Auxotrophy for uridine increases the sensitivity of *Aspergillus niger* to weak-acid preservatives

Petter Melin,^{1,2} Malcolm Stratford,¹ Andrew Plumridge¹ and David B. Archer¹

Correspondence

Peter Melin

petter.melin@mikrob.slu.se

¹School of Biology, University of Nottingham, University Park, Nottingham NG7 2RD, UK

²Department of Microbiology, Swedish University of Agricultural Sciences, PO Box 7025, SE-750 07 Uppsala, Sweden

Weak-acid preservatives such as sorbic acid are added to foods to prevent fungal spoilage. The modes of action of weak-acid preservatives are only partially understood and, in this paper, further insight is presented into the mechanisms by which weak acids inhibit the growth of fungi. Uridine-requiring strains of *Aspergillus niger* were shown to be more sensitive to weak acids (including sorbic, acetic and benzoic acids) than wild-type (WT) strains. In contrast, sensitivity to other, non-acidic, antifungal substances was similar in mutant and WT strains. By complementing a *pyrG*[−] strain of *A. niger* with an intact *pyrG* gene, WT-like resistance to weak-acid preservatives was restored. Using ¹⁴C-labelled uridine, sorbic acid was shown to completely inhibit uridine uptake in germinating conidia in a non-competitive manner. It is therefore proposed that the additional weak-acid sensitivity of the *pyrG*[−] strains was caused by weak-acid inhibition of uridine uptake. Several other auxotrophic strains of *A. niger* were screened for sensitivity to acetic, sorbic and decanoic acids. Strains auxotrophic for either adenine or uridine were found to have enhanced sensitivity but, in contrast, amino acid auxotrophs showed resistance comparable to that of the WT. Uridine auxotrophs of *Saccharomyces cerevisiae* were not more sensitive to weak acids compared to WT strains. In conclusion, this study describes a previously unknown mechanism of action of weak acids against the filamentous fungus *A. niger*, which may fundamentally affect our understanding of the preservation of food against spoilage fungi.

Received 25 October 2007

Revised 18 January 2008

Accepted 24 January 2008

INTRODUCTION

Food spoilage caused by fungi, including both yeasts and the filamentous moulds, results in economic losses throughout the world and has raised concerns over safety due to the possible production of mycotoxins in foods. Spoilage yeast or mould species constitute only a small fraction of the total number of yeast and mould species (Pitt & Hocking, 1997) because most yeasts and moulds are unable to grow in the food or packaging environment. *Aspergillus niger* is a recognized spoilage mould causing post-harvest decay of fruit, and is also among the most common fungi isolated from nuts, especially peanuts, cereals, meat products and cheese (Pitt & Hocking, 1997). *A. niger* shows resistance to sorbic acid through the degradation of this preservative by germinating spores (Plumridge *et al.*, 2004, 2008). *A. niger* is also an important industrial organism in citric acid production and has a large number of mutant strains available and a published complete genome sequence (Bos *et al.*, 1988; Pel *et al.*, 2007; Schuster *et al.*, 2002).

To prevent the growth of microbes in food, a very limited range of chemical compounds, with a proven history of safe consumption, has been approved in Europe for use in

foods as preservatives (Anon, 1995). Many such preservatives are weak acids, and are collectively known as weak-acid preservatives. These include sorbic and benzoic acids, used in confectionery, dressings and soft drinks, acetic acid which is used as an acidulant in pickles, and propionic acid in bread-based foods. Most commonly, weak organic acids are used to inhibit fungal growth in low-pH foods, although several fungal species, notably in the genus *Zygosaccharomyces*, show a high level of resistance to preservatives (James & Stratford, 2003). To optimize the dosage and effectiveness of preservatives and to develop improved preservative strategies, it is important to elucidate the mechanisms underlying the antifungal activity of weak-acid preservatives. At present, the exact mechanisms behind the antifungal activity of these acids remain debatable. The classical weak-acid theory suggests that, at low pH, weak acids are uncharged and pass by diffusion through the fungal cell membrane. Once inside the cell, the acids ionize to the charged anionic form, e.g. sorbate, benzoate or acetate, which are unable to diffuse back through the cell membrane and therefore accumulate and acidify the cytoplasm (Neal *et al.*, 1965; Freese *et al.*, 1973; Krebs *et al.*, 1983). This does not explain the widely differing toxicity of acetic and sorbic

acids which have identical pK_a values, or the far greater toxicity of longer-chain, more lipophilic acids. An effect of weak acids on the cell membrane has been suggested as a contributory toxic mechanism (Stratford & Anslow, 1998).

The *A. niger* *pyrG* gene encodes an ornithine decarboxylase enzyme, essential for the biosynthesis of uracil, and subsequent formation of uridine and RNA. *pyrG*[−] mutants are therefore cultured in the presence of excess uracil or uridine. In a previous study *pyrG* was used as a selectable marker for disruption of the *padA1* gene in a *pyrG*[−] strain of *A. niger* (Plumridge *et al.*, 2008) in order to confirm that the PadA1 enzyme catalysed the degradation of sorbic acid to 1,3-pentadiene, thereby conferring enhanced resistance to sorbic acid. In this study, we show that *pyrG*[−] strains of *A. niger* are more sensitive to sorbic acid than the wild-type (WT) and we investigate the underlying reason.

METHODS

Fungal maintenance and spore preparation. All *A. niger* and *S. cerevisiae* strains used in this study are listed in Table 1. All *A. niger* strains are derived from the same parent strain (N402) referred to here as the WT strain (Bos *et al.*, 1988). The strains of *S. cerevisiae* used were CEN.PK derivatives (van Dijken *et al.*, 2000). *A. niger* mutant strains were maintained on *Aspergillus* minimal medium [AMM: (all l^{−1}) NaNO₃, 6 g; KCl, 0.52 g; MgSO₄·7H₂O, 0.52 g; KH₂PO₄, 1.52 g; FeSO₄·7H₂O, 0.5 mg; ZnSO₄·7H₂O, 0.5 mg; glucose, 20 g; agar, 20 g] plates with the required supplements. The yeast strains were cultured on YPD agar (1 %, w/v, yeast extract, 1 %, w/v, peptone, 2 %, w/v, glucose, 1.5 %, w/v, agar). Conidial suspensions of *A. niger* were prepared in water containing 0.01 % (v/v) Tween 80 from freshly grown agar plates, whereas yeast inocula were from broth cultures.

Measurement of biomass and growth inhibition. Minimal inhibitory concentration (MIC) experiments with *A. niger* were performed in AMM or *Aspergillus* complete medium (ACM; AMM supplemented with Casamino acids, 1.5 g, bacto-peptone, 2.0 g and yeast extract, 1.5 g) at 28 °C. In both media, the pH was accurately adjusted to 4.0 (AMM was buffered with 10 mM succinate). Unless otherwise indicated, the growth medium was supplemented with 10 mM uridine, uracil, adenine, arginine, histidine, leucine, lysine, methionine, proline or tryptophan. For growth of the *A. niger* *nicA1* mutant, the medium was supplemented with nicotinic acid (81 µM). Yeasts and moulds were cultured on 20 ml agar plates, or in 10 ml broth cultures in 30 ml McCartney bottles and incubated for 28 days without shaking, or in 10 ml broth cultures in 125 ml Erlenmeyer flasks with shaking at 160 r.p.m. for 72 h. For biomass estimations, the volume in the shaking flasks was doubled to 20 ml. To agar plates, a suspension of 10², 10³, 10⁴ or 10⁵ conidia was added to the agar plates, and a 100 µl conidial suspension (10⁷ ml^{−1}) was added to 9.9 ml of supplemented broth in the shaking flasks (125 ml), giving a 10⁵ ml^{−1} inoculum. *S. cerevisiae* cells (WT and uridine auxotrophs) were also grown from an inoculum of 10⁵ ml^{−1} in YPD broth at 28 °C. MICs were determined as the concentrations when no germlings could be observed (microscopically) or, for yeast cells, when no increase in optical density (600 nm) could be detected. Weak acids and similar compounds were dissolved in methanol prior to addition. The final methanol concentration never exceeded 1 % (v/v). Control experiments showed that this does not affect the growth of cultures. Hygromycin B was supplemented as a solution in PBS, and amphotericin B was dissolved in DMSO.

Transformation of *A. niger* to restore the *pyrG*⁺ phenotype.

Transformation of *A. niger* AB4.1 was performed with an *EcoRI*-linearized plasmid (van Hartingsveldt *et al.*, 1987) containing the full-length *A. niger* *pyrG* gene. *A. niger* conidia (10⁷ ml^{−1}) were incubated in 400 ml AMM (containing 0.2 %, w/v, glucose, 10 mM uridine) at 28 °C for 16 h. Germlings were collected and washed on a Miracloth filter (Calbiochem). Protoplasting using Glucanex (Sigma) and transformation using PEG and CaCl₂ were carried out as described by Melin *et al.* (2003). Uridine prototrophs from each strain were

Table 1. *A. niger* and *S. cerevisiae* strains used in this study

Strain	Genotype	Origin/reference*
<i>A. niger</i> N402	WT	Bos <i>et al.</i> (1988)
<i>A. niger</i> AB4.1	<i>pyrG1</i>	van Hartingsveldt <i>et al.</i> (1987)
<i>A. niger</i> A734	<i>metB10</i>	FGSC
<i>A. niger</i> A738	<i>nicA1</i>	FGSC
<i>A. niger</i> A741	<i>leuA1</i>	FGSC
<i>A. niger</i> A742	<i>pyrA5</i>	FGSC
<i>A. niger</i> A799	<i>argA1</i>	FGSC
<i>A. niger</i> A877	<i>proC3</i>	FGSC
<i>A. niger</i> A878	<i>lysB2</i>	FGSC
<i>A. niger</i> A881	<i>hisA1</i>	FGSC
<i>A. niger</i> A890	<i>adeC3</i>	FGSC
<i>A. niger</i> N469	<i>trpA1</i>	Goosen <i>et al.</i> (1989)
<i>A. niger</i> AB4.1 <i>pyrG</i> ⁺	WT	This study
<i>S. cerevisiae</i> CEN.PK113-7D	WT (haploid)	van Dijken <i>et al.</i> (2000)
<i>S. cerevisiae</i> CEN.PK113-5D	Δ <i>ura3</i> (haploid)	van Dijken <i>et al.</i> (2000)
<i>S. cerevisiae</i> CEN.PK113-13D	Δ <i>ura3</i> (haploid)	van Dijken <i>et al.</i> (2000)
<i>S. cerevisiae</i> CEN.PK122	WT (diploid)	van Dijken <i>et al.</i> (2000)

*FGSC, Fungal Genetics Stock Center, Kansas City, USA (McCluskey, 2003).

selected and subcultured at least three times, followed by selection of one colony after plating a diluted spore suspension.

Uridine uptake experiments. Fresh suspensions of WT *A. niger* conidia (10^7 ml⁻¹ in 200 ml ACM, pH 4.0, supplemented with 0.1 mM uridine) were incubated for 5 h at 28 °C to allow the spores to initiate growth and nutrient uptake. At this stage the conidia were swollen but no emerging germ tubes could be observed. The culture was then divided equally into four 50 ml tubes and the conidia were collected by centrifugation. Each pellet was resuspended and washed three times in succinate buffer (30 mM succinic acid, pH 4.0, supplemented with 0.2%, w/v, glucose). Finally, all conidia were collected and the volume was adjusted to 4 ml and divided into four 2.0 ml Eppendorf tubes that were kept on ice for up to 40 min. One millilitre of the conidial suspension (about 5×10^7 ml⁻¹) was added to 9.0 ml pre-warmed (28 °C) succinate buffer supplemented with 0.01 mM uridine and [2-¹⁴C]uridine (1.7×10^4 Bq; 0.10 µM; Sigma). Temperature was maintained at 28 °C using a water bath and the spore suspension was kept homogeneous with a magnetic stirrer. Samples of 2.0 ml were taken after 1, 10, 20 and 30 min. Sorbic acid was added to a final concentration of 5.0 mM after 15 min. Samples (2.0 ml) were filtered through 2.5 cm glass fibre filters (Whatman GF/B; pore size 1.0 µm). Filters were pre-treated before sampling with 1 ml ice-cold succinate buffer containing 1.0 mM uridine. After each sample had been filtered, the filter was washed five times with 1 ml ice-cold succinate buffer containing 1.0 mM uridine. For analysis, filters were placed into scintillation vials with 10 ml Emulsifier-Safe (Perkin-Elmer) scintillation fluid. Activities were estimated using a liquid scintillation analyser (Tri-Carb 2100TR; Packard).

RESULTS

Growth of the *A. niger* WT and AB4.1 strains in the presence of sorbic acid

The *A. niger* AB4.1 *pyrG*⁻ strain was more sensitive to sorbic acid than the WT strain N402 (Fig. 1). After 72 h, both the mutant and the WT formed large colonies on the control plates. However, on plates supplemented with 1.0 mM sorbic acid, a *pyrG* mutant colony was only observed at the highest inoculum level, whereas the WT colonies were only slightly smaller than on the control plate (Fig. 1). At 2.0 mM sorbic acid, no growth was observed for the AB4.1 mutant, while the WT grew at all inoculation levels (data not shown).

The *A. niger* AB4.1 strain was originally derived from WT strain N402 following UV radiation. To confirm that the sensitivity to sorbic acid in AB4.1 was a result of the inability to synthesize uridine, and not due to any hidden mutation involved in the mutagenesis procedure, AB4.1 was transformed with an intact *A. niger pyrG* gene. All of the restored transformants regained the ability to grow on uracil- or uridine-free media. Six transformants were subcultured and subjected to sorbic acid treatment. All of these isolates showed resistance to sorbic acid at similar concentrations to the WT strain. The MIC values of sorbic acid for *A. niger* WT N402, the uridine-requiring mutant AB4.1 and the complemented mutant in ACM (pH 4.0) after 72 h incubation at 28 °C and shaking (150 r.p.m.) were 4.5, 3.0 and 4.5 mM, respectively. The recovery of WT resistance to sorbic acid was also observed in all tested

Fig. 1. Agar plates (ACM, pH 4.0) inoculated with conidia (10^2 – 10^5 ; anticlockwise starting in the lower left corner) and incubated for 72 h at 28 °C. (a) WT, control plate; (b) AB4.1 control; (c) WT + 1.0 mM sorbic acid; (d) AB4.1 + 1.0 mM sorbic acid.

deletion mutants, restored using the *A. oryzae pyrG* gene to regain uridine prototrophy (data not shown).

Since MIC values alone do not provide full information on whether the growth (rate or yield) of an organism is reduced at subinhibitory concentrations, we complemented this study by estimating growth rates over 96 h, and biomass yields after 16 days. This study was also carried out using a sorbic acid analogue, hexanoic acid, which is not a substrate for PadA1 and therefore eliminates any potential experimental complications due to weak-acid degradation (Plumridge *et al.*, 2008). The growth rates of *A. niger* WT and AB4.1 strains were similar in control media (i.e. without weak-acid supplementation). The AB4.1 strain had a much slower growth rate than WT when cultured in the presence of either 1.0 mM sorbic or hexanoic acids (Fig. 2a and b). However, if given sufficient time, the slow-growing AB4.1 strain in the presence of 1.0 mM sorbic or hexanoic acids eventually reached the same biomass yield as WT strains (Fig. 2c). Therefore, while the amount of uridine may not limit the yield of biomass, uptake of this base appears to limit the growth rate in strain AB4.1.

It is possible that sorbic acid could bind uridine, thus reducing the effective concentration, or that sorbic acid could competitively inhibit the uptake of uridine into cells. In AMM with a supplemented concentration of uridine ranging from 1.0 to 60 mM, only slight changes in the MIC values for sorbic acid and hexanoic acid were observed (Fig. 3a), showing only a marginal increase in resistance

Fig. 2. Dry weight estimations from WT N402 and AB4.1 (uridine-requiring) strains of *A. niger* grown at 28 °C in 20 ml ACM (pH 4.0) and shaking (150 r.p.m.). (a) Biomass yields from cultures grown in the presence of 1.0 mM sorbic acid for 96 h, sampling every 24 h. ○, N402 (control); ●, N402 (sorbic acid); □, AB4.1 (control); ■, AB4.1 (sorbic acid). (b) Yield after growth in 1.0 mM hexanoic acid. ○, N402 (control); ●, N402 (hexanoic acid); □, AB4.1 (control); ■, AB4.1 (hexanoic acid). (c) Total biomass yields after 16 days growth at different concentrations of sorbic acid. The error bars are the SD calculated from three independent cultures. The high deviations observed in (c) are because growth only occurred in one out of the three flasks [4.5 and 5.0 mM sorbic acid for N402 (○) and 3.5 mM sorbic acid with AB4.1 (●)].

with increased uridine. Replacing uridine with uracil in the medium, however, resulted in increased resistance to sorbic and hexanoic acids in the uridine auxotroph AB4.1 compared to the WT strain (Fig. 3b).

Fig. 3. (a) MICs of sorbic (light grey) and hexanoic acids (dark grey) for the uridine auxotroph AB4.1 grown for 28 days in McCartney bottles at 28 °C in 10 ml AMM (pH 4.0) supplemented with different concentrations of uridine (1.0–60 mM). (b) MICs of sorbic or hexanoic acids for WT and AB4.1 in medium supplemented with either 10 mM uridine (dark grey) or 10 mM uracil (light grey) (b). The error bars are the SD calculated from three independent cultures.

Effect of sorbic acid on uridine uptake

To test directly whether the increased sensitivity of uridine auxotrophs to weak-acid preservatives was due to inhibition of uridine uptake from the growth medium, the uptake of radiolabelled uridine was examined in swollen conidia of *A. niger*. The rate of uridine uptake was found to be linear over the 30 min duration of the experiment. In the presence of 5.0 mM sorbic acid, the uptake of uridine was completely inhibited. Uptake stopped immediately after supplementing the spores with sorbic acid at 15 min (Fig. 4). At the end of the control experiment, less than 10% of the added radiolabelled uridine had been incorporated into the swollen spores. There was no evidence of leakage of radiolabel from cells after sorbic acid inhibition of uptake. The concentration of sorbic acid used in this experiment (5.0 mM) was the minimal concentration required to prevent germination of AB4.1

Fig. 4. Uptake of [^{14}C]uridine into swollen WT *A. niger* conidia ($5 \times 10^7 \text{ ml}^{-1}$), with (●) or without (○) sorbic acid (5 mM) in succinate buffer (see Methods). The acid was added to spore suspensions after 15 min incubation with radiolabel. The error bars indicate the SD between three replicate samples.

in an overnight experiment using a conidial concentration of $5 \times 10^7 \text{ ml}^{-1}$ (data not shown).

Sensitivity of *A. niger* strains to aliphatic fatty acids of different chain lengths

Previous experiments had shown that the uridine-requiring strain AB4.1 had increased sensitivity to sorbic and hexanoic acids (Fig. 3). To learn whether the increased sensitivity (compared to WT) was dependent on the hydrophobicity or chain length of the fatty acid, we exposed cells to the aliphatic series of acids from C_2 (acetic acid) up to C_{10} (decanoic acid). For both the WT and strain AB4.1, the MIC values were reduced with increased length, and hydrophobicity, of the acid. Strain AB4.1 was sensitive to all acids tested (Table 2). The MIC value of the WT strain for decanoic acid was above the solubility limit at this pH, preventing a numerical comparison.

MIC of acetic, sorbic and decanoic acids with auxotrophic mutants of *A. niger* and *S. cerevisiae*

Results thus far indicate that the uridine-requiring AB4.1 strain of *A. niger* is overly sensitive to sorbic acid due to its inability to transport uridine in the presence of sorbic acid. To test whether this phenomenon is unique to uridine uptake, several other auxotrophic *A. niger* strains were tested for sensitivity to acetic, sorbic and decanoic acids. Two uridine-requiring strains, AB4.1 and A742, which contain different mutations responsible for the auxotrophy, showed enhanced sensitivity relative to the WT strain (N402) to the three acids. A similar oversensitivity was observed in the adenine-auxotrophic strain A890 (Table 3). In contrast, the nicotinic-acid- and all amino-acid-

Table 2. MICs of fatty acids of different lengths after 28 days' growth in ACM, pH 4.0

Values presented are the means of three independent measurements.

Weak acid	N402 (WT) (mM)	AB4.1 (uridine-requiring) (mM)
Acetic acid	72	32
Propionic acid	16	6
Butyric acid	10	4.5
Valeric acid	3.6	1.6
Hexanoic acid	3.7	2.1
Heptanoic acid	3.0	2.0
Octanoic acid	2.2	1.6
Nonanoic acid	1.2	0.8
Decanoic acid	—*	0.3
Sorbic acid	4.3	2.3

*Decanoic acid precipitates at concentrations above 0.4 mM at pH 4.0 resulting in inaccurate MIC estimations above that concentration.

auxotrophic strains tested (see Table 1) showed an overall similar level of resistance as the WT (data not shown). In addition we could not observe any significant difference in biomass yield compared to the WT at subinhibitory concentrations (data not shown).

To determine whether the sensitivity to weak acids observed in *A. niger* adenine and uridine auxotrophs occurred in another fungus, we examined WT (haploid and diploid) and uridine-auxotrophic strains (Δura3) of *S. cerevisiae*. Using a similar experimental procedure as used for *A. niger*, the MIC values of acetic, sorbic and decanoic acids were found to be similar in the *S. cerevisiae* WT strains and uridine auxotrophs (data not shown), suggesting that uridine uptake is not inhibited by weak acids in *S. cerevisiae*.

MIC of acids, aldehydes, alcohols and antibiotics

The adenine-auxotrophic *A. niger* strain A890 and uridine-auxotrophic strains were more sensitive to fatty acids than the WT. Further tests examined their sensitivities towards aromatic weak acids, alcohols, aldehydes and two antibiotics. The inhibitory effect of all tested acids, including the structurally unrelated benzoic acid, was stronger in both the adenine and uridine auxotrophs than in the WT (Table 3), and reduced growth at subinhibitory concentrations was also observed (data not shown). In contrast, when we treated strains with sorbic aldehyde and decanol, two compounds that both showed strong antifungal activity and are structurally related to sorbic and decanoic acid, respectively, the observed MICs were similar in the mutants and the WT (Table 3). Furthermore, no additional sensitivity to the two antibiotics hygromycin B and amphotericin B could be observed in the auxotrophs (Table 3).

Table 3. MICs (mM) for other antifungal substances in shaking flasks (ACM, pH 4.0; 72 h)

Values presented are the highest MIC values observed from three independent measurements.

<i>A. niger</i> strain	Acetic acid	Sorbic acid	Decanoic acid	Hexanoic acid	Sorbic-aldehyde	Benzoic acid	Decanol	Hygromycin B	Amphotericin B
N402	55	4.5	3.0	3.5	0.75	3.0	1.0	1.0	0.005
AB4.1	35	3.0	0.20	2.0	1.0	1.5	1.0	1.0	0.005
A890	35	2.0	0.20	1.5	0.75	1.5	0.75	0.75	0.005

DISCUSSION

In this study, *A. niger* uridine- and adenine-auxotrophic strains were shown to be more sensitive than the WT strain to a variety of weak acids, but not to other inhibiting molecules, including hydrophilic alcohols or aldehydes. Reintroducing the *pyrG* gene into uridine-auxotrophic strains restored the WT level of resistance to weak acids, strongly indicating that the effect was due to the *pyrG*[−] phenotype, and not due to a mutation elsewhere in the genome. Weak acids that varied in chain length were examined, and all showed a much-enhanced effect against an *A. niger* uridine-auxotrophic strain. Two different uridine auxotrophs and the tested adenine-requiring strain showed similar properties, whereas all other auxotrophic strains (with requirements for nicotinic acid and for several amino acids) were not overly sensitive to weak acids. This indicates a common phenomenon in auxotrophs for nucleotide precursors. The structure of acids appeared to have a limited effect, the uridine- and adenine-auxotrophic strains being sensitive to both aromatic and aliphatic acids, with or without unsaturation in the carbon chain. This is an important finding because it presents an additional functionality, unique to weak acids, that adds complexity to the mode of action of weak-acid preservatives.

In contrast to *A. niger*, uridine-auxotrophic strains of *S. cerevisiae* did not show abnormal sensitivity to weak acids, in accordance with a previous report where the authors screened the *S. cerevisiae* gene-deletion strain collection and did not observe any hypersensitivity to sorbic acid in strains deleted in any gene involved in nucleotide synthesis (Mollapour *et al.*, 2004). It therefore appears that the uridine transporter in *A. niger* is sensitive to weak-acid inhibition, whereas the *S. cerevisiae* homologue is not. It is probable that the adenine transporter is also weak-acid-sensitive in *A. niger*. Conversely, there may be other transporters in *S. cerevisiae* that are specifically weak-acid-sensitive. It has been reported that a tryptophan auxotroph in *S. cerevisiae* was abnormally sensitive to sorbic acid (Bauer *et al.*, 2003) although, in this instance, supplying the medium with tryptophan in excess could circumvent the effect. Since the *trp* marker is commonly used for selection in yeast, this led to several erroneous reports of gene interactions in weak-acid resistance. Here, no such general sensitivity to a variety of weak acids was seen in the *trpA* auxotroph of *A. niger*. It has also been observed that in

nitrogen-starved *Penicillium chrysogenum* mycelia, weak acids inhibit leucine transport (Hunter & Segel, 1973), but this study is hard to interpret and compare with ours since no acid concentrations were given. Weak acids have also been shown to reduce the uptake of several amino acids in the bacterium *Bacillus subtilis* (Sheu *et al.*, 1972), but this appears to be a general effect on active transport (Freese *et al.*, 1973).

The mechanisms behind the observed sensitivity to weak acids of *A. niger* strains auxotrophic for nucleotide bases are likely to be due to inhibited uptake of the nucleotide, since we could not observe any uridine uptake into sorbic-acid-treated swollen spores. In *S. cerevisiae*, there exist separate but homologous transporters for uridine and uracil, and an unrelated, non-homologous adenine transporter (Nelissen *et al.*, 1997) and homologous proteins in *A. niger* can be found in the recently annotated genome sequence (Pel *et al.*, 2007). The data presented here indicate a strong effect of sorbic acid on the *A. niger* uridine transporter and an as yet unproven effect on the adenine and uracil transporters.

The mechanism by which weak acids inhibit the transporters remains speculative at present. Weak acids or their hydrophilic anions could interact directly with the aqueous portions of transport proteins protruding from either face of the plasma membrane. Alternatively, the observed effect could be caused by the weak acids integrating into the lipid bilayer of the membrane, thereby interacting with the hydrophobic portions of the transporters. The partition coefficients of lipophilic weak acids suggest that they may accumulate to significant levels in membranes, depending on the acid (Leo *et al.*, 1971). Since it is known that transporters may be affected by their lipid environment (Keenan *et al.*, 1982), it is tempting to speculate that the altered environment of a weak-acid-containing membrane may inhibit the activity of the *A. niger* uridine transporter. In that case, some of the transporters (e.g. uridine) would be more sensitive to the lipid environment than others (e.g. amino acids). Other mechanisms whereby weak acids may impair the uptake of uridine may also be involved, but data are not available to inform a choice between possibilities. At present, the most likely explanation is that the lipophilic weak acids affect the environment of transmembrane transporters within the membrane and that some transporters (e.g. for uridine) are more sensitive than others.

ACKNOWLEDGEMENTS

Dr Peter Kötter is thanked for the *S. cerevisiae* CEN.PK strains and Dr Fons Debets is thanked for the *A. niger* tryptophan auxotroph. This work was largely financed by the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning as a personal postdoctoral stipend to P.M. In addition, funding from a Defra BBSRC Link project (FQS69) is gratefully acknowledged.

REFERENCES

- Anon (1995). EC Directive 95/2/EC; Food additives other than colours and sweeteners. *Off J Eur Communities* **L61** 18 March, 1–40.
- Bauer, B. E., Rossington, D., Mollapour, M., Mamnun, Y., Kuchler, K. & Piper, P. W. (2003). Weak organic acid stress inhibits aromatic amino acid uptake by yeast, causing a strong influence of amino acid auxotrophies on the phenotypes of membrane transporter mutants. *Eur J Biochem* **270**, 3189–3195.
- Bos, C. J., Debets, A. J. M., Swart, K., Huybers, A., Kobus, G. & Slakhorst, S. M. (1988). Genetic analysis and the construction of master strains for assignment of genes to 6 linkage groups in *Aspergillus niger*. *Curr Genet* **14**, 437–443.
- Freese, E., Sheu, C. W. & Galliers, E. (1973). Function of lipophilic acids as antimicrobial food additives. *Nature* **241**, 321–325.
- Goosen, T., van Engelenburg, F., Debets, F., Swart, K., Bos, K. & van den Broek, H. (1989). Tryptophan auxotrophic mutants in *Aspergillus niger*: inactivation of the *trpC* gene by cotransformation mutagenesis. *Mol Gen Genet* **219**, 282–288.
- Hunter, D. R. & Segel, I. H. (1973). Effect of weak acids on amino acid transport by *Penicillium chrysogenum*: evidence for a proton or charge gradient as the driving force. *J Bacteriol* **113**, 1184–1192.
- James, S. A. & Stratford, M. (2003). Spoilage yeasts with emphasis on the genus *Zygosaccharomyces*. In *Yeasts in Food, Beneficial and Detrimental Aspects*, pp. 171–191. Edited by T. Boekhout & V. Robert. Hamburg: Behr's.
- Keenan, M. H. J., Rose, A. H. & Silverman, B. W. (1982). Effect of plasma-membrane unsaturation on solute transport into *Saccharomyces cerevisiae* NCYC 366. *J Gen Microbiol* **128**, 2547–2556.
- Krebs, H. A., Wiggins, D., Stubbs, M., Sols, A. & Bedoya, F. (1983). Studies on the mechanism of the antifungal action of benzoate. *Biochem J* **214**, 657–663.
- Leo, A., Hansch, C. & Elkins, D. (1971). Partition coefficients and their uses. *Chem Rev* **71**, 525–616.
- McCluskey, K. (2003). The fungal genetics stock center: from molds to molecules. *Adv Appl Microbiol* **52**, 245–262.
- Melin, P., Schnürer, J. & Wagner, E. G. H. (2003). Characterization of *phiA*, a gene essential for phialide development in *Aspergillus nidulans*. *Fungal Genet Biol* **40**, 234–241.
- Mollapour, M., Fong, D. N., Balakrishnan, K., Harris, N., Thompson, S., Schuller, C., Kuchler, K. & Piper, P. W. (2004). Screening the yeast deletant mutant collection for hypersensitivity and hyper-resistance to sorbate, a weak organic acid food preservative. *Yeast* **21**, 927–946.
- Neal, A. L., Weinstock, J. O. & Lampen, J. O. (1965). Mechanisms of fatty acid toxicity for yeast. *J Bacteriol* **90**, 126–131.
- Nelissen, B., DeWachter, R. & Goffeau, A. (1997). Classification of all putative permeases and other membrane plurispansers of the major facilitator superfamily encoded by the complete genome of *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* **21**, 113–134.
- Pel, H. J., de Winde, J. H., Archer, D. B., Dyer, P. S., Hofmann, G., Schaap, P. J., Turner, G., de Vries, R. P., Albang, R. & other authors (2007). Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nat Biotechnol* **25**, 221–231.
- Pitt, J. I. & Hocking, A. D. (1997). *Fungi and Food Spoilage*, 2nd edn. London: Blackie Academic and Professional.
- Plumridge, A., Hesse, S. J. A., Watson, A. J., Lowe, K. C., Stratford, M. & Archer, D. B. (2004). The weak acid preservative sorbic acid inhibits conidial germination and mycelial growth of *Aspergillus niger* through intracellular acidification. *Appl Environ Microbiol* **70**, 3506–3511.
- Plumridge, A., Stratford, M., Lowe, K. C. & Archer, D. B. (2008). The weak-acid preservative sorbic acid, is decarboxylated and detoxified by a phenylacrylic acid decarboxylase, PadA1, in the spoilage mold *Aspergillus niger*. *Appl Environ Microbiol* **74**, 550–552.
- Schuster, E., Dunn-Coleman, N., Frisvad, J. C. & van Dijck, P. W. M. (2002). On the safety of *Aspergillus niger* – a review. *Appl Microbiol Biotechnol* **59**, 426–435.
- Sheu, C. W., Konings, W. N. & Freese, E. (1972). Effects of acetate and other short-chain fatty acids on sugar and amino acid uptake of *Bacillus subtilis*. *J Bacteriol* **111**, 525–530.
- Stratford, M. & Anslow, P. A. (1998). Evidence that sorbic acid does not inhibit yeast as a classic 'weak acid preservative'. *Lett Appl Microbiol* **27**, 203–206.
- van Dijken, J. P., Bauer, J., Brambilla, L., Duboc, P., Francois, J. M., Gancedo, C., Giuseppin, M. L., Heijnen, J. J., Hoare, M. & other authors (2000). An inter-laboratory comparison of physiological and genetic properties of four *Saccharomyces cerevisiae* strains. *Enzyme Microb Technol* **26**, 706–714.
- van Hartingsveldt, W., Mattern, I. E., van Zeijl, C. M. J., Pouwels, P. H. & van den Hondel, C. (1987). Development of a homologous transformation system for *Aspergillus niger* based on the *pyrG* gene. *Mol Gen Genet* **206**, 71–75.

Edited by: H. A. B. Wösten