The *SPI1* Gene, Encoding a Glycosylphosphatidylinositol-Anchored Cell Wall Protein, Plays a Prominent Role in the Development of Yeast Resistance to Lipophilic Weak-Acid Food Preservatives

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The *Saccharomyces cerevisiae* *SPI1* gene encodes a member of the glycosylphosphatidylinositol-anchored cell wall protein family. In this work we show results indicating that *SPI1* expression protects the yeast cell from damage caused by weak acids used as food preservatives. This is documented by a less extended period of adaptation to growth in their presence and by a less inhibited specific growth rate for a parental strain compared with a mutant with *SPI1* deleted. Maximal protection exerted by Spi1p against equivalent concentrations of the various weak acids tested was registered for the more lipophilic acids (octanoic acid, followed by benzoic acid) and was minimal for acetic acid. Weak-acid adaptation was found to involve the rapid activation of *SPI1* transcription, which is dependent on the presence of the Msn2p transcription factor. Activation of *SPI1* transcription upon acetic acid stress also requires Haa1p, whereas this recently described transcription factor has a negligible role in the adaptive response to benzoic acid. The expression of *SPI1* was found to play a prominent role in the development of yeast resistance to 1,3-β-glucanase in benzoic acid-stressed cells, while its involvement in acetic acid-induced resistance to the cell wall-lytic enzyme is slighter. The results are consistent with the notion that Spi1p expression upon weak-acid stress leads to cell wall remodeling, especially for the more lipophilic acids, decreasing cell wall porosity. Decreased cell wall porosity, in turn, reduces access to the plasma membrane, reducing membrane damage, intracellular acidification, and viability loss.

Weak acids used as food preservatives inhibit microbial growth, causing highly extended lag phases. However, spoilage yeasts, such as *Zygosaccharomyces bailii* and *Saccharomyces cerevisiae*, can sometimes grow in acidic food and beverages in the presence of acid concentrations near the legal limits (22). Therefore, understanding of the molecular mechanisms that may contribute to countering the effects of organic acids as fungistatic agents is crucial to guide suitable preservation strategies. In this work we examined the role of the *SPI1* gene in *S. cerevisiae* adaptation to weak acids used as food preservatives. *SPI1* encodes a member of the glycosylphosphatidylinositol (GPI)-anchored cell wall protein (CWP) family. This class of cell wall proteins (GPI-CWP) is covalently linked to 1,6-β-glucan through a trimmed form of their original GPI anchor (21). The highly branched 1,6-β-glucan chains, found at the external face of the 1,3-β-glucan network, can be further linked to 1,3-β-glucan or chitin, resulting in a strong covalent attachment of GPI-CWP to the external layer of the cell wall (7, 21). Global expression analysis carried out in recent years has indicated that yeast cells respond to environmental stress by modifying the organization of the cell wall. This cell wall remodeling is highly dynamic and constitutes a mechanism for protection against environmental stresses and other cell wall-destabilizing agents or cell wall mutations (13, 15, 17, 18, 19). The porosity of stationary-phase yeast cells is much lower than the porosity of exponential-phase cells, as assessed by comparing resistance to a cell wall-lytic enzyme (8). This modification is accompanied by the increased expression of Spi1p and Sed1p, another GPI-CWP (23, 26, 27). However, the specific role of Spi1p and of other cell wall proteins in the yeast response to stress remains unclear.

In previous work, *SPI1* emerged as a major determinant of yeast resistance and adaptation to the lipophilic weak-acid herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) among 13 Msn2p- and Msn4p-regulated genes (27). Yeast adaptation to the herbicide was then proposed to involve Spi1p modification of cell wall architecture, suggested by the increased resistance to 1,3-β-glucanase (27). In the present study, we show evidences indicating that *SPI1* expression reduces the duration of the initial phase of adaptation to growth under stress imposed by equivalent concentrations of different weak acids used as food preservatives. *SPI1* expression also allows a less inhibited growth rate, following an earlier growth resumption in the presence of weak acids. The protective effect of *SPI1* against weak acids was correlated with their liposolubility: it was maximal for the more lipophilic acid tested (octanoic acid, C8) and minimal for acetic acid (C2). The transcriptional activation of *SPI1* during the early response to weak-acid stress and the maintenance of high *SPI1* mRNA levels during the exponential growth of cells adapted to the acid were also demonstrated. Besides the expected role of the Msn2p and Msn4p transcription factors in *SPI1* transcription activation by weak acids (27),
we demonstrated that this response to acid stress also requires a functional Haa1p. Msn2p and Msn4p are involved in the general stress response in yeast, and the transcription factor Haa1p was recently implicated in the yeast response to weak acids (12).

The antimicrobial effect of weak acids at low pHs relies on the undissociated liposoluble toxic form, leading to plasma membrane permeabilization to different extents, depending on the weak-acid concentration and lipophilicity. The liposoluble acid form significantly reduces plasma membrane spatial organization, affecting the function of the plasma membrane as a matrix for enzymes and a selective barrier, thereby leading to a decrease in the internal pH of cells suspended in an acidified medium suitable for yeast growth and to the dissipation of the proton motive force across the membrane (22). Following the passive diffusion of the undissociated acid into the cell, its dissociation in the approximately neutral cytoplasm also leads to internal acidification and to the accumulation of the counter-ion, which can cross the plasma membrane only by active export through specific transporters (22). In this study, we also examined the mechanism behind the protection exerted by Spi1p against the deleterious effects of weak acids of increasing lipophilicity. The presence of Spi1p was found to lead to cell wall remodeling reducing cell wall porosity, especially for the more lipophilic acids; this finding was based on the decreased level of resistance to lyticase in weak-acid-stressed cells. We also demonstrated that SPI1 expression leads to reductions in weak-acid-induced plasma membrane damage, intracellular acidification, and viability loss. Based on this evidence, we hypothesize that yeast cell wall remodeling in response to acid stress, mediated by Spi1p, reduces the access of weak acids to the plasma membrane.

Northern blot analysis. RNA extraction from yeast cells cultured under acidic or benzoic acid stress was performed by the hot phenol method, and Northern blot hybridizations were carried out as reported previously (12). The total RNA concentration in each sample used for Northern blotting was approximately constant (20 μg). The specific DNA probe used to detect SPI1 transcripts was prepared by PCR amplification (primer sequences, 5′-AACTTCTCGAA GTTCCGATTG-3′ and 5′-TGCAGTACGAGCAGATTGTT-3′). This probe showed no significant homology to the rest of the genome, and its specificity was tested using total RNA extracted from cells of the corresponding deletion mutant, previously exposed to weak acids under activation conditions. The ACTI mRNA level was used as the internal control. Hybridization signals in nitrocellulose membranes were detected in a screen exposed to the hybridized membrane by a laser-based imaging system, using Typhoon Trio equipment from Amersham Pharmacia Biotech (Carnaxide, Portugal). The relative intensities in laser-based images were quantified by densitometry using ImageMaster 1D Elite software (Amersham Pharmacia Biotech, Carnaxide, Portugal).

1.3-β-Glucanase sensitivity assay. To monitor structural changes in the yeast cell wall, a lysis of the 1,3-β-glucanase from Arthrobacter luteus; Sigma) sensitivity assay was conducted as described by Shimoi et al. (26). Wild-type yeast cells and Δmnr2, Δmnr1, and Δspi1 deletion mutants were cultivated in MM4 liquid medium, either supplemented or not with acetic acid (60 mM) or benzoic acid (0.9 mM), and were harvested following 0 or 3 h of incubation. The harvested cells were washed with distilled water and resuspended in 0.1 mM sodium phosphate buffer (pH 7.5). After the addition of 20 μg/ml of lyticase, cell lysis was followed by a decrease in the initial OD600 of the cell suspensions (expressed as a percentage).

Assessment of pHi values. Intracellular pH (pHi) was compared by fluorescence microscopy in cell populations of S. cerevisiae BY4741 and the Δspi1 mutant, harvested by filtration after 20 min of growth in the absence or presence of weak acids. Cell pellets were washed twice with CF buffer (50 mM glycine, 10 mM NaCl, 5 mM KCl, 1 mM MgCl2 in 40 mM Tris–100 mM morpholinethanesulfonic acid [MES], pH 4.0) and resuspended in 2 ml of CF buffer to an OD600 of 10. For pHi staining, the probe 5′(and 6′)-carboxyfluorescein diacetate, succinimidyl ester [5(6)-CFDA,SE] (45 mM in dimethyl sulfoxide; Molecular Probes Europe BV, Leiden, The Netherlands) was added to the cell suspension to a final concentration of 20 μM (further information may be found at http://probes.invitrogen.com/handbook/boxes/0428.html). The mixture was vortexed in one burst of 10 s and incubated for 20 min at 30°C, with orbital agitation (250 rpm). After being washed twice with CF buffer, 5(6)-CFDA,SE-loaded cells were resuspended in 2 ml of CF buffer and examined immediately with a Zeiss Axioplan microscope equipped with adequate epifluorescence interference filters (Zeiss BPS50-490 and Zeiss LPS20). Fluorescent emission was collected with a cooled charge-coupled device (CCD) camera (Cool SNAPFX, Roper Scientific Photometrics). Bright-field images for determination of pH were obtained continuously and recorded at 1-min intervals; each experiment was finished within 15 min. The images were analyzed using MetaMorph, version 3.5. The fluorescence images were background corrected using dark-current images. The pHi values were calculated for cells per experiment, taking into account the number of cells that were selected using regions of interest obtained from bright-field images recorded before or after the experiment. The value of fluorescence intensity emitted by each cell was obtained pixel by pixel in the region of interest. To estimate average pHi values, an in vivo calibration curve was prepared using cell suspensions grown in the absence of acids. Cells were loaded with 5(6)-CFDA,SE as described above and incubated at 30°C for 10 min with 0.5 mM carbonyl cyanide m-chlorophenylhydrazone (CCCP, Sigma), to dissipate the plasma membrane pH gradient, before adjustment of the external pH (in the range of 3.0 to 7.0) by the addition of HCl or NaOH at 2 M. Membrane permeability and cell viability during acid stress. Membrane permeability during weak-acid stress was measured by the uptake of the fluorescent nuclear stain ethidium bromide, which is largely excluded by yeast cells with intact plasma membranes (5, 6). Cells were harvested by filtration after 20 min of growth in the absence or presence of weak acids. After being washed twice with 25 mM MES (pH 4), cells were resuspended in 1 ml of MES buffer to an OD600 of 5. Following this, 10 μg of ethidium bromide was added to 1 ml aliquots of these cells. After incubation at 30°C for 15 min, with orbital agitation (250 rpm), a 6-μl aliquot of the cell sample was dispensed in a clean microscope slide and examined immediately with a Zeiss AxioPlan microscope (Carl Zeiss MicroImaging, Inc.) equipped with adequate epifluorescence interference filters (Zeiss BPS50 and Zeiss LPS20). Fluorescent emission was collected with a cooled CCD camera (Cool SNAPFX, Roper Scientific Photometrics). The membrane integrity was estimated by capturing low-magnification epifluorescent and phase-contrast images to calculate the percentage of fluorescent cells in the population (at least 300 cells were analyzed for each condition). The concentra-
tion of viable cells in the cell populations harvested for measurement of membrane permeability was also assessed as the number of CFU on YPD solid medium following incubation for 3 days at 30°C.

RESULTS

**SPI1** expression is required for a more rapid yeast adaptation to weak acids, and this effect is more evident as the lipophilicity of the acid increases. Comparison of the growth curves of yeast populations not previously adapted to weak acids, either expressing the **SPI1** gene or not, indicates that this gene is required for a more rapid adaptation to growth in weak-acid-supplemented media. The work was carried out using equivalent inhibitory concentrations of the different acids tested. These concentrations were selected so that the durations of the parental-strain growth latency periods were similar. The elimination of the **SPI1** gene led to an increased duration of the period of latency, and this increase was correlated with the lipophilicity of the weak acid (Fig. 1). Specifically, a lag phase of approximately 18 h, observed for the parental strain, was extended to 22 h or 138 h when the Δ**spi1** population was incubated in the same medium supplemented with acetic acid or octanoic acid, respectively (Fig. 1). **SPI1** deletion also led to a more reduced specific growth rate following adaptation to growth under acid stress. This protective effect of **SPI1** was also more evident for the more lipophilic acids, butyric, benzoic, and octanoic acids, in that order (Fig. 1).

Transcriptional activation of **SPI1** in weak-acid-stressed cells depends on Msn2p/Msn4p and Haa1p. After 1 h of exposure of an unadapted cell population of *S. cerevisiae* BY4741 to stress-inducing concentrations of acetic or benzoic acid, or during exponential adapted growth in the presence of the acids, the mRNA level of the **SPI1** gene was significantly above the level exhibited by unstressed cells (Fig. 2 and 3). The rapid **SPI1** transcriptional activation induced by acetic or benzoic acid is fully dependent on the presence of Msn2p; the homologous transcription factor Msn4p has a marginal role in this activation (Fig. 2 and 3 and data not shown). The level of the rapid transcriptional activation observed during the early yeast response to acute stress promoted by benzoic acid was twice the activation level detected with an equivalent concentration of acetic acid. Although they maintained high **SPI1** mRNA levels during exponential growth, cells growing in the presence of benzoic acid exhibited **SPI1** mRNA values below those registered during the initial phase of adaptation, as reported previously for the highly lipophilic herbicide 2,4-D (27). However, exponential-phase cells, adapted to growth with an equivalent concentration of the more hydrophilic acetic acid, exhibited levels of **SPI1** mRNA above those estimated during latency. An additional difference in the yeast cell responses to these two weak acids is related to the effect that the presence of the transcription factor Haa1p had on the level of **SPI1** transcriptional activation under acid stress: whereas **SPI1** activation is fully dependent on the presence of Haa1p during the...
early response to acetic acid, the effect of HAA1 deletion in benzoic acid-challenged cells is slight. Interestingly, this differential yeast response to these two acids is consistent with the level of protection exerted by Haa1p against equivalent concentrations of these acids (Fig. 2 and 3) (12).

**Decreased sensitivity to lyticase in weak-acid-stressed cells: role of Spi1p, Haa1p, and Msn2p.** The different levels of SPI1 transcriptional activation during the early responses to acetic and benzoic acids and the differential involvement of Haa1p in this activation prompted us to monitor the alterations in yeast cell wall architecture induced by these acids, as well as the role that Spi1p and the transcription factors found to influence SPI1 transcription activation may play in the process. The very simple 1,3-β-glucanase sensitivity assay, which has been shown to be valuable in monitoring cell wall alterations (15, 26, 27), was used. Yeast cells grown for 3 h in a culture medium at pH 4 in the absence of weak acids are slightly more susceptible to lyticase when Spi1p is absent (the Δspi1 mutation) (Fig. 4A). Cells incubated at pH 4 are even more susceptible to lyticase when a functional Msn2p transcription factor is not present...
periodically. Data are means ± standard deviations from at least three independent experiments.

(Fig. 4A), consistent with the notion that Msn2p regulates other cell wall-related genes besides SPI1 (4, 14, 19). It has been suggested previously that yeast cells respond to low pH (pH 4.0) by favorably altering the yeast cell wall (15). Three hours of incubation with either acetic acid or benzoic acid led to a remarkably higher resistance of wild-type cells to lyticase activity, and this effect was even more evident for an equivalent concentration of the more lipophilic benzoic acid (Fig. 4B and C). The elimination of SPI1 had a major effect on the benzoic acid-induced increase in yeast resistance to the cell wall-lytic enzyme, contrasting with the slight effect that Spi1p had on acetic acid-induced resistance. This observation is consistent with the major role of the SPI1 gene in conferring protection against benzoic acid compared with its minor effect as a determinant of resistance to acetic acid (Fig. 1). For both weak acids, the presence of Msn2p was important for providing maximal resistance to lyticase, suggesting similar effects on the yeast responses to these acids, consistent with indications from Northern blot experiments (Fig. 2 and 3). The role of Haa1p in providing resistance to lyticase for yeast cells exposed to both acids was minor. This finding is in agreement with the minor role of the SPI1 gene in counteracting these deleterious effects, were examined (Fig. 5). For this purpose, wild-type and Δspi1 deletion mutants, toward acetic acid (Fig. 4B); in the case of benzoic acid, for which SPI1 is a relevant resistance determinant (Fig. 1), this finding is in agreement with the minor effect of Haa1p in SPI1 transcriptional activation in response to benzoic acid stress (Fig. 3).

SPI1 expression reduces intracellular acidification under weak-acid stress. The effects of equivalent inhibitory concentrations of weak acids of increasing lipophilicity have on the acidification of the cell interior, and the role of SPI1 expression in counteracting these deleterious effects, were examined (Fig. 5). For this purpose, wild-type and Δspi1 mutant cells, which had not previously been exposed to the acid, were incubated in a growth medium either supplemented or not with concentrations of weak acids identical to those used to prepare the growth curves shown in Fig. 1. The pHi values of these cell populations were determined after 20 min of incubation, and the results indicate that weak-acid stress leads to intracellular acidification. The pHi was reduced in the presence of the acids, and this effect was markedly increased for the Δspi1 mutant compared with the parental strain population and was more evident as the lipophilicity of equivalent concentrations of the weak acid increased (Fig. 5). The alterations occurring in pHi under acid stress were monitored by using an adaptation of the fluorescence microscopy image-processing technique described by Viegas et al. (30). This technique allows a clear-cut picture of the pHi values of individual cells, giving information about the distribution of the pHi values of the yeast cell population (Fig. 5A) instead of only an estimate of the average value for the whole population. The results obtained indicate that even in the absence of acid, the yeast cell populations exhibited significant heterogeneity, as reported previously for another cell population used as an inoculum (30). Cells cultivated in the basal medium without acid supplementation showed pHi values in the optimal range (90% exhibited pHi values above 6.5), with an estimated average pHi of approximately 6.9. Incubation in the presence of the acids tested led to an increase in the percentage of cells with pHi values below 6.0 (Fig. 5A). In the case of the parental cell population, the average pHi underwent only a moderate decrease, maintaining values in the range of 6.3 to 6.0 (Fig. 5B), but for the Δspi1 mutant, the decrease was more drastic, reaching pH 5.3 with propionic acid and pH 4.6 with octanoic acid, the most lipophilic acid tested (Fig. 5B). These results indicate that SPI1 expression is essential to help the cell counteract the intracellular acidification induced by weak acids and that this protective effect is more evident as the lipophilicity of the acids increases.

SPI1 expression reduces the level of weak-acid-induced plasma membrane damage. Since SPI1 expression was found to reduce weak-acid-induced intracellular acidification, we then tested the hypothesis that this cell wall protein may protect the cell from plasma membrane permeabilization by the lipophilic weak acids. The poorly permeant ethidium bromide molecule, which enters the cells only upon plasma membrane damaging and is able to bind nucleic acids, becoming strongly fluorescent, was used to test this hypothesis. This method is valuable in monitoring cell permeabilization by membrane-disturbing compounds (5, 6). Observation of the percentage of
fluorescent cells present after 20 min of incubation with the different acids clearly suggests that these acids lead to membrane destabilization (Fig. 6A). The membrane-damaging effects of the weak acids at equivalent inhibitory concentrations were much more evident for the Δspi1 population, strongly increasing as the molecular mass and lipophilicity of the acids tested increased (Fig. 6A). The increase in cell permeability (Fig. 6B) correlates with the loss of cell viability under acid stress, suggesting that Spi1p provides cell protection by helping to reduce membrane damage resulting from the action of lipophilic weak acids.

**DISCUSSION**

The cell wall surrounding the yeast cell prevents the lysis of the protoplast in a hypotonic environment, protects the cell from mechanical injury, and determines cellular shape. Another function of the cell wall, based on its porosity, is to limit the size of compounds that may come in contact with the plasma membrane (31). Therefore, this highly adaptable organelle protects yeast cells from harsh environments by building a dynamic interface with the environment (11, 18, 20). The cell shape and its physical strength depend on a complex structure consisting of β-glucan cross-linked with chitin and mannoproteins, forming a resilient 3-dimensional structure (18, 20). The largest class of covalently linked CWPs, linked to 1,6-β-glucan, are the GPI-CWPs. Since 1,6-β-glucan can be further linked to 1,3-β-glucan or chitin, this results in a strong covalent attachment of GPI-CWPs to the cell wall (7, 11, 18).
Global expression analysis carried out in recent years has indicated that yeast cells respond to environmental stress by modifying the organization of the cell wall (13, 19). This cell remodeling may compensate for the damage produced in the cell wall, may increase protection against diverse stresses, and may result in the rapid induction of resistance to cell wall-lytic enzymes (2, 4, 8, 13, 14, 15, 23, 25, 28, 29). Nevertheless, the specific role of cell wall-associated proteins in this stress response and their contribution to the development of stress resistance remain unclear. In particular, the mechanisms underlying the involvement of the GPI-CWP SpI1p, examined in the present study, in yeast response to stress certainly deserve attention. In fact, the inspection of results from microarray analysis obtained in recent years reveals that SPI1 transcription, together with the transcription of other genes encoding proteins that have a structural role in cell architecture, is activated in response to a number of environmental stress conditions, including osmotic stress, lipid peroxidation, low external pH, stress induced by the herbicide 2,4-D, and heat stress (2, 4, 14, 15, 23, 25, 29). SPI1 is also up-regulated in response to cell wall-destabilizing conditions, involving cell wall mutations or the application of hydrolytic enzymes such as Zymolyase (13, 19). Moreover, the important role of SpI1p in yeast adaptation and resistance to stress imposed by the highly lipophilic acid herbicide 2,4-D has been demonstrated (27). In the present work, we extend this result to other weak acids, used as food preservatives. Our results clearly indicate that SpI1p is required for more rapid adaptation to the weak acids tested and for reduced growth inhibition by those acids. Cell protection mediated by SpI1p became much more evident as the size of the carbon chain (and lipophilicity) of the weak acid increased, from acetic acid (C2) to octanoic acid (C8), with benzoic acid having a deleterious effect intermediate between those of butyric acid (C4) and octanoic acid.

Yeast adaptation and resistance to weak acids was proved to involve the rapid alteration of the cell wall, mediated by (at least) the increased expression of SPI1. This adaptation is accompanied by the acquisition of resistance to cell lysis by 1,3-β-glucanase digestion, a physiological response that is more evident for benzoic acid-stressed cells than for cells challenged with an equivalent inhibitory concentration of acetic acid. The prominent role that SpI1p plays in the rapid acquisition of resistance to lyticase in benzoic acid-stressed cells, compared with cells incubated with an equivalent inhibitory concentration of acetic acid, is in agreement with the level of protection exerted by this cell wall protein. Interestingly, the activation of SPI1 transcription during the early response to stress induced by equivalent concentrations of weak acids is also twofold higher for benzoic acid than for acetic acid. Increased resistance to lyticase by cells adapted to the acid may result from limitation of the accessibility of the internal glucan layer to 1,3-β-glucanase, due to an acid-induced decrease in the permeability of the external protein layer mediated by SpI1p. The modification of cell wall molecular organization as a result of the decrease in the external pH, from 5.5 to 3.5, with cells becoming increasingly resistant to 1,3-β-glucanase lysis, has been observed previously (15). The DNA microarrays analysis carried out in this study also revealed the increased transcription, at low pH, of four genes encoding cell wall proteins, including the SPI1 gene (15). These cell responses were attributed by the authors to the decrease in external pH from 5.5 to 3.5. However, it is likely that this may not be caused by the low pH itself but by the presence in the buffered incubation medium of succinic acid. The same response may not be observed if a strong acid is used as the acidifying agent, as reported before (3).

The transcriptional activation of SPI1 during acute acid stress is dependent on the presence of the transcription factor Haa1p, recently implicated in yeast adaptation to weak acids (12). However, in contrast to SPI1, Haa1p exerts maximal cell protection against short-chain weak acids, in particular against propionic acid and, more strongly, against acetic acid (12). Remarkably, although the transcriptional activation of SPI1 under acetic acid stress is fully dependent on the presence of Haa1, the role of Haa1p in this response to benzoic acid is slight, indicating that there are differences in the sensing regulations involved in the responses to stress induced by the two acids. The transcription of the Haa1p target genes identified by Keller et al. (16) is activated under conditions of weak-acid stress (12). The known responsive genes, which did not include SPI1, encode plasma membrane and cell wall proteins of unknown or poorly characterized function. This is the case for the TPO2 and TPO3 genes, encoding two plasma membrane multidrug transporters of the major facilitator superfamily (1), and for YGP1, encoding a cell wall glycoprotein (10). However, the effect of Haa1p on the increase in resistance to cell wall-lytic enzymes is very moderate, suggesting that cell wall remodeling, essential for conferring resistance to acids, involves other genes and processes that are independent of Haa1p regulation. In fact, although transcriptional activation of SPI1 for the short-chain acetic acid is Haa1p dependent, the protective effect exerted by SpI1p against this acid is not very significant. Moreover, the more important role of SPI1 against benzoic acid is not accompanied by a significant effect of Haa1p on SPI1 transcription. As observed before with 2,4-D, the transcriptional activation of SPI1 by acetic acid or benzoic acids is fully dependent on the presence of Msn2p. Consistently, the effect of MSN2 expression on the increase in cell wall resistance to lytic enzymes under weak-acid stress is significant and apparently independent of the acid.

The role of SpI1p in counteracting the deleterious effects of weak acids may conceivably be attributed to its putative action in structuring the cell wall in such a way that cell wall porosity decreases. According to this hypothesis, SpI1p plays a role in preventing the permeation across the cell wall of the weak-acid molecules in weak-acid-adapted cells, especially the permeation of the more lipophilic acids, with larger sizes, thus rendering more difficult their access to the plasma membrane. Keeping in mind the mode of action of weak acids at the plasma membrane levels, whose functions are crucial to cell physiology, this hypothesis is consistent with increased membrane damage, higher intracellular acidification, and decreased viability, observed for a Δspi1 population compared with the wild-type population when both populations were exposed to equivalent concentrations of the weak acids. Although the molecular-weight limit for easy penetration through the intact yeast cell wall seems to be about 700 (24), this value depends markedly on the yeast strain and physiological conditions (9). Experimental evidence set forth in this work strongly supports the notion that cell wall porosity is rapidly and markedly re-
duced in weak-acid-stressed cells in a SpI1p-dependent process. This adaptive response will limit the size and type of molecules that may penetrate the cell wall and come in contact with the plasma membrane. The decrease in cell wall porosity was more equivalent for equivalent inhibitory concentrations of benzoic acid than of acetic acid; the role of SpI1p is prominent in benzoic acid-induced-remodeling of the cell wall and minor in acetic acid-induced cell wall modifications. The higher protection exerted by SpI1p against benzoic acid than against acetic acid cannot, therefore, be essentially attributed to differences in the molecular mass and structure of the weak acids examined but may be determined by the yeast cell adaptive responses to these differently liposoluble compounds. The results obtained in this work indicate that the response to the more lipophilic compounds is markedly dependent on SpI1p, while for the more hydrophilic weak acids, the yeast cell response is essentially mediated by other mechanisms.

It is now clear that cell wall proteins play a crucial role in yeast biology, particularly under stress conditions. The experimental evidence obtained during this work strongly supports the notion that the increased expression of the SpI1 gene under weak-acid stress is required for decreasing cell wall porosity, thus providing protection to the cell membrane against the deleterious effects of these food preservatives, especially against those with higher molecular mass and lipophilicity.

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