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Outline of Research Result

Creation and Characterization of Lactic Acid Tolerant Yeasts for Efficient Production of Lactic Acid

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Research aim

Plant- and crop-based renewable plastics including polylactic acid have been receiving increasing attention as carbon neutral materials that contribute to reduced atmospheric CO_2 emissions. *Saccharomyces cerevisiae* can grow and survive at a pH lower than the lactic acid bacteria that are widely used for production of lactic acid as a monomer for polylactic acid. This offers the potential to reduce cost because the process of desalination of lactate is costly. A genetically-engineered *S. cerevisiae* strain was recently evaluated for efficient production of L-lactic acid and was found to produce high levels in the absence of pH control[1]. However, it was also observed that production was lower at pH values of ≤2.8. To increase production of lactic acid without a requirement for pH control, the acid tolerance of *S. cerevisiae* must be enhanced. To this end, research was undertaken to isolate lactic acid-tolerant strains using the following approaches. i) Isolation and characterization of multicopy genes that enhance lactic acid tolerance, ii) evaluation of a screening method to select strains with enhanced tolerance by chromosome splitting, and iii) identification of genes required for lactic acid resistance.

Methods

A yeast genomic DNA library in a YEp13 multicopy vector (ATCC 37323) was used to screen for genes conferring lactic acid resistance when overexpressed. Chromosome splitting was performed according to a PCR-mediated Chromosome Splitting (PCS) method[2] that allows repeated splitting of chromosomes by a 2-step PCR procedure followed by a single transformation. Lactic acid tolerance and sensitivity were assayed by spotting cells on YPDA media containing 6.5% and 4% (w/v) L-lactic acid, respectively.

Results

i) Isolation and characterization of genes that enhance lactic acid tolerance when overexpressed.

Based on the premise that specific overexpressed genes will confer protection from damage caused by high concentrations of lactic acid, tolerant multicopy transformants of a sensitive parent strain were sought. *S. cerevisiae* strain BY4742 was transformed with the YEp13-based multicopy genomic library and transformants were screened for tolerance to 6% lactic acid (pH 2.6). Of over 60,000 transformants screened, 61 tolerant strains were isolated. Three genomic regions in plasmids were identified that conferred lactic acid tolerance. Subcloning revealed that *WHI2*, *HAA1*, and *ESBP6* were responsible for the lactic acid tolerance. *WHI2*, *HAA1*, and *ESBP6* encode a factor required for full activation of the general stress response, a transcription factor for genes encoding multidrug resistance permeases, and a monocarboxylate permease-like protein, respectively.

To examine whether *WHI2*, *HAA1*, and *ESBP6* are required for normal tolerance to lactic acid, we constructed Δ*whi2*, Δ*haa1*, and Δ*esbp6* strains and analyzed the effect of *WHI2*, *HAA1*, and *ESBP6* disruptions on 5% lactic acid media. The disruption mutants exhibited growth defects on 5% lactic acid media although no growth defect was seen in the absence of lactic acid. To determine if these genes also confer increased tolerance to other stresses, multicopy transformants were exposed to heat shock, oxidative (H_2O_2) stress, acetic acid, ethanol, and high salt (NaCl). Only transformants with multicopy *HAA1* exhibited acetic acid tolerance, suggesting that *WHI2*, *HAA1*, and *ESBP6* are specifically implicated in the acid stress response.

HAA1 and *ESBP6* alleles were constructed under the control of the *TDH3* promoter and were expressed at their respective chromosomal loci. The resulting strains exhibited lactic acid tolerance, suggesting that overproduction of proteins encoded by *HAA1* and *ESBP6* confer tolerance, likely resulting from hyperactivation of the lactic acid stress response.

ii) Evaluation of a chromosome splitting-based screening method to select strains highly tolerant to lactic acid.

We and others have identified gene disruption mutants that exhibit lactic acid tolerance by screening the *S. cerevisiae* nonessential gene knockout collection. A chromosome splitting method was applied to evaluate the effect of combining deletions of *SED1*, *DSE2*, *EAF3*, *SCW11*, and *IRC8* that individually confer high lactic acid tolerance.

Short chromosomal fragments (7–31 kb), containing the individual genes noted above were converted into minichromosomes by splitting. A yeast strain having 5 minichromosomes was constructed and sub-cultured in media containing lactic acid to induce minichromosome loss based on the

observation that chromosomes less than 50 kb exhibit high mitotic instability. Cultures were plated on 4% lactic acid media and clones giving rise to the largest colonies were selected and evaluated for loss events. Of the 50 clones tested thus far, all were found to have lost only the *IRC8*-containing minichromosome and to exhibit modest lactic acid tolerance relative to wild-type. However, a Δ*sed1* Δ*dse2* Δ*eaf3* Δ*scw11* Δ*irc8* quintuple disruptant constructed as a control strain by conventional methods was found to have higher lactic acid tolerance than a Δ*irc8* single disruptant, suggesting that the other minichromosomes contained genes required for lactic acid tolerance and thus were not lost.

iii) Identification of genes required for lactic acid resistance.

In order to identify additional genes required for lactic acid tolerance, a *S. cerevisiae* nonessential gene knockout collection was screened for sensitivity to 4% lactic acid. Disruption of more than 180 genes was found to result in differential sensitivity to 4% lactic acid. Although the genes found necessary for resistance are functionally diverse, they were also found to function in shared processes, including telomere maintenance, vacuole organization and acidification, vesicle-mediated transport, signal transduction, chromatin modification, and lipid metabolism. These results suggest that these functional units comprise the lactic acid stress response pathway, and that functional enhancement of these components may further improve tolerance.

Conclusions

Three approaches were used to isolate and construct lactic acid tolerant strains of *S. cerevisiae*. i) Overexpression of *WHI2*, *HAA1*, and *ESBP6* was found to enhance lactic acid tolerance. Stable lactic acid tolerant strains were constructed by chromosomal integration of constitutively-expressed alleles of *HAA1* and *ESBP6* under the control of the *TDH3* promoter. Evaluation of lactic acid production by these strains is in progress. ii) A screening method to select the most tolerant strains was evaluated by means of combinatorial loss of minichromosomes harboring 4 to 26 genes, including the five genes whose disruption was found to result in enhanced lactic acid tolerance. Because only one minichromosome was found to have been lost in strains recovered in the lactic acid tolerance screen, it was presumed that all the other minichromosomes harbored genes necessary for tolerance, indicating a quantitative trait. iii) Screening of a *S. cerevisiae* nonessential gene knockout collection revealed that more than 180 genes are involved in conferring lactic acid resistance. These new findings not only provide a better understanding of the cellular response to lactic acid, but will also contribute to efforts to breed strains with increased tolerance for the purpose of using *S. cerevisiae* to produce lactic acid.

References

1) Ishida, N., Saitoh, S., Tokuhiro, K., Nagamori, E., Matsuyama, T., Kitamoto, K., and Takahashi, H. Efficient production of L-lactic acid by metabolically engineered *Saccharomyces cerevisiae* with a genome-integrated L-lactate dehydrogenase gene. *Appl. Environ. Microbiol.* **71**(4): 1964–1970 (2005).
2) Sugiyama, M., Ikushima, S., Nakazawa, T., Kaneko, Y., and Harashima, S. PCR-mediated repeated chromosome splitting in *Saccharomyces cerevisiae*. *Biotechniques* **38**(6): 909–914 (2005).