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Decarboxylation of Sorbic Acid by Spoilage Yeasts Is Associated with the *PAD1* Gene[▽]

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The spoilage yeast *Saccharomyces cerevisiae* degraded the food preservative sorbic acid (2,4-hexadienoic acid) to a volatile hydrocarbon, identified by gas chromatography mass spectrometry as 1,3-pentadiene. The gene responsible was identified as *PAD1*, previously associated with the decarboxylation of the aromatic carboxylic acids cinnamic acid, ferulic acid, and coumaric acid to styrene, 4-vinylguaiacol, and 4-vinylphenol, respectively. The loss of *PAD1* resulted in the simultaneous loss of decarboxylation activity against both sorbic and cinnamic acids. Pad1p is therefore an unusual decarboxylase capable of accepting both aromatic and aliphatic carboxylic acids as substrates. All members of the *Saccharomyces* genus (sensu stricto) were found to decarboxylate both sorbic and cinnamic acids. *PAD1* homologues and decarboxylation activity were found also in *Candida albicans*, *Candida dubliniensis*, *Debaryomyces hansenii*, and *Pichia anomala*. The decarboxylation of sorbic acid was assessed as a possible mechanism of resistance in spoilage yeasts. The decarboxylation of either sorbic or cinnamic acid was not detected for *Zygosaccharomyces*, *Kazachstania* (*Saccharomyces* sensu lato), *Zygotorulaspora*, or *Torulaspora*, the genera containing the most notorious spoilage yeasts. Scatter plots showed no correlation between the extent of sorbic acid decarboxylation and resistance to sorbic acid in spoilage yeasts. Inhibitory concentrations of sorbic acid were almost identical for *S. cerevisiae* wild-type and Δ*pad1* strains. We concluded that Pad1p-mediated sorbic acid decarboxylation did not constitute a significant mechanism of resistance to weak-acid preservatives by spoilage yeasts, even if the decarboxylation contributed to spoilage through the generation of unpleasant odors.

Of the near 800 yeast species described to date (1), only a small number are regarded as spoilage yeasts (34). Foods characteristically spoiled by yeasts include those with low levels of water activity, acidic and preserved foods, and those containing substantial concentrations of fermentable sugar, e.g., 1 to 60% (wt/wt) (15, 43). The most notorious spoilage yeasts have a number of physiological properties in common, typically being highly fermentative, vitamin requiring, osmotolerant, and resistant to preservatives (10). These include *Zygosaccharomyces bailii*, which is able to proliferate in 60% (wt/wt) glucose (1, 47) and in concentrations of food preservatives far in excess of the legal limitations in Europe (42, 46). *Z. bailii* is also reputed to be able to cause spoilage from an inoculum of 1 cell/liter or per container (10, 49). Other preservative-resistant spoilage yeasts include *Issatchenkia orientalis* (*Candida krusei*), *Pichia membranifaciens*, *Schizosaccharomyces pombe*, and certain strains of *Saccharomyces cerevisiae* (34).

Preservative agents commonly encountered in foods are almost without exception weak acids and include acetic acid, propionic acid, SO_2 (added as sulfite, bisulfite, or metabisulfite), sorbic acid, and benzoic acid (44). All of these weak-acid preservatives are much more potent inhibitors of spoilage at acidic pHs because the proportion of acid molecules rises exponentially as the pH decreases. The classic "weak-acid preservative" theory proposes that the antimicrobial action of weak acids is initiated by the rapid diffusion of undissociated lipophilic acid molecules through the cytoplasmic membrane and into the cytosol. Because it is close to neutrality, the yeast cytoplasmic pH causes lipophilic weak-acid molecules to dissociate into anions and protons; being charged, these anions and protons are lipid insoluble and accumulate in the cytoplasm. The theory suggests that metabolism is inhibited though proton accumulation sufficient to cause a catastrophic decline in intracellular pH. Such an acidification of the cytoplasm has been demonstrated in yeast and is caused by acetic acid (28), sulfite (31), and benzoic acid (17).

Certain spoilage yeast species show resistance to several weak-acid preservatives and include *Z. bailii* and its close relatives, *Zygosaccharomyces bisporus* and *Zygosaccharomyces lentus* (41, 42). In *S. cerevisiae*, a moderately preservative-resistant yeast, the deletion of the *PDR12* gene has been shown to cause sensitivity to sorbic and benzoic acids (32). It has been proposed that Pdr12p, a member of the major facilitator superfamily, carries an anion pump that ejects sorbate and benzoate anions from the cytoplasm (33). Such a mechanism does not appear to be present in more-resistant species such as *Z. bailii* (25). Resistance to sorbic and benzoic acids by *Z. bailii* may be caused by reduced preservative uptake combined with the metabolism of preservatives (25, 26).

Fungal degradation of food preservatives was first demonstrated through the disappearance of sorbic acid from cheese wrappers contaminated with mold (23, 24). Several species in the genus *Penicillium* were reported to detoxify sorbic acid by decarboxylating it to the volatile hydrocarbon 1,3-pentadiene (16, 22). Other reports indicated the conversion of sorbic acid to trans-4-hexenoic acid and ethyl sorbate by *Geotrichum* sp. (18) and to trans-4-hexenol by *Mucor* sp. (19). Several authors have considered the metabolism of preservatives by yeasts to

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TABLE 1. Yeast strains used in this study

Species (strain designation)	Collection	Origin[a]
Candida albicans (212)		Soft drink factory
Candida albicans (309)		Spoilage, soft drink
Candida dubliniensis (632)	NCYC 2670	Type strain
Candida glabrata (LP34)		Spoilage, yogurt
Candida parapsilosis (625)		Soft drink factory
Debaryomyces hansenii var. *fabryii* (558)		Soft drink factory
Dekkera bruxellensis (319)		Spoilage, soft drink
Issatchenkia occidentalis (523)	NCYC 3322	Soft drink factory
Kluyveromyces marxianus (563)		Contaminated culture of NCYC 733
Pichia anomala (612)		Soft drink factory
Pichia membranifaciens (210)		Soft drink factory
Saccharomyces bayanus (24)		Spoilage, carbonated soft drink
Saccharomyces bayanus (59)		Cider apples
Saccharomyces bayanus (565)	NCYC 2669	Substitute for type strain
Saccharomyces bayanus var. *uvarum* (97)		Soil/pepper mixture, acetic acid tolerant
Saccharomyces cariocanus (511)	NCYC 2890	Type strain
Saccharomyces cerevisiae (62)		Wine yeast
Saccharomyces cerevisiae (51)	NCYC 957	Haploid strain X2180-1B
Saccharomyces cerevisiae (633)	NCYC 1451	Spoilage, spiced buns
Saccharomyces cerevisiae (634)	NCYC 1379	Phenolic flavor in beer
Saccharomyces cerevisiae (635)	NCYC 1380	Phenolic flavor in beer
Saccharomyces cerevisiae, wild-type *PAD1*	Y00000	Euroscarf BY4741
Saccharomyces cerevisiae, deleted *pad1*	Y05833	Euroscarf YDR538w::kanMX4
Saccharomyces cerevisiae AP001 *PAD1*		Y05833 transformed with pPad1
Saccharomyces cerevisiae AP002 *pad1*		Y05833 transformed with pESC
Saccharomyces exiguus (55)		Spoilage, mayonnaise-based salad
Saccharomyces kudriazvzevii (514)	NCYC 2889	Type strain
Saccharomyces martiniae (567)	NCYC 2703	Type strain
Saccharomyces mikatae (515)	NCYC 2888	Type strain
Saccharomyces paradoxus (513)	NCYC 2600	Type strain
Saccharomyces pastorianus (201)		Spoilage, orange soft drink
Saccharomyces pastorianus (510)	NCYC 392	Type strain
Saccharomyces servazzii (568)	NCYC 2577	Type strain
Saccharomycodes ludwigii (508)	NCYC 734	Cider factory
Schizosaccharomyces pombe (162)	NCYC 1346	Genetic strain
Torulaspora delbrueckii (624)		Soft drink factory
Zygosaccharomyces bailii (1)	NCYC 1766	Spoilage, black current and grape juice
Zygosaccharomyces cidri (46)	NCYC 2875	Type strain
Zygosaccharomyces fermentati (45)	NCYC 2508	Type strain
Zygosaccharomyces florentinus (44)		Fermenting apple juice
Zygosaccharomyces rouxii (596)		Spoilage, dried fruit

[a] Cultures were either isolated directly or obtained from culture collections. Yeasts are available from the NCYC (http://www.ncyc.co.uk/) or from Euroscarf (http://web.uni-frankfurt.de/fb15/mikro/euroscarf).

be a mechanism of resistance. Stratford et al. (45) demonstrated the complete removal of SO_2 by the sulfite-resistant yeast *Saccharomycodes ludwigii* prior to growth. Acetic acid was removed by metabolism in *Z. bailii*, even in the presence of glucose (39, 40). *Z. bailii* has also been shown to metabolize benzoic acid and sorbic acid in the presence of the Zb*YME2* gene, with benzoic acid being converted via 4-hydroxybenzoic acid (25, 26). Sorbic acid decarboxylation to 1,3-pentadiene was reported for *Debaryomyces hansenii* and *Zygosaccharomyces rouxii* (4, 3).

For this paper, the decarboxylation of sorbic acid was demonstrated in the yeast *S. cerevisiae*, the gene responsible for sorbic acid decarboxylation was identified as *PAD1*, and the role of degradation of preservatives was examined as a mechanism of preservative resistance among spoilage yeasts.

MATERIALS AND METHODS

Yeast strains. The strains used in this study are listed on Table 1. The identity of all yeast strains was confirmed by sequencing the D1/D2 region of the 26S rRNA genes by using the method described by Kurtzman (20). Yeasts were stored on ceramic beads at −80°C (Microbank) and on malt extract agar slopes at 4°C.

Media and growth conditions. Two growth media were routinely used in this study: yeast extract-peptone-dextrose (YEPD), comprising yeast extract (10 g/liter), bacteriological peptone (20 g/liter) and glucose (20 g/liter), and ACM, comprising glucose (20 g/liter), bacteriological peptone (2 g/liter), yeast extract (1.5 g/liter), Casamino Acids (1.5 g/liter), sodium nitrite (6 g/liter), $MgSO_4 \cdot 7H_2O$ (0.53 g/liter), KCl (0.52 g/liter), KH_2PO_4 (1.52 g/liter), $FeSO_4 \cdot 7H_2O$ (2 mg/liter), $ZnSO_4 \cdot 7H_2O$ (2 mg/liter), biotin (0.02 mg/liter), *p*-aminobenzoic acid (1 mg/liter), pyridoxine (5 mg/liter), thiamine (5 mg/liter), nicotinic acid (10 m g/liter), and riboflavin (10 mg/liter). Where indicated, ACM and YEPD were adjusted to pH 4.0 with 10 M HCl prior to autoclaving.

Yeast starter cultures were grown in 10 ml YEPD in 30-ml McCartney bottles without shaking for 2 days at 28°C. Cultures were vortexed, and cells were counted using a hemocytometer before inoculation at either 10^6 or 10^3 cells per ml.

Decarboxylation of weak-acid preservatives. For the detection of the decarboxylation of weak-acid preservatives, new 30-ml McCartney bottles without any defects to the rims were selected. The rubber seals of the aluminum caps were smeared with silicone grease and fitted with aluminum foil inserts to prevent contact between the bottle contents and the rubber seals. Untreated caps resulted in more than 99% of volatiles being adsorbed by the rubber seals (data not

shown). Ten-milliliter aliquots of ACM, pH 4.0, were added to each bottle, supplemented with 1 mM sorbic acid or 0.5 mM cinnamic acid from 100-mM stock solutions in methanol. Control experiments showed that methanol had no effect on yeast growth at the concentrations used. Bottles were inoculated with yeast at 10^6 cells/ml before being tightly capped. Bottles were then incubated at 28°C while being shaken at 120 rpm on an orbital shaker for 10 h. A standard, 1,3-pentadiene or styrene, was added to uninoculated ACM and was similarly incubated. All chemicals were supplied by Sigma-Aldrich, unless otherwise stated.

Detection of volatile metabolites. At designated times, 1-ml headspace samples were removed by piercing the rubber cap septa with a syringe needle. Samples were immediately injected into stainless steel thermal desorption tubes (inside diameter, 89 mm by 5 mm) packed with 200 mg Tenax TA 60-80 mesh (Phase Separations, United Kingdom), followed by a 10-ml air flush. Tubes were then tightly capped and stored at 4°C before analysis.

Less-volatile metabolites, such as 4-vinylphenol, were detected from the media, rather than from the headspace. Media samples were centrifuged rapidly to remove yeast, and supernatants were stored frozen in capped ampoules. One-milliliter samples were warmed to 30°C, and volatiles were drawn off in a stream of nitrogen at 300 ml/min for 20 min. The nitrogen stream was passed through a thermal desorption tube, and the volatiles were trapped as before. Standard 4-vinylphenol was obtained from Lancaster Research Chemicals, Morecambe, Lancashire LA3 3BN, United Kingdom.

Headspace analysis was carried out by gas chromatography-mass spectrometry (GCMS). Thermal desorption tubes were placed on an autosampler from a Perkin Elmer ATD400 thermal desorption system. The volatiles were passed from the sample tubes to the gas chromatograph in a two-stage process. Primary desorption was carried out by purging the tube at 250°C for 10 min with a helium flow of 40 ml/min. We used a split ratio of 1:1 to retain volatiles in a trap (25 mg Tenax TA) held at −30°C. Secondary desorption was carried out by rapidly heating and holding the cold trap at 250°C for 2 min, with a helium flow of 20 ml/min and a split ratio of 19:1. A BPX5 column (30 mm by 0.32 millimeter; 0.25-μm film thickness) installed in a Carlo Erba GC8000 gas chromatograph was used to achieve separation and the introduction of volatiles to the mass spectrometer (Finnigan Voyager MD800). The column temperature was held at 40°C for 2 min and then increased at 2°C/min to 80°C and then 25°C/min to 200°C. The column outlet was coupled via a heated transfer line at 250°C into the ion source of the mass spectrometer operating at 70 eV in electron ionization mode. The source temperature was 200°C, and the detection photomultiplier was set at 500 V. Full-scan MS data acquisition was carried out from *m/z* 30 to 300, with a 0.6-s scan and a 0.05-s interscan delay. Control experiments showed that very low concentrations of 1,3-pentadiene or styrene formed in experimental cultures lacking sorbic or cinnamic acid were due to carryover (adsorption and release of 1,3-pentadiene in rubber components of the sampling system and in the GCMS cold trap). Blank uninoculated control cultures were therefore used with each individual experiment and subtracted from all subsequent readings. Typically, blank peak areas for 1,3-pentadiene were 0 to 5 and blank peak areas for styrene were 20 to 50 relative to positive readings with peak areas up to 25,000 units.

Complementation of *pad1*Δ with the native *PAD1* gene of *S. cerevisiae*. The *PAD1* open reading frames with 0.990 and 0.909 kb of upstream and downstream flanking sequences, respectively, were PCR amplified from *S. cerevisiae* BY4741 genomic DNA using primers pad1esc1, 5′-GGAAGATCTGCTATAAAAAGC TTATAAAATAGCCCGACTCCGTAGTC-3′, and pad1esc2, 5′-GGACTAGT CCGTCTGAAACATGTAGATATGGTGTTG-3′, containing artificial BglII and SpeI sites (underlined), respectively. PCR conditions were as follows: 98°C for 2 min; 35 cycles of 98°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min; and then 1 cycle of 72°C for 10 min. Following confirmation by sequencing, the *S. cerevisiae PAD1* gene PCR product was ligated into BglII/SpeI-digested pESC (Invitrogen) to form pPad1. This was then transformed into *S. cerevisiae* Euroscarf strain Y05833 (*pad1*Δ) by using standard techniques, forming strain AP001, and thus restoring the *PAD1* gene under its native promoter.

RNA isolation, radiolabeling, and Northern analysis. Total RNA was isolated from *S. cerevisiae* cultures by using the hot phenol method (37), was fractionated by gel electrophoresis, and was blotted onto HybondN+ (Amersham) before being hybridized to a ^{32}P-radiolabeled probe by using standard protocols (36). A 520-bp fragment of the *PAD1* gene (+172 to +691 bp) was radiolabeled by using a Megaprime labeling kit (Amersham) according to the manufacturer's instructions.

Effect of *PAD1*/*pad1* on weak-acid resistance in *S. cerevisiae*. The effect of *PAD1* disruption on the ability of *S. cerevisiae* to grow in the presence of weak-acid preservatives was investigated by the determination of the MIC of each acid required to completely prevent yeast growth. Series of McCartney bottles were prepared containing aliquots of 10 ml YEPD, pH 4.0, each holding a progressively higher concentration of preservative. Tubes were inoculated at 10^3 cells/ml and incubated for 14 days at 28°C. Cultures were then vortexed, and yeast growth was assessed by the optical density at 600 nm. The MIC was the lowest concentration of preservative at which no growth was detectable.

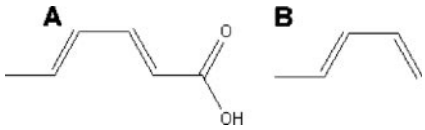

FIG. 1. Chemical structures of (A) sorbic acid (2,4-hexadienoic acid) and (B) 1,3-pentadiene. Sorbic acid could be converted into 1,3-pentadiene by removal of the carboxylic acid group.

RESULTS

Breakdown of sorbic acid to 1,3-pentadiene in *S. cerevisiae*. The ability of spoilage yeasts to degrade the preservative sorbic acid was investigated for a collection of spoilage yeast species, which are listed in Table 1. GCMS headspace analysis of yeast cultures was carried out to detect volatile products of degradation. A prominent GCMS peak in the headspace of *S. cerevisiae* cultures treated with 1 mM sorbic acid was positively identified as 1,3-pentadiene. Cultures forming 1,3-pentadiene smelled strongly of kerosene. Headspace samples from uninoculated cultures or from cultures not treated with sorbic acid did not contain detectable 1,3-pentadiene. A direct causal link between the presence of the preservative sorbic acid and the generation of 1,3-pentadiene was supported by an examination of their respective chemical structures (Fig. 1). The elimination of the carboxylic acid head group of sorbic acid by decarboxylation would yield the volatile hydrocarbon 1,3-pentadiene. These results strongly suggested that sorbic acid was degraded by *S. cerevisiae* to 1,3-pentadiene directly in a single decarboxylation step. This is the first report of the generation of 1,3-pentadiene from sorbic acid by the yeast *S. cerevisiae*.

Sorbic acid decarboxylation is mediated by *PAD1*. The genome of *S. cerevisiae* has been fully sequenced and contains 12 putative decarboxylase genes (http://www.yeastgenome.org). One of these 12 is the *PAD1* gene, encoding a phenyl acrylic acid decarboxylase, whose accepted substrates are cinnamic acid (phenylacrylic acid), ferulic acid (3-methoxy-4-hydroxycinnamic acid), and coumaric acid, (4-hydroxycinnamic acid) (9). The decarboxylation of these substrates, cinnamic acid, ferulic acid, and coumaric acid, yields styrene, 4-vinylguaiacol, and 4-vinylphenol, respectively.

In the current study, the results showed that the deletion of the *PAD1* gene prevented not only the conversion of cinnamic acid to styrene but also the decarboxylation of sorbic acid to 1,3-pentadiene. A time course of the formation of 1,3-pentadiene was carried out for *S. cerevisiae* strain Y05833 (*pad1*Δ) and *S. cerevisiae* strain BY4741 (wild type) during growth in 1 mM sorbic acid (Fig. 2). Over 96 h, growth levels in replicate cultures of wild-type and *pad1*Δ strains were near identical. 1,3-pentadiene formation occurred only in wild-type cultures and reflected the growth of cultures after a delay of a few hours (Fig. 2). In these long-term experiments, headspace sampling was complicated by gas formation by fermenting cultures. This complication necessitated the equalization of the pressure in cultures before the removal of a fixed fraction of the total

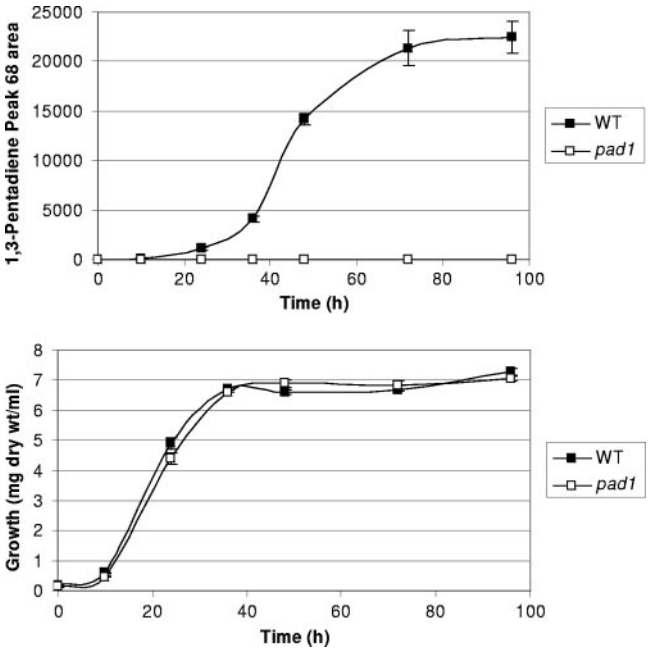

FIG. 2. Time course of growth and 1,3-pentadiene formation by *S. cerevisiae* BY4741 (wild type [WT]) and *S. cerevisiae* Y05833 (*pad1*Δ) in multiple replicates of 30-ml bottles containing 10 ml YEPD, pH 4.0, 1 mM sorbic acid incubated and shaken at 120 rpm and 28°C. Each point indicates the mean and standard deviation of two replicate samples at each time point. Following pressure equalization, 10% of the headspace was sampled, and 1,3-pentadiene was detected by GCMS. Yeast cell density was determined by the optical density at 600 nm and converted to dry weight by calibration curve.

headspace volume. In subsequent tests, sampling was carried out at a fixed time point, 10 h, a time at which growth had been initiated and 1,3-pentadiene was easily detectable, without the complication of pressure equalization.

Figure 3 shows that wild-type *S. cerevisiae* strain BY4741 generated both styrene and 1,3-pentadiene over 10 h, while *S. cerevisiae* strain Y05833 (*pad1*Δ) did not form detectable styrene or 1,3-pentadiene over this period. The proportion of acids decarboxylated was calculated from the GCMS peak areas of hydrocarbons formed by yeast activity and the GCMS peak areas of control styrene and 1,3-pentadiene added at equimolar concentrations to their respective acids (100% conversion). This result showed that only a small proportion of weak acids was decarboxylated over 10 h by strain BY4741 (2% of 0.5 mM cinnamic acid and 0.3% of 1 mM sorbic acid). Confirmation of the role of *PAD1* in sorbic acid decarboxylation was obtained by the restoration of *PAD1* in *S. cerevisiae* strain Y05833 (*pad1*Δ) under its native promoter. Figure 3 shows that the decarboxylation of each weak acid was restored by the transformation of the *pad1*Δ strain with *PAD1* (strain AP001). Control experiments showed no restoration of decarboxylation when *S. cerevisiae* strain Y05833 (*pad1*Δ) was transformed with the empty vector (strain AP002). Unexpectedly, the decarboxylation of coumaric acid to 4-vinylphenol could not be detected in any yeast strain over the 10-h duration of these experiments.

The induction and expression of the *PAD1* gene in the presence of weak acids was examined by Northern analysis. We found (Fig. 4) that *PAD1* expression closely coincided with the growth of the cultures. *PAD1* expression was greatest at 10 and 24 h in 1 mM sorbic acid (growth curves are shown in Fig. 2), when cultures were in exponential growth. *PAD1* expression fell to a low level in late growth and stationary phase. *PAD1* expression also occurred in growing control cultures without sorbic acid treatment, showing that sorbic acid was not required for *PAD1* induction.

To confirm that *PAD1* expression in actively growing cells correlated with decarboxylation activity, tests were carried out to determine the rate (activity) of decarboxylation of cinnamic acid (0.5 mM) at different stages of growth, necessitating a large cell inoculum. At successive time points, replicate bottles were removed and the concentration of styrene was determined by GCMS. The rate of formation (concentration of styrene per milligram of cells per hour) was determined by the subtraction of the previous time point for the concentration of styrene and division by the milligrams of dry weight of yeast present and the time between sampling points. Results (Fig. 5) showed that the rate of styrene formation was initially low during lag phase but increased substantially during exponential growth, declining steeply thereafter into stationary phase.

Pad1p homologues and sorbic acid decarboxylation in other yeast species. Complete genome sequences have been published for a limited number of yeast species (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism = fungi). Putative Pad1p homologues were found in 8 out of 23 yeast genome sequences. These were *S. cerevisiae*, *Candida albicans* (58.0% amino acid identity over the entire sequence of *S. cerevisiae* Pad1p), *C. dubliniensis* (58.8% identity), *Saccharomyces kudriavzevii*, (81.5% identity), *Saccharomyces mikatae* (87.2% identity), *Saccharomyces paradoxus* (90.9% identity), *Saccharomyces bayanus* (83.1% identity), and *Debaryomyces hansenii* (57.4% identity). The decarboxylation of sorbic and cinnamic acids was examined in a range of yeast species containing, or lacking, recognized Pad1p homologues. The results showed a striking coincidence between those species able to decarboxylate sorbic and cinnamic acids and those species containing Pad1p homologues. All species carrying Pad1p homologues, *C. albicans*, *C. dubliniensis*, *D. hansenii*, *S. cerevisiae*, *S. kudriavzevii*, *S. mikatae*, *S. bayanus*, and *S. paradoxus*, were able to decarboxylate both sorbic acid and cinnamic acids. No examples of yeasts able to act on one acid but not the other were found. No yeast lacking a Pad1p homologue, *C. glabrata*, *Kluyveromyces marxianus*, *Saccharomyces exiguus*, *Saccharomyces servazzii*, *Schizosaccharomyces pombe*, *Yarrowia lipolytica*, or *Zygosaccharomyces rouxii*, decarboxylated sorbic or cinnamic acids. The only possible exception was *S. bayanus*. The initial reported genome sequence of *S. bayanus* (strain 623-6C) lacked a *PAD1* homologue, but a more recently completed *S. bayanus* (MCYC 623) sequence indeed contains a *PAD1* homologue, with 83.1% amino acid identity with *S. cerevisiae* Pad1p. Test results for Pad1p activity in four *S. bayanus* strains were positive, but activity was low in strain 24. It is possible that this result reflects strain variation in *S. bayanus*. The type strain of *S. bayanus* is known to be a hybrid between *Saccharomyces uvarum* and *Saccharomyces pastorianus*, and the existence of *S. bayanus* itself as a species has been questioned (30). It is possible that all strains of *S. bayanus* are hybrid variations between several specific genome sequences.

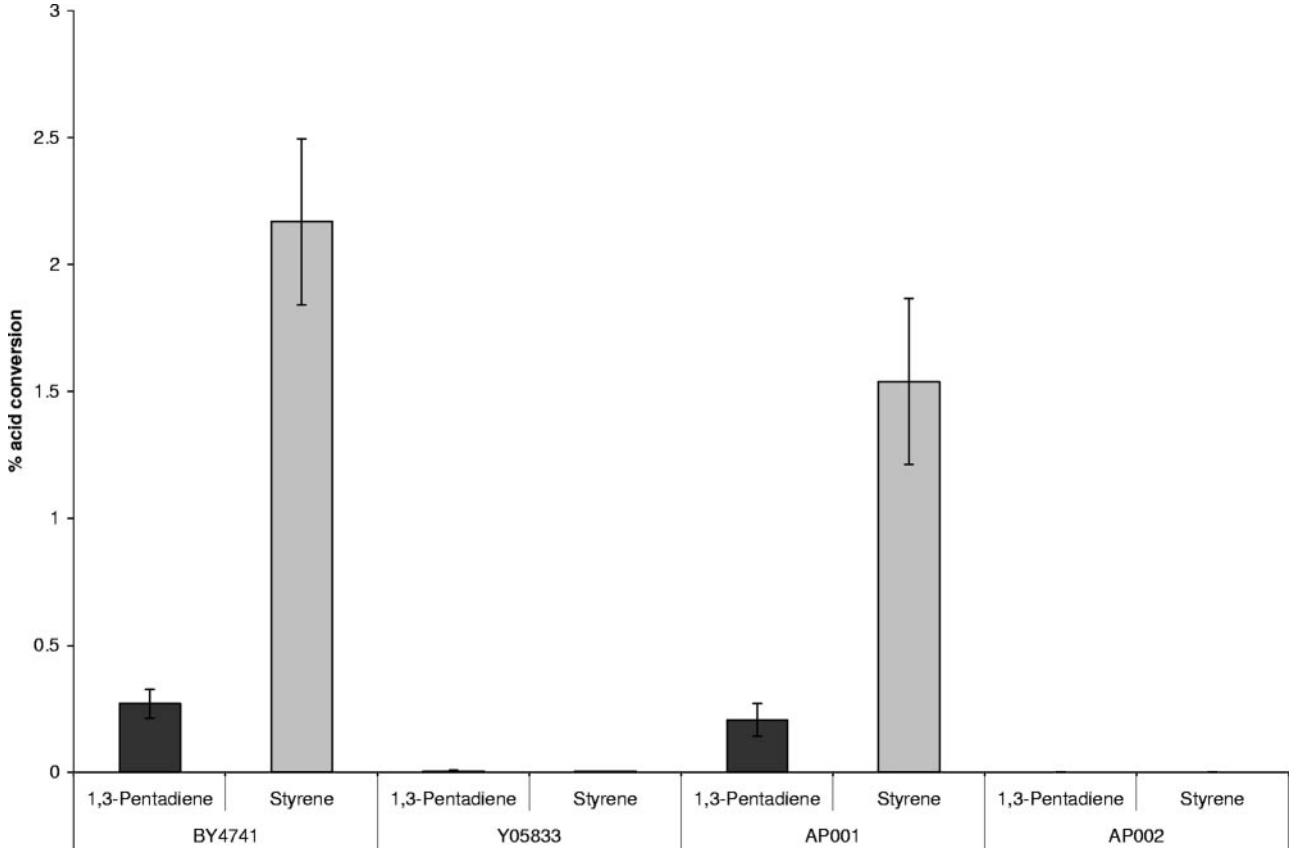

FIG. 3. Percentages of decarboxylation of sorbic acid to 1,3-pentadiene (dark histograms) and cinnamic acid to styrene (light histograms) in *S. cerevisiae* strains containing or lacking *PAD1*. Strains were *S. cerevisiae* BY4741 (wild type), Y05833 (*pad1*Δ), AP001 (Y05833 transformed with pPad1), AP002 (Y05833 transformed with pESC). Tests were carried out over 10 h at 28°C. Data are based on two independent determinations. Error bars indicate standard deviations.

Tests were carried out with a range of yeast species to determine whether the decarboxylation of weak acids was a common feature of spoilage species. It was found that of 13 spoilage species (34), only 2, *S. cerevisiae* and *Debaryomyces hansenii*, were able to decarboxylate sorbic and cinnamic acids. Of particular note is that the extremely preservative-resistant spoilage species *Z. bailii* was not able to decarboxylate sorbic acid.

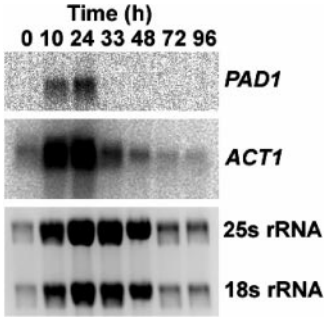

FIG. 4. Northern blot analysis of *PAD1* mRNA levels measured at 0, 10, 24, 33, 48, 72, and 96 h during growth in the presence of 1 mM sorbic acid. Growth of cultures during this experiment is shown in Fig. 2. Maximum *PAD1* expression was found to coincide with exponential growth. *ACT1* expression and 26S and 18S rRNA are used as loading controls.

The taxonomic significance of *PAD1* and the ability to decarboxylate weak acids is most obvious in the yeast species most closely related to *S. cerevisiae*. All of the species currently recognized within the genus *Saccharomyces* (20) had the ability to degrade sorbic and cinnamic acids. However, species in closely related genera, such as *Kazachstania* (formerly *Saccharomyces* sensu lato), *Zygosaccharomyces*, *Zygotorulaspora*, *Nakaseomyces*, *Torulaspora*, *Lachancea*, *Kluyveromyces*, and *Saccharomycodes*, lacked that ability. It is therefore possible that *PAD1* may represent a taxonomic marker for the genus *Saccharomyces*, absent in neighboring taxa.

Cinnamaldehyde conversion to styrene. Several strains of *S. cerevisiae* have been reported to form styrene from the complementary aldehyde of cinnamic acid, cinnamaldehyde (8, 27). This occurs naturally in oil of cinnamon and in cinnamon flavors in many foods. Six strains of *S. cerevisiae*, including strain NCYC 1451, were tested for their ability to degrade cinnamaldehyde to styrene. NCYC 1451 has been reported to produce styrene from cinnamaldehyde in spiced-bun production (27). Tests showed that all five wild-type strains of *S. cerevisiae* were able to form styrene from cinnamaldehyde, in addition to forming styrene from cinnamic acid and 1,3-pentadiene from sorbic acid (Table 2). How-

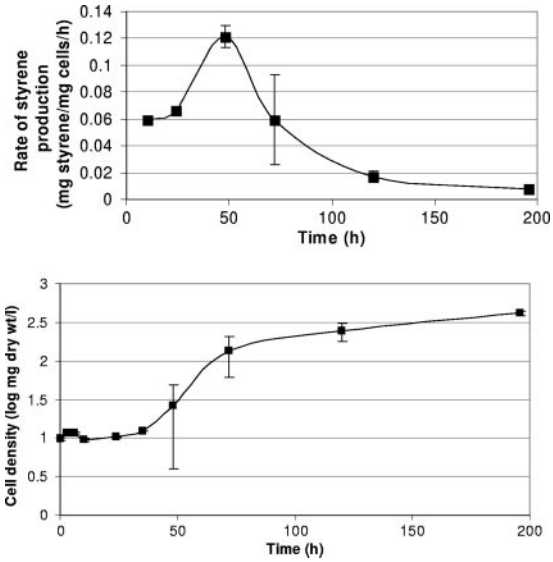

FIG. 5. Long-term growth (bottom) and rate of formation of styrene (top) by *S. cerevisiae* BY4741 in closed 30-ml bottles (multiple replicates) containing 10 ml YEPD, pH 4.0, 0.5 mM cinnamic acid incubated, with shaking at 120 rpm and 28°C. Following pressure equalization, styrene formation was detected by GCMS and expressed in milligrams against styrene standards. Yeast cell density was determined by optical density at 600 nm and converted to dry weight by calibration curve. Each point represents the mean of two cultures sampled at that time point. Error bars indicate standard deviations.

ever, *S. cerevisiae* strain Y05833 (*pad1*Δ) did not form styrene from cinnamaldehyde, indicating that the conversion of cinnamaldehyde to styrene was also mediated by Pad1p.

Role of *PAD1* as a mechanism of resistance to preservatives. Tests were carried out to determine the effect of *PAD1* on the resistance of yeasts to sorbic or cinnamic acid as preservatives. Results showed that the inhibitory concentrations of either acid were not altered by the presence or absence of *PAD1* in *S. cerevisiae*. The growth of yeast at subinhibitory concentrations of sorbic acid was also unaffected by *PAD1* or Δ*pad1* (Fig. 6). At subinhibitory concentrations of cinnamic acid, the presence of *PAD1* enabled significantly higher growth yields to be obtained (Fig. 6). The role of acid decarboxylation was also examined in other spoilage yeasts. Scatter plots of the inhibitory concentrations of cinnamic or sorbic acid against levels of acid decarboxylation in a wide variety of spoilage yeasts showed no positive correlation (data not shown).

TABLE 2. Molar percent conversion of styrene from cinnamaldehyde and 1,3-pentadiene from sorbic acid by strains of *Saccharomyces cerevisiae*

Yeast species (strain designation)	% Conversion (±SD) for:	
	Styrene	1,3-Pentadiene[a]
S. cerevisiae (62)	11.1 ± 1.5	1.03 ± 0.11
S. cerevisiae (633)	6.5 ± 1.5	0.99 ± 0.06
S. cerevisiae (634)	13.9 ± 4.0	0.91 ± 0.38
S. cerevisiae (635)	13.2 ± 4.1	0.83 ± 0.01
S. cerevisiae BY4741, wild-type *PAD1*	6.1 ± 1.9	0.31 ± 0.04
S. cerevisiae Y05833, deleted *pad1*	0	0

[a] Tests were carried out in sealed bottles holding 10 ml ACM, pH 4.0, containing 1 mM sorbic acid or 0.1 mM cinnamaldehyde incubated for 10 h at 28°C. Data are based on two independent determinations of each value and the subtraction of uninoculated blanks.

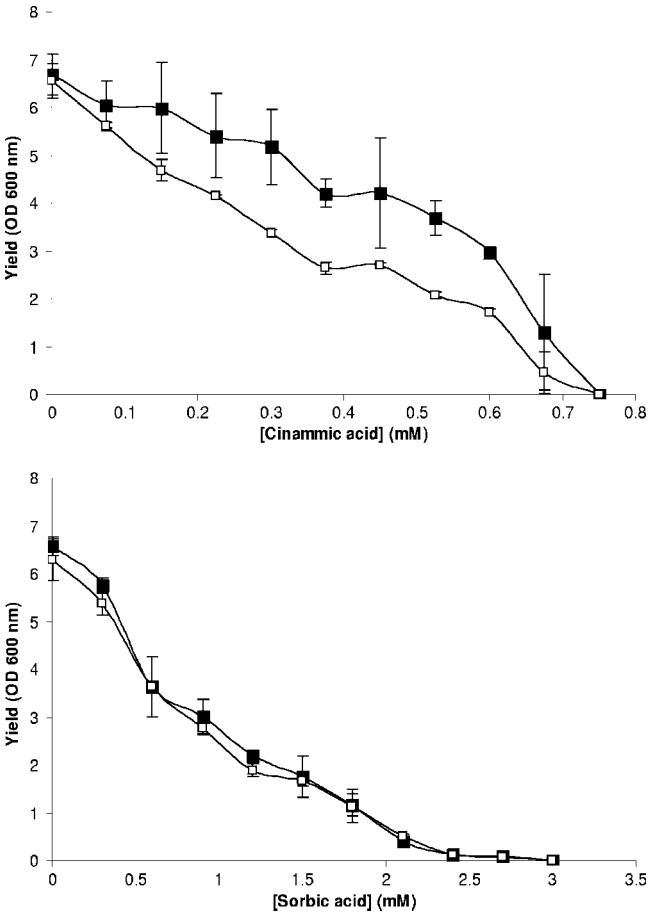

FIG. 6. Growth and inhibition of *S. cerevisiae* BY4741 (closed symbols) and *S. cerevisiae* Y05833 (open symbols) by cinnamic acid or sorbic acid in YEPD, pH 4.0, after 14 days of incubation at 28°C. Data are based on the means of three independent determinations, and error bars indicate standard deviations. OD_{600}, optical density at 600 nm.

If *PAD1* is a potential resistance mechanism to preservative acid toxicity, growth would be predicted to occur after the removal of a substantial proportion of the acids. Tests showed that when yeast growth was first detected (24 h) in cultures containing 0.5 mM cinnamic acid, only 3 to 4% of cinnamic acid had been removed. This proportion later increased to circa 50%, but only after the growth of yeast to high cell concentrations in stationary phase. For sorbic acid, the fraction removed was smaller, with less than 1% converted to 1,3-pentadiene when growth was detected.

DISCUSSION

In this paper, two possible new substrates are described for Pad1p, sorbic acid and cinnamaldehyde. Sorbic acid is an unsaturated, aliphatic, monocarboxylic acid, distinguished from other Pad1p substrates, which are based on aromatic acids, cinnamic acid (phenylacrylic acid), and 4-hydroxy-

substituted cinnamic acids, ferulic, and coumaric acids (9). The name *PAD1* itself reflects the predicted substrate, as it is an acronym for phenyl acrylic acid decarboxylase. Despite the structural difference between aliphatic and aromatic compounds, sorbic acid and cinnamic acid share a number of chemical similarities. Both compounds are monocarboxylic weak acids, with pK_a values of 4.76 and 4.44 for sorbic and cinnamic acids, respectively. Both are moderately lipophilic, with $logP_{oct}$ values of 1.41 and 1.96 for sorbic and cinnamic acids, respectively (ChemOffice Ultra 2002, version 7.0; CambridgeSoft, Cambridge, MA). Furthermore, both are also unsaturated at position 2, below the carboxylic acid head group. Clearly, the aromatic ring at position 4 in cinnamic acid is not obligatory for recognition by Pad1p and the substrate specificity of this enzyme is less stringent than was previously thought.

While fungal Pad1p homologues have received little attention, a number of other bacterial enzymes with sequence homology to Pad1p have been described in the literature. Bacterial Pad1p homologues act predominantly on 4-hydroxy-substituted cinnamic acid analogues, such as ferulic acid and coumaric acid. The constitutive enzyme in an *Aerobacter* sp. acted primarily on coumaric acid, but it also acted on caffeic and ferulic acids (11). No action on cinnamic acid was detected. A ferulic acid decarboxylase characterized in *Pseudomonas fluorescens* (14) also acted on coumaric and ferulic acids but not cinnamic acid. A ferulic acid decarboxylase in *Bacillus pumilus* (50) and a less-specific phenolic acid decarboxylase in *Bacillus subtilis* acting on ferulic, coumaric and caffeic acids (7) have been reported. The phenolic acid decarboxylase gene from *B. subtilis* was overexpressed in *S. cerevisiae* (38), and the recombinant strain had high activity against coumaric acid. *Lactobacillus plantarum* has also been shown to contain decarboxylases with specificity towards coumaric acid and ferulic acid (2, 5, 6, 38, 48). No aliphatic acids such as sorbic acid appear to have been tested on any bacterial phenolic acid decarboxylases.

Less information has been reported for eukaryotes. Goodey and Tubb (12) examined the generation of phenolic off-flavors by yeasts in beer and demonstrated ferulic acid decarboxylation to 4-vinylguaiacol mediated by the *PAD1* gene (then named the *POF1* gene) with lesser action on coumaric and cinnamic acids. However, the decarboxylation of ferulic and coumaric acids by *S. cerevisiae* in laboratory cultures has been reported by other authors (7, 13, 48) to be slow and with low activity, a suggestion that we confirm with a lack of activity detected by Pad1p on coumaric acid over 10 h.

Cinnamaldehyde may also be a substrate of Pad1p in *S. cerevisiae* strains. The presence of cinnamaldehyde in growing yeast cultures resulted in styrene production. This result does not prove ipso facto that cinnamaldehyde is a substrate for Pad1p, since cinnamaldehyde has a tendency to auto-oxidize to cinnamic acid. Cinnamaldehyde is an oily yellow liquid, but white crystals around the necks of previously opened bottles are composed of cinnamic acid. It is also possible that a small fraction of cinnamaldehyde was auto-oxidized to cinnamic acid during the 10-h duration of experiments. However, in the studies of Chen and Peppler (8), no cinnamic acid was detected in the medium and they concluded that styrene was formed directly from the aldehyde. This is not conclusive, as Pad1p activity could potentially remove cinnamic acid as quickly as it was formed.

In the results shown here, substantial quantities of styrene were formed from cinnamaldehyde (newly purchased and stored under nitrogen), although the quantities were less than those formed from cinnamic acid. Such quantities are significantly greater than we expected from a small fraction of cinnamaldehyde oxidized to cinnamic acid, indicating that cinnamaldehyde probably constitutes a new substrate for Pad1p.

The role of *PAD1* as a mediator of resistance to weak-acid preservatives is equivocal. *PAD1* was originally identified as conferring resistance to cinnamic acid (9) by the small size of *pad1*Δ mutant colonies on cinnamic acid agar. The overexpression of *PAD1* was reported to increase the growth rate in the presence of phenylacrylic acids (21). The data shown here revealed no increase in the MIC of cinnamic acid when *PAD1* was present, but *PAD1* increased growth yield at subinhibitory concentrations of cinnamic acid. This increased yield could easily give rise to larger colonies on agar as reported by Clausen et al. (9). Increased growth yield at subinhibitory concentrations of cinnamic acid may constitute increased resistance or may rather reflect the amelioration of the symptoms of cinnamic acid toxicity at subinhibitory concentrations. It appears that one aspect of cinnamic acid toxicity inhibits growth. This aspect is unaffected by the presence or absence of Pad1p. Cinnamic acid also causes a reduction in growth yield; this symptom of cinnamic acid toxicity is ameliorated by the presence of Pad1p.

For the food preservative sorbic acid, the presence or absence of *PAD1* gave no change in MIC and also no significant change in growth yield or in colony size on agar. A smaller proportion of sorbic acid was decarboxylated by Pad1p, and its expression was not affected by sorbic acid. Furthermore, many spoilage yeasts do not contain *PAD1* homologues and there appeared to be no relationship between the decarboxylation of sorbic acid and resistance to it in a number of yeast strains. The most sorbic acid-resistant yeast, *Z. bailii*, showed no decarboxylation of sorbic acid. There is therefore no evidence to support any role for *PAD1* in a resistance mechanism of spoilage yeasts against sorbic acid. This lack of evidence is in direct contrast to the role of PadA1 in *Aspergillus niger*, which confers very significant resistance to sorbic acid by mold spores (A. Plumridge et al., unpublished data).

While *PAD1* does not increase resistance to sorbic acid in yeasts, it is possible that *PAD1* may contribute to food spoilage in a different way, e.g., through the generation of off-flavors and odors. Pad1p activity in spoilage yeasts will generate styrene if cinnamic acid or cinnamaldehyde is present, 4-vinylphenol or 4-vinylguaiacol from coumaric acid or ferulic acid, or 1,3-pentadiene from sorbic acid. Styrene has a plastic-like smell, 4-vinylphenol and 4-vinylgaiacol have a medicinal phenolic smell, and the odor of 1,3-pentadiene is gasoline/petrol/kerosene like. The taste and odor thresholds for styrene have been determined in water (29, 35). The taste threshold for styrene is circa 120 ppb, and the odor threshold lies between 4 to 2,600 ppb (35), depending on the temperature. In the experiments shown here, up to 0.25 mM styrene was produced following long-term growth of yeast cultures. This result rep-

resents 17 ppm styrene, and all such cultures smelled strongly of plastic. Similarly, cultures decarboxylating sorbic acid smelled strongly of kerosene. The major impact of Pad1p in spoilage yeast growing in foods containing sorbic or cinnamic acids is therefore most likely to increase perceived spoilage due to the generation of offensive odors.

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