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Evolutionary engineering reveals divergent paths when yeast is adapted to different acidic environments

Eugene Fletcher[a,c], Amir Feizi[a,c], Markus M.M. Bisschops[a,c], Björn M. Hallström[b], Sakda Khoomrung[a,c], Verena Siewers[a,c], Jens Nielsen[a,b,c,d,*]

[a] Department of Biology and Biological Engineering, Chalmers University of Technology, Kemivägen 10, SE-412 96 Gothenburg, Sweden
[b] Science for Life Laboratory, Royal Institute for Technology, SE-171 21 Stockholm, Sweden
[c] Novo Nordisk Foundation Center for Biosustainability, Chalmers University of Technology, Kemivägen 10, SE-412 96 Gothenburg, Sweden
[d] Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kemitorvet Bygning 220, DK-2820 Lyngby, Denmark

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ABSTRACT

Tolerance of yeast to acid stress is important for many industrial processes including organic acid production. Therefore, elucidating the molecular basis of long term adaptation to acidic environments will be beneficial for engineering production strains to thrive under such harsh conditions. Previous studies using gene expression analysis have suggested that both organic and inorganic acids display similar responses during short term exposure to acidic conditions. However, biological mechanisms that will lead to long term adaptation of yeast to acidic conditions remains unknown and whether these mechanisms will be similar for tolerance to both organic and inorganic acids is yet to be explored. We therefore evolved *Saccharomyces cerevisiae* to acquire tolerance to HCl (inorganic acid) and to 0.3 M L-lactic acid (organic acid) at pH 2.8 and then isolated several low pH tolerant strains. Whole genome sequencing and RNA-seq analysis of the evolved strains revealed different sets of genome alterations suggesting a divergence in adaptation to these two acids. An altered sterol composition and impaired iron uptake contributed to HCl tolerance whereas the formation of a multicellular morphology and rapid lactate degradation was crucial for tolerance to high concentrations of lactic acid. Our findings highlight the contribution of both the selection pressure and nature of the acid as a driver for directing the evolutionary path towards tolerance to low pH. The choice of carbon source was also an important factor in the evolutionary process since cells evolved on two different carbon sources (raffinose and glucose) generated a different set of mutations in response to the presence of lactic acid. Therefore, different strategies are required for a rational design of low pH tolerant strains depending on the acid of interest.

1. Introduction

Microbial hosts used in industrial processes encounter extreme environments, often including low pH, which severely inhibit their growth and productivity. This is particularly the case during fermentation processes where the yeast cells are washed in an aqueous solution of an inorganic acid to eliminate bacterial contamination and are recycled for the next batch of fermentation (De Melo et al., 2010). Alternatively, fermentations can be carried out at low pH to prevent bacterial contamination (Kádár et al., 2007). Furthermore, stress at low pH is a key factor to consider in the design of a consolidated bioprocess involving harsh acid pre-treatment of lignocellulosic feedstocks to release fermentable sugars for conversion into industrially relevant products such as ethanol or lactic acid (Brodeur et al., 2011). Ethanol serves as an important biofuel whereas lactic acid is a key platform chemical that can be converted to biodegradable plastics and other valuable products. Major investments have gone into lactic acid production with an annual global production estimated to reach one million tons by 2020 (Jem et al., 2010), and following this success there is much interest in the production of other organic acids that can be used for polymer production, e.g. succinic acid and 3-hydroxypropionic acid (Chen and Nielsen, 2016), and the yeast *Saccharomyces cerevisiae* is one of the preferred platform cell factories for producing these chemicals (Nielsen, 2015; Chen and Nielsen, 2016). Microbial production of organic acids, however, is challenging since the pKa of these acids (3.86 for lactic acid; 4.21 for succinic acid; 4.51 for 3-hydroxypropionic acid) is below the optimal pH (pH 5.0–5.5) for growth of *S. cerevisiae* (Verduyn et al., 1990). Thus, large amounts of neutralizing agents such as $CaCO_3$ are required to control the pH of the bioreactors which in effect lead to generation of large amounts of gypsum waste,

* Corresponding author at: Department of Biology and Biological Engineering, Chalmers University of Technology, Kemivägen 10, SE-412 96 Gothenburg, Sweden.
E-mail address: nielsenj@chalmers.se (J. Nielsen).

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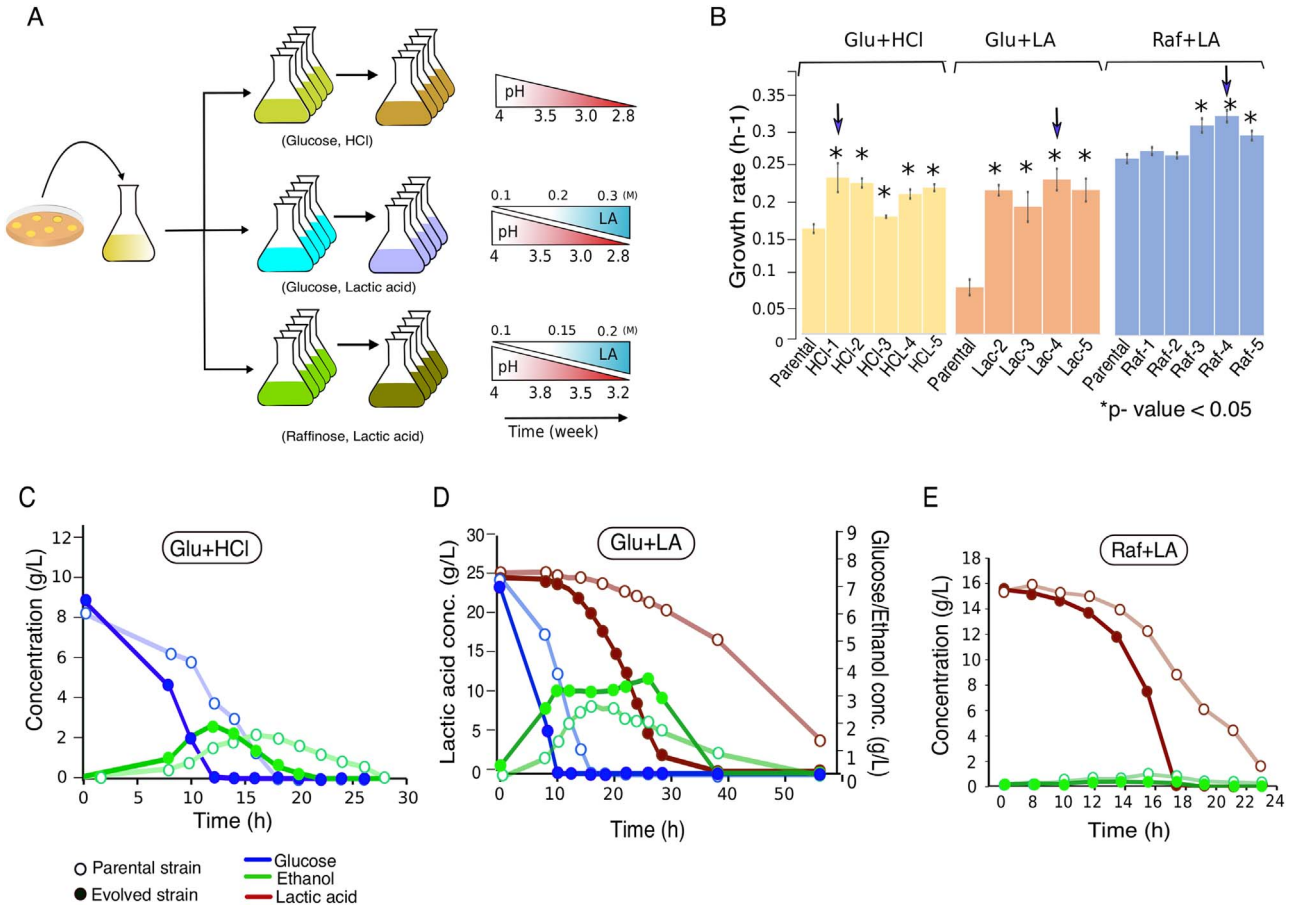

Fig. 1. Overview of evolution experiment and characterization of tolerant strains. Three parallel lines of evolution (Glu+HCl, Glu+LA and Raf+LA) were started from an isogenic yeast population. During the evolution experiment, the pH of the cultures was dropped stepwise from pH 4 while the concentration of lactic acid (LA) was increased in the cultures evolving on lactic acid starting with a concentration of 88 mM. The time scale reflects the course of the evolution experiment (A). Bar plots represent specific growth rates of the evolved strains isolated at the end of the evolution experiment at low pH (and high lactic acid concentrations) in comparison to the parental strain. The arrows indicate the top performing strains (HCl-1, Lac-4 and Raf-4). The error bars represent the standard deviation of three biological replicates (B). The plots in C-E show batch fermentation in bioreactors of HCl-1 in minimal media (pH 2.8) with glucose as the carbon source (C), Lac-4 in minimal medium (pH 2.8) containing glucose as the carbon source and 0.3 M lactic acid (D) and Raf-4 in minimal media containing raffinose as the carbon source and 0.2 M lactic acid (E).

making downstream processing costly (Datta and Henry, 2006). Even though yeast can grow at low pH organic acids are toxic at these pH values as the undissociated form diffuses freely into the cytoplasm of the cell where it dissociates (due to the higher pH of the cytoplasm) and thereby acidifies the cytoplasm (Narendranath et al., 2001). Furthermore, accumulation of the anionic form of the acid following dissociation in the cytoplasm also results in a considerable amount of toxicity (Abbott et al., 2008). Hence, the cells employ an energy-intensive mechanism to maintain proton homeostasis by extruding excess intracellular protons to the extracellular space or into the vacuoles by using membrane H^+-ATPases, which drains the cell of its free energy (ATP) and compromises cell growth (Zhou et al., 2011).

While this process of proton efflux from cells by membrane H^+-ATPases is well-established, recent efforts have elucidated other mechanisms of tolerance to low pH and organic acids, particularly acetic acid, at the molecular level using transcriptomic analysis (Abbott et al., 2008), functional screening (Kawahata et al., 2006a) and quantitative trait locus (QTL) analysis (Geng et al., 2016) in both yeasts and bacteria. By using these approaches several genes that encode metabolic enzymes (*sdhABCD*) or proteins involved in biofilm formation (*gatAB*) were found to be highly upregulated in bacteria (Kannan et al., 2008). While in yeasts, upregulation of metal metabolism (Aft1p), a weak acid transcriptional regulator (Haa1p), proline and glutathione production, among others, have been shown to be linked with acid stress response (Kawahata et al., 2006a; Mira et al., 2010; Nugroho et al., 2015; Meijnen et al., 2016). An acid stress response involves rapid changes in gene expression as a consequence of short term exposure to acidic conditions (Kawahata et al., 2006a). Some of these upregulated genes may not necessarily lead to prolonged tolerance to acidic conditions. For example, the cell wall glycoprotein gene *SED1* and the weak acid inducible multidrug transporter gene *PDR12* have been shown to be upregulated during acid shock response but a depletion of these genes was beneficial for resistance to acidic conditions in *S. cerevisiae* (Kawahata et al., 2006a). Therefore, identifying mechanisms involved in long term tolerance to low pH will be crucial for engineering microbial cell factories to thrive and be productive under acidic conditions.

Microbial populations can adapt to long term exposure to extreme environments by acquiring key driver mutations to alter specific biological processes associated with the stress as illustrated by the ability of yeast to evolve to grow at increased temperatures (Caspeta et al., 2014), in the presence of inhibitory compounds (Tomás-Pejó et al., 2010), acetic acid (Narayanan et al., 2016) and in high ethanol concentrations (Stanley et al., 2010). We were, therefore, interested in probing the possibility of adapting yeast to grow at low pH both in the absence and in the presence of an organic acid, and further identify the possible molecular mechanisms underlying such an adaptation. Hence, we evolved *S. cerevisiae* to grow in the presence of HCl and in high concentrations of L-lactic acid at low pH and determined whether the adaptive mechanism to these two acids were the same since they both induce an acid stress response (Brandao et al., 2014).