developed the stressostat (STress Resistance Evolution in Substrate Surplus) as a novel continuous ALE method. Stressostat cultivation applies end-product concentrations as constant evolutionary pressure on microorganisms in the presence of substrate surplus. In this study, we improved the lactate resistance of *Lactococcus lactis* FM03P in 35 days of stressostat cultivations. The lactate concentrations increased over time from 530 to 675 mM, indicating the successful selection for variants with improved lactate resistance. Thirty-four variants were isolated and grouped into four clusters based on their growth rates at high lactate concentrations. In the high-throughput screening without pH control, most isolated variants could grow at high lactate concentrations (870–928 mM), while the wild type was completely inhibited. The variants grew slower than wild type at low lactate media indicating possible evolutionary trade-off. However, in pH-controlled batch cultivations, most variants produced more biomass than the wild type. In conclusion, stressostat cultivation is a valuable method to obtain *L. lactis* variants with improved end-product resistance and further characterization is needed to elucidate underlying resistance mechanisms and potential industrial applications.

Introduction

In the starter culture production industry and many other biotechnological processes, inhibition by the metabolic end-product is the major limiting factor of microbial biomass formation in pH-controlled batch cultures. These metabolites are mainly organic acids, such as lactic acid [1]. Their presence not only inhibits microbial growth, but can also cause complete cell arrest and/or even cell lysis. Several inhibitory mechanisms have been proposed for organic acids. First, it is assumed that the protonated acids passively diffuse across the cell membrane into the cytoplasm. Upon entry, the protonated weak acids are dissociated inside the cell due to the higher intracellular pH, releasing protons and anions. Accumulation of protons inside the cells disrupts pH homeostasis, which causes intracellular acidification leading to growth inhibition and ultimately complete cell arrest or even cell lysis [2,3]. Secondly, the accumulation of the dissociated acid (anion) increases cellular osmolarity leading to lethal turgor pressure [2,4]. Moreover, the anion accumulation may also induce a direct feedback inhibition of important metabolic pathways [5]. This may result in the depletion of important nutrients for growth and/or accumulation of toxic intermediates, and thus growth inhibition.

End-product inhibition can be tackled by utilizing adaptive laboratory evolution (ALE). ALE emerged over the past decades as a powerful tool for metabolic engineering of microbial strains aimed at phenotype optimization [6,7], in this case to improve stress resistance. Using ALE, evolutionary phenomena can be studied in controlled laboratory settings. Microorganisms are cultivated for prolonged periods under a selective and defined environment. This allows for the selection of beneficial mutations in specific environments [7–9].

Two methods are mainly used for ALE: (1) serial batch cultivation and (2) continuous cultivation. Serial batch cultivation is performed by serial transfer over a period of time in the specific environment. Serial

Abbreviations: ALE, adaptive laboratory evolution; AU, arbitrary unit; CDM, chemically defined medium; CFU, colony forming unit; EP, end-product; HTS, high-throughput screening; WT, wild type.

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batch cultivation mainly selects variants with an increased maximum growth rate when the transfer occurs in exponential phase, or with a better survival in starvation when the transfer is performed in stationary phase. This method is simple and easy to execute, but it lacks the constancy of the selective pressure due to the dynamics in environmental conditions (e.g., pH, nutrient abundance, oxygen availability) [10,11].

In contrast, continuous cultivation could provide a constant selection pressure for prolonged times and depending on the settings, continuous cultivation can be used for various selection pressures. Although the setup is complex and high-throughput execution is less feasible, continuous ALE offers precision in the selection pressure because it strictly controls the environmental conditions and growth characteristics. One of the most used continuous ALE methods is a chemostat cultivation. In chemostat cultivation, fresh medium is constantly added at a fixed rate to the growing culture and spent medium with cell biomass is removed at the same rate. This combination results in a fixed and controllable growth rate of the culture. Moreover, the medium for chemostat cultivations is usually designed to have one specific nutrient at a growth limiting concentration. Therefore, this method is commonly used to obtain variants with increased nutrient affinity [8,11–14]. However, chemostat cultivation can also be used for other evolutionary purposes, such as adaptation at low pH [15] or growth in the presence of abiotic stress [16–19]. Another variation of continuous ALE is the morbidostat, a technology used to study antibiotic resistance in microorganisms [20,21]. In this system, an antibiotic of interest is automatically added when the optical density (OD) increases above a pre-defined threshold. This control algorithm allows to adjust the antibiotic concentration according to the actual rate at which antibiotic resistance evolves. In this way, the cell densities and growth rate will remain constant despite of evolutionary adaptation because the antibiotic concentration increases to the required inhibitory level after emergence of protective mutations.

Despite all the existing methodological variations of continuous cultivation, continuous ALE to improve the end-product resistance has never been described. Therefore, inspired by the concept of the morbidostat, we developed the stressostat (STress Resistance Evolution in Substrate Surplus) as a novel continuous ALE method to improve end-product resistance. Stressostat offers a relatively simpler setup compared to morbidostat as it does not require an algorithm, nor a separate reservoir with the inhibitory compound of interest, nor control of the inhibitory compound concentration (Fig. 1A). In the stressostat, the feed medium contains an end-product of growth as inhibitory compound and in contrast to a conventional chemostat all nutrients are supplied in surplus (Fig. 1B). The feed is added to the culture at constant dilution rate that determines the growth rate. During microbial growth, the inhibitory end-product concentration increases in situ by metabolism of the substrate. Substrate consumption increases every time a mutation arises, which improves the fitness of the microorganism. This results in a higher end-product concentration increasing the stress condition in the culture. The surplus of substrate ensures that growth remains limited by the end-product and increased end-product concentrations indicate the presence of variants with higher end-product resistance.

In this study, the stressostat was used to select for *Lactococcus lactis* variants with increased end-product (lactic acid) resistance. Isolated variants were phenotypically clustered based on a high-throughput screening. Representative variants for each cluster were grown in pH-controlled fermentations to mimic starter culture production, demonstrating improved lactate resistance and biomass production.

Material and methods

Strain and pre-culture condition

Lactococcus lactis biovar *diacetylactis* FM03P [22] was used in this study. For each cultivation, *L. lactis* was streaked on M17 plates (BD Difco, Saint Ferréol, France) supplemented with 0.5% (w/v) lactose (LM17) and incubated at 30 °C for 72 h. A single colony was inoculated into LM17 broth and incubated overnight at 30 °C. These pre-cultures were used for further experiments.

Chemically defined media

The chemically defined media (CDM) contained lactose monohydrate (VWR Chemical, Radnor, PA, USA) and tryptone (BD Difco and Oxoid, Basingstoke, UK) as the main carbon and nitrogen sources, and K_2HPO_4 (Merck, Darmstadt, Germany) as buffer. The concentration of these 3 compounds and pH of the CDM varied per experiment (Table 1). Moreover, various amounts of potassium lactate (60% w/w) (Merck, Darmstadt, Germany) were added depending on the experiment. Finally, all media contained per kg: 10 g 100x metal stock, 10 g 100x nucleotide stock and 10 g 100x vitamin stock [23]. In addition, 0.1 g riboflavin was added into the 100x vitamin stock solution. All media were sterilized by filtration through a 0.2 µm membrane filter.

Stressostat evolution

Stressostat cultivations

Two independent stressostat cultivations were executed in a bioreactor (Multifors, Infors HT, Bottmingen, Switzerland) with a working

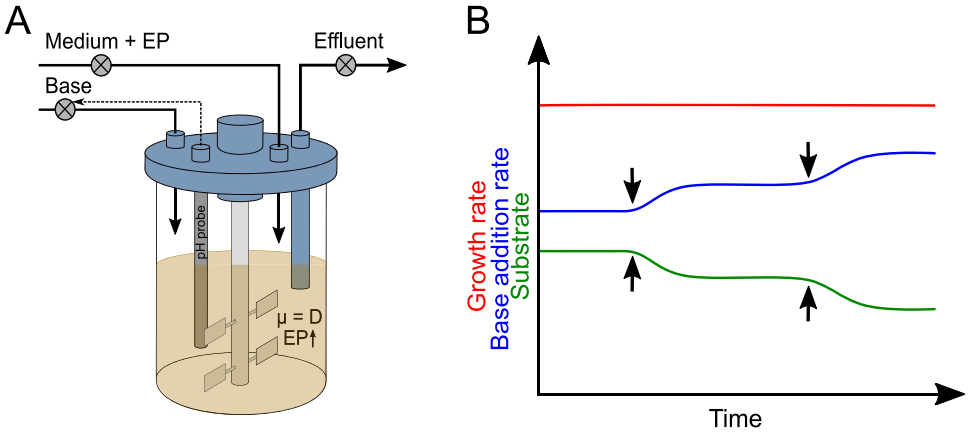

Fig. 1. Stressostat concept. A: Schematic overview of the stressostat cultivation setup. Medium containing end-product is continuously supplied at fixed rate. pH is maintained at fixed value by addition of counter-ion. B: Expected outcome of stressostat cultivation. Substrate consumption increases every time a mutation occurs that improves the fitness of the microorganism (black arrows). This results in higher end-product levels thereby increasing the stress condition in the culture. This adaptive process can be monitored online by measuring base addition rate over time when the end-product is an organic acid. EP = end-product.

Table 1
Chemically defined media composition in different experiments.

Experiment	Lactose (mM)	Tryptone (g kg^{-1})	K_2HPO_4 (mM)	Potassium lactate (mM)	pH
Stressostat cultivation	143	20[a]	16	402	6.5
High-throughput screening	57	10[a]	47	0, 754, 812, 870, 928	7.0[c]
pH-controlled fermentation	285	40[b]	16	0	6.5

[a] supplied by BD Difco, France
[b] supplied by Oxoid, UK
[c] initial pH

volume of 0.25 L. The conditions were set as follows: temperature at 30 °C, stirring speed at 200 rpm, and pH at 6.5. The pH was maintained by automatic addition of 15 M KOH. In addition, the headspace was flushed with nitrogen gas at a flow of 0.1 L min^{-1} to maintain anaerobic condition. The bioreactor was inoculated (4% v/v) with a pre-culture as described before. After 24 h incubation in a pH-controlled batch, fresh medium was continuously supplied (Table 1) to the culture at a constant dilution rate of 0.17 ± 0.01 h^{-1} for 35 days. The rate of KOH addition was continuously monitored online using software controlling the bioreactor (Iris 6.0, Infors HT). Samples were taken every 3–4 days to measure the OD at 600 nm and the extracellular metabolite concentrations. Moreover, glycerol stocks (culture mixed with 30% (v/v) glycerol) were made and stored at −80 °C until variant isolation.

Variant isolation

The variants were isolated after 7, 13, 20 or 24, and 35 days of stressostat cultivation. The stored glycerol stocks were streaked on 2 different isolation plates: LM17 with 1.5% (w/w) agar and CDM supplemented with 481 mM potassium lactate and 1% (w/w) agar. LM17 plates were incubated for 72 h at 30 °C, while CDM plates were incubated for 14 days at 30 °C. Two or three (at the end point) single colonies from each plate were transferred into LM17 broth and incubated overnight at 30 °C. Glycerol stocks were made from the overnight culture and stored at −80 °C for further use. In total, 17 variants were isolated from each stressostat cultivation.

High-throughput screening (HTS) for growth at high lactate

To analyze the growth performance of the isolated variants at high lactate concentrations, each well of a 96-well plate was filled with 180 µL CDM containing different lactate concentrations (Table 1). At least 2 independent overnight cultures from the wild type and each variant (preparation as previously described) were added to the plate with an inoculum level of 0.1% (v/v). The plate was incubated in a Versamax plate reader (Molecular Devices, Sunnyvale, CA, USA) at 30 °C for 99 h. The OD was continuously measured every 10 min at 600 nm with 5 s shaking between each read. The measurement was automatically recorded in SoftMax Pro software (Molecular Devices). The maximum growth rate of the wild type and variants in each condition was calculated by using the R package "growthrates" [24] using the OD data as input. The growth rate was determined at OD below 0.3.

pH-controlled batch cultivations

pH-controlled batch cultivations were executed in bioreactors with a working volume of 1 L using the CDM described in Table 1 and with the same conditions as the stressostat cultivations. The bioreactor was inoculated (0.1% v/v) with an overnight culture as previously described. The culture was incubated until the stationary phase was reached (24 – 200 h). The rate of KOH addition was continuously monitored online (Iris 6.0) and the OD was continuously measured every 10 s using an internal probe (EXcell 231 with EXpert software Version 2.1.7, Exner Process Equipment GmbH, Ettlingen, Germany). Samples were taken several times from the exponential phase to the late stationary phase for OD calibration, extracellular metabolites analysis, and dry cell weight measurement. Lactate formation was calculated with linear regression of measured lactate in extracellular metabolite analysis against KOH addition (Suppl. Fig. 1).

Extracellular metabolites analysis

Cells were removed from the samples by centrifugation (13300 ×*g* for 2 min at 4 °C) and the supernatant was stored at −20 °C until analysis. High-performance liquid chromatography (HPLC) was performed to quantify lactose, lactate, acetate, ethanol, formate, pyruvate, and acetoin on an Ultimate 3000 (Dionex, Germering, Germany) equipped with an Aminex HPX-87 H column (300 × 7.8 mm) with precolumn (Bio-Rad, Hercules, CA, USA) kept at 40 °C. As mobile phase, 5 mM sulphuric acid was used at 0.6 ml min^{-1}. Compounds were detected and quantified by a refractive index detector (RefractoMax520, ERC Inc, Kawaguchi-Shi, Japan).

Dry cell weight determination

Samples volume of 2–5 ml were passed through a pre-weighted membrane filter with a pore size of 0.2 µm (Pall Corporation, Port Washington, NY, USA) by a vacuum filtration unit. Sample sizes were determined by weighing. The membrane filters were washed with at least 50 ml demineralized water, dried at 80 °C for at least 48 h, and weighed again on an analytical balance. Dry weights were determined in technical duplicate for each sample.

Viable cell count

Samples were serially diluted in peptone physiological salt solution (Tritium Microbiology BV, Eindhoven, The Netherlands) and appropriate dilutions were plated on LM17 plates. Plates were incubated for 72 h at 30 °C after which colonies were counted to quantify the number of colony forming units (CFU).

Heatmap

The heatmap to cluster all isolated variants was generated by using R package "pheatmap" [25]. Maximum growth rates calculated in high-throughput screening experiment were converted into log scale and the variants were clustered according to the growth similarity at increasing lactate concentration. Row annotations indicating the replicate, cultivation time, isolation plate, and metabolism were added into the constructed heatmap.

Results

Lactococcus lactis biovar diacetylactis FM03P was evolved in stressostat cultivations to obtain variants that can grow at a high lactate concentration. Two independent cultivations were executed for 35 days at a dilution rate of 0.17 h^{-1}, reaching approximately 200 generations. During the cultivations, base addition rate was monitored online, while the OD and metabolite concentrations were determined every three to four days (run 1, Fig. 2 and run 2, Suppl. Fig. 2).

In the cultivation, the first quasi steady state was reached 24 h after the feed started as the base addition rate remained constant. The base addition rate sharply increased after seven days reaching a second quasi steady state, and it gradually increased again after approximately 20 days to a third quasi steady state (Fig. 2A). The increasing base addition rate indicates that more lactic acid was produced as a result of a higher lactose utilization (Fig. 2C). The lactate concentration gradually increased in both stressostat cultivations from 530 mM to approximately

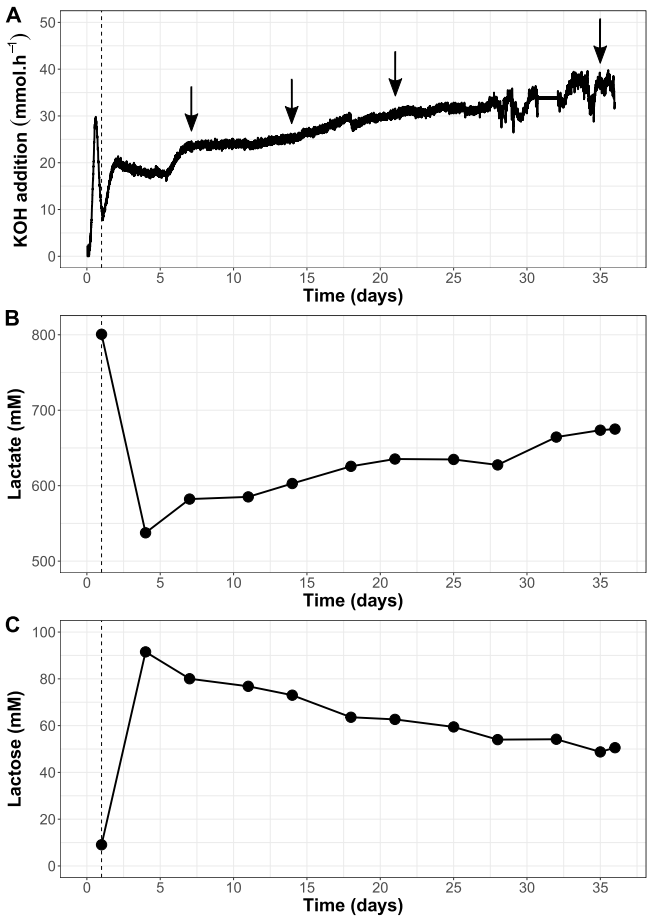

Fig. 2. Growth of L. lactis FM03P in one of the stressostat cultivations in lactose CDM containing 402 mM potassium lactate at pH 6.5. Time 0 indicates the inoculation of the bioreactor. Dashed vertical line indicates the start of the stressostat by addition of fresh medium at a dilution rate of 0.17 h^{-1} after 24 h of batch cultivation. Black arrows indicate the time of variants isolation. A: Addition rate of 15 M KOH to maintain a constant pH. B: Lactate concentration during stressostat cultivation. C: Residual lactose concentration during stressostat cultivation. The other stressostat cultivation replicate can be found in supplementary figure 2.

675 mM at the end of the cultivations, while the production of other metabolic products remained low (<5%). Meanwhile, residual lactose concentrations gradually decreased from 95 mM to 50 mM. This surplus of lactose indicated that growth was not limited by substrate limitation as in a conventional chemostat. The higher lactose utilization is expected to be caused by mutations that increased the lactate resistance. When the variants became the dominant population and their growth was inhibited again at a higher lactate concentration, a new quasi steady state occurred. Despite the increased lactose utilization rate, the biomass concentration in the stressostat remained relatively stable throughout the cultivations (Suppl. Fig. 2B).

Seventeen individual variants were isolated as single colony isolates from 4 different time points (Fig. 2A, black arrows; Suppl. Fig. 2) in the stressostat cultivations. To determine their lactate resistance, the wild type and all isolated variants were grown in different concentrations of lactate (754, 812, 870, and 928 mM) (Suppl. Fig. 3). According to a constructed heatmap, the variants were divided into four clusters based on their μ_{max} in different lactate concentrations (Fig. 3).

The wild type could only grow up to 812 mM of lactate, while the variants from cluster I could still grow at 870 mM lactate, but not at 928 mM lactate. Variants from cluster II grew slow in the control medium without lactate, but they had similar growth rate at higher lactate concentrations, even at 928 mM lactate. This indicated that lactate did not inhibit the growth of variants in this cluster. Interestingly, all of the variants in cluster II were isolated from CDM plates containing 481 mM lactate and from various cultivation times in the stressostat (from one to five weeks). Variants from cluster III had a higher μ_{max} at 870 mM lactate than the variants from cluster I. The variants in cluster III also grew at 928 mM, albeit slow. Interestingly, variants from cluster I were isolated mostly during the first two weeks of stressostat cultivation, while variants from cluster III were isolated at later time points. This indicated that a longer stressostat cultivation allowed *L. lactis* to evolve to a higher lactate resistance. Finally, cluster IV consisted of only three variants. These variants had higher μ_{max} than all other variants at all tested concentrations, including 928 mM lactate. In contrast to the cluster II variants, the variants in cluster IV still grew fast in the control media and showed no significant different μ_{max} compared to the wild type (Suppl. Fig. 4). We expected that the variants in cluster IV to have the highest potential for increased biomass yield in starter culture production, considering their fast growth and ability to grow at high lactate concentrations.

It is known that at low growth rates *L. lactis* shifts from fast but inefficient homolactic fermentation (mainly lactate) to more energy-efficient mixed acid metabolism (combination of lactate, acetate, formate, and ethanol) [26]. Therefore, the observed slow growth of variants in cluster II led to the hypothesis that these variants might have shifted their metabolism. To confirm this hypothesis, the main-fermentation products (lactate, formate, acetate, ethanol, acetoin, and pyruvate) were determined after the variants were grown in batch cultures in CDM with 57 mM lactose for 7 days. Fermentation end-product concentrations were converted into pyruvate consumption fluxes and the fluxes were normalized to 100% for each variant to compare differences in pyruvate dissipation pathways (Fig. 4).

L. lactis FM03P wild type as well as variants from clusters I, III and IV converted pyruvate mainly to lactic acid using lactate dehydrogenase (>90%). In contrast, variants from cluster II produced mainly a mixture of acetate, ethanol and formate using pyruvate formate lyase (~50–80%), while the flux through lactate dehydrogenase was low (<40%). Interestingly, when variants were grown in CDM supplemented with 113 mM glucose, all variants grew fast and metabolism remained homolactic (data not shown), indicating that the slow growth of variants from cluster II was caused by the inability to rapidly metabolize lactose. Determination of the plasmid content revealed that all variants from cluster II, but none of the other variants, lost plasmid pLd8. This plasmid carries an operon for lactose utilization [22], and its loss caused slow growth on lactose, resulting in a metabolic shift to mixed acid fermentation.

To further assess the ability of variants to produce biomass in a starter culture setting, two representative variants of each cluster were grown in two independent pH-controlled batch fermentations. The OD at 600 nm was measured continuously to monitor biomass formation (Fig. 5A). Ratio of cell dry weight (g kg^{-1}) per OD (AU) was not significantly different between variants, except for FM105 (Suppl. Fig. 5).

FM03P wild type reached the end of exponential phase after 12 h cultivation, after which the OD and viable cell count immediately decreased (Fig. 5A). In contrast, none of the variants showed such decreases. Notably, most of the variants reached a higher OD and viable cell count than the wild type. However, except for FM115, the variants had a longer time to detection than the wild type and required more time to reach the stationary phase, indicating a longer lag phase and/or a lower growth rate. Next to biomass, the lactate concentration was measured at specific time points and correlated to the addition of KOH to estimate lactic acid production over time (Fig. 5B). Most variants (except FM113) reached a higher lactate concentration than the wild type and they stopped growing at a significantly higher lactate concentration than the wild type (growth rate reduced to 0.005 h^{-1}; Suppl. Fig. 7).

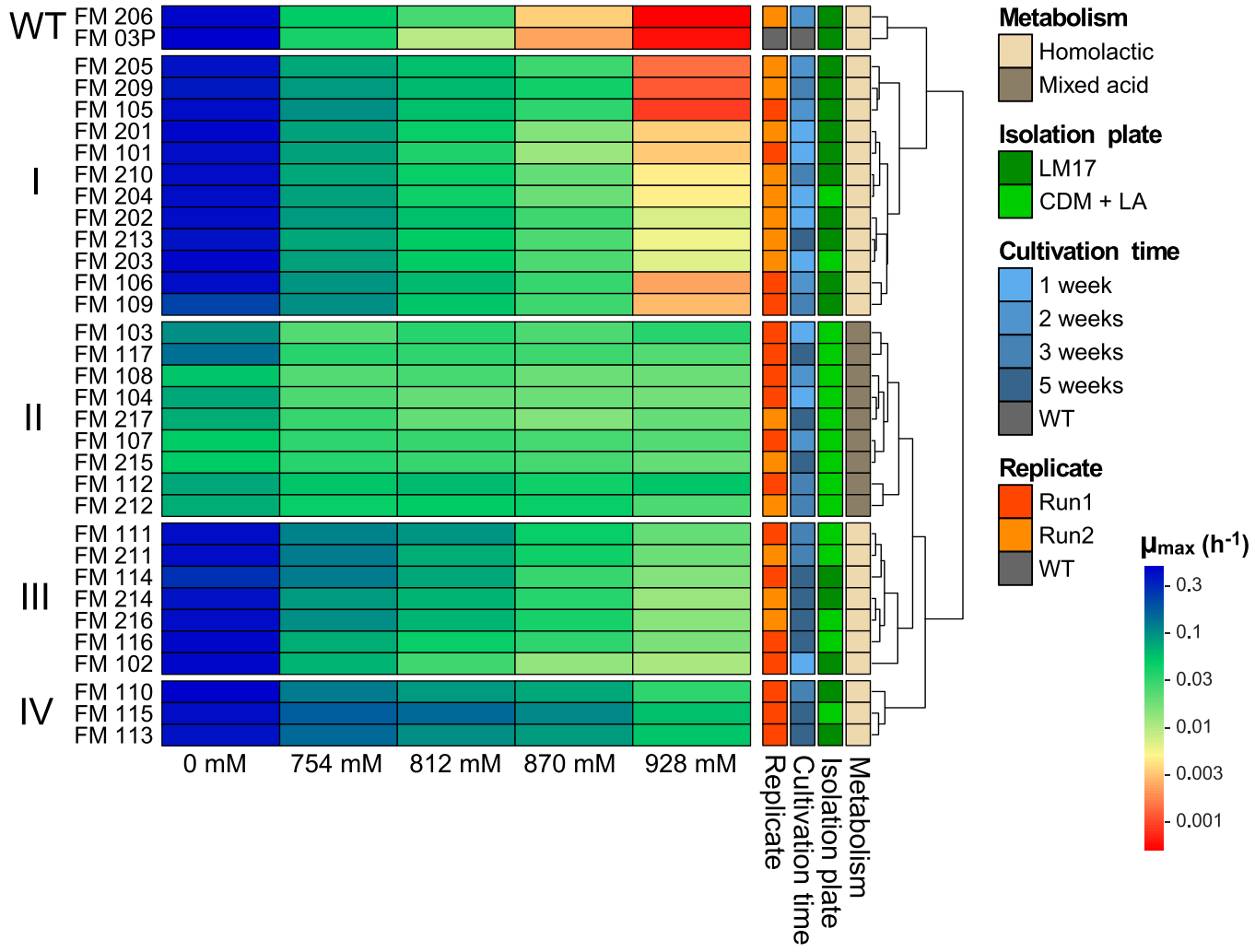

Fig. 3. Comparison of the maximum growth rates (μ_{max}) of L. lactis FM03P variants isolated from stressostat cultivations grown in different concentrations of lactate. μ_{max} are indicated by color from high (dark blue) to low (dark red). Annotation: (1) replicate indicates the 2 independent stressostats; (2) cultivation time represents the time of isolation of variants from the stressostats; (3) isolation plate describes the type of plate used for isolation of the the variants (LM17 or CDM + 481 mM lactate); (4) metabolism of the variants (homolactic or mixed acid fermentation).

Discussion

In this study, stressostat cultivations were used to obtain *L. lactis* FM03P variants with improved growth at high lactate concentrations. High throughput screening (HTS) of growth rates revealed that the wild type had a growth rate of ~0.2 h^{-1} at 522 mM lactate (Suppl. Fig. 8), which corresponds to the initial condition in the stressostat. Furthermore, the variants which were isolated at the end of stressostat cultivation had a growth rate of ~0.2 h^{-1} at 696 mM lactate. This corresponds with the condition at the end of stressostat cultivation, which was 0.17 h^{-1} with a lactate concentration close to 700 mM. This demonstrated the successful selection of lactate resistant variants of *L. lactis* FM03P by using stressostat cultivation.

Stressostat cultivation offers a simple but biologically sophisticated setup. It has the same setup as the chemostat, but the feed medium is designed in such a way that the substrate will not be limiting throughout the cultivation, but instead the end-product limits the growth. In the stressostat a constant selection pressure is maintained by in situ production of the end-product by the microorganism. When a beneficial mutation that improves the fitness (e.g., end-product resistance) of a variant takes place, this variant will start dominating the culture, causing more substrate to be utilized. This results automatically in an increased end-product concentration and thus higher selection pressure.

The outcome of ALE is determined by the selected ALE method. ALE methods determine the phenotypes present in the population by applying a particular evolutionary pressure. Sequential batch propagation is the most used and easiest ALE method. Batch ALE selects mainly for fast growth and possibly also for a better survival in the stationary phase depending on the experimental setup. However, this method lacks environmental control causing inconsistency of the evolutionary pressure [10,11]. In addition, the growth characteristics of a microorganism change when ALE is performed for a prolonged time due to the growth benefit from adaptation mechanisms (e.g. shorter lag phase). This causes variations in selection pressure even when the transfer is done at the same duration. All aforementioned factors cause uncertainty in the phenotype of the isolated variants. Batch ALE of *L. lactis* in CDM supplemented with 400 mM potassium lactate resulted in variants with similar biomass production and growth inhibition by lactate as the wild type when grown in a pH-controlled batch cultivation (data not shown). Interestingly, like in the stressostat variants, these batch ALE variants survived better in the stationary phase than the wild type, indicating that the batch ALE selected for variants with increased survival in stationary phase rather than increased growth.

In addition, choice of the agar plates used for isolation of the variants

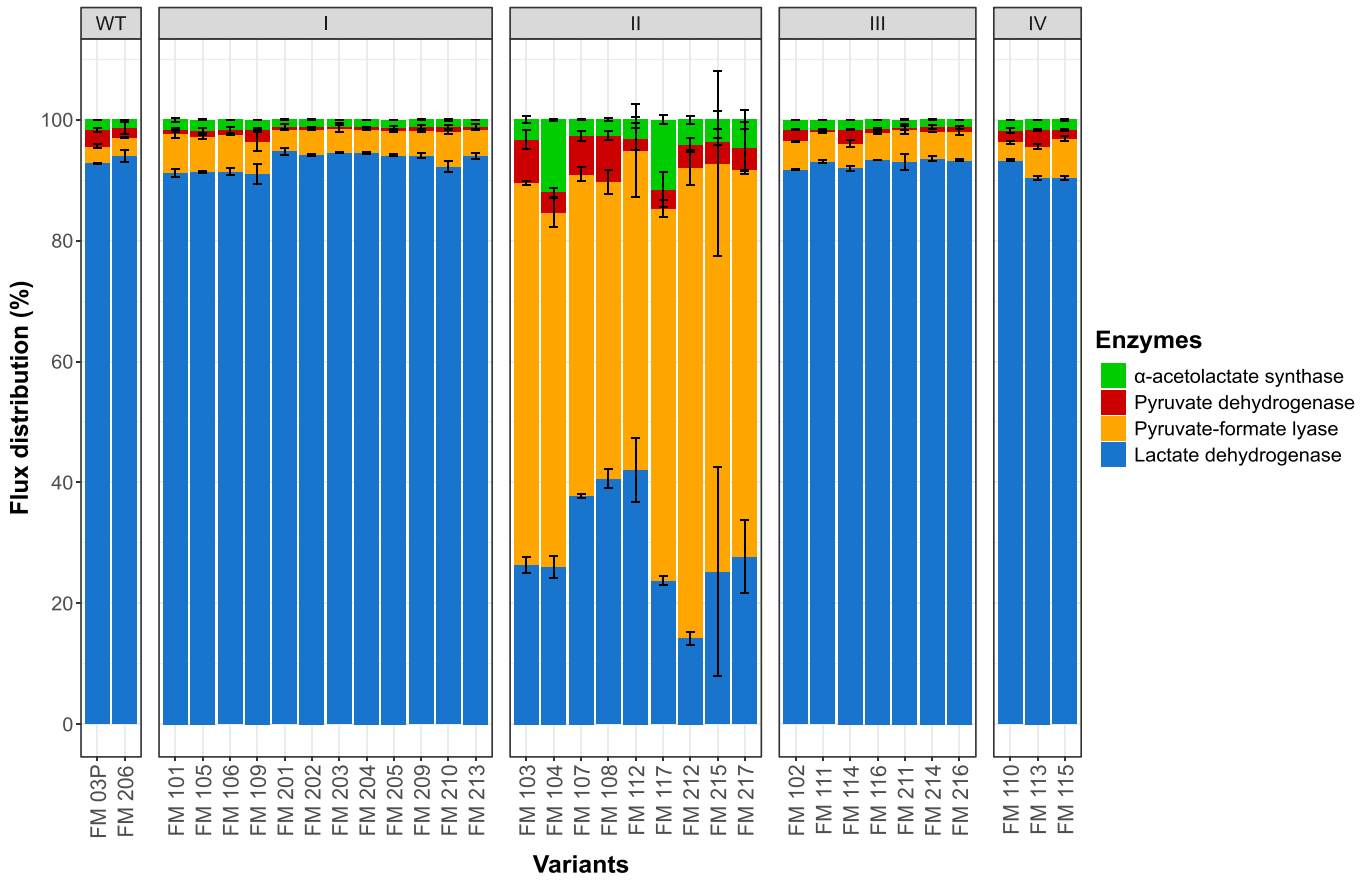

Fig. 4. Flux distribution of the main pyruvate dissipation pathways in variants isolated from stressostat cultivations when grown in CDM with 57 mM lactose. Reactions are normalized based on the consumption of 1 pyruvate. Error bars represent the standard deviation of 2 biological replicates.

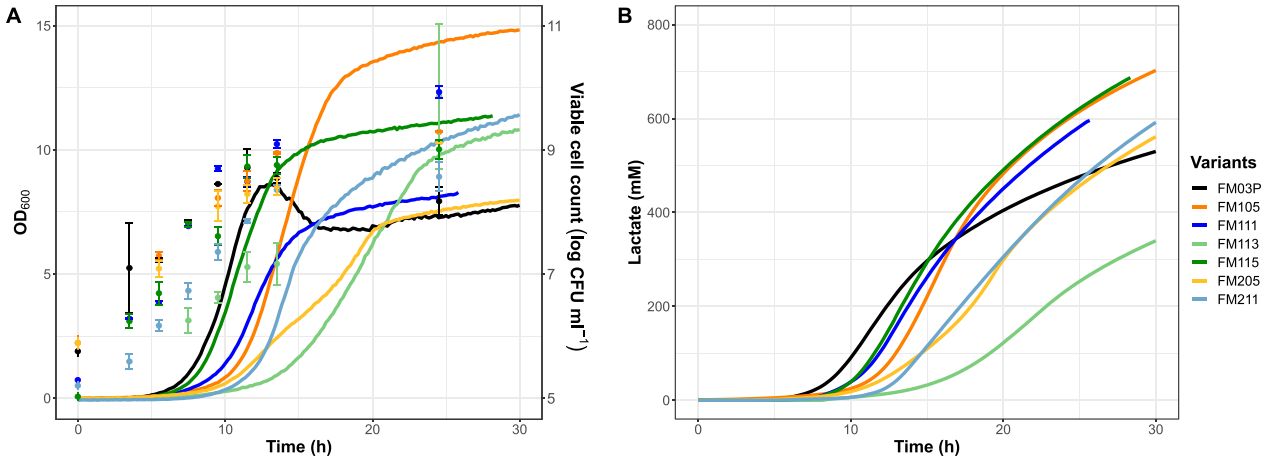

Fig. 5. Growth performance of L. lactis FM03P and its variants in pH-controlled batch cultivations with CDM containing 285 mM lactose. A: Biomass formation measured by OD_{600} (lines) and viable cell count (circles). B: Lactate formation calculated from linear correlation with KOH addition. The data shown was the average of 2 biological replicates. Standard deviation of the replicates are shown as a shading in supplementary Figure 6A and B. FM112 and FM212 are given in supplementary Figure 6 because these variants grew slow and had OD lower than 2.5 after 50 h cultivation.

of interest also influences the outcome of ALE. Next to an unselective rich plate medium (LM17), we also used a selective plate (CDM + 481 mM lactate) to isolate variants from the stressostat cultivations. Interestingly, mixed acid variants (Fig. 4, Cluster 3) were mainly isolated from selective plates. These variants appeared to have lost plasmid pLd8 encoding a lactose operon and had a lower growth rate (Fig. 3) than the stressostat dilution rate (0.17 h^{-1}) due to inability to utilize lactose and therefore were expected to be washed out during the stressostat cultivation. Since it is known that plasmids can be easily lost by *L. lactis* [22], it is hypothesized that plasmid loss was a recurrent process during the stressostat cultivation, resulting in the presence of such variants throughout the cultivation despite washout. The strict selection on our lactate containing CDM plates resulted in favorable conditions for the mixed acid variants despite having a lower fitness and abundance in

the population. This outcome was undesirable and demonstrated how the choice of isolation plate can affect the outcome of ALE.

In the evolution experiments, the possible evolutionary trade-off is an important factor to consider. Trade-offs occur at the single protein level (stability vs activity), at the whole proteome level (stress protein vs growth protein), and at the cellular level (growth rate vs functionality), all of which can change the phenotype of the evolved microorganism [8]. Various phenotypes were observed in the isolated variants of the stressostat. One key phenotype of the variants (except cluster 4) was the higher maximum growth rate (μ_{max}) compared to the wild type at high lactate concentrations, but significantly lower μ_{max} compared to the wild type in a control medium without lactate (Suppl. Fig. 4). This could be caused by adaptation at either the whole proteome level (e.g., more stress-related proteins compared to growth-related proteins) or the single protein level (e.g., improved enzyme activity in osmotic stress). The trade-off between growth rate and functionality in *L. lactis* has been reviewed and it has been shown that the growth rate in *L. lactis* affects cellular metabolism, physiology, and robustness [8,26–28]. For example, a high growth rate could negatively affect flavor formation [29] and freezing survival [30], which are both important characteristics for the application of starter cultures or their production, respectively. Therefore, careful assessment of the functionalities of stressostat variants is required to ensure that the variants with improved growth performance can still function as starter culture in the food industry.

To conclude, it was demonstrated that the stressostat can be applied as novel adaptive evolution tool to select for variants with increased end-product resistance. In particular, it was shown that stressostat cultivation can select for variants of *L. lactis* with improved growth at high lactate concentrations. These variants also produced more biomass during pH-controlled batch cultivations. Follow-up research is required to elucidate the resistance mechanisms of the variants and their functionalities in industrial applications. In addition, application of the stressostat to other microorganisms, in particular those with other end-products, remains to be explored.

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Declaration of Competing Interest

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Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2023.10.010.

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