Doctoral Dissertation

Strategy for enhancement of lactic-acid resistance of *Saccharomyces cerevisiae* to produce lactic acid without neutralization

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Chapter 1

General Introduction

1.1 Lactic acid production

Lactic acid (2-hydroxypropionic acid, $CH_3CHOHCOOH$) is widely used in foods (acidulants, preservatives, flavors, pH regulators, etc.), cosmetics (moisturizers, skin-lightening agents, etc.), pharmaceutical agents (mineral preparations, dialysis solution, etc.), and industrial chemical products (descaling agents, chiral intermediates, etc.). Lactic acid is also one of the major metabolic intermediates in most organisms from prokaryotes to human (Vickroy, 1985; Datta *et al.*, 1995). Lactic acid was first isolated from sour milk by Scheele, C. W. in 1780 and was commercially produced in 1881 by Avery, C. E. in Littleton, Massachusetts, USA (Vickroy, 1985). Lactic acid can be produced by chemical synthesis or carbohydrate fermentation. Chemical synthesis is mainly by hydrolysis of lactonitrile using a strong acid; this method produces a racemic mixture of D- and L-lactic acid. Production of lactic acid by fermentation has several advantages compared to chemical synthesis, such as low cost of substrates, low production temperature, low energy consumption, and high product specificity, *i.e.*, it can produce the desired stereoisomer, either pure L-(+)- or D-(−)-lactic acid, in an optically pure form (Pandey *et al.*, 2001).

The commercial production of lactic acid uses homolactic bacteria, such as *Lactobacillus delbrueckii*, *L. bulgaricus*, and *L. leichmanii* (Datta *et al.*, 1995). Homolactic bacteria almost exclusively produce a single fermentation product, *i.e.*, lactic acid, whereas heterolactic bacteria produce a mix of products including ethanol, diacetyl, formate, acetoin, acetic acid, and carbon dioxide along with lactic acid. Homolactic- and heterolactic bacteria utilize pyruvic acid, which is the end product of Embden–Meyerhof pathway, and convert it to lactic acid. L- or D-lactate dehydrogenase (LDH) plays a key role in this conversion. Lactic-acid yields of approximately 90% (w/w) from a glucose equivalent of carbohydrate are obtained. The pH of the culture medium drops during fermentation, affecting growth of the bacteria and their productivity of L-lactic acid. To counteract this, calcium carbonate is added to the culture medium to neutralize the pH during fermentation by producing calcium lactate. The culture solution containing calcium lactate is filtered to remove cells with carbon treatment, evaporated, and acidified with sulfuric acid to convert the calcium lactate salt into lactic acid and a precipitate of calcium sulfate, which is removed by filtration. The filtrate is further purified over carbon columns and ion exchange, and subsequently evaporated to produce technical- and food-grade lactic acid. The technical-grade lactic acid is esterified and the ester is recovered by distillation, hydrolyzed and evaporated, producing a highly pure product.

1.2 Poly-lactic acid and a novel method of lactic-acid production

The application of lactic acid to its polymer is recently desired. Poly-lactic acid is a semicrystalline polymer has high tensile strength, low elongation with high modulus, a melting point of 175–178°C, and slow degradation time. These physical properties are suitable for medical applications (orthopedic, vascular, dental, and intestinal application and sutures, etc.) (Wee *et al.*, 2006). Furthermore, the application of this polymer to environmental-friendly alternative products is recently desired because shift of petroleum-based society to carbon-neutral society is needed to solve serious problems of global warming and escalating petroleum costs. Poly-lactic acid is being developed as a renewable alternative to conventional petroleum-based plastics (Arntzen and Dale, 1999; Chotani *et al.*, 2000; Ohara *et al.*, 2001). Poly-lactic acid has been receiving increasing attention as a carbon neutral material that can contribute to reducing atmospheric CO_2 emissions. Although this type of plastic should be applicable globally to products such as films and chassis, among others, the relatively high production costs of producing poly-lactic-acid plastics in comparison to petroleum-based plastics such as polyethylene prevented their widespread application.

Lactic-acid bacteria can produce lactic acid in high yields of more than 0.9 g lactic acid per gram of glucose consumed (Wee *et al.*, 2006), based on a maximum theoretical yield of 1.0 g lactic acid per gram of glucose. However, the optical purity of the lactic acid produced by some lactic-acid bacteria is not 100% because some lactic-acid bacteria have both the L-*LDH* and D-*LDH* genes (Hofvendahl and Hahn-Hägerdal, 2000). The low optical purity is not suitable for the regulation of the physical properties of poly-lactic acid (Tsuji, 2002). It was also reported that

stereocomplex-type poly lactic acid has a high melting temperature point compared with L- or D-homopolymers (Ikada *et al.*, 1987). However, because poly-DL-lactic acid is not crystalline and soften at a low glass-transition point (54°C) (Tsuji, 2002), it is important that poly-L-lactic acid and poly-D-lactic acid of high optical purity are blended during the synthesis of the stereocomplex polymer. In addition, because of the difficulties in cultivating lactic-acid bacteria at high density and their highly auxotrophic growth requirements (Hofvendahl and Hahn-Hagerdal, 2000), some other organisms are now being used for lactic-acid production, for example, *Escherichia coli* (Dien *et al.*, 2001), *Rhizopus oryzae* (Bai *et al.*, 2008), and *Bacillus coagulans* (Sakai and Ezaki, 2006). It has been recognized that *Saccharomyces cerevisiae* has advantages for lactic-acid production and new methods for the large-scale production of L-lactic acid with engineered yeast have been developed (Dequin and Barre, 1994; Porro *et al.*, 1995). *S. cerevisiae* is more tolerant to low pH than lactic-acid bacteria (Skory, 2003) and can grow on inexpensive synthetic media. However, wild-type *S. cerevisiae* hardly produce lactic acid owing to the conversion of pyruvate to ethanol by pyruvate decarboxylase (PDC) and alcohol dehydrogenase; however, genetically engineered yeast with an introduced exogenous L-*LDH* gene can produce lactic acid through the glycolytic pathway (Dequin and Barre, 1994; Porro *et al.*, 1995; Ishida *et al.*, 2005). Genetically engineered yeast can produce very high optical purity of L-lactic acid (at least 99.9%) (Saitoh *et al.*, 2005). These advantages offer the potential to reduce cost because the engineered yeast can produce lactic acid without requiring the process of desalination of lactate. Although, as mentioned above, *S. cerevisiae* is more

tolerant to low pH than lactic-acid bacteria, the decrease in yield of L-lactic acid due to the decrease in culture pH resulting from the accumulation of lactic acid still occurs (Ishida *et al.*, 2005).

1.3 Effect of organic acid on cell mechanisms

In *S. cerevisiae*, the monocarboxylate permease Jen1 mediates the transport of lactic, pyruvic, acetic, and propionic acid (Casal *et al.*, 1999) but is repressed in the presence of glucose (Chambers *et al.*, 2003). It has been suggested that undissociated lactic-acid molecule (pK_a = 3.86) freely diffuse through the plasma membrane (Cássio *et al.*, 1987). Under acidic conditions, undissociated organic acids can diffuse through the plasma membrane and acidify the cytoplasm following its dissociation inside the cell (Mollapour *et al.*, 2008). The dissociated anions accumulate in the cytoplasm, their negative charge prevents them from easily crossing the plasma membrane, and such a high anion pool can potentially generate abnormally high turgor pressure (Hatzixanthis *et al.*, 2003). Cells can counteract this intracellular acidification by extruding protons through the plasma membrane ATPase, but this is performed at the expense of ATP and leads to a reduction in growth yield (Viegas *et al.*, 1998).

Organic-acid stress is not only because of the toxic effect of a high hydrogen ion concentration, but also depends on the specific chemical nature of the organic acid to which the organism is exposed (Bayrock and Ingledew, 2004). For example, acetic acid is far less inhibitory than the more lipophilic sorbic acid, even though these two carboxylate compounds have a pK_a = 4.76 (Piper *et al.*, 1998;

Bracey *et al.*, 1998). In addition, benzoic acid inhibits nitrogen starvation-induced macroautophagy, whereas sorbic acid does not (Hazan *et al.*, 2004). Because of the effects of low extracellular pH on the cell wall, inhibition of growth by weak-organic acids has been proposed to be due to a number of actions, including membrane disruption, inhibition of essential metabolic reactions, stress on intracellular pH homeostasis, and the accumulation of toxic anions (Holyoak *et al.*, 1999).

1.4 Response to weak-organic acid

The adaptation and resistance of *S. cerevisiae* to weak-organic acids involves several mechanisms. (i) The plasma membrane H^+-ATPase, Pam1, pumps protons out of the cell in an ATP-dependent manner (Eraso and Gancedo, 1987; Viegas and Sà-Correia, 1991). Under acidic conditions, the vacuolar ATPase also plays a role in cytosolic pH homeostasis (Kane, 2006). (ii) The transcription factors Msn2/Msn4 are involved in the general stress response (Schüller *et al.*, 2004). Most genes up-regulated by Msn2 and Msn4 in response to weak acid stress encode proteins of the environmental stress response such as molecular chaperones (*e.g.*, Hsp26, Sse2), enzymes involved in carbohydrate metabolism (*e.g.*, Hxk1, Gpd1), and antioxidant defense system (*e.g.*, Ctt1, Gpx1) (Schuller *et al.*, 2004; Simões *et al.*, 2006). (iii) War1 is required for weak acid-induced transcriptional activation of *PDR12*, which encodes a plasma membrane ABC efflux pump and is presumably involved in the efflux of benzoic and sorbic acid (Piper *et al.*, 1998; Piper *et al.*,

2001; Schüller *et al.*, 2004). Pdr12 catalyzes the active efflux of weak organic-acid anions from the cytosol and mediates broad resistance to water-soluble weak acids, including monocarboxylic acids of aliphatic chain length from C1–7 (Holyoak *et al.*, 1999). (iv) Extracellular undissociated acetic acid enters glucose-repressed cells primarily by facilitated diffusion through the plasma membrane aquaglyceroporin Fps1 (Mollapour and Piper, 2007). Hog1 is transiently activated by acetic-acid stress (Mollapour and Piper, 2006) and activated Hog1 directly phosphorylates Fps1 (Mollapour and Piper, 2007). This phosphorylation is the signal for Fps1 to become ubiquitinated and endocytosed into the vacuole (Mollapour and Piper, 2007). This removal of Fps1 from the plasma membrane seems to be essential for downregulating the acetic-acid influx to the cell (Mollapour and Piper, 2007). (v) Haa1, a transcription factor, is required for adaptation and resistance to low pH, particularly to the acids that are more hydrophilic, such as acetic and propionic acids (Fernandes *et al.*, 2005). A number of genes are activated directly or indirectly by Haa1 in response to acetic acid (Mira *et al.*, 2010). Expression of *SAP30* and *HRK1* gene is the most significant protective effect (Mira *et al.*, 2010). *SAP30* encodes a subunit of the Rpd3L histone deacetylase complex, whereas *HRK1* encodes a protein kinase dedicated to the posttranslational regulation of plasma membrane transporters (Goossens *et al.*, 2000). Other Haa1-regulated genes that encode the drug:H^+ antiporters Tpo2 and Tpo3 which propose to mediate acetate export (Fernandes *et al.*, 2005) and the cell wall-related protein Ygp1 which may contribute to the acid-induced remodeling of the cell-wall structure (Fernandes *et al.*, 2005) also confer resistance to acetic acid.

(vi) Rim101, which is involved in response to alkaline pH and cell-wall assembly is also required for resistance to benzoic, propionic, and acetic acid (Mira *et al.*, 2009). For example, Rim101 was found to regulate a small subset of the genes transcriptionally activated in response to propionic acid, including *KNH1* and *CWP1* which are involved in cell-wall integrity, and the uncharacterized gene *YIL029c* (Mira *et al.*, 2009).

1.5 Objective of the study

The fermentation of lactic acid under low pH leads to minimization of the risk of contamination (van Maris *et al.*, 2006) and elimination of the necessity for neutralization process. To increase the productivity of L-lactic acid without requiring pH control, the resistance of *S. cerevisiae* to L-lactic acid must be enhanced. To uncover the detailed mechanisms underlying the adaptation of *S. cerevisiae* to lactic-acid stress, I perform a functional genomic analysis. This study deals with the response to lactic acid among weak organic acids and the finding in this study may be applicable to the response of other weak organic acids.

In chapter 1, I describe the background information for this study: lactic-acid production and its effects on the cell. In chapter 2, I systematically screen for the yeast haploid collection for single disruptants that are resistant to 6% lactic acid, construct the lactic acid resistant strains, and then evaluate the lactic-acid productivity of these resistant strains in the absence of neutralization. Because there is currently no effective method for enhancing the lactic-acid resistance of *S. cerevisiae*, I adapt a way of the combining gene disruptions which cause lactic-acid

resistance, expecting improvement of the lactic-acid resistance. In chapter 3, I systematically screen the single disruptants that are hypersensitive to lactic acid stress and attempt to examine the intracellular mechanisms which are important for adaptation to lactic acid stress. In chapter 4, I summarize the results obtained in this study and discuss the validity of strain constructed in this study for lactic-acid production and the approach to further enhancement of lactic-acid resistance.

Chapter 2

Disruption of multiple genes whose deletion causes lactic-acid resistance improves lactic-acid resistance and productivity in *S. cerevisiae*

2.1 Introduction

While wild-type *S. cerevisiae* produces very little lactic acid, genetically engineered yeast harboring an exogenous L-lactate dehydrogenase gene (L-*LDH*) can produce lactic acid through the glycolytic pathway (Dequin and Barre, 1994; Porro *et al.*, 1995; Ishida *et al.*, 2005). A genetically engineered strain that has L-*LDH* also produces ethanol (Porro *et al.*, 1995; Adachi *et al.*, 1998). Because pyruvic acid is consumed to produce ethanol during fermentation, the production of ethanol will affect any improvement in lactic-acid yield. In *S. cerevisiae*, the pyruvate decarboxylase genes *PDC1*, *PDC5*, and *PDC6* are responsible for conversion of pyruvic acid into ethanol. Pdc1 is the main PDC that functions during glucose fermentation, whereas Pdc5 is expressed only in the absence of *PDC1* (Seeboth *et al.*, 1990) or under thiamine limitation (Muller *et al.*, 1999). Pdc6 is not expressed during glucose fermentation and relates to the activity of pyruvate decarboxylase in an ethanol-based medium (Hohmann[a], 1991). It has been reported that disruption of *PDC1* by replacement with L-*LDH* results in the efficient production of L-lactic acid (Ishida *et al.*, 2005). To increase the productivity of L-lactic acid without relying on pH control, the L-lactic acid

resistance of *S. cerevisiae* must be enhanced. However, findings about the mechanisms of adaptation to lactic acid are limited. For example, it was reported that the concentration of intracellular ATP was decreased by lactic acid (Thomsson and Larsson, 2006), and *FIT2*, *ARN1* and *ARN2*, involved in metal metabolism regulated by Aft1 which is a transcription factor that responds to intracellular iron, were induced by lactic-acid stress (Kawahata *et al.*, 2006). In addition, the transcription factor Ace2 and Swi5 involved in cell cycle progression from M to G1 and target genes of transcription factor Haa1, *TPO2*, *TPO3*, *YGP1*, and *YRO2*, were highly induced by lactic acid while Haa1 was not strongly induced by lactic acid and disruptants of these target genes had no effect of growth rates in the presence of lactic acid (Abbott *et al.*, 2008).

To identify the cellular mechanisms which prevent the exhibition of lactic-acid resistance toward construction of superior lactic-acid resistant yeast, I performed functional genomic analysis. I found that 94 gene disruptants showed resistance to 6% lactic acid and that lactic-acid resistance was further enhanced by combining the disruption of several genes. I have evaluated the effect of those multiple disruptions on the production of lactic acid and found that, when those disruptions were incorporated in a strain with an exogenous bovine L-*LDH* gene, quadruple disruptant (*Δdse2Δscw11Δeaf3Δsed1*) showed improved productivity of lactic acid as compared with the wild-type strain. The findings in this chapter strongly suggest that a combination of disruptions that show lactic-acid resistance is an effective approach to improve lactic-acid resistance and that this approach contributes to improvements in lactic-acid productivity without neutralization.

2.2 Materials and Methods

2.2.1 Strains and media

The nonessential genes deletion collection (Openbiosystems, Huntsville, AL, USA), which contains 4828 yeast strains with all nonessential ORFs disrupted by the *kanMX4* cassette (http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html), was used to screen for disruptants that showed superior lactic-acid resistance. Other strains used in this chapter are listed in Table 1. The parental strain of the deletion collection, BY4742 (*MATα leu2Δ0 his3Δ1 ura3Δ0 lys2Δ0*) (Brachmann *et al.*, 1998), was used as a wild-type strain. The standard complete medium YPDA consisted of 1% (w/v) yeast extract (Sigma-Aldrich, St. Louis, MO, USA), 2% (w/v) peptone (Sigma-Aldrich, St. Louis, MO, USA), 2% (w/v) glucose (Sigma-Aldrich, St. Louis, MO, USA) and 0.04% (w/v) adenine (Wako Pure Chemical Industries, Osaka). L-Lactic acid (NACALAI TESQUE, Kyoto) was added to YPDA after autoclaving. Agar (2%) was added to media to make culture plates.

Table 1. Yeast strains used in this chapter.

Yeast strains	Genotype	Origin
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Euroscarf
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
SH6704	*MATα Δdse2::kanMX his3Δ1 leu2Δ0 ura3Δ0*	OpenBio systems
SH6705	*MATα Δscw11::loxP-CgLEU2-loxP his3Δ1 leu2Δ0 ura3Δ0*	This chapter

SH6706	*MATα Δeaf3::loxP-CgHIS3-loxP his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0*	This chapter
SH6707	*MATα Δsed1::loxP*-zeocin-*loxP his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0*	This chapter
SH6708	*MATα Δdse2::kanMX Δscw11::loxP-CgLEU2-loxP his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	This chapter
SH6709	*MATα Δdse2::kanMX Δeaf3::loxP-CgHIS3-loxP his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	This chapter
SH6710	*MATα Δdse2::kanMX Δsed1::loxP*-zeocin-*loxP his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*	This chapter
SH6711	*MATα Δscw11::loxP-CgLEU2-loxP Δeaf3::loxP-CgHIS3-loxP his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0*	This chapter
SH6712	*MATα Δscw11::loxP-CgLEU2-loxP Δsed1::loxP*-zeocin-*loxP his3Δ1 leu2Δ0 ura3Δ0*	This chapter
SH6713	*MATα Δeaf3::loxP-CgHIS3-loxP Δsed1::loxP*-zeocin-*loxP his3Δ1 leu2Δ0 ura3Δ0*	This chapter
SH6714	*MATα Δdse2::kanMX Δscw11::loxP-CgLEU2-loxP Δeaf3::loxP-CgHIS3-loxP his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	This chapter
SH6715	*MATα Δdse2::kanMX Δscw11::loxP-CgLEU2-loxP Δsed1::loxP-zeocin-loxP his3Δ1 leu2Δ0*	This chapter
SH6716	*MATα Δdse2::kanMX Δeaf3::loxP-CgHIS3-loxP Δsed1::loxP*-zeocin-*loxP his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*	This chapter
SH6717	*MATα Δscw11::loxP-CgLEU2-loxP Δeaf3::loxP-CgHIS3-loxP Δsed1::loxP*-zeocin-*loxP his3Δ1 leu2Δ0 ura3Δ0*	This chapter
SH6718	*MATα Δdse2::kanMX Δscw11::loxP-CgLEU2-loxP Δeaf3::loxP-CgHIS3-loxP Δsed1::loxP*-zeocin-*loxP his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*	This chapter
SH6764	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 Δpdc1::LDH-HPH*	This chapter
SH6765	*MATα Δdse2::kanMX his3Δ1 leu2Δ0 ura3Δ0 Δpdc1::LDH-HPH*	This chapter
SH6766	*MATα Δscw11::loxP-CgLEU2-loxP his3Δ1 leu2Δ0 ura3Δ0 Δpdc1::LDH-HPH*	This chapter
SH6767	*MATα Δeaf3::loxP-CgHIS3-loxP his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 Δpdc1::LDH-HPH*	This chapter
SH6768	*MATα Δsed1::loxP*-zeocin-*loxP his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 Δpdc1::LDH-HPH*	This chapter
SH6769	*MATα Δdse2::kanMX Δscw11::loxP-CgLEU2-loxP his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 Δpdc1::LDH-HPH*	This chapter
SH6770	*MATα Δdse2::kanMX Δeaf3::loxP-CgHIS3-loxP his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 Δpdc1::LDH-HPH*	This chapter
SH6771	*MATα Δdse2::kanMX Δsed1::loxP*-zeocin-*loxP his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 Δpdc1::LDH-HPH*	This chapter

SH6772	*MATα Δscw11::loxP-CgLEU2-loxP Δeaf3::loxP-CgHIS3-loxP his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 Δpdc1::LDH-HPH*	This chapter
SH6773	*MATα Δscw11::loxP-CgLEU2-loxP Δsed1::loxP*-zeocin-*loxP his3Δ1 leu2Δ0 ura3Δ0 Δpdc1::LDH-HPH*	This chapter
SH6774	*MATα Δeaf3::loxP-CgHIS3-loxP Δsed1::loxP*-zeocin-*loxP his3Δ1 leu2Δ0 ura3Δ0 Δpdc1::LDH-HPH*	This chapter
SH6775	*MATα Δdse2::kanMX Δscw11::loxP-CgLEU2-loxP Δeaf3::loxP-CgHIS3-loxP his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 Δpdc1::LDH-HPH*	This chapter
SH6776	*MATα Δdse2::kanMX Δscw11::loxP-CgLEU2-loxP Δsed1::loxP*-zeocin-*loxP his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 Δpdc1::LDH-HPH*	This chapter
SH6777	*MATα Δdse2::kanMX Δeaf3::loxP-CgHIS3-loxP Δsed1::loxP*-zeocin-*loxP his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 Δpdc1::LDH-HPH*	This chapter
SH6778	*MATα Δscw11::loxP-CgLEU2-loxP Δeaf3::loxP-CgHIS3-loxP Δsed1::loxP*-zeocin-*loxP his3Δ1 leu2Δ0 ura3Δ0 Δpdc1::LDH-HPH*	This chapter
SH6779	*MATα Δdse2::kanMX Δscw11::loxP-CgLEU2-loxP Δeaf3::loxP-CgHIS3-loxP Δsed1::loxP*-zeocin-*loxP his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 Δpdc1::LDH-HPH*	This chapter

2.2.2 Construction of plasmid vectors

Integration vector pBHPH-PDC1P-LDHKCB (Fig. 1), which was based on pBTRP-PDC1P-LDHKCB plasmid (Ishida *et al.*, 2005), consist of the *PDC1* promoter, L-*LDH* from bovine (GeneBank No. D90141), *TDH3* terminator, hygromycin B phosphotransferase (*HPH*, GenBank No. K01193) (Gritz and Davies, 1983), and *PDC1*-3′ fragment. *HPH* fragment was isolated by PCR using the genomic DNA of the *Escherichia coli* K12 strain as a template and fused between the *S. cerevisiae* *TDH3* promoter and *CYC1* terminator. This fragment was replaced by *TRP1* fragment of pBTRP-PDC1P-LDHKCB plasmid. For construction of plasmid having *loxP*-zeocin-*loxP* cassette, zeocin resistant gene was amplified from pREMI-Z (van Dijk *et al.*, 2001) using Zeocin F (Table 2), containing a *Bgl*II restriction site (underlined) and Zeocin R (Table 2), containing a *Xho*I restriction site (underlined). The amplified fragment treated with *Bgl*II and *Xho*I, was ligated to pUG6 vector to yield plasmid pUG6-zeocin^r.

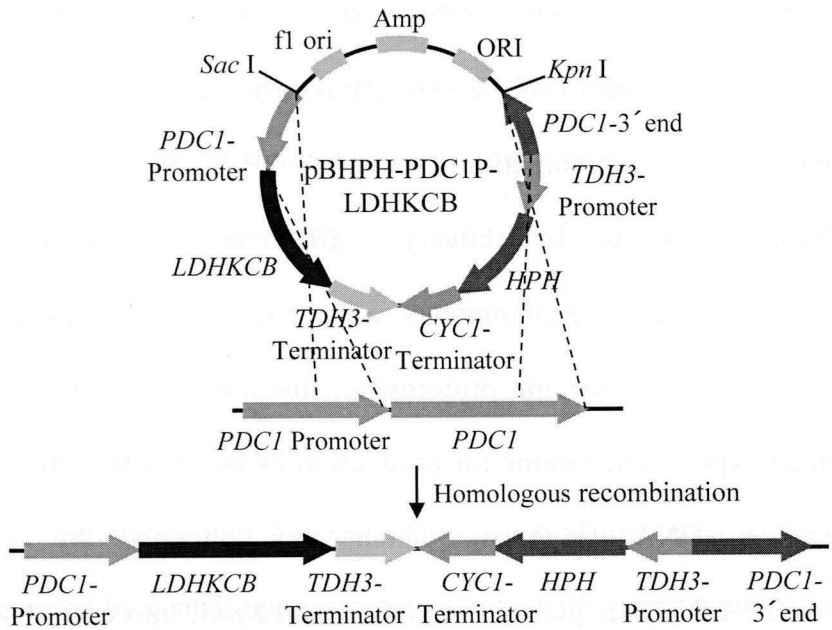

Figure 1. Structure of the plasmid pBHPH-PDC1P-LDHKCB and breeding of transgenic *S. cerevisiae*. The constructed DNA fragment, which was obtained by digesting the plasmid pBHPH-PDC1P-LDHKCB with *Sac* I and *Kpn* I, was integrated into the *PDC1* ORF region of the *S. cerevisiae* disruptant strains by homologous recombination.

2.2.3 Construction of single and multiple disruptants

For construction of single disruptants, *DSE2*, *SCW11*, *EAF3*, or *SED1* ORF was replaced with *kanMX4* (amplified from the genomic DNA of SH6704 using the primers DSE2 F and DSE2 R, Table 2), *loxP-CgLEU2-loxP* cassette [amplified from p3008 (Sugiyama *et al.*, 2005) using the primers SCW11 F and SCW11 R, Table 2], *loxP-CgHIS3-loxP* cassette [amplified from p3009 (Sugiyama *et al.*, 2005) using the primers EAF3 F and EAF3 R, Table 2], and *loxP*-zeocin-*loxP* cassette (amplified from pUG6-zeocin^r using the primers SED1 F and SED1 R, Table 2), respectively. Multiple disruptants were constructed by the following procedure: (i) *DSE2* and *SCW11* of BY4742 were disrupted with homologous recombination using *kanMX4*

and *loxP-CgLEU2-loxP* cassette, respectively. (ii) *EAF3* and *SED1* of BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) (Brachmann *et al.*, 1998) were disrupted with homologous recombination using *loxP-CgHIS3-loxP* cassette and *loxP*-zeocin-*loxP* cassette, respectively. (iii) These double disruptants were crossed, and multiple gene disruptants were constructed by tetrad analysis as showing G418 resistant, leucine protorophic, histidine protorophic, and/or zeocin resistant phenotype. The strains for production of lactic acid were constructed by transforming a DNA fragment, which was obtained by digesting the pBHPH-PDC1P-LDHKCB plasmid with *Sac*I and *Kpn*I, into each disruptant strain (Fig. 1).

Table 2. Sequence of the deoxy-oligonucleotides used in this chapter.

Oligo-nucleotide	Sequence
Zeocin F	5′-CTCAGATCTCCCACACACCATAGCTTCAA-3′
Zeocin R	5′-CTCCTCGAGCCAGCTTGCAAATTAAAGCC-3′
DSE2 F	5′-CAGAGTAGAAATAAAGCCACTCCTTTAACAAATTACAAAGAAATGCTTCGTACGCTGCAG-3′
DSE2 R	5′-GAAAACAAATAGAAAAGAATGCACCACCATTATGCCTAAGCGCTAGCCACTAGTGGATCT-3′
SCW11 F	5′-TTACGCTACACTCATTGATATAATATCTAATAGAAAACCATCATGCTTCGTACGCTGCAG-3′
SCW11 R	5′-CTGATCTTTTATATGCATGTTTCTCTATTCGATTTAGAAAACTTAGCCACTAGTGGATCT-3′
EAF3 F	5′-GTGAGGCCTCGTCACTGGATTTACCCTATTGAAGAACGTATAATGCTTCGTACGCTGCAG-3′
EAF3 R	5′-AACTAAATACTAGAAATAATCCCAAGCTAGAATATAAACGTCTCAGCCACTAGTGGATCT-3′
SED1 F	5′-ACTACAAAGACAAGCAAAATAAAATACGTTCGCTCTATTAAGATGCTTCGTACGCTGCAG-3′
SED1 R	5′-GAAAGAAAGCATTAAGAAGGCGGATGTGTCAAACACCACCGTTTAGCCACTAGTGGATCT-3′

Underline represents restriction site.

2.2.4 Screening for L-lactic-acid-resistant disruptants

To screen for L-lactic-acid-resistant strains, the nonessential genes deletion collection was stamped onto YPDA and 6% L-lactic acid plates with a 96-prong

replicator. Growth of the disruptants was scored visually by comparison with that of the parent strain (BY4742), which was stamped similarly onto YPDA and 6% L-lactic acid plates. Before screening, single-gene disruptants were tested for growth on YPDA medium containing 300 mg/l of G418 disulfate (Wako Pure Chemical Industries, Osaka).

2.2.5 Spot dilution growth assay

Yeast cells were grown in YPDA at 30°C to mid-log phase (OD_{660} = 1.0). Cells were harvested by centrifugation, washed once with water, and resuspended in water. The cell density was normalized to 1×10^6 cells/5 μl. A 10-fold serial dilution of this culture was made, and 5 μl of each dilution was spotted onto YPDA medium or lactic-acid medium. Cells were incubated at 30°C and scored visually by comparison with that of BY4742.

2.2.6 Measurement of lactic acid production

Fermentation experiments were performed according to the method of Ishida *et al.* (2005). The inoculum of each strain was prepared by cultivating overnight in YPDA 5 ml at 30°C. Overnight culture (OD_{660} = 1.2) 2 ml was transferred to 30 ml YPD10 medium (1% Bacto yeast extract, 2% Bacto peptone, 10% D-glucose). Each culture was incubated by static culture at 30°C for 72 h without neutralization. The glucose, lactic acid, and ethanol concentrations were measured with a biosensor BF-5 instrument (Oji Keisoku Kiki, Hyogo). The *post hoc* Tukey's test was used for multiple comparisons between strains with statistical significance set at a p

value lower than 0.05 (Toothaker, 1993). Data analysis was carried out with IBM SPSS Statistics version 21 (IBM SPSS Inc., Chicago, USA).

2.3 Results

2.3.1 Genome-wide screening for *S. cerevisiae* L-lactic-acid-resistant disruptants

The purpose of this chapter was to construct a superior lactic-acid-resistant strain by combining gene disruptions that lead to lactic-acid resistance, and to investigate the possibility of enhancing lactic-acid productivity without neutralization. Although Kawahata *et al.* (2006) reported 46 genes whose disruption led to resistance to 5.1% lactic acid, in this chapter I intended to screen for strains showing stronger resistance under more severe conditions. A concentration of at least 5% lactic acid in culture media (pH 2.7) seems to exert toxic effects on yeast (Ishida *et al.*, 2005). To obtain more highly lactic-acid-resistant *S. cerevisiae* strains, I first systematically screened for viable yeast haploid single disruptants (4828 strains) that were resistant to 6% L-lactic acid stress (pH 2.6), a condition in which the wild-type strain (BY4742) hardly grew (Fig. 2). Cell suspensions of freshly grown cultures of all of the disruption mutants were transferred to YPDA plates containing 0% and 6% L-lactic acid by using a 96-prong replicator and incubated for 1 day (0%) or 6 days (6%), and growth was assessed visually. The screen was carried out in triplicate. Disruption alleles of candidate disruptants were structurally confirmed by PCR

analysis (data not shown).

As a result of screening, I identified 94 genes (1.9% of the total) that, when disrupted, led to a phenotype resistant to 6% L-lactic acid (Table 3). These genes were categorized by gene function according to the Munich Information Center for Protein Sequences (MIPS, http://mips.gsf.de/) (Mewes *et al.*, 2002) and *Saccharomyces* Genome Database (SGD, http://www.yeastgenome.org/) (Dwight *et al.*, 2002). Although 25 of the 94 genes had previously been reported to increase resistance to 5.1% lactic acid in a synthetic glucose minimal medium (Kawahata *et al.*, 2006), my analysis newly identified 69 genes whose gene disruption increased resistance to 6% lactic acid in YPDA glucose-rich medium. Although my screening condition was more severe than that used in Kawahata's work (2006), not all genes that have previously been reported (Kawahata *et al.*, 2006) were identified in my screening. One reason for this may be that I used a glucose-rich medium (YPDA) containing lactic acid as a screening medium, whereas Kawahata *et al.* (2006) used a synthetic glucose minimal medium containing lactic acid.

Table 3. Genes whose loss results in resistance to 6% L-lactic acid.

ORF	Description
Cellular transport, transport facilities and transport routes	
i) vacuolar/lysosomal transport	
ATG22	Breakdown of autophagic vesicles inside the vacuole
SYN8	Endosomal SNARE protein
VPS62	Vacuolar protein sorting
VPS70	Protein involved in vacuolar sorting
VPS74	Protein involved in vacuolar sorting
VTC3	Subunit of the vacuolar transporter chaperone complex
ii) vesicular transport (Golgi network, etc.)	
ALY1	α arrestin that controls intracellular sorting of Gap1
ERP2	P24 protein involved in membrane trafficking
ERV25	Component of the COPII-coated vesicles
MVB12	ESCRT-I subunit
iii) transport facilities	
ATP4	F1F0-ATPase complex
THI72	Transporter of thiamine or related compound
iv) nuclear transport	
YHL010c	Protein of unknown function
Protein fate (folding, modification, estination)	
i) protein modification	
FRK1	Putative protein kinase
MNN2	Type II membrane protein
MSG5	Dual-specifity protein phosphatase
PTC7	2C protein phosphatase
RUP1	Protein involved in regulation of Rsp5
SHR5	Subunit of a almitoyltransferase
YPL150w	Putative Ser/Thr protein kinase
YPL236c	Protein kinase localised to vacuolar membrane
ii) protein/peptide degradation	
CDH1	Substrate-specific activator of APC-dependent proteolysis
MMS2	Ubiquitin-conjugating enzyme
RKR1	Nuclear RING domain E3 ubiquitin ligase
TRE1	Transferrin receptor-like protein
UBP2	Ubiquitin-specific proteinase
Transcription	
SRO9	La motif-containing proteins that modulate mRNA translation
i) RNA synthesis	
ACE2	Metallothionein expression activator
ASH1	Negative regulator of HO transcription
CBF1	Centromere binding factor 1
EAF3	Esa1p-associated factor
EAF5	Esa1p-associated factor
EAF6	Esa1p-associated factor
SFL1	Transcriptional repressor and activator
YAF9	Subunit of the NuA4 and the SWR1 complex
ii) RNA processing	

CWC15	Protein involved in mRNA splicing
ISY1	Pre-mRNA splicing factor
Metabolism	
i) lipid, fatty acid and isoprenoid metabolism	
ACB1	Acyl-coenzyme-A-binding protein
FEN1	Fatty acid elongase
LDH1	Serine hydrolase
PSD2	Lipid, fatty acid and isoprenoid metabolism
ii) C-compound and carbohydrate metabolism	
GND1	6-phosphogluconate dehydrogenase
TCO89	TOR complex 1
iii) nucleotide/nucleoside/nucleobase metabolism	
URA5	Orotate phosphoribosyltransferase
YND1	Nucleoside diphosphatase
iv) metabolism of vitamins, cofactors, and prosthetic groups	
SIS2	Negative regulatory subunit of Ppz1 and a subunit of PPCDC
v) amino acid metabolism	
TKL1	Transketolase 1
Cell cycle and DNA processing	
i) cell cycle	
BUD3	Budding protein
CTS1	Endochitinase
CYK3	SH3-domain protein
DSE2	Daughter cell-specific secreted protein with similarity to glucanases
EGT2	Cell-cycle regulation protein
SCW11	Cell wall protein with similarity to glucanases
SPO7	Meiotic protein
ii) DNA processing	
MEI4	Meiosis-specific protein
iii) DNA repair	
UBC13	E2 ubiquitin-conjugating enzyme
Cell type differentiation	
i) fungal/microorganismic cell type differentiation	
AXL2	Protein required for axial pattern of budding
BUD4	Budding protein
DIT1	Spore wall maturation protein
RBD2	Protein of unknown function
Biogenesis of cellular components	
i) cell wall	
LRG1	GTPase-activating protein of the Rho/Rac family
SED1	Abundant cell surface glycoprotein
ii) eukaryotic plasma membrane	
SEY1	Synthetic enhancement with *YOP1*
Cellular communication/Signal transduction mechanism	
VAC17	Vacuole-specific receptor of Myo2
i) cellular signaling	
GIS4	CAAX box containing protein
Cell fate	
i) cell growth	
CKA2	α catalytic subunit of casein kinase II
ii) cell aging	
SUN4	Protein involved in the aging process

Cell rescue, defense and virulence	
i) stress response	
SIP5	Protein facilitates the interaction between Glc7 and Snf1
Interaction with the environment	
i) homeostasis of cations	
FRE6	Putative ferric reductase
Unclassified proteins	
CEX1	Protein of unknown function
FMP49	Questionable protein
IRC8	Protein of unknown function
IRC9	Questionable protein
MRN1	RNA-binding protein
NBA1	Protein of unknown function
SLP1	Protein of unknown function
SVF1	Protein with a potential role in cell survival pathways
TOS6	Protein of unknown function
YBR062c	Protein of unknown function
YBR187w	Protein of unknown function
YCL062w	Questionable protein
YDR357c	Protein of unknown function
YHR078w	Conserved hypothetical protein
YHL017w	Protein of unknown function
YIL141w	Questionable protein
YKL063c	Protein of unknown function
YKR073c	Questionable protein
YLR346c	Protein of unknown function
YMR294w-A	Questionable protein
YNR005c	Questionable protein
YOR139c	Questionable protein
YOR289w	Protein of unknown function
YPL191c	Protein of unknown function
ZRG8	Protein of unknown function

Underline represents newly identified gene whose disruption results in resistance to lactic acid. Other genes were also identified by Kawahata *et al.* (2006).

2.3.2 Multiple disruptants show superior lactic-acid resistance

My analysis identified disruptants of *DSE2*, *SCW11*, *EAF3*, and *SED1*, which belonged to the group showing strong resistance to 6% lactic acid as compared with the parental strain BY4742 (Fig. 2). Kawahata *et al.* (2006) reported that Δ*scw11* and Δ*eaf3* disruptants showed 0.5% acetic-acid resistance while Δ*dse2*, Δ*scw11*, Δ*eaf3*, and Δ*sed1* disruptants did not show 0.28% hydrochloric-acid resistance. The lactic-acid-resistant phenotype of these disruptants was subsequently confirmed to be due to a single disruption, as indicated by tetrad analysis of 6–10 asci, which showed 2 lactic-acid-resistance/2 lactic-acid-sensitive segregation, and cosegregation with the disruption marker (*kanMX*) (data not shown). In the following experiments, I primarily used these 4 disruptants as lactic-acid resistance had been confirmed to be due to a single disruption.

I expected that multiple disruptants harboring various combinations of the Δ*dse2*, Δ*scw11*, Δ*eaf3*, and Δ*sed1* disruptions might show more superior lactic-acid resistance. Therefore, I constructed single, double, triple, and quadruple disruptants of these genes by tetrad analysis as described in Materials and methods (Table 1), and examined the lactic-acid resistance of these multiple disruptants (Fig. 2). There was no significant difference between the growth curve of BY4742 and that of 15 disruptants (SH6704–SH6718) in YPDA without lactic acid (data not shown). As shown in Fig. 2, all of the multiple disruptants showed resistance to 7% lactic acid (pH 2.5), and almost all disruptants except for Δ*dse2*Δ*scw11* (SH6708) and Δ*dse2*Δ*sed1* (SH6710) displayed enhanced lactic-acid resistance as compared with the single-gene disruptants.

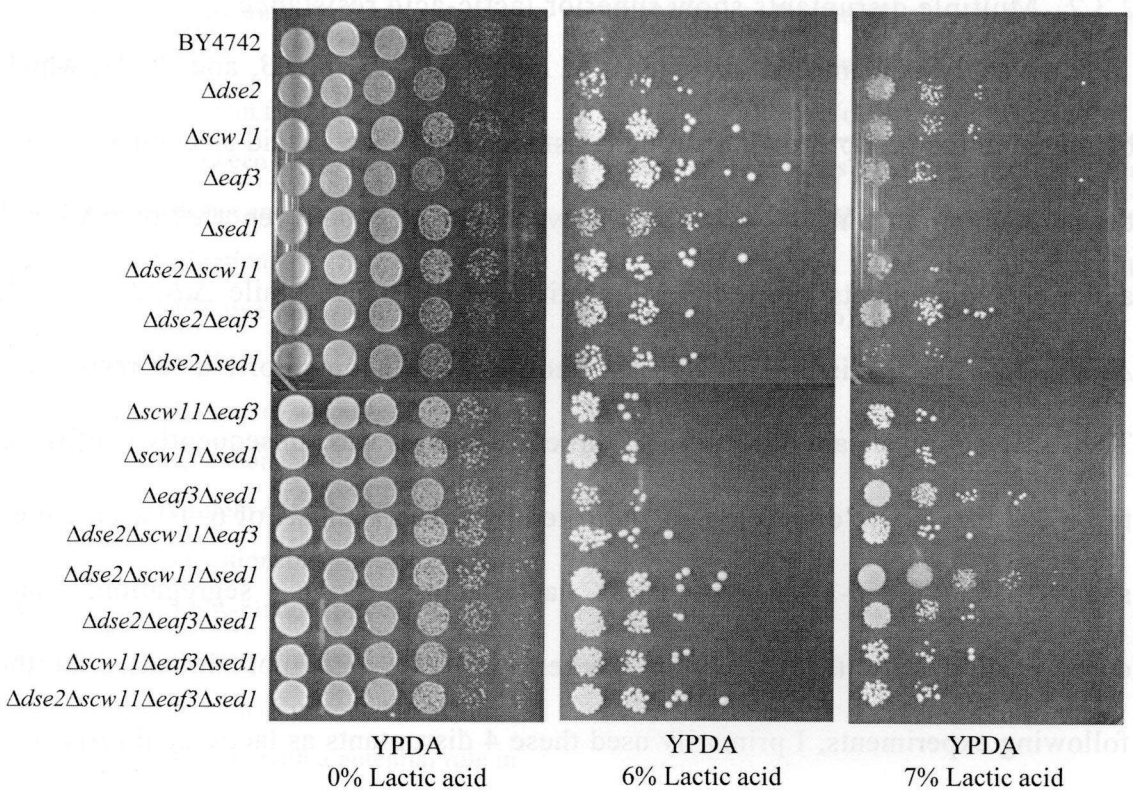

Figure 2. Assay of growth resistance of single and multiple disruptants to lactic acid. A ten-fold dilution of each yeast strain was spotted onto YPDA medium, 6% lactic-acid medium (pH 2.6), and 7% lactic-acid medium (pH 2.5) as described in Materials and methods. Cells were incubated at 30°C for 24 h (YPDA medium), 5 days (6% lactic-acid medium), and 10 days (7% lactic-acid medium). The strains assayed were Δ*dse2* (SH6704), Δ*scw11* (SH6705), Δ*eaf3* (SH6706), Δ*sed1* (SH6707), Δ*dse2*Δ*scw11* (SH6708), Δ*dse2*Δ*eaf3* (SH6709), Δ*dse2*Δ*sed1* (SH6710), Δ*scw11*Δ*eaf3* (SH6711), Δ*scw11*Δ*sed1* (SH6712), Δ*eaf3*Δ*sed1* (SH6713), Δ*dse2*Δ*scw11*Δ*eaf3* (SH6714), Δ*dse2*Δ*scw11*Δ*sed1* (SH6715), Δ*dse2*Δ*eaf3*Δ*sed1* (SH6716), Δ*scw11*Δ*eaf3*Δ*sed1* (SH6717), and Δ*dse2*Δ*scw11*Δ*eaf3*Δ*sed1* (SH6718).

The multiple disruptant showing the highest lactic-acid resistance seemed to be the Δ*dse2*Δ*scw11*Δ*sed1* disruptant (SH6715) judging from Fig. 2 since it showed the highest number of colonies in each serial dilution spots on 7% lactic-acid medium. However, its colony size was very small when compared with those of other multiple disruptants. This may come from a complex effect of highest survival

rate but slower growth rate that the Δ*dse2*Δ*scw11*Δ*sed1* disruptant displays against lactic-acid stress. Since the difference of level of resistance among multiple disruptants appeared to be obscure on lactic-acid plate and weak organic acid such as sorbic acid causes the extended lag phase (Lambert *et al.*, 1999), I also examined the growth behavior of the multiple disruptants in liquid culture under 6% lactic-acid condition to see what extent of the lactic-acid resistance of the multiple disruptants was enhanced as compared with the single-gene disruptants (Fig. 3). Six strains, Δ*eaf3* (SH6706), Δ*dse2*Δ*eaf3* (SH6709), Δ*dse2*Δ*sed1* (SH6710), Δ*scw11*Δ*eaf3* (SH6711), Δ*dse2*Δ*scw11*Δ*eaf3* (SH6714), and Δ*dse2*Δ*eaf3*Δ*sed1* (SH6716), showed almost the same pattern of growth as other strains and are not shown in Fig. 3. That is, the growth curve of Δ*eaf3* (SH6706) was almost the same as that of Δ*scw11* (SH6705). Similarly, Δ*dse2*Δ*eaf3* (SH6709) and Δ*scw11*Δ*ead3* (SH6711) were the same as Δ*dse2*Δ*scw11* (SH6708); Δ*dse2*Δ*sed1* (SH6710) and Δ*dse2*Δ*scw11*Δ*eaf3* (SH6714) were the same as Δ*scw11*Δ*sed1* (SH6712); and Δ*dse2*Δ*eaf3*Δ*sed1* (SH6716) was the same as Δ*scw11*Δ*eaf3*Δ*sed1* (SH6717). Since pH of culture media of strains including quadruple disruptant was not changed from the initial pH value (pH 2.6) during 120 h cultivation, I believe that the resistance was not acquired by raising the pH of culture media. As shown in Fig. 3, all of the single-gene disruptants showed lower cell density than the multiple disruptants after 120 h incubation and the specific growth rate (μ) of Δ*sed1* (SH6707), Δ*scw11*Δ*sed1* (SH6712), Δ*dse2*Δ*scw11*Δ*sed1* (SH6715), Δ*scw11*Δ*eaf3*Δ*sed1* (SH6717), Δ*dse2*Δ*scw11*Δ*eaf3*Δ*sed1* (SH6718) was almost same [μ = 0.085 h^{-1} (SH6704), 0.074 h^{-1} (SH6706), 0.10 h^{-1} (SH6707), 0.072 h^{-1} (SH6708), 0.10 h^{-1} (SH6712),

0.082 h^{-1} (SH6713), 0.10 h^{-1} (SH6715), 0.10 h^{-1} (SH6717), 0.11 h^{-1} (SH6718)]. The quadruple disruptant (*Δdse2Δscw11Δeaf3Δsed1*; SH6718) displayed both the shortest lag phase and the highest cell density than any other disruptants after 120 h of incubation. Based upon these results, I concluded that the highest resistant-strain to 6% lactic acid was *Δdse2Δscw11Δeaf3Δsed1* quadruple disruptant (SH6718). I also examined the resistance of all the 15 disruptants (SH6704-SH6718) against hydrochloric acid (0.4%). Hydrochloric-acid resistance of all of 15 disruptants was not stronger than that of wild-type strain (BY4742) (data not shown). This result suggests that all the 15 disruptants show the resistance to the effect of lactic-acid anion but not proton. I show that a strategy of constructing strains with multiple disruptions of genes whose disruption leads to lactic-acid resistance is an effective way to enhance resistance to lactic acid.

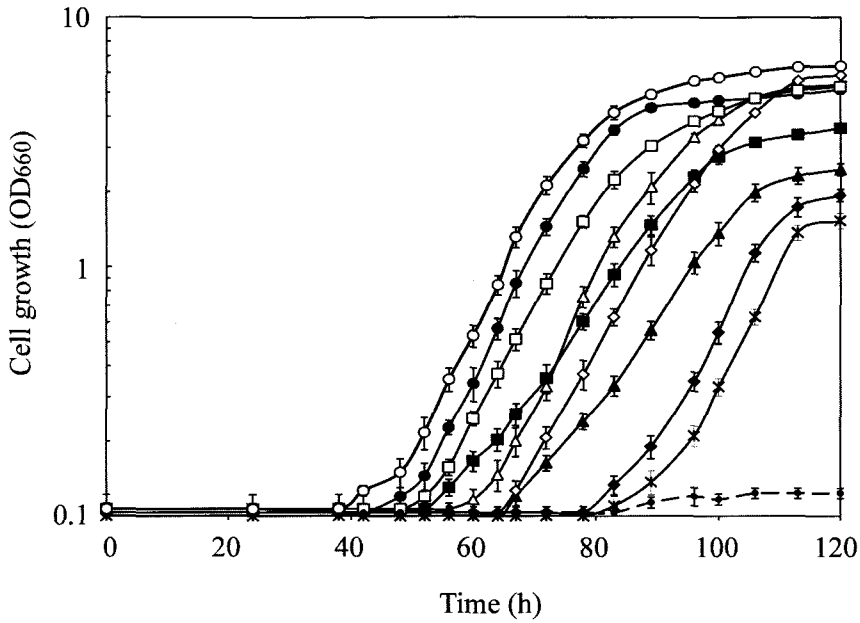

Figure 3. Growth curve of single and multiple disruptants under 6% lactic acid without neutralization. Each strain was pre-cultured on YPDA at 30 °C and transferred to 6% lactic-acid medium (OD_{660} = 0.1); the OD_{660} of the culture was then monitored periodically for 120 h. Symbols: closed diamond (Δ*dse2*, SH6704), closed triangle (Δ*scw11*, SH6705), asterisk (Δ*sed1*, SH6707), closed square (Δ*dse2*Δ*scw11*, SH6708), open triangle (Δ*scw11*Δ*sed1*, SH6712), open diamond (Δ*eaf3*Δ*sed1*, SH6713), closed circle (Δ*dse2*Δ*scw11*Δ*sed1*, SH6715), open square (Δ*scw11*Δ*eaf3*Δ*sed1*, SH6717), open circle (Δ*dse2*Δ*scw11*Δ*eaf3*Δ*sed1*, SH6718). Broken line indicates the growth curve of the wild-type strain (BY4742). Error bars show the standard deviation of three independent experiments.

2.3.3 A lactic-acid-resistant strain that produces lactic acid more efficiently than wild type

I expected that lactic-acid production in the lactic-acid resistant strains would be improved as compared with that in the wild-type strain under non-neutralizing conditions. *S. cerevisiae* converts pyruvic acid to ethanol and Pdc1 is the main pyruvate decarboxylase. Therefore, to decrease the production of ethanol and to generate efficient production of lactic acid, the bovine L-lactate dehydrogenase gene (L-*LDH*) was introduced into the *PDC1* locus of the wild-type strain (BY4742),

as well as single and multiple disruptants, by homologous recombination with a DNA fragment obtained by digesting the plasmid pBHPH-PDC1P-LDHKCB (Fig. 1). The *LDH*-harboring BY4742 (SH6764) strain produced lactic acid and, as expected, the ethanol productivity of this strain decreased as compared with that of BY4742 (data not shown).

Because the *LDH*-harboring strains acquired the ability to intrinsically produce lactic acid, I examined to what extent single and multiple disruptants harboring the L-*LDH* gene displayed lactic-acid resistance (Fig. 4). As shown in Fig. 4, all single and multiple disruptants with bovine *LDH* showed resistance to 6% lactic-acid, whereas the wild-type strain harboring *LDH* could not. Interestingly, the *LDH*-harboring *Δdse2Δscw11* (SH6769), *Δdse2Δeaf3* (SH6770), *Δscw11Δeaf3Δsed1* (SH6778), and *Δdse2Δscw11Δeaf3Δsed1* (SH6779) strains displayed high resistance to 7% lactic acid, although for almost all strains the degree of resistance to 6% and 7% lactic acid was different from that shown in Fig. 2.

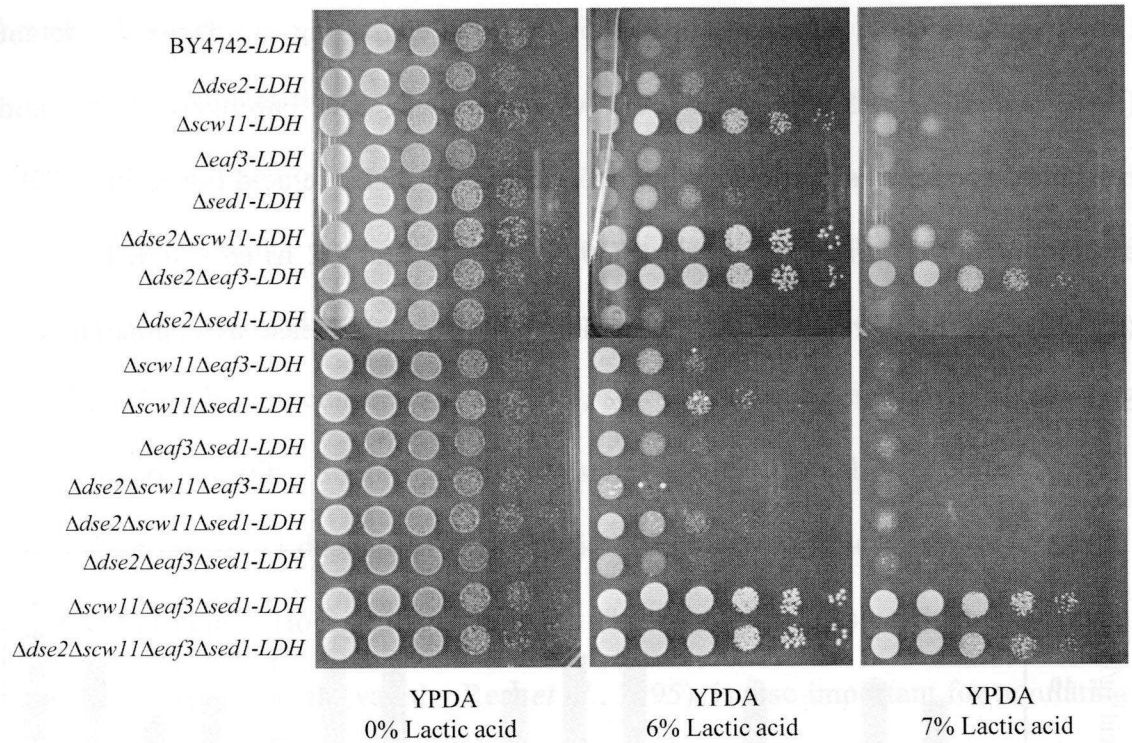

Figure 4. Assay of growth resistance of *LDH*-harboring single and multiple disruptants to lactic acid. A ten-fold dilution of each yeast strain was spotted onto YPDA medium, 6% lactic-acid medium (pH 2.6), and 7% lactic-acid medium (pH 2.5) as described in Materials and methods. Cells were incubated at 30°C for 24 h (YPDA medium) and 6 days (6% and 7% lactic-acid medium). The strains assayed were BY4742-*LDH* (SH6764), Δ*dse2-LDH* (SH6765), Δ*scw11-LDH* (SH6766), Δ*eaf3-LDH* (SH6767), Δ*sed1-LDH* (SH6768), Δ*dse2*Δ*scw11-LDH* (SH6769), Δ*dse2*Δ*eaf3-LDH* (SH6770), Δ*dse2*Δ*sed1-LDH* (SH6771), Δ*scw11*Δ*eaf3-LDH* (SH6772), Δ*scw11*Δ*sed1-LDH* (SH6773), Δ*eaf3*Δ*sed1-LDH* (SH6774), Δ*dse2*Δ*scw11*Δ*eaf3-LDH* (SH6775), Δ*dse2*Δ*scw11*Δ*sed1-LDH* (SH6776), Δ*dse2*Δ*eaf3*Δ*sed1-LDH* (SH6777), Δ*scw11*Δ*eaf3*Δ*sed1-LDH* (SH6778), and Δ*dse2*Δ*scw11*Δ*eaf3*Δ*sed1-LDH* (SH6779).

I then examined the productivity of lactic acid in the disruptants harboring *LDH* without neutralization after 72 h cultivation, at which time all glucose in medium was consumed in almost all disruptants (Fig. 5). The multiple comparisons of productivity between strains were carried out by Tukey's test

(Toothaker, 1993). As shown in Fig. 5, the *LDH*-harboring quadruple disruptant (*Δdse2Δscw11Δeaf3Δsed1*; SH6779) which showed the strongest lactic-acid resistance produced significantly high concentration of lactic acid (48 g/litter, 72h) as compared with wild-type strain (SH6764) (38 g/litter, 72 h) (p = 0.024), while that of other 14 disruptants did not exhibit significant increase as compared with SH6764.

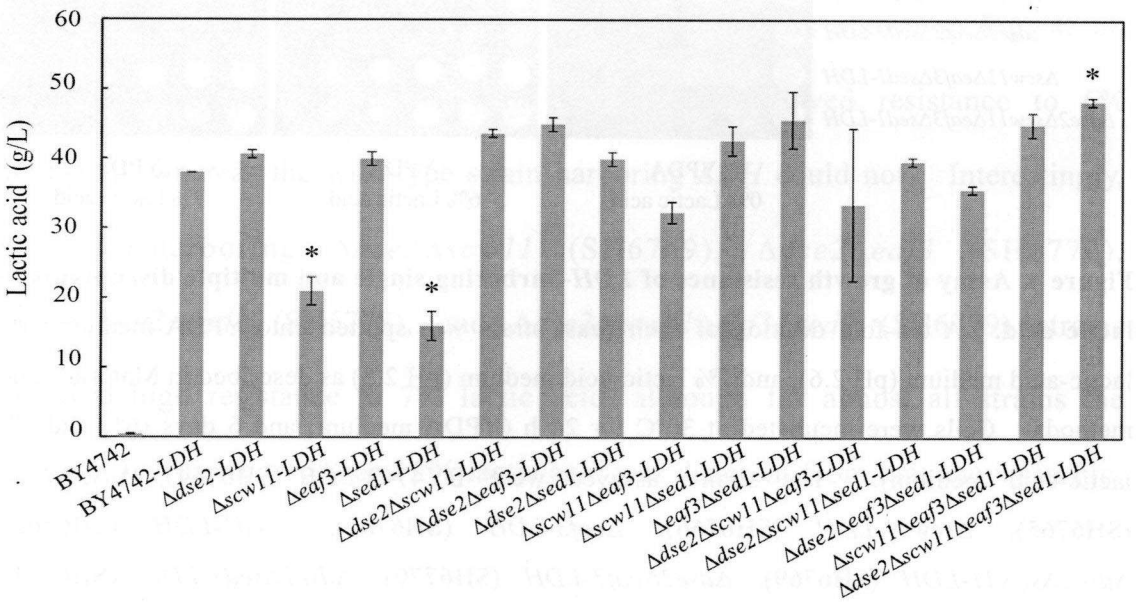

Figure 5. Lactic-acid productivity of LDH-harboring single and multiple disruptants. The amount of lactic acid was measured as described in Materials and methods. Error bars show the standard deviation of three independent experiments. Asterisk represents a significant difference (p < 0.05) from *LDH*-harboring wild-type strain (SH6764) (Tukey's multiple comparisons). The strains assayed were BY4742-*LDH* (SH6764), *Δdse2-LDH* (SH6765), *Δscw11-LDH* (SH6766), *Δeaf3-LDH* (SH6767), *Δsed1-LDH* (SH6768), *Δdse2Δscw11-LDH* (SH6769), *Δdse2Δeaf3-LDH* (SH6770), *Δdse2Δsed1-LDH* (SH6771), *Δscw11Δeaf3-LDH* (SH6772), *Δscw11Δsed1-LDH* (SH6773), *Δeaf3Δsed1-LDH* (SH6774), *Δdse2Δscw11Δeaf3-LDH* (SH6775), *Δdse2Δscw11Δsed1-LDH* (SH6776), *Δdse2Δeaf3Δsed1-LDH* (SH6777), *Δscw11Δeaf3Δsed1-LDH* (SH6778), and *Δdse2Δscw11Δeaf3Δsed1-LDH* (SH6779).

2.4 Discussion

The newly identified genes belong to a variety of functional categories. For example, the deletion of genes related to ubiquitination (*RUP1*, *MMS2*, *RKR1*, and *UBC13*) was found for the first time to lead to lactic-acid resistance. Ubiquitination is involved in resistance to various types of stress, such as UV, canavanine, high temperature (39°C), LiCl, sorbitol, ethanol, and H_2O_2 (Hofmann and Pickart, 1999; Seufert and Jentsch, 1990; Hiraishi *et al.*, 2006). Ubiquitin/proteasome-dependent proteolysis consumes ATP in order to degrade abnormal proteins (Hochstrasser, 1995). The role of ATPase, which consumes much ATP during growth (van der Rest *et al.*, 1995), is also important for regulating intracellular pH under acidic conditions. Therefore, it may be advantageous for the survival of yeast cells in a high concentration of lactic acid if ATP is utilized to remove protons from the cytosol via ATPase rather than to degrade proteins by the ubiquitin-proteasome pathway, although an accumulation of misfolded proteins in the endoplasmic reticulum causes endoplasmic reticulum stress (Schröder and Kaufman, 2005).

Δ*dse2*, Δ*scw11*, Δ*eaf3*, and Δ*sed1* disruptants showed strong resistance to 6% lactic acid. According to the SGD database, Dse2 and Scw11 both function as glucanases that are involved in the separation of daughter cells from mother cells (Cappellaro *et al.*, 1998; Colman-Lerner *et al.*, 2001; Ufano *et al.*, 2004). Because the continuous synthesis of glucan may lead to wall thickening (Dominguez *et al.*, 1978), the Δ*scw11* and Δ*dse2* disruptants may have a thick cell wall and therefore show increased resistance to lactic acid and/or other substances. Eaf3 is a

component of the NuA4 acetyltransferase complex. In the Δ*eaf3* disruptant, histone acetylation is decreased at promoter regions and increased at coding regions, and the transcriptional level of a few yeast genes increase (Reid *et al.*, 2004). *FET3*, which is negatively regulated by Eaf3, is involved in high-affinity iron uptake, and it has been suggested that iron metabolism may be important for growth under acidic conditions (Kawahata *et al.*, 2006). Therefore, enhancement of lactic-acid resistance in the Δ*eaf3* strain may be caused by increased expression of Eaf3-downregulated genes such as *FET3*. Sed1 is a major stress-induced GPI-cell wall glycoprotein that associates with translating ribosomes and is involved in mitochondrial genome maintenance (van der Vaart *et al.*, 1996; Shimoi *et al.*, 1998). Because the Δ*sed1* disruptant shows somewhat increased resistance to calcofluor white and Congo red (Caro *et al.*, 1998), this disruption may lead to an increase in the maintenance of cell-wall integrity and thus to enhancement of lactic-acid resistance.

Lactic-acid resistance of the Δ*dse2*Δ*eaf3* double disruptant was higher than that of the Δ*dse2*Δ*scw11*Δ*eaf3* and Δ*dse2*Δ*eaf3*Δ*sed1* triple disruptants in experiment shown in Fig. 4. However, I did not see clear difference in the level of the resistance among these three disruptants in Fig. 2. Therefore, I think that the difference in resistance between the Δ*dse2*Δ*eaf3* double disruptant and the Δ*dse2*Δ*scw11*Δ*eaf3* and Δ*dse2*Δ*eaf3*Δ*sed1* triple disruptants shown in Fig. 4 seems to come from a consequence of the expression of the *LDH* that was integrated into the genome of those strains shown in Fig. 4. Although the mechanisms are unknown, one possible interpretation would be that this difference in resistance in

Fig. 4 was caused by the alteration of pyruvate metabolism. Since at least some amount of pyruvate is fluxed to the synthesis of lactic acid in *LDH*-containing disruptant, TCA cycle may be slow down and consequently ATP synthesis may decrease compared with disruptant without *LDH* gene. The amount of ATP required for growth under lactic acid may differ with disruption genotype. Another possibility would be that the detrimental effects of intracellularly produced lactic acid in addition to extracellularly added lactic acid to cells may differ with disruption genotype.

In conclusion, I showed that lactic-acid resistance can be enhanced by combining multiple gene disruptions, each of which causes lactic-acid resistance, and also showed that the quadruple disruptant displaying highest lactic-acid resistance led to significant improvement in lactic-acid production without neutralization as compared with wild-type strain.

2.5 Summary

To create strains that have high productivity of lactic acid without neutralization, a genome-wide screening for strains showing hyper-resistance to 6% L-lactic acid (pH 2.6) was performed using the gene deletion collection of *S. cerevisiae*. I identified 94 genes whose disruption led to resistance to 6% lactic acid in rich medium. I also found that multiple combinations of Δ*dse2*, Δ*scw11*, Δ*eaf3*, and Δ*sed1* disruption led to enhanced resistance to 7% lactic acid (pH 2.5). In particular, the quadruple disruptant Δ*dse2*Δ*scw11*Δ*eaf3*Δ*sed1* grew well in 6% lactic acid with a short lag phase. I then generated strains harboring these multiple

disruptions along with an exogenous lactate dehydrogenase gene (*LDH*) gene, and found that some of the multiple disruptants that showed stronger lactic-acid resistance displayed high productivity of lactic acid. In particular, the *LDH*-harboring quadruple disruptant, which showed the strongest lactic-acid resistant strains, showed an increase in lactic-acid productivity of approximately 27% as compared with the *LDH*-harboring wild-type strain (BY4742) (48 g/litter, 72 h). These observations suggest that disruption of multiple genes each of whose deletion leads to lactic-acid resistance is an effective way to enhance resistance to lactic acid, leading to high lactic-acid productivity without neutralization.

Chapter 3

Lactic-acid stress causes vacuolar fragmentation and impairs intracellular amino-acid homeostasis in *S. cerevisiae*

3.1 Introduction

In *S. cerevisiae*, it is well known that adaptation to lipophilic weak organic acids such as sorbic and benzoic acids involves the extraction of organic acid anions by the Pdr12p plasma membrane ATP-binding cassette transporter whose expression is regulated by the War1p zinc-finger transcription factor (Mollapour *et al.*, 2008; Holyoak *et al.*, 1999). On the other hand, adaptation to acetic acid that is more hydrophilic than sorbic or benzoic acid involves the attenuation of influx of acetic acid into the cell through degradation of acetic acid uptake channel Fps1 on the plasma membrane (Mollapour *et al.*, 2008; Mollapour and Piper, 2007). Although in chapter 2 I described that enhancement of lactic-acid resistance contributes to improvements in lactic-acid productivity without neutralization, the detailed mechanism of adaptation response to lactic-acid stress in *S. cerevisiae* remains unclear, and factors and mechanisms involved in this response are not fully elucidated.

To elucidate the cellular mechanisms which are injured by lactic-acid, I performed functional genomic analysis. I identified 107 genes that when disrupted reduced the resistance of yeast cells to 4% lactic acid. Of these 107 genes, 34 (>30% in my analysis) had not been mentioned in previous studies and were newly

identified to be involved in mechanisms for adaptation response to lactic acid. Intracellular transport including vacuolar transport and amino acid metabolism were suggested to be involved in adaptation to lactic-acid stress. The analysis of this chapter revealed that vacuole of wild-type strain fragments under lactic- and hydrochloric-acid conditions and the amount of intracellular amino acids significantly reduced by addition of lactic acid. These data suggest for the first time that lactic-acid stress impairs the vacuole integrity and intracellular amino acids are related to the growth under lactic-acid stress.

3.2 Materials and Methods

3.2.1 Strains, plasmids, and media

The nonessential genes deletion collection (OpenBiosystems, Huntsville, AL, USA), which contains 4828 yeast strains with all nonessential ORFs disrupted by the *kanMX4* cassette (http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html), was used for screening in this chapter. A parental strain of the deletion collection, BY4742, (*MATα leu2*Δ0 *his3*Δ1 *ura3*Δ0 *lys2*Δ0) (Brachmann *et al.*, 1998) was used as a wild-type strain in this chapter. The standard complete medium, YPDA, consisted of 1% (weight per volume, w/v) yeast extract, 2% Bacto-peptone, 2% glucose, and 0.04% adenine. L-Lactic acid (4%) or hydrochloric acid (0.13% and 0.3%) was added to YPDA after autoclaving. The pH of both the 4% lactic-acid and the 0.13% hydrochloric-acid media was 2.8. Agar (2%) was added to media to make culture plates.

3.2.2 Screening for L-lactic acid hypersensitive mutants

To screen for L-lactic-acid-hypersensitive strains, the nonessential genes deletion collection was replica stamped onto YPDA and 4% L-lactic acid plates with a 96-prong replicator. Growth of the disruptants was scored visually by comparison with that of the parent strain (BY4742), which was stamped similarly on YPDA and 4% L-lactic acid plates. Before screening, single-gene disruptants were tested for their growth on YPDA medium containing 300 mg/l G418 disulfate (Wako Pure Chemical Industries, Osaka). The L-lactic-acid-sensitive phenotype of all of the disruptants identified in the screen was subsequently confirmed to be due to a single disruption, as indicated by tetrad analysis of 6-10 asci, which showed 2 lactic-acid-resistance/2 lactic-acid-sensitive segregation and cosegregation with the disruption marker (*kanMX4*). After screening, the sensitivity of the disruptants to 1% (pH 3.7), 2% (pH 3.0), 3% (pH 2.9), and 4% (pH 2.8) lactic acid and 0.3% hydrochloric acid (pH 2.3) was tested and scored.

3.2.3 Spot dilution growth assays

Yeast cells were grown in YPDA at 30 °C to mid-log phase (OD_{660} = 1.0). Cells were harvested by centrifugation, washed once with water, and resuspended in water. The cell density was normalized to 1×10^6 cells/5 μl. A 10-fold serial dilution of this culture was made, and 5 μl of each dilution was spotted onto YPDA medium, 4% lactic-acid medium, or 4% lactic-acid medium supplemented with amino acids (10 mM Ile, 50 mM Tyr, 50 mM Thr, 50 mM Gly, or 50 mM Leu).

Each amino acid-supplemented medium was adjusted to pH 2.8 with hydrochloric acid. Cells were incubated at 30 °C and scored visually by comparison with that of parent strain (BY4742).

3.2.4 Active staining of the vacuole with quinacrine

In vivo quinacrine staining of the vacuole was performed by a slightly modified method from Phelan *et al.* (2006). Yeast cells were grown in YPDA at 30 °C to mid-log phase (OD_{660} = 1.0), and 1 ml of the sample was collected. The cell pellet was resuspended in 1 ml HEPES-glucose buffer [100 mM HEPES/KOH (pH 7.5), 3% (w/v) glucose] containing 200 μM quinacrine (Sigma-Aldrich, St. Louis, MO, USA), and incubated for 5 min in the dark at room temperature. Cells were then washed twice with HEPES-glucose buffer. After centrifugation, the cell pellet was resuspended in HEPES-glucose buffer and photographed by fluorescence microscopy (Olympus BX61; Olympus, Tokyo) within 1 h after quinacrine staining. To quantify quinacrine staining, a culture grown in YPDA was divided into two, and the quinacrine fluorescence in 1 ml of cell suspension processed as described above was measured directly using a spectrofluorometer (Hitachi F-2500; Hitachi, Tokyo), while the remainder of the cells was collected and measured for optical density. Relative fluorescence was then determined as total fluorescence per number of cells.

3.2.5 Labeling the vacuolar membrane with FM4-64

FM4-64 [*N*-(3-triethylammoniumpropyl)-4-(ρ-diethylaminophenyl-hexatrienyl)

pyridinium dibromide; Invitrogen, Carlsbad, CA, USA] labeling was performed by a slightly modified method described by Conibear *et al.* (2002). Yeast cells were grown in YPDA at 30 °C to mid-log phase (OD_{660}= 0.8), and 1 ml of the sample was collected. The cell pellet was resuspended in 100 μl YPDA. FM4-64 was added to final concentration of 40 μM [from a 16 mM stock in dimethyl sulfoxide (DMSO)], and cells were incubated for 15 min in the dark at room temperature. Cells were then washed once with YPDA. After centrifugation, the cell pellet was resuspended in YPDA and incubated for 30 min in the dark at 30 °C. Finally, cells were collected, resuspended in YPDA, and photographed by fluorescence microscopy (Olympus BX61). In the lactic-acid or hydrochloric-acid condition, cells were similarly incubated for 30 min in the dark at 30 °C, transferred to 4% lactic acid or 0.13% hydrochloric acid medium, and then photographed over time.

3.2.6 Quantification of intracellular amino acids

In the non-stressed condition, yeast cells were grown to mid-log phase (OD_{660}= 1.0) in YPDA and then collected. In the lactic-acid condition, yeast cells were grown to mid-log phase in YPDA, collected and then incubated for 2 h or 24 h in YPDA medium supplemented with 4% lactic acid. Collected cells were suspended in 0.5 N perchloric acid and disrupted with glass beads. The homogenate was kept on ice for 10 min, and then the supernatant was collected by centrifuging at 13,200 rpm for 15 min. The sample was adjusted to pH 2.2 with lithium hydroxide and was kept on ice for 10 min. Next, to remove the precipitate, the sample was centrifuged at 13,200 rpm for 15 min. Free-form amino acids were quantified by

an amino acid analyzer (Hitachi L-8500; Hitachi, Tokyo). To normalize the content of amino acids, total protein was extracted by the TCA method and determined with a Quant-iT™ Protein Assay Kit (Invitrogen, Carlsbad, CA, USA).

3.2.7 Cell viability

Cell viability was determined by exclusion of the vital dye trypan blue, based on previously published protocols (Xu *et al.*, 1999). Mid-log phase cultures were centrifuged at 3300 rpm for 5 min at room temperature, re-suspended in YPDA with 4% L-lactic acid, and incubated with aeration at 30 °C. Samples of each culture were taken at 0, 24 and 48 hours of induction. Samples were once washed with phosphate buffered saline (PBS), mixed with equal volumes of 0.4% trypan blue (Sigma-Aldrich, St. Louis, MO, USA) in PBS, incubated at room temperature for 15 min and washed two times with PBS. The numbers of dead (blue) and live (no color) cells were determined by counting using microscope.

3.3 Results

3.3.1 A genome-wide screening for lactic-acid-hypersensitive alleles

To gain more insight into the adaptation response to lactic-acid stress, I systematically screened the *S. cerevisiae* haploid collection for single disruptants that were hypersensitive to L-lactic acid stress. I used YPDA medium with 4% L-lactic acid (pH 2.8) as the screening medium because a concentration of at least 5% L-lactic acid in culture media (pH 2.7) seems to exert strong toxic effects on

yeast (Ishida *et al.*, 2005). Cell suspensions of freshly grown cultures of all of the deletion mutants (4828 strains) were transferred to YPDA plates containing 0% and 4% L-lactic acid media by using a 96-prong replicator and incubated for 1 day (0%) or 2 days (4%), and growth was assessed visually. In these conditions, the 4% lactic acid medium caused growth retardation (of about 16 h) of the wild-type strain as compared to the 0% medium. The screen was carried out in triplicate, and all lactic-acid-sensitive disruptants identified in the screening were subsequently confirmed by tetrad analysis (data not shown). Disruption alleles were also structurally confirmed by PCR (data not shown).

As a result of screening, 107 genes (2.2% of the total genes) were identified to lead to a hypersensitive phenotype to L-lactic acid when deleted (Table 4). Of the 107 genes, 34 (> 30%) had not been mentioned in a previous study (Kawahata *et al.*, 2006). For example, I found for the first time that the depletion of Uba4 that is involved in protein urmylation (Goehring *et al.*, 2003) caused lactic-acid hypersensitivity, suggesting that protein urmylation appears to be associated with mechanisms underlying adaptation to lactic-acid stress. A N-terminal acetyltransferase, Nat3 (Caesar *et al.*, 2006), has also never been reported to be involved in lactic acid adaptation mechanisms. I also found that disruptions of 6 genes (*RNY1*, *YEL059w*, *GUP1*, *RIC1*, *YKR041w*, and *MRF1*) reported by Kawahata *et al.* (2006) did not cosegregate with the 4% L-lactic-acid-sensitive phenotype as judged by the disruption marker in tetrad analysis. Therefore, those disruptants were excluded for further analysis. In addition, I evaluated degree of the sensitivity of the 107 disruptants to 1% (pH 3.7), 2% (pH 3.0), 3% (pH 2.9), and 4%

(pH 2.8) lactic acid (Table 4). I examined the sensitivity of 107 disruptants to 0.5% acetic acid and 0.3% hydrochloric acid. Nine disruptants showed hydrochloric-acid sensitivity and 21 disruptants showed acetic acid sensitivity. Seven disruptants (Δ*clc1*, Δ*vps15*, Δ*vps34*, Δ*ppa1*, Δ*hsp150*, Δ*ypr022c*, Δ*ecm3*) did not show the sensitivity to both acetic acid and hydrochloric acid (data not shown), indicating that these 7 genes play a role for the response to lactic-acid anion. *PMA1* that should be important for adaptation to acidic condition (Viegas *et al.*, 1998) is an essential gene for yeast growth, thereby was not employed in my screening. In my screening, disruption of *WAR1*, *PDR12*, *AFT1*, or *HAA1*, which has been reported to be involved in weak-acid stress responses (Mollapour *et al.*, 2008; Kawahata *et al.*, 2006; Abbott *et al.*, 2008; Gregory *et al.*, 2008; Hatzixanthis *et al.*, 2003) did not cause sensitivity to at least 4% lactic acid stress.

Table 4. Genes whose loss results in sensitivity to 4% L-lactic acid.

ORF	Description	Lactic acid sensitivity
ADA2	General transcriptional adaptor or co-activator	- -
ALF1	Protein implicated in folding of alpha tubulin	- - - -
ANP1	Protein required for protein glycosylation in the golgi	-
ARG82	Arginine metabolism transcription factor	-
ARO1	Arom pentafunctional enzyme	- - -
ARO2	Chorismate synthase	- - -
ARO7	Chorismate mutase	- - - -
BAP2	Broad-specificity amino-acid permease	- -
BCK1	Ser/Thr protein kinase of the MEKK family	- - -
BRP1	Questionable protein	- -
BST1	Negative regulator of COPII vesicle formation	- -
BUD16	Protein involved in bud-site selection	-
BUD19	Questionable protein	- - - -
BUD20	Protein involved in bud-site selection	- - - -
BUD23	Protein involved in bud-site selection	- - - -
BUD32	Protein involved in bud-site selection, putative *O*-sialoglyco -endopeptidase	- - - -
CAX4	Protein required for full levels of dolichol-linked oligosaccharides in the endoplasmic reticulum	-
CCH1	Calcium channel (α subunit) of the plasma membrane	-
CHC1	Clathrin heavy chain	- - - -
CLC1	Clathrin light chain	- - -
CNB1	Calcineurin B, regulatory subunit	-
COG1	Conserved oligomeric golgi complex	-
CSG2	Calcium dependent regulatory protein	- -
DEF1	Coordinates repair and RNA pol II proteolysis in response to DNA damage	- -
ECM3	Protein involved in cell wall biogenesis and architecture	- -
EFG1	Essential protein required for maturation of 18S rRNA	- - - -
ERG2	C-8 sterol isomerase	-
ERG3	C-5 sterol desaturase	- -
ERG4	Sterol C-24 reductase	- -

ERG6	*S*-adenosyl-methionine delta-24-sterol-c-methyltransferase	- - -
ERG24	C-14 sterol reductase	-
FYV8	Protein required for survival upon exposure to K1 killer toxin	-
GAL11	DNA-directed RNA polymerase II holoenzyme and Kornberg's mediator subcomplex subunit	- -
GAS1	Glycophospholipid-anchored surface glycoprotein	-
GLY1	L-threonine aldolase	- - - -
HFI1	Transcriptional coactivator	- - -
HOG1	Ser/Thr protein kinase of MAP kinase family	-
HSP150	Member of the Pir1/Hsp150/Pir3 family	-
ILV1	Anabolic serine and threonine dehydratase precursor	- - -
KEM1	Multifunctional nuclease	- -
LEM3	Membrane protein of the plasma membrane and ER	- -
MDJ1	Heat shock protein-chaperone	- - - -
MID1	Mechanosensitive Ca^{2+}-permeable channel of the plasma membrane	-
MON2	Peripheral membrane protein with a role in endocytosis and vacuole integrity	- -
NAT3	*N*-acetyltransferases	-
NHX1	Na^{+}/H^{+} exchanger of the prevacuolar compartment	-
OCH1	α-1,6-mannosyltransferase	- - - -
PBS2	Tyrosine protein kinase of the MAP kinase kinase family	-
PDX3	Pyridoxamine-phosphate oxidase	- - - -
PEP3	Vacuolar membrane protein	- - -
PEP5	Vacuolar biogenesis protein	- - -
PEP7	Vacuolar segregation protein	- -
PHO85	Cyclin-dependent protein kinase	-
POP2	Protein required for glucose derepression	- - - -
PPA1	H^{+}-ATPase V_0 domain	- -
RAD6	E2 ubiquitin-conjugating enzyme	- -
RCY1	Protein involved in recycling of the SNARE Snc1p	- - - -
RGD1	GTPase activating protein (GAP) (putative)	-
RGP1	Reduced growth phenotype protein	- -
RPB9	DNA-directed RNA polymerase II	- - - -
RPL1B	60S large subunit ribosomal protein	- -

RRG1	Protein of unknown function localized to mitochondria, required for vacuolar acidification	- -
RTF1	Regulates DNA binding properties of TBP	- - - -
SAC1	Recessive suppressor of secretory defect	- - -
SAP30	Subunit of the histone deacetylase B complex	-
SFP1	Zinc finger protein	- - - -
SNF5	Component of SWI/SNF transcription activator complex	- -
SNF12	Component of SWI/SNF global transcription activator complex	- -
SHE4	Protein required for mother cell-specific gene expression	- - - -
SHP1	Potential regulatory subunit for Glc7	- -
SLA2	Cytoskeleton assembly control protein	- - - -
SLT2	Ser/Thr protein kinase of MAP kinase family	- - -
SPT20	Member of the TBP class of SPT proteins that alter transcription site selection	- - - -
SRV2	Adenylate cyclase-associated protein	- -
SWI3	Transcription regulatory protein	- -
TFP3	H^+-ATPase V_0 domain	-
THR1	Homoserine kinase	- -
THR4	Threonine synthase (*o*-*p*-homoserine *p*-lyase)	-
TLG2	Member of the syntaxin family of t-SNAREs	-
TRK1	Potassium transporter I	- - - -
TYR1	Prephenate dehydrogenase	- - -
UBA4	Protein that activates Urm1 before its conjugation to proteins	-
VAM10	Protein required for normal tethering of vacuoles prior to fusion	-
VMA1	Encodes 3 region protein which is self-spliced into Tfp1 and PI-SceI	- -
VMA4	H^+-ATPase V_1 domain	-
VMA5	H^+-ATPase V_1 domain	-
VMA6	H^+-ATPase V_0 domain	-
VMA7	H^+-ATPase V_1 domain	- -
VMA8	H^+-ATPase V_1 domain	-
VMA10	H^+-ATPase V_1 domain	- -
VMA22	Vacuolar ATPase assembly protein	- - - -
VPS1	Member of the dynamin family of GTPases	- - -
VPS3	Vacuolar sorting protein	- -
VPS5	Protein involved in Golgi retention and vacuolar sorting	- -

VPS15	Ser/Thr protein kinase	- - - -
VPS16	Vacuolar sorting protein	- - -
VPS20	Vacuolar protein sorting	- -
VPS34	Phosphatidylinositol 3-kinase	- - - -
VPS35	Protein-sorting protein	- -
VPS45	Vacuolar protein sorting-associated protein	- - -
VPS51	Subunit of VP51–54 complex, required for protein sorting at the yeast late Golgi	- - -
VPS52	Subunit of VP51–54 complex, required for protein sorting at the yeast late Golgi	- - -
VPS54	Subunit of VP51–54 complex, required for protein sorting at the yeast late Golgi	- - -
VRP1	Verprolin	- -
YCL007c	Questionable protein	- -
YPR022c	Protein of unknown function localized to cytoplasm and nucleus	- -
YPT6	GTP-binding protein of the Rab family	-

Underline represents newly identified genes in this chapter whose disruption causes lactic acid hypersensitivity. Others were also identified in Kawahata *et al.* (2006). Scores for lactic acid sensitivity were assigned as follows: "- - - -", "- - -", "- -", and "-" represent hypersensitivity to >1%, to >2%, to >3%, and to >4% lactic acid when compared with wild type, respectively.

I initially classified the 107 genes into functional categories using Munich Information Centre for Protein Sequence (MIPS) database with FunSpec program (Robinson *et al.*, 2002). These classifications are useful for understanding which biological functions are important for adaptation responses to lactic acid. Table 5 lists the representative functional categories of genes whose deletions caused lactic acid sensitivity ($p < 0.01$). A variety of cellular functions were suggested to be involved in the adaptation response to lactic acid. The p value is a good statistical indicator of overrepresentation of genes in a given functional category (Robinson *et al.*, 2002). The category associated with vacuolar transport containing 23 genes

(*VPS15*, *PEP7*, *NHX1*, *VPS3*, *VMA8*, *VPS45*, *CHC1*, *VMA7*, *CLC1*, *VMA10*, *VPS35*, *VMA5*, *VPS1*, *VPS51*, *PEP3*, *VPS34*, *VMA6*, *VPS20*, *PEP5*, *MON2*, *TLG2*, *VMA4*, and *VPS16*) exhibited lowest *p* values (Table 5), suggesting that many cellular functions, especially functions associated with vacuolar transport play important roles in adaptation response under lactic acid stress condition.

Table 5. Representative functional categories of genes whose disruption causes sensitivity to 4% L-lactic acid stress.

MIPS functional category[a]	No. of genes identified in this chapter	No. of total genes	*p*-value[b]
Vacuolar transport [20.09.13]	23	153	$< 1 \times 10^{-14}$
Cation transport (H^+, Na^+, K^+, Ca^{2+}, NH_4^+, etc.) [20.01.01.01]	10	68	8.3×10^{-8}
Vesicular transport (Golgi network, etc.) [20.09.07]	10	72	1.4×10^{-7}
Homeostasis of protons [34.01.01.03]	8	47	5.5×10^{-7}
Protein targeting, sorting and translocation [14.04]	15	281	3.0×10^{-5}
Vacuole [42.25]	6	44	5.7×10^{-5}
Intra Golgi transport [20.09.07.05]	5	33	1.5×10^{-4}
Transport ATPases [20.03.22]	6	53	1.7×10^{-4}
Osmosensing and response [34.11.03.13]	5	35	2.0×10^{-4}
Metabolism of tryptophan [01.01.09.06]	3	8	2.0×10^{-4}
Tetracyclic and pentacyclic triterpenes metabolism [01.06.06.11]	5	36	2.3×10^{-4}
Electron transport [20.01.15]	7	83	3.0×10^{-4}
Budding, cell polarity and filament formation [43.01.03.05]	14	312	3.5×10^{-4}
Cellular import [20.09.18]	7	90	5.0×10^{-4}
Biosynthesis of threonine [01.01.06.04.01]	2	3	7.3×10^{-4}
MAPKKK cascade [30.01.05.01.03]	4	27	7.7×10^{-4}
Metabolism of phenylalanine [01.01.09.04]	3	13	9.7×10^{-4}
Metabolism of tyrosine [01.01.09.05]	3	14	1.2×10^{-3}
Phosphate metabolism [01.04]	15	401	1.4×10^{-3}
Degradation of threonine [01.01.06.04.02]	2	4	1.4×10^{-3}
Exocytosis [20.09.16.09.03]	4	33	1.7×10^{-3}

Modification by phosphorylation, dephosphorylation, autophosphorylation [14.07.03]	9	186	2.5×10^{-3}
Transcriptional control [11.02.03.04]	15	426	2.6×10^{-3}
Pheromone response, mating-type determination, sex-specific proteins [34.11.03.07]	9	189	2.8×10^{-3}
Homeostasis of cations [34.01.01]	3	19	3.1×10^{-3}
Protein binding [16.01]	14	391	3.1×10^{-3}
Polyphosphoinositol mediated signal transduction [30.01.09.11]	3	20	3.6×10^{-3}
Regulation of C-compound and carbohydrate metabolism [01.05.25]	7	126	3.6×10^{-3}
Actin cytoskeleton [42.04.03]	6	96	3.9×10^{-3}
Modification by acetylation, deacetylation [14.07.04]	5	69	4.5×10^{-3}
Cell wall [42.01]	9	213	6.2×10^{-3}
Metabolism of serine [01.01.09.02]	2	8	6.5×10^{-3}

[a] Number in parenthesis is MIPS functional category number.

[b] *p*-values represent the probability that the intersection of a given list with any given category occurs by chance (Robinson *et al.*, 2002).

3.3.2 Almost half of the lactic-acid-sensitive disruptants show vacuolar acidification defect.

Many disruptants for genes involved in the vacuolar transport such as *VPS* genes are known to show defects of vacuole (Rothman and stevens, 1986; Rothman *et al.*, 1989; Seeley *et al.*, 2002). The yeast vacuole, an acidic compartment responsible for the degradation of cellular components, is essential for many cellular processes including endocytosis, pH homeostasis, metabolite storage, and sequestration of toxic substances such as excess metals (Corson *et al.*, 1999; Klionsky *et al.*, 1990; Kane, 2006). Vacuolar acidification is important for stress-related functions. Strains with a defect in acidification of the vacuole show a growth defect in medium of inadequate pH (Nelson and Nelson, 1990). Vacuolar acidification activity is also essential for efficient cytosolic pH homeostasis that plays important roles in expressing proper activity of many cellular proteins

(Martínez-Muñoz and Kane, 2008). Furthermore, vacuolar acidification defect causes acutely sensitivity to a wide variety of heavy metals (Corson *et al.*, 1999) and hypersensitivity to many different stresses including ethanol (Teixeira *et al.*, 2009), acids (Kawahata *et al.*, 2006), and air-drying (Shima *et al.*, 2008). When the 107 genes were classified into yeast biological process categories using the FunSpec program (Robinson *et al.*, 2002), the category associated with vacuolar acidification exhibited lowest p value (1.2×10^{-10}) (data not shown). In addition, 18 of the 23 lactic-acid-sensitive disruptants for genes classified in vacuolar transport category were found to exhibit abnormal acidification of vacuole even in the absence of lactic acid by staining with quinacrine which is an indicator of the acidic interior of a vacuole (Conibear and Stevens, 2002) (Table 6). Because these data suggested importance of maintenance of vacuolar functions especially vacuolar acidification for adaptation for lactic-acid stress, I next examined the presence of vacuolar acidification defects in all of the lactic-acid-sensitive disruptants with quinacrine.

Table 6. Vacuolar acidification and morphology of L-lactic acid sensitive disruptants under lactic-acid free condition.

Acidification	Morphology	Genes
Normal	Fragmented	*VAM10, CCH1, SLA2, MON2, VPS35, VPS5, BST1, SAP30, CHC1*
Decreased	Normal	*ECM3, BUD19, BUD23, RGD1, MID1, VMA8, VPS1, COG1, CSG2, PDX3, CAX4, CNB1, SWI3, SFP1, VPS20, NHX1, VPS3, VPS34, SRV2*
Decreased	Fragmented	*OCH1, YPT6, ALF1, BUD32, VPS54, VRP1*
Lost	Normal	*PPA1, VPS45, PEP5, SHP1, KEM1, VPS15, VMA22, PEP3, VMA5, VMA7, VMA6, VMA1, VPS16, PEP7, VPS52, VMA10, VMA4, RRG1*
Lost	Fragmented	*TFP3, VPS51, ANP1, MDJ1, RPL1B*

Interestingly, I found that 48 of the lactic-acid-sensitive disruptants (45% of the total) exhibited abnormal acidification of vacuole, even in the absence of lactic-acid stress (Table 6). In this analysis, 20 genes (*ECM3*, *BUD19*, *BUD23*, *RGD1*, *MID1*, *COG1*, *CSG2*, *PDX3*, *CAX4*, *CNB1*, *SWI3*, *SRV2*, *SFP1*, *OCH1*, *ALF1*, *BUD32*, *KEM1*, *ANP1*, *MDJ1*, and *RPL1B*; Table 6) were newly found to be involved in vacuole acidification by confirming that these abnormal vacuolar phenotypes were indeed caused by disruption of the corresponding genes by tetrad analysis (data not shown). For example, the vacuole of the Δ*kem1* and Δ*mdj1* disruptants did not show positive quinacrine staining, and the quinacrine fluorescence intensity of the Δ*cnb1* and Δ*och1* disruptants was significantly reduced as compared with the parental strain, indicating that these disruptants failed to acidify the vacuole properly (Fig. 6A). The decrease in fluorescence intensity of quinacrine in these disruptants was also confirmed by using a spectrofluorometer (Fig. 6B). I also found that the vacuole stained with FM4-64, which was used for visualization of the vacuolar membrane (Conibear and Stevens, 2002), of the Δ*och1* and Δ*mdj1* disruptants was fragmented (Fig. 6A). Because vacuolar acidification is important for adaptation to many stress conditions (Phelan *et al.*, 2006; Corson *et al.*, 1999; Teixeira *et al.*, 2009), it may be suggested that these disruptants displaying vacuolar acidification defect even under lactic acid free condition showed sensitivity to lactic acid due to its vacuolar acidification defect. Moreover, disruptants showing defects in vacuolar acidification may be suggested to have some defects in intracellular pH homeostasis which is important to maintain the activity of intracellular proteins because proper acidification of vacuole was

essential for stable localization of Pma1, an essential proton pump, at the plasma membrane (Martínez-Muñoz and Kane, 2008; Perzov *et al.*, 2000; Hirata and Takatsuki, 2001).

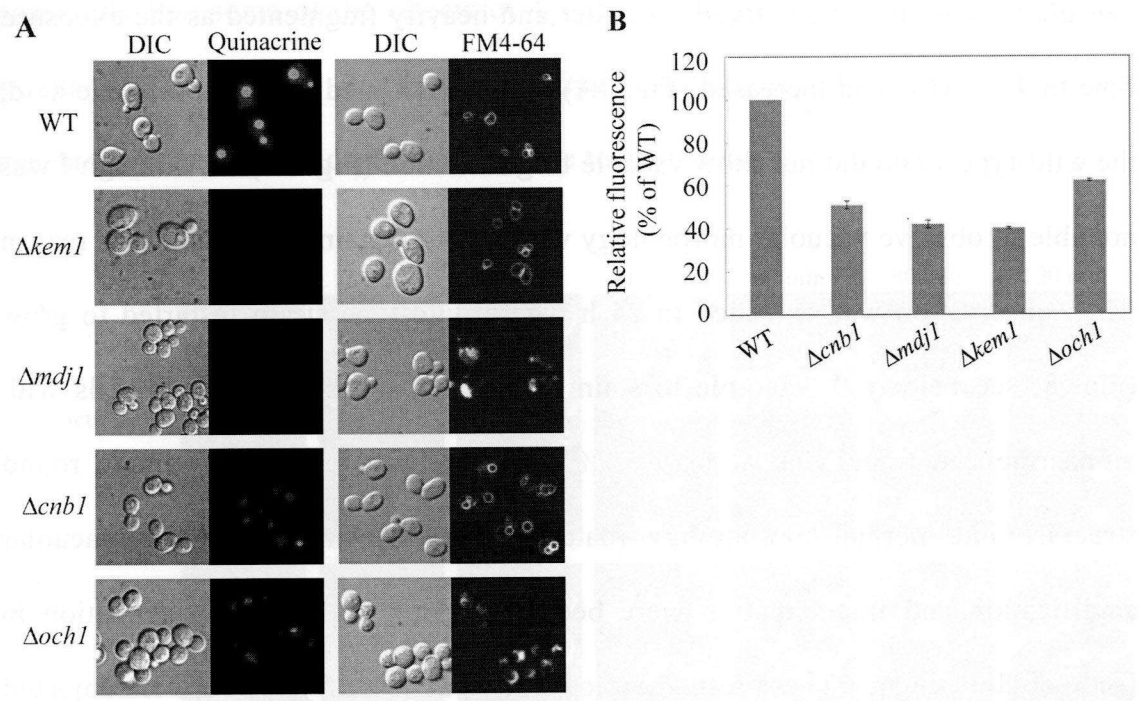

Figure 6. Vacuolar acidification and morphology of lactic-acid-sensitive disruptants. (A) Quinacrine and FM4-64 staining. BY4742 (WT) and lactic-acid-sensitive disruptants (Δ*cnb1*, Δ*mdj1*, Δ*kem1*, and Δ*och1*) were stained as described in Materials and methods, and examined by differential interference contrast (DIC) and fluorescence microscopy. A decrease in fluorescence intensity indicates defective vacuolar acidification. (B) Quantification of quinacrine fluorescence intensity. Fluorescence intensity values were calculated as described in Materials and methods and were expressed relative to those of the BY4742 (WT) strain (defined as 100%); error bars show the standard deviation of three independent experiments.

3.3.3 Vacuole is subjected to fragmentation by lactic-acid stress

I next examined vacuolar integrity from the view point of vacuolar morphology during adaptation period to lactic-acid stress in the wild-type strain

since fragmentation of the vacuole also causes increased sensitivities against low and high pH (Corson *et al.*, 1999) and ethanol and heat shock (Matsuura and Takagi, 2005). When I observed the vacuolar morphology of the wild-type strain with FM4-64 under the 4% lactic-acid condition in a time-dependent manner, I found that the vacuole became markedly smaller and heavily fragmented as the exposure time to 4% lactic acid increased (Fig. 7A). In YPDA medium without lactic acid, the wild-type strain did not show vacuole fragmentation (Fig. 7B). Although I was not able to observe vacuolar morphology with FM4-64 staining by unknown reason when observation was extended to 24 h where wild-type strain restarted to grow (Fig. 8; see below), I was able to stain vacuole of a portion of 24 h cells with quinacrine and found that at least 53% of cells had vacuole as a single round structure like normal morphology (data not shown), indicating that vacuolar acidification and fragmentation were becoming normal after 24 h incubation in lactic-acid medium. These results indicate that vacuole of yeast cells is subjected to fragmentation by exposure to lactic acid but the fragmented vacuole is gradually being restored to normal state through adaptation to lactic acid.

Osmotic stress (0.4 M NaCl or 0.5 M sorbitol) is also reported to induce vacuolar fragmentation (Bonangelino *et al.*, 2002). This response may serve to regulate the surface area to volume ratio of the vacuole by changing the number and size of the vacuole lobes under conditions where the vacuole loses volume due to loss of water (Bonangelino *et al.*, 2002). Thus, it is possible that the vacuolar fragmentation caused by 4% lactic acid (0.44 M) might be due to osmotic stress. Therefore, I tested whether another acid stress that has microbial growth inhibitory

effects at a lower concentration than lactic acid also causes vacuole fragmentation. The results revealed that 0.13% (35 mM) hydrochloric acid (pH 2.8) stress that causes similar growth retardation to 4% lactic acid stress (data not shown) also causes vacuolar fragmentation (Fig. 7C). Although I cannot fully rule out the possibility that some of the effects of 4% lactic acid are due to osmostress, these results highly suggest that vacuolar fragmentation seems to be a common mechanism underlying adaptation response to acid stress.

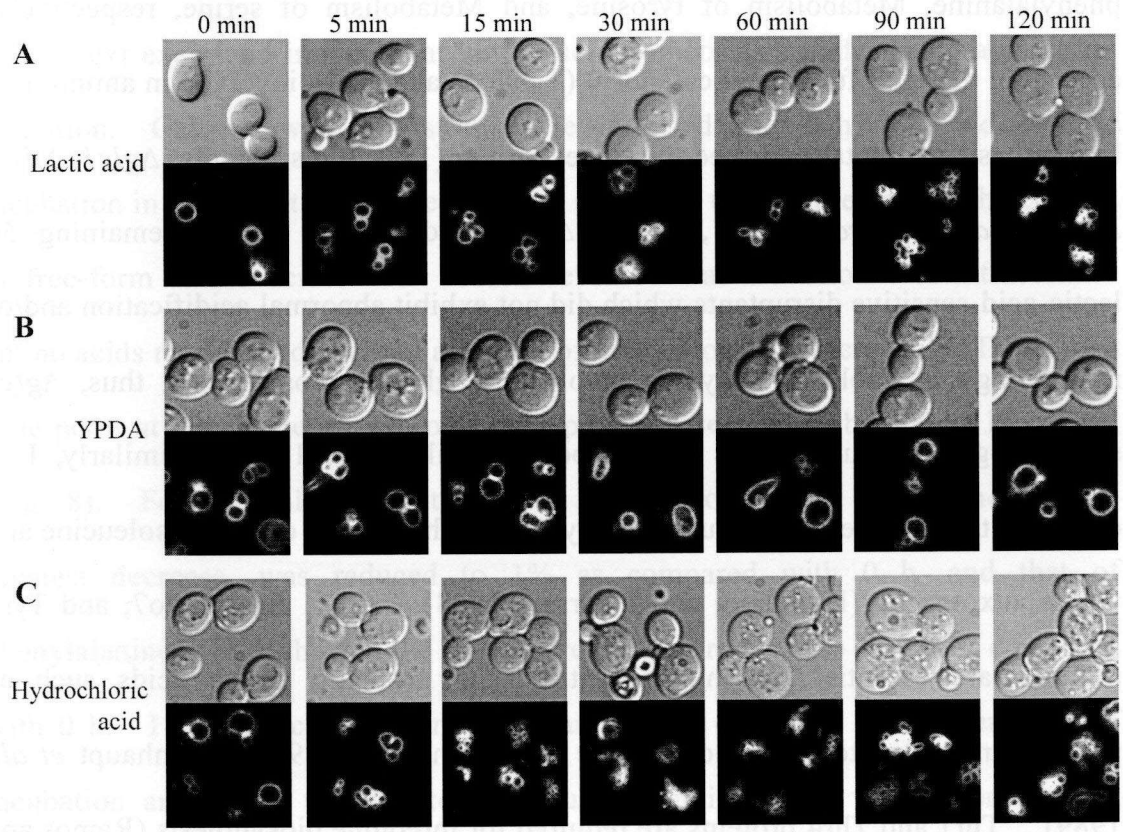

Figure 7. Time-dependent change in vacuolar morphology caused by acid stress. BY4742 cells were stained with FM4-64 as described in Materials and methods, transferred to 4% lactic acid (A), fresh YPDA (B), and 0.13% hydrochloric acid (C) medium (time = 0 min), and then examined by differential interference contrast (upper panel) and fluorescence microscopy (lower panel) for 120 min.

3.3.4 Amount of intracellular amino acid is important for the growth under lactic-acid stress

To facilitate my understanding of mechanisms involved in adaptation to lactic-acid stress in *S. cerevisiae*, I next examined the involvement of intracellular amino acid homeostasis in the adaptation because the category associated with tryptophan, threonine, phenylalanine, tyrosine, and serine metabolisms also exhibited extremely low *p* values ($p = 2.0 \times 10^{-4}$, 7.3×10^{-4}, 9.7×10^{-4}, 1.2×10^{-3}, 6.5×10^{-3} for Metabolism of tryptophan, Biosynthesis of threonine, Metabolism of phenylalanine, Metabolism of tyrosine, and Metabolism of serine, respectively, according to MIPS functional category) (Table 5) and genes involved in amino-acid biosynthesis and uptake formed the largest group (18%, 9 disruptants, Δ*gly1*, Δ*ilv1*, Δ*aro1*, Δ*aro2*, Δ*aro7*, Δ*thr1*, Δ*thr4*, Δ*tyr1*, and Δ*bap2*) in the remaining 50 lactic-acid-sensitive disruptants which did not exhibit abnormal acidification and/or morphology in Table 4. Gly1 is involved in glycine biosynthesis; thus, Δ*gly1* shows a glycine auxotrophic phenotype (McNeil *et al.*, 1994). Similarly, Ilv1 catalyzes the first step in isoleucine biosynthesis; thus, Δ*ilv1* displays isoleucine and valine auxotrophy (Holmberg and Petersen, 1988). Aro1, Aro2, Aro7, and Tyr1 proteins are essential for the biosynthesis of aromatic amino acids such as phenylalanine, tryptophan, and tyrosine (Jones and Fink, 1982; Mannhaupt *et al.*, 1989). Thr1 and Thr4 proteins are required for threonine biosynthesis (Ramos and Calderon, 1994). *BAP2* encodes a plasma membrane permease for a branched-chain amino acid (leucine, isoleucine, and valine).

To see the relationship between intracellular amount of amino-acid and growth retardation under lactic-acid stress condition, growth of wild-type strain under

lactic-acid condition was examined (Fig. 8). After addition of 4% lactic-acid stress, the growth of wild-type cells seemed to be hampered within 2 h, whereas the cells began to grow slowly after 12 h incubation and to grow more robustly after 24 h incubation (Fig. 8). I also examined the cell viability of wild-type strain after 2 h and 24 h incubation. Results showed that the cell viability decreased to 23.8% ±4.2 (2 h incubation), whereas it recovered to 44.7% ±4.1 (24 h incubation) compared with 0 h incubation. This suggests that the cell growth increased as the cell viability recovered.

I next examined intracellular amino-acid homeostasis under lactic-acid stress condition. Cells of wild-type strain were collected after 0 h, 2 h, and 24 h of incubation in 4% L-lactic acid medium and subjected to measurement of the content of free-form amino acids. The results revealed that the amount of intracellular amino acids markedly decreased after 2 h of incubation with lactic acid (Table 7), a time point at which the growth of wild-type strain seemed to be almost hampered (Fig. 8). For example, the intracellular amount of lysine, which showed the greatest decrease, was reduced to 1% as compared with 0 h, and that of phenylalanine, which showed the least decrease, was reduced to 13.2% as compared with 0 h. I also checked the intracellular amount of amino acids after 24 h of incubation and found that the total amount of amino acids was restored about 3.7-fold as compared with 2 h (Table 7). These results indicate that the cell viability (23.8% ±3.1 for 2 h incubation and 44.7% ±8.1 for 24 h incubation compared with 0 h incubation) showed a link with the intracellular amino acid concentration during the adaptation period to lactic-acid stress. The disruptant of

BAP2, which encodes a high-affinity branched-chain amino acid permease and causes lactic-acid sensitivity when deleted, displayed a much smaller amount of intracellular amino acids including not only branched-chain amino acid but also other amino acids after 0 h, 2 h, and 24 h of incubation than did the wild-type strain (Table 7). Importantly, the growth of the Δ*bap2* disruptant was still suppressed even in the 24 h of incubation under lactic-acid stress condition (Fig. 8) and the cell viability of the Δ*bap2* disruptant was also much less than that of the wild-type strain (0.4% ± 0.03 for 2 h incubation and 1.8% ±0.11 for 24 h incubation compared with 0 h incubation). These results indicate that intracellular amino acids reduce by addition of lactic acid, suggesting that the amount of intracellular amino acid is important for the growth under lactic-acid stress.

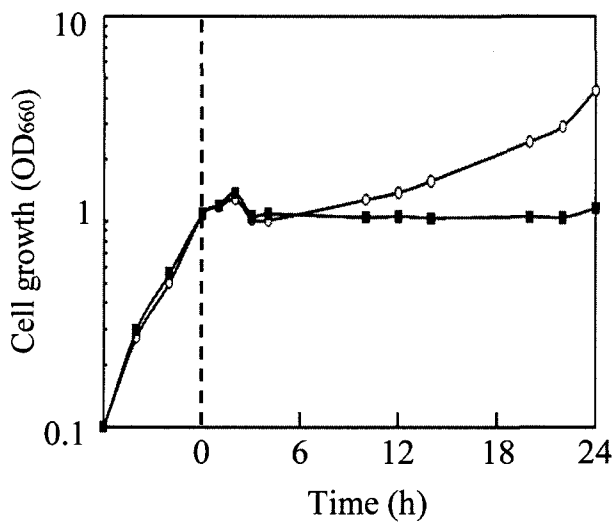

Figure 8. Effect of lactic-acid stress on cell growth. BY4742 (WT; open circles) and Δ*bap2* (closed square) strains were grown in YPDA at 30 °C to mid-log phase (OD_{660} = 1.0). Cells were harvested by centrifugation and resuspended in 4% lactic-acid medium, and then the OD_{660} of the culture was monitored periodically for 24 h.

Table 7. Amount of intracellular amino acids (nmol/mg protein).

Amino acid	BY4742			*Δbap2*		
	– Lactic acid	+ Lactic acid 2 h	+ Lactic acid 24 h	– Lactic acid	+ Lactic acid 2 h	+ Lactic acid 24 h
Asp	865.3	15.2	93.3	450.3	4.1	4.8
Thr	591.3	11.0	48.9	263.5	3.2	4.3
Ser	542.9	15.4	89.2	280.5	4.9	6.2
$AspNH_2$	184.5	8.1	20.2	77.9	2.6	3.5
Glu	4250.4	82.6	451.5	2744.1	14.4	14.9
$GluNH_2$	921.2	22.9	110.9	96.0	N.D.	N.D.
Gly	861.5	15.3	56.0	521.8	5.4	7.0
Ala	964.7	32.0	65.4	523.3	12.0	13.0
Val	271.3	13.8	24.1	115.1	5.0	6.5
Met	56.9	4.4	8.3	39.2	1.8	2.1
Ile	143.6	10.6	11.2	70.6	4.5	5.8
Leu	266.9	25.2	29.1	125.5	10.2	11.7
Tyr	88.9	9.5	4.5	51.8	3.9	3.6
Phe	151.1	20.0	15.8	80.7	8.3	9.0
Orn	248.7	5.8	24.7	160.7	0.9	1.8
Lys	4610.2	68.9	405.6	2528.0	12.5	11.4
His	536.0	8.2	42.5	278.6	2.4	2.6
Arg	3512.6	121.1	313.3	2031.0	36.0	32.9
Pro	242.3	5.4	25.3	90.3	1.3	2.1
Total	19310.2	495.4	1839.9	10528.6	133.4	143.2

N.D., not detected

3.3.5 Amino acid supplementation recovers adaptation deficiency to lactic acid

To further investigate an idea that the amount of intracellular amino acid is important for the growth under lactic-acid stress, I examined whether addition of an amino acids to the media could rescue the growth defect of 9 disruptants (*Δgly1*, *Δilv1*, *Δaro1*, *Δaro2*, *Δaro7*, *Δthr1*, *Δthr4*, *Δtyr1*, and *Δbap2*) under the lactic-acid stress condition. A serial dilution-spot assay revealed that amino-acid supplementation alleviated the lactic-acid sensitivity of those disruptants with a defect in amino-acid biosynthesis and transport (Fig. 9). For example, the

lactic-acid sensitivity of the Δ*aro1*, Δ*aro2*, Δ*aro7*, and Δ*tyr1* disruptants, which cannot synthesize Tyr, was alleviated by adding 50 mM Tyr to the 4% lactic-acid medium. Similarly, the sensitivity of disruptants requiring Ile (Δ*ilv1*), Thr (Δ*thr1* and Δ*thr4*) or Gly (Δ*gly1*, Δ*thr1*, and Δ*thr4*) was alleviated by adding 10 mM Ile, 50 mM Thr, or 50 mM Gly, respectively. The sensitivity of the Δ*bap2* disruptant was also alleviated by adding 50 mM Leu to the 4% lactic-acid medium. On the other hand, the addition of 10 or 50 mM Val, 10 or 50 mM Trp, or 5, 10, or 50 mM Phe did not relieve the sensitivity of disruptants requiring Val (Δ*bap2*), Trp (Δ*aro1*, Δ*aro2*, and Δ*aro7*) or Phe (Δ*aro1*, Δ*aro2*, Δ*aro7*, and Δ*tyr1*), respectively (data not shown). Since *ARO1*, *ARO2*, *ARO7*, and *TYR1*, or *BAP2* and *ILV1* are involved in biosynthesis of Tyr, Trp, and Phe, or transport of Leu, Ile, and Val, respectively, the growth of their disruptants was examined on 4% lactic-acid media containing Tyr (5 mM or 50 mM), Trp (5 mM or 10 mM), and Phe (1 mM or 3 mM), or Leu (50 mM or 25 mM), Ile (10 mM or 5 mM), and Val (10 mM or 5 mM), simultaneously. However, the growth of the Δ*aro1*, Δ*aro2*, Δ*aro7*, Δ*tyr1*, Δ*bap2*, and Δ*ilv1* disruptants on those media was not enhanced further when compared with those on the media containing single amino acid, *i.e.*, Tyr (*ARO1*, *ARO2*, and *ARO7*) or Leu (*BAP2* and *ILV1*) (data not shown). These results suggest that specific amino acids but not any amino acids are important for the growth under lactic-acid stress. Taken together, the amount of intracellular amino acids, especially for Thr, Gly, Ile, Tyr, and Leu, may be important for mitigation of the growth defect by lactic-acid stress.

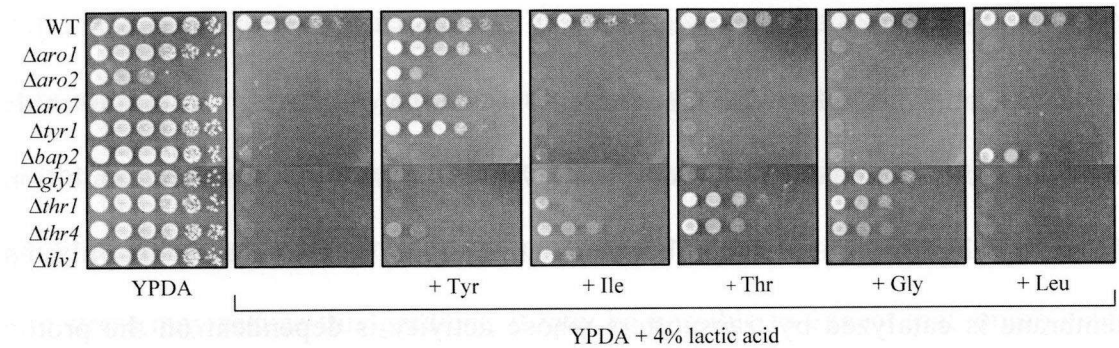

Figure 9. Growth assay of lactic-acid-sensitive disruptants on lactic-acid medium supplemented with amino acids. A ten-fold serial dilution of each yeast strain was spotted onto YPDA medium, 4% lactic-acid medium (pH 2.8), and 4% lactic-acid medium supplemented with the indicated amino acids (50 mM Tyr, 10 mM Ile, 50 mM Thr, 50 mM Gly, or 50 mM Leu) (pH 2.8) as described in Materials and methods. Cells were incubated at 30 °C for 2 days.

3.4 Discussion

In this chapter, my analysis newly identified 34 genes (>30% in my analysis) (Table 4) that were not mentioned previously but are involved in lactic-acid resistance. For example, the depletion of Uba4 was found for the first time to cause lactic-acid hyper-sensitivity. Since Uba4 was suggested to regulate the function of the antioxidant protein Ahp1 through its urmylation in response to oxidative stress (Goehring *et al.*, 2003), it may be possible to speculate that Uba4 is also involved in the urmylation of proteins that are important for adaptation to lactic-acid stress. A N-terminal acetyltransferase, Nat3 (Caesar *et al.*, 2006), was also found to be involved in lactic acid adaptation mechanisms. Since Nat3-mediated N-terminal acetylation of proteins is involved in many physiological processes such as regulation of actin cytoskeleton or cell cycle (Caesar *et al.*, 2006), N-terminal acetylation of specific targets is also suggested to play important roles in

adaptation responses of lactic acid.

In this analysis, I found that remarkable reduction of intracellular amino acids occurred under lactic acid condition (Table 7). This may be caused by inhibition of the uptake of amino acids because amino-acid uptake across the plasma membrane is catalyzed by transporters whose activity is dependent on the proton electrochemical gradient (Seaston *et al.*, 1973), and acid stress likely reduces this gradient. Consistent with this idea, it has been reported that sorbic acid inhibits the uptake of aromatic amino acids (Bauer *et al.*, 2003). Disturbance of vacuolar integrity, which is suggested to be important for recycling of membrane proteins (Bugnicourt *et al.*, 2004), may also impair the correct localization of amino-acid transporters, thereby leading to amino-acid starvation. Moreover, vacuolar fragmentation caused by lactic acid is likely to affect the intracellular amount of amino acids because the vacuole is involved in storage of amino acids, and a fragmented vacuole loses storage capacity and activity of vacuolar enzymes (Klionsky *et al.*, 1990; Jacquemin-Faure *et al.*, 1994; Kitamoto *et al.*, 1988). In addition, since yeast cells can utilize peptides that are contained in much amount in YPD broth as a source of amino acids by transporting and hydrolyzing them (Perry *et al.*, 1994; Hauser *et al.*, 2001), my result (Table 7) suggests that decreased transport and hydrolysis activities toward peptides may also occur under lactic-acid condition. Since amino-acid starvation caused by lactic-acid stress may in turn cause a general reduction in protein synthesis, which is important for producing stress-response proteins, maintaining amino acid homeostasis plays an important role in adaptation responses against lactic-acid stress. As observed in Fig. 9, the

lactic-acid sensitivities of disruptants for genes involved in amino-acid metabolism were alleviated but partially even when excess amount of amino acids were added to the media. Since the proton electrochemical gradient is important for amino-acid uptake activity across the plasma membrane (Seaston *et al.*, 1973) and acid stress may reduce this gradient, it may be possible to expect that amino acids were not transported sufficiently into the cells, thereby I think that the disruptants did not show complete recovery.

Lactic acid sensitive disruptants having vacuolar acidification defects possibly had insufficient sequestration abilities of proton at the vacuole, thereby showing the sensitivity. Moreover, since proper acidification of vacuole was essential for stable localization of Pma1 at the plasma membrane (Martínez-Muñoz *et al.*, 2008; Perzov *et al.*, 2000; Hirata and Takatsuki, 2001), lactic acid sensitive disruptants showing defects in vacuolar acidification may also likely have some defects in intracellular pH homeostasis which is important to maintain the activity of intracellular proteins. It is also known that fragmentation of vacuoles causes shortage of storage capacity of amino acids (Kitamoto *et al.*, 1988). Attenuation of the uptake activity of amino acids is also imagined since amino-acid uptake across the plasma membrane is catalyzed by transporters whose activity is dependent on the proton electrochemical gradient (Seaston *et al.*, 1973), and lactic-acid stress may reduce this gradient across the plasma membrane. Therefore, severe depletion of amino acids should be caused subsequently as observed in this chapter. In the stressed condition, maintenance of intracellular amino acid homeostasis is critical because cells have to produce a variety of stress response

proteins using intracellular amino acids. Starvation of amino acids also causes a marked accumulation of oxygen radicals (Eisler *et al.*, 2004), thereby producing an additional stress. Moreover, as reported in bacteria, for example *Escherichia coli* (Foster, 2004), amino acids play important roles to maintain intracellular pH by enhancing consumption of protons through decarboxylation of glutamate or arginine under acidic condition. In addition to decreased ATP level resulting from activation of Pma1 under acidic condition (Viegas *et al.*, 1998), these may prevent the proliferation of yeast. However, yeast cell is supposed to acquire abilities to generate ATP by progressing the phase of stress adaptation, for example, the sequestration of the proton and cell-wall reorganization that may attenuate the influx of lactic acid into the cell (Kawahata *et al.*, 2006) and by possibly increasing pH of the medium through consumption of proton. ATP may be important for active efflux of the anion from cell by unknown transporters and for import of peptides or amino acids into cell. These effects would further facilitate the adaptation responses, thereby supporting the restart of yeast growth under lactic-acid condition.

3.5 Summary

To gain more insight into adaptation response to lactic-acid stress in yeast, a genome-wide screening for genes whose disruption cause hypersensitivity to 4% L-lactic acid (pH 2.8) was performed using the gene deletion collection of *S. cerevisiae*. I identified 107 genes that contributed significantly to the ability of yeast cells to adapt lactic-acid stress. More than 30% of the genes identified in

this screening were newly identified to be involved in mechanisms for adaptation response to lactic acid. For example, I found for the first time that protein urmylation by Uba4 and N-terminal acetylation by Nat3 were involved in lactic acid adaptation mechanisms. Functional categorization of the genes followed by microscopic analysis revealed that a variety of cellular functions were involved in adaptation response to lactic acid and function associated with vacuolar transport played important roles in adaptation response to lactic acid. I also found that vacuole fragmented immediately upon exposure to lactic- and hydrochloric-acid stress. In addition, my analysis revealed that the amount of intracellular amino acids significantly reduced by addition of lactic acid. Amino acid supplementation recovered the adaptation deficiency to lactic acid, suggesting that the amount of intracellular amino acids may be important for mitigation of the growth defect by lactic-acid stress.

Chapter 4

General discussion and conclusion

In this study, I first screened single gene disruptants which showed resistance to 6% lactic acid (Chapter 2) or hypersensitivity to 4% lactic-acid stress (Chapter 3) although Kawahata *et al.* (2006) reported the lactic-acid sensitive or resistant disruptants screened under different concentration of lactic acid (3.1% and 5.1%, respectively) from those (4% and 6%) used in this study. Lactic acid (5%) exerts toxic effect to yeast cells (Ishida *et al.*, 2005) and wild-type strain showed transient growth retardation in the presence of 4% lactic acid (Chapter 3). Kawahata *et al.* (2006) identified 46 genes whose disruption led to resistance to 5.1% lactic acid and 128 genes whose disruption led to sensitivity to 3.1% lactic acid. This study aimed to identify novel genes involved in resistance and sensitivity against lactic acid by employing more severe conditions than previous studies. This screen identified 69 additional disruptants that were resistance to lactic acid and 34 additional disruptants sensitive to lactic acid. Because many genes involved in severe lactic-acid stress were newly identified, it is considered that understanding the function of these genes will help to elucidate the detailed response mechanisms to lactic acid in yeast.

In chapter 2, the lactic-acid productivity of the *LDH*-harboring quadruple disruptant (*Δdse2Δscw11Δeaf3Δsed1*; SH6779), which showed the strongest lactic-acid resistance, significantly increased (approximately 27%, 48 g/litter, 72h)

compared with wild-type strain (SH6764; 38 g/litter, 72 h) (Fig. 5). Table 8 compares lactic-acid productivity with those reported in previous studies which were performed in batch cultures, with or without neutralization. As shown in Table 8, although *LDH*-harboring quadruple disruptant (*Δdse2Δscw11Δeaf3Δsed1*; SH6779) did not show the highest productivity and yield among all cases, SH6779 had the highest productivity and yield of any published strain grown without neutralization (Dien *et al.,* 2001 and Gao *et al.* 2011), and despite the non-neutralization condition, SH6779 productivity was comparable to studies employing neutralization. Thus, I propose that SH6779 has great potential and its lactic-acid productivity could be additionally enhanced with other refinements.

Table 8. Comparison of lactic-acid production

Organism	*LDH* (copy number)	Neutralization	Initial glucose	Lactic acid (g/l)	Productivity (g/l/h)	Yield (g/g)	Disruption of EtOH pathway	Reference
L. casei	Native (1)	$CaCO_3$	9% (9 g)	82.0	1.71	0.91	—	Hujanen (1996)
R. oryzae	Native (1)	NaOH	15% (15 g)	76.2	2.21	0.51	—	Bai (2003)
E. coli	*S. bovis* (low copy)	KOH NaOH	10% (35 g)	64.0	0.40	0.64	—	Dien (2001)
E. coli	*S. bovis* (low copy)	—	10% (35 g)	11.4	0.07	0.11	—	Dien (2001)
S. cerevisiae	*R. oryzae* (over expression)	NaOH	9% (2.7 g)	38.0	1.27	0.42	—	Skoy (2003)
S. cerevisiae	*L. plantarum* (over expression)	KOH	20% (200 g)	58.0	0.64	0.29	Δ*pdc1*	Colombie (2003)
S. cerevisiae	Bovin (2)	$CaCO_3$	10% (4 g)	55.6	0.76	0.56	Δ*pdc1*	Ishida (2005)
S. cerevisiae	Bovin (2)	$CaCO_3$	10% (4 g)	82.3	0.38	0.82	Δ*pdc1* Δ*pdc5*	Ishida (2006)
S. cerevisiae	Bovin (4)	NaOH	10% (4 g)	74.2	1.55	0.74	Δ*pdc1* Δ*adh1*	Tokuhiro (2009)
S. cerevisiae	Bovin (2)	—	10% (10 g)	36.0	0.50	0.36	Δ*pdc1*	Gao (2011)
S. cerevisiae (SH6779)	Bovin (1)	—	10% (3 g)	48.0	0.67	0.48	Δ*pdc1*	This study

All single and multiple disruptants (SH6765–SH6779) retained the *PDC5* gene. Because Pdc1 and Pdc5 are key enzymes for ethanol production (Hohmann and Cederberg, 1990) and *PDC5* transcription is enhanced about five-fold in Δ*pdc1* disruptants (Hohmann[b], 1991), Pdc5 activity may allow ethanol production in the *LDH*-harboring disruptants. Consistent with this, ethanol was detectable in all single and multiple disruptants (SH6765–SH6779; data not shown). However, because the Δ*pdc1*Δ*pdc5* double disruptant failed to grow using glucose as a sole

carbon source (Hohmann[b], 1991), additional disruption of *PDC5* may not be suitable for construction of an industrial strain. It has been also reported that the lactic-acid productivity of a double Δ*pdc1*Δ*adh1* disruptant is higher than that of a single Δ*pdc1* disruptant, although growth rate of the Δ*pdc1*Δ*adh1* disruptant on glucose slightly decreased as compared with the Δ*pdc1* disruptant (Tokuhiro *et al.*, 2009). To minimize the negative effect on growth, other approaches may be suitable to increase the productivity of lactic acid. For example, lactic-acid production by the *S. cerevisiae* isogenic diploid strain is higher than the haploid strain (Skory, 2003). In addition, increasing the *LDH* gene copy number on genome to six enhances LDH activity 2.8-fold versus two gene copies (Saitoh *et al.*, 2005). However, as noted in Fig. 4, the resistance of some disruptants, such as Δ*dse2*Δ*scw11*Δ*eaf3* and Δ*dse2*Δ*eaf3*Δ*sed1*, differed from the disruptants in Fig. 2, possibly related to expression of *LDH*. Therefore, although increasing the *LDH* copy number is an effective way to enhance the lactic-acid productivity, it should be kept in mind that this may also decrease the degree of lactic-acid resistance in some disruptants. On the basis of these findings, it appears appropriate to construct industrial strains by either disruption of *ADH1*, a construction of isogenic diploid strain, and/or increasing the *LDH* copy number of *LDH* gene. In addition, because undissociated lactic acid freely diffuses through the cell membrane, lactic acid production at a pH lower than 3.86, which is the pK_a of lactic acid, may facilitate higher lactic-acid concentrations in the medium owing to increased efflux. Therefore, I propose that the enhancement of lactic-acid resistance is an important and viable method for breeding yeast strains for lactic-acid production.

Fragmentation of the vacuole occurred rapidly under lactic acid conditions (Fig. 7). Undissociated lactic acid diffuses through the plasma membrane and dissociates in the cytoplasm because of higher intracellular pH, which generates protons that acidify the cytoplasm and also increases concentration of lactate anion, which in turn increases turgor pressure. Upon exposure to lactic-acid stress, the growth of yeast cells was inhibited and the cell viability decreased (Fig. 8). Intracellular amino acid homeostasis was also disturbed severely after addition of lactic acid (Table 7). However, after incubation in lactic acid-containing medium for 24 h, the yeast cells resumed growth and survival rate was recovered significantly (Fig. 8). Intracellular amino acid content was also restored in comparison to the level after 2 h exposure to lactic-acid (Table 7). During this period, the vacuole began to fragment (Fig. 7) but seemed to be restored to normal after 24 h incubation in lactic-acid medium (data not shown). Although the mechanisms for the induction of the vacuolar fragmentation under lactic-acid conditions is not clear, vacuolar fragmentation may be an adaptive response to lactic-acid stress: the resulting increase in the surface area/volume ratio may maximize sequestration of proton and/or the anion by the vacuolar proton-ATPase and/or anion transporters which are not yet identified on the vacuolar membrane. This kind of response has been reported in the calcium and sodium-stress responses to increase the uptake efficiency of ions by vacuolar transporters (Kellermayer *et al.*, 2003). In the present study, vacuolar morphology in the SH6718 lactic acid resistant strain (*Δdse2Δscw11Δeaf3Δsed1*) under 4% lactic acid was examined, considering that the vacuole of lactic acid resistant strain was not fragmented.

Although the vacuole of the *Δdse2Δscw11Δeaf3Δsed1* disruptant was fragmented after 2 h incubation in 4% lactic acid, the ratio of the fragmented cells was slightly lower than in wild-type strain (unpublished data).

The amount of intracellular amino acids decreased after 2 h of incubation with lactic acid (Table 7) and addition of excess amino acids to lactic-acid media could rescue the growth defect of disruptants (Fig. 9). However, it was not clear whether the decrease in intracellular amino acids played a role in lactic-acid sensitivity. It is possible that lactic-acid sensitivity was caused by some other factor and as a result, the amount of intracellular amino acids decreased. However, when the amino-acid requirements were complemented by introducing each of three plasmids bearing have *LEU2*, *HIS3*, or *LYS2* as a selection marker into BY4742 (*MATα leu2Δ0 his3Δ1 ura3Δ0 lys2Δ0*), the transformants showed higher lactic-acid resistance in 6% lactic-acid medium as compared with BY4742 and the total amount of intracellular amino acids of these strains was higher than that of BY4742 after 2 h incubation with 4% lactic acid (unpublished data). It is also known that acetic-acid sensitivity of *S. cerevisiae* was alleviated by adding of Tyr to acetic-acid medium and that acetic-acid resistance is enhanced by overexpression of *TAT2*, which encodes a tyrosine permease (Shima *et al.*, unpublished data). These data suggest the possibility that the amount of intracellular amino acids is one factors affecting the resistance of cells to acid stress and the improvement of cellular mechanisms to increase amino acid availability, such as the storage capacity of vacuole and uptake of amino acids may contribute to increased resistance to acid-stress.

This study is beneficial not only for breeding of yeast strains with high lactic acid production without neutralization but also for a full-scale investigation of the response mechanisms to organic and inorganic acid stress in *S. cerevisiae*. The goal of this study was to establish a strategy to improve lactic-acid resistance in yeast and construct a superior lactic acid resistant yeast strain producing lactic acid efficiently without neutralization. The results show successful construction of yeast strains with improved lactic-acid resistance and suggest that lactic-acid stress causes vacuolar fragmentation and impairs intracellular amino-acid homeostasis.

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List of Publications

1. **Suzuki, T., Sakamoto, T., Sugiyama, M., Ishida, N., Kambe, H., Obata, S., Kaneko, Y., and Harashima, S.:** Disruption of multiple genes whose deletion causes lactic-acid resistance improves lactic-acid resistance and productivity in *Saccharomyces cerevisiae*. J. Biosci. Bioeng. (In press).

2. **Suzuki, T., Sugiyama, M., Wakazono, K., Kaneko, Y., and Harashima, S.:** Lactic-acid stress causes vacuolar fragmentation and impairs intracellular amino-acid homeostasis in *Saccharomyces cerevisiae*. J. Biosci. Bioeng., **113**, 421–430 (2012).

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