Genetic manipulation of 6-phosphofructo-1-kinase and fructose 2,6-bisphosphate levels affects the extent to which benzoic acid inhibits the growth of *Saccharomyces cerevisiae*

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The mechanisms by which the weak acid preservative benzoic acid inhibits the growth of *Saccharomyces cerevisiae* have been investigated. A reduction in the pyruvate kinase level, which decreases glycolytic flux, did not increase the sensitivity of yeast to benzoic acid. However, a decrease in 6-phosphofructo-1-kinase (PF1K), which does not affect glycolytic flux, did increase sensitivity to benzoic acid. Also, resistance was increased by elevating PF1K levels. Hence, resistance to benzoic acid was not dependent upon optimum glycolytic flux, but upon an adequate PF1K activity. Benzoic acid was shown to depress fructose 2,6-bisphosphate levels in YKC14, a mutant with low PF1K levels. This effect was partially suppressed by overexpressing constitutively active 6-phosphofructo-2-kinase (Pfk26Asp644) or by inactivating fructose-2,6-bisphosphatase (in a ∆fbp26 mutant). The inactivation of PF2K (in a ∆pfk26 ∆pfk27 mutant) increased benzoic acid sensitivity. Therefore, the antimicrobial effects of benzoic acid can be relieved, at least in part, by the genetic manipulation of PF1K or fructose 2,6-bisphosphate levels.

**Keywords:** Yeast physiology, benzoic acid, phosphofructokinase, glycolysis

**INTRODUCTION**

Weak acids such as benzoic acid are used to preserve foods and beverages (Booth & Kroll, 1989). At an acidic external pH they exist in a non-dissociated, uncharged state that is able to diffuse across the plasma membrane. Once they enter the neutral pH of the cytoplasm, they dissociate to release an anion and a proton. The pH gradient across the plasma membrane drives the intracellular accumulation of protons and anions, reducing the intracellular pH and inhibiting the growth of the micro-organism (Booth & Kroll, 1989). However, *Saccharomyces cerevisiae* cells are able to adapt to weak acids and growth can resume after a period of recovery (Lambert & Stratford, 1999). The exact mechanisms by which benzoic acid inhibits growth and by which cells adapt to weak acids remain to be clarified.

Two main mechanisms have been proposed to account for the effects of weak acids upon the growth of yeast. The first model suggests that growth inhibition is due to the intracellular depletion of ATP, rather than the inhibition of glycolytic enzymes such as 6-phosphofructo-1-kinase (PF1K) (Warth, 1988, 1991). In response to acidification, the plasma membrane H⁺-ATPase (Pma1) pumps protons out of the cell in an ATP-dependent manner (Eraso & Gancedo, 1987; Ramos et al., 1989; Viegas & Sá-Correia, 1991). Growth in the presence of a weak acid has been shown to depend upon Pma1 activity (Holyoak et al., 1996), and the length of the adaptation period, before growth can resume, has been predicted to depend upon the time taken to raise the intracellular pH to neutrality (Lambert & Stratford, 1999). Recently, it was demonstrated that normal levels of resistance to weak acids depend upon an ABC transporter (Pdr12; Piper et al., 1998). This transporter pumps benzoate from the yeast cell in an ATP-dependent manner...
manner (Holyoak et al., 1999). Hence, both Pma1 and Pdr12 might contribute to ATP depletion as they combat intracellular accumulation of both protons and benzoate anions, respectively.

A second model suggests that benzoic acid blocks glycolysis, thereby inhibiting growth (Krebs et al., 1983). These authors showed that benzoic acid addition leads to a reduction in the rate of glucose fermentation and the accumulation of glycolytic intermediates before PF1K. This was consistent with a block in glycolysis at PF1K. Their basic model was supported and extended by François et al. (1986, 1988). They showed that PF1K activity decreased following benzoic acid addition and that there was also a reduction in the levels of fructose-2,6-bisphosphate, a potent activator of PF1K. Benzoic acid was also shown to inhibit purified 6-phosphofructo-2-kinase (PF2K) in vitro (François et al., 1988). Furthermore, it has been reported that growth in the presence of a weak acid depends upon optimal glycolytic flux (Holyoak et al., 1996). However, a direct causal relationship between the inhibition of glycolysis and the antimicrobial effects of the weak acid remains to be proven.

In this study we have further examined the relationship between glycolysis and benzoic acid tolerance, and in particular, the influence of PF1K and its activator, fructose-2,6-bisphosphate. We show that (a) wild-type rates of glycolytic flux are not required for growth in the presence of sublethal concentrations of benzoic acid, (b) yeast cells become more sensitive to benzoic acid when PF1K or PF2K levels are lowered artificially, and (c) benzoic acid sensitivity can be suppressed by overexpressing a subunit of PF2K or by inactivating fructose-2,6-bisphosphatase (F26B). In this way we have confirmed a causal relationship between the inhibition of glycolysis and the antimicrobial effects of benzoic acid.

**METHODS**

**Growth conditions.** Strains were grown at 30 °C in Meltzaine medium (2%, w/v, glucose; 0.67%, w/v, yeast nitrogen base without amino acids; 0.2 M K$_2$HPO$_4$; 0.1 M citric acid, pH 4.6) (Roe et al., 1998).

**Strain construction.** Strain genotypes are presented in Table 1. YKC strains represent a congenic set of PFK1 and PFK2 mutants that were made in S. cerevisiae W303-1B (Pearce et al., 2000). Congenic Afp26 mutants were made in W303-1B and YKC14 by targeted gene disruption, as described by Wach et al. (1998). Briefly, a Afp26::kanMX4 cassette was made by PCR amplifying the kanMX4 marker using the primers 5'-TGGGTCGCGCCAATGCGCTCACAAAT CTATTCT and 5'-TTTGGTGACCTTTGTGCCATA. The PCR product, which deletes from −191 to +1180 of the 1356 bp FBP26 ORF, was transformed into W303-1B and YKC14 by homologous recombination. The construction of the congenic strains VW-1B and VW.EB-13B was described previously (Müller et al., 1997). To construct the multicopy plasmid YEpPFK26, YPlacPFK26 was ligated to the LEU2, 2 µm HindIII fragment from pMA91 (Mellor et al., 1985).
Southern and Western blotting. *S. cerevisiae* DNA was isolated according to the protocol of Hoffman & Winston (1987) and subjected to Southern blotting (Church & Gilbert, 1984; Wicksteed *et al*., 1994). Hybridization probes were as follows: FBP26, a 1·07 kbp PCR product, corresponding to +237 to +830, generated using the primers 5'-GTGCCGTC- AAATCGCTACAAAATCTAT-3' and 5'-GTCCACAGGGTCAAATT-3'. Random-primed labelling of DNA fragments was carried out using the Pharmacia Ready-To-Go DNA Labelling Kit ([γ-32P]dCTP), based on the method of Feinberg & Vogelstein (1983). Published methods were used for the preparation of protein extracts from yeast and for Western analysis (Towbin *et al*., 1979; Pearce *et al*., 2000). Nitrocellulose membranes were probed using a polyclonal antiserum against yeast Pfk (Heinisch *et al*., 1996). The second antibody was anti-rabbit IgG–alkaline phosphatase conjugate (Boehringer Mannheim).

**Assays.** Pyruvate kinase (Pyk1) assays were carried out as described previously (Hunsley & Sueter, 1969; Yun *et al*., 1976). PF1K assays were carried out in duplicate using procedures adapted from Reibstein *et al*. (1986). Reaction mixtures contained 3·6 mM ATP, 2·7 U aldolase, 48 U triosephosphate isomerase, 47 U α-glyceroisophosphate dehydrogenase, 1·4 mM fructose 6-phosphate, 0·1 mM KCl, 8 mM MgCl₂, 10 mM NH₄Cl, 2·5 mM 2-mercaptoethanol, 5 mM fructose 2,6-bisphosphate and 50 mM PIPES, pH 7·2. Protein extracts were added and after equilibration for 1 min at 23°C, reactions were initiated by addition of NADH to 0·2 mM. Rates of decrease in A₅40 from 0·1 to 0·0 min⁻¹ were measured and expressed relative to the amount of total protein used in the assay. Protein determinations were performed using the Bradford assay (Bradford, 1976).Fructose 2,6-bisphosphate was measured according to Van Schaftingen *et al*. (1982).

**RESULTS**

**Effect of reducing Pyk1 or PF1K upon benzoic acid tolerance**

It has been suggested that optimal glycolytic flux is required for growth of *S. cerevisiae* in the presence of the weak acid preservative sorbic acid (Holyoak *et al*., 1996). To test the generality of this observation, we examined the effects of well-defined glycolytic mutants upon the tolerance to benzoic acid. First, we defined the benzoic acid tolerance of the parental strain W303-1B. Concentrations of benzoic acid above 2·5 mM inhibited growth over the 8 h period analysed, while concentrations above 0·25 mM resulted in a significant delay before growth was resumed (Fig. 1a). To examine whether this delay involved adaptation to the weak acid, cells were allowed to resume growth following exposure to benzoic acid, and then reincoculated into fresh medium containing the same concentration of benzoic acid (Fig. 1b). No additional delay was observed, confirming that the yeast cells had adapted to the weak acid. Benzoic acid concentrations of up to 0·75 mM did not significantly affect the growth rate after cells had pre-adapted to the weak acid. A slight reduction in specific growth rate was observed in the presence of 1 mM benzoic acid (Fig. 1). Subsequent experiments examined the mechanisms by which *S. cerevisiae* can grow in the presence of these sublethal concentrations of this weak acid.

Benzoic acid tolerance was examined in the YKC set of congenic glycolytic mutants (Table 1). These mutants expressed altered levels of wild-type Pyk1, Pfk1 and/or Pfk2 proteins (Pearce *et al*., 2000). Pyk1 levels were reduced about fourfold as a result of the PGK1Δ767−PYK1 fusion in YKC11; this caused a significant decrease in specific growth rate and glycolytic flux even in the absence of benzoic acid (Pearce *et al*., 2000). Addition of benzoic acid at concentrations up to 1 mM did not have a major impact upon the growth of this strain (Fig. 2a). Therefore, growth in the presence of benzoic acid did not depend upon wild-type rates of glycolytic flux. The PGK1−PFK1 and PGK1−PFK2 promoter fusions in YKC4 increased PF1K levels about twofold, whereas in YKC14, the PGK1Δ767−PFK1 and PGK1Δ767−PFK2 fusions reduced PF1K levels significantly, to about 75% of normal (Pearce *et al*., 2000). Glycolytic flux was not significantly affected by these changes in PF1K levels (Pearce *et al*., 2000). However, benzoic acid tolerance

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**Fig. 1.** Effect of benzoic acid upon growth of W303-1B. (a) Growth of W303-1B in McIlvaines medium, pH 4.6, containing different concentrations of benzoic acid: 0 mM, ◆; 0·05 mM, ■; 0·25 mM, ▲; 0·75 mM, ▼; 1·00 mM, ★; 2·5 mM, ○; 5·0 mM, +; 10 mM, □. (b) Adaptation to different benzoic acid concentrations was examined by transferring exponentially growing W303-1B cells to fresh McIlvaines medium, pH 4·6, containing the same concentration of benzoic acid (arrow) and monitoring growth thereafter. 0 mM, ◆; 0·05 mM, ■; 0·25 mM, ▲; 0·75 mM, ▼. (c) Specific growth rate of W303-1B grown in triplicate at different benzoic acid concentrations once cells had entered the exponential growth phase.
Fig. 2. Effect of reducing PF1K or Pyk1 levels upon benzoic acid tolerance. Specific growth rates were measured in triplicate in McIlvaines medium, pH 4.6, containing different concentrations of benzoic acid once strains had entered the exponential growth phase. (a) Effects of altering PF1K or Pyk1 levels upon benzoic acid tolerance. W303-1B (wild-type), •; YKC4 (PGK1p–PFK1 PGK1p–PFK2), ●; YKC11 (PGK1576–PYK1), ■; YKC14 (PGK1576–PFK1 PGK1576–PFK2), ▲. (b) Effects of reducing the levels of individual PF1K subunits upon benzoic acid tolerance. W303-1B (wild-type), •; YKC12 (PGK1576–PFK1), ▲; YKC13 (PGK1576–PFK2), ▼. (c) Effects of inactivating individual PF1K subunits upon benzoic acid tolerance. W303-1B (wild-type), •; YKC22 (Δpfk1), ■; YKC23 (Δpfk2), ▲.

Fig. 4. Effect of PF2K inactivation upon benzoic acid tolerance. Specific growth rates were measured in triplicate in McIlvaines medium, pH 4.6, containing 1 mM benzoic acid. (a) Growth of strains. VW-1B (wild-type), ○, ●; VW.EB-13B (Δpfk26 Δpfk27), □, ■; no benzoic acid, ○, □; 1 mM benzoic acid, ●, ■. (b) Specific growth rates of strains analysed in (a).

was affected in these strains: it was increased slightly in YKC4 and significantly decreased in YKC14 (Fig. 2a). Therefore, the effect of 1 mM benzoic acid upon growth seemed to correlate with PF1K level, rather than with glycolytic flux.
The influence of PF1K upon benzoic acid tolerance was examined further by testing the contributions of individual PF1K subunits. The levels of these α and β subunits are decreased in YKC12 and YKC13, respectively, whereas these subunits are inactivated in YKC22 and YKC23 (Table 1; Pearce et al., 2000). Decreasing the level of, or inactivating the α subunit (encoded by PFK1) had little effect upon benzoic acid tolerance (Fig. 2b, c). In contrast, benzoic acid sensitivity was increased by inactivating or reducing the level of the β subunit of PF1K (encoded by PFK2). Hence, yeast cells relying on the PF1K α subunit for growth were more sensitive to this weak acid.

**Effect of benzoic acid upon PF1K and fructose 2,6-bisphosphate levels**

The differing contributions of the PF1K α and β subunits to benzoic acid tolerance could have been due to differential effects of the weak acid upon the expression levels of the individual subunits. However, Western analysis revealed that the levels of these proteins were not significantly affected by exposure to benzoic acid (Fig. 3). This analysis reconfirmed the reduction in PF1K level in YKC14 compared to its wild-type parent (Fig. 3; Pearce et al., 2000).

Fructose 2,6-bisphosphate is an important activator of PF1K (Avigad, 1981; Bartrons et al., 1982; Heinisch et al., 1996), and benzoate addition has been reported to reduce fructose 2,6-bisphosphate levels (François et al., 1986). Also, the levels of this activator are significantly elevated in YKC14 (Pearce et al., 2000). Therefore, we measured intracellular fructose 2,6-bisphosphate concentrations following benzoic acid treatment (Fig. 3b). Fructose 2,6-bisphosphate levels remained low in W303-1B throughout these experiments (<0·5 M), but in YKC14 they decreased rapidly following addition of benzoic acid. Normal growth of YKC14 is thought to depend upon elevated fructose 2,6-bisphosphate levels that compensate for the reduced PF1K levels in this strain (Pearce et al., 2000). Hence, the sensitivity of YKC14 to benzoic acid might be due, at least in part, to the effect of this weak acid upon fructose 2,6-bisphosphate levels.

**Effect of modulating PF2K levels upon benzoic acid tolerance**

If benzoic acid sensitivity is influenced by fructose 2,6-bisphosphate concentration, one would expect changes in PF2K to affect benzoic acid tolerance. In *S. cerevisiae*, the two PF2K isozymes are encoded by *PFK26* and *PFK27* (Kretschmer & Fraenkel, 1991; Boles et al., 1996). Therefore, we examined the effects of deleting both genes upon growth in the presence and absence of benzoic acid (Fig. 4). Even in the absence of benzoic acid, the double Δpfk26 Δpfk27 mutation slowed growth in acidic medium (pH 4·6), and this effect was exacerbated by addition of benzoic acid. Therefore, PF2K is required for normal growth under acidic conditions.

If the fructose 2,6-bisphosphate concentration influences benzoic acid tolerance, one might expect the elevation of...
Effect of F26BPase inactivation upon benzoic acid tolerance

Fructose-2,6-bisphosphatase (F26BPase), which is encoded by \( FBP26 \) (Paravicini & Kretchmer, 1992), catalyses the conversion of fructose 2,6-bisphosphate to fructose 6-phosphate. Therefore, if the inhibitory effect of benzoic acid upon YKC14 is influenced by the intracellular level of fructose 2,6-bisphosphate, one would expect \( FBP26 \) inactivation to suppress the benzoic acid sensitivity of YKC14. To test this prediction we deleted the \( FBP26 \) gene in both W303-1B and YKC14 to generate the strains YAKP1 and YAKP2, respectively (Table 1). The absence of \( FBP26 \) sequences in these strains was confirmed by Southern analysis (Fig. 6a). The \( \Delta fbp26 \) mutation did not affect the growth of the control strain in the presence of benzoic acid (Fig. 6b). Again, this was expected because 1 mM benzoic acid only has a small effect upon the growth of W303-1B. Significantly, the \( \Delta fbp26 \) mutation suppressed the benzoic acid sensitivity of YKC14 (Fig. 6c, d). Therefore, F26BPase inactivation prevented the inhibitory effects of benzoic acid upon a strain with low PF1K levels.

**DISCUSSION**

In this study we have investigated the mechanisms by which the weak acid preservative benzoic acid inhibits the growth of \( S. \) cerevisiae. We have specifically examined the effects of sublethal concentrations of this weak acid. It has been suggested that growth in the presence of a
weak acid depends upon optimal glycolytic flux (Holyoak et al., 1996). However, a yeast Pyk1 mutant that displays reduced rates of glycolytic flux (Pearce et al., 2000) was relatively insensitive to sublethal concentrations of benzoic acid (YKC11, Fig. 2). Also, a PF1K mutant that displays wild-type rates of glycolytic flux (Pearce et al., 2000) was relatively sensitive to benzoic acid (YKC14, Fig. 2). These data indicate that suboptimal glycolytic flux is sufficient for yeast to tolerate at least 1 mM benzoate. They also support previous reports suggesting that PF1K represents a potential target for this weak acid preservative (Krebs et al., 1983; François et al., 1986).

The two PF1K subunits displayed differential sensitivities to benzoic acid (Fig. 2). Mutants that only contained the $\alpha$ subunit were relatively sensitive, whereas those that only contained the $\beta$ subunit were not. This suggests that the PF1K $\alpha$ subunit is inhibited by benzoic acid (Fig. 2). Heinisch et al. (1996) have reported that the PF1K $\alpha$ and $\beta$ subunits are activated to similar extents by fructose 2,6-bisphosphate. Here we report that the weak acid did not significantly affect the level of either subunit (Fig. 3). Hence, benzoic acid appears to exert a direct effect upon the activity, rather than the synthesis or degradation, of the PF1K $\alpha$ subunit.

Benzoic acid addition led to a dramatic decline in fructose 2,6-bisphosphate concentration in YKC14 (Fig. 3), confirming a previous report by François et al. (1986). This suggested that, in addition to direct inhibition of the PF1K $\alpha$ subunit, benzoic acid might also exert an indirect effect upon glycolysis by reducing the levels of this important allosteric activator of PF1K. Yeast mutants with altered PF1K levels display compensatory changes in the concentration of the allosteric activator, fructose 2,6-bisphosphate (Davies & Brindle, 1992; Pearce et al., 2000). Fruuctose 2,6-bisphosphate levels are abnormally high in YKC14, and this is thought to allow normal rates of glycolytic flux in this strain, despite its low PF1K levels (Pearce et al., 2000). Therefore, the sensitivity of YKC14 to benzoic acid might be due to the combined effects of this weak acid upon the fructose 2,6-bisphosphate concentration and the PF1K $\alpha$ subunit.

Further examination of the relationship between benzoic acid and fructose-2,6-bisphosphate confirmed this view. Acid sensitivity was increased by inactivating PF2K ($\Delta pfk26 \Delta pfk27$; Fig. 4). In addition, the benzoic acid sensitivity of YKC14 was suppressed by overexpressing PF2K (PF2K$^{\Delta pfk26 \Delta pfk27}$; Fig. 5), or by inactivating F26BPase ($\Delta fbp26$; Fig. 6). These data strongly suggest that benzoic acid mediates its effects upon yeast growth, at least in part, by decreasing fructose 2,6-bisphosphate levels. This decrease is probably mediated by the stimulation of F26BPase combined with the inhibition of PF2K (François et al., 1986, 1988).

Müller et al. (1997) showed that yeast cells lacking PF2K ($\Delta pfk26 \Delta pfk27$) are able to grow at normal rates under fermentative conditions, suggesting that under these conditions they are able to sustain glycolytic flux in the absence of fructose 2,6-bisphosphate. Our data indicate that the stimulatory activity of fructose 2,6-bisphosphate is required to maintain adequate glycolytic flux when yeast cells are exposed to weak acid stress.

Our data support the model of Krebs et al. (1983), which suggests that the antimicrobial effects of benzoic acid are mediated, at least partly, by inhibition of glycolysis at PF1K. However, our data do not invalidate the alternative view that growth is inhibited as a result of the intracellular depletion of ATP (Warth, 1988). Pma1 (Eraso & Gancedo, 1987; Ramos et al., 1989; Viegas and Sá-Correia, 1991; Serrano, 1991) and Pdr12 (Piper et al., 1998; Holyoak et al., 1999), probably expend a large proportion of total cellular ATP pumping protons and benzoate anions from the cell.

Hence, the antimicrobial effects of benzoic acid appear to be mediated by a combination of factors. The growth of yeast cells is probably inhibited by their reduced capacity to generate ATP (via the inhibition of glycolytic flux), combined with their need to expend considerable amounts of ATP in their attempt to maintain homeostasis.

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