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Extreme resistance to weak-acid preservatives in the spoilage yeast *Zygosaccharomyces bailii* ☆

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ABSTRACT

Weak-acid preservatives, such as sorbic acid and acetic acid, are used in many low pH foods to prevent spoilage by fungi. The spoilage yeast *Zygosaccharomyces bailii* is notorious for its extreme resistance to preservatives and ability to grow in excess of legally-permitted concentrations of preservatives. Extreme resistance was confirmed in 38 strains of *Z. bailii* to several weak-acid preservatives. Using the brewing yeast *Saccharomyces cerevisiae* as a control, tests showed that *Z. bailii* was ~3-fold more resistant to a variety of weak-acids but was not more resistant to alcohols, aldehydes, esters, ethers, ketones, or hydrophilic chelating acids. The weak acids were chemically very diverse in structure, making it improbable that the universal resistance was caused by degradation or metabolism. Examination of *Z. bailii* cell populations showed that extreme resistance to sorbic acid, benzoic acid and acetic acid was limited to a few cells within the population, numbers decreasing with concentration of weak acid to <1 in 1000. Re-inoculation of resistant sub-populations into weak-acid-containing media showed that all cells now possessed extreme resistance. Resistant sub-populations grown in any weak-acid preservative also showed ~100% cross-resistance to other weak-acid preservatives. Tests using ^{14}C-acetic acid showed that weak-acid accumulation was much lower in the resistant sub-populations. Acid accumulation is caused by acid dissociation in the higher pH of the cytoplasm. Tests on intracellular pH (pH_i) in the resistant sub-population showed that the pH was much lower, ~pH 5.6, than in the sensitive bulk population. The hypothesis is proposed that extreme resistance to weak-acid preservatives in *Z. bailii* is due to population heterogeneity, with a small proportion of cells having a lower intracellular pH. This reduces the level of accumulation of any weak acid in the cytoplasm, thus conferring resistance to all weak acids, but not to other inhibitors.

1. Introduction

Many foods form ideal substrates for the growth of fungi, both yeasts and moulds, due to their carbohydrate, protein and vitamin content. If left untreated, fungal growth will result in spoilage, due to alterations in visual appearance, texture, taste, aroma, and the formation of fungal biomass and in some cases, a variety of mycotoxins. In order to prevent microbial spoilage, many foods are sterilised using heat, while others are treated with preservatives of proven safety of which the great majority are weak-acids. Soft drinks may contain limited concentrations of sorbic acid (2,4-hexadienoic acid) or benzoic acid (Anon., 1995) while acetic acid, commonly used as a preservative in salad dressings, pickles and vinegars, is legally recognised as an acidulant within the EU (Anon., 1995).

Preservatives inhibit the great majority of yeast and mould species, but a few species are able to proliferate in preserved foods (Pitt and Hocking, 1997). These are the spoilage fungi, and their physiological properties largely define their spoilage behaviour. The most dangerous spoilage yeasts (Group 1) were characteristically preservative-resistant (Davenport, 1996), osmotolerant, vitamin-requiring and highly fermentative, leading to excessive gas formation, bottle explosions, and occasional physical injury (Grinbaum et al., 1994). The majority of yeast species were Group 3 (hygiene indicators, not causing spoilage) while Group 2 were opportunistic yeasts able to cause spoilage following mistakes in manufacturing (Davenport, 1997, 1998). The most notorious of the Group 1 spoilage fungi, due to its outstanding degree of preservative resistance, was a yeast species known as *Zygosaccharomyces bailii*.

Z. bailii, reviewed by Thomas and Davenport (1985) and James and Stratford (2011), is a yeast naturally-occurring in mummified dried fruits, readily forming moderately heat-resistant ascospores. It is osmotolerant (Tilbury, 1976) and grows preferentially on fructose

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(Emmerich and Radler, 1983). This species is similar in some respects to the brewing yeast *Saccharomyces cerevisiae*, fermenting in aerobic conditions (Merico et al., 2003; Rodrigues et al., 2001) and in anaerobic conditions with suitable nutritional supplementation (Rodrigues et al., 2001). Spoilage by *Z. bailii*, reviewed by Fleet (1992), includes soft drinks (Sand, 1973), cordials and tomato sauce (Pitt and Richardson, 1973), high-sugar syrups (Tokuoka, 1993), acetic preserves (Dennis and Buhagiar, 1980), wine (Goswell, 1986) and cider (Beech, 1993). *Z. bailii* is reported to be highly resistant to sorbic, benzoic, acetic and propionic acids (Ingram, 1960; Malfeito-Ferreira et al., 1997; Neves et al., 1994; Pitt, 1974) and to sulphite (Goswell, 1986; Goto, 1980; Hammond and Carr, 1976) and hydroxycinnamic acids (Stead, 1995). It is also reported to be resistant to ethanol and other alkanols (Fujita et al., 2008; Goswell, 1986; Thomas and Davenport, 1985) and to carbonation (Ison and Gutteridge, 1987) and low pH (Betts et al., 1999).

The causes of resistance in *Z. bailii* have been investigated on several occasions and the overall results can be circumscribed by two possible hypotheses; 1. degradation and metabolism of the preservatives, and 2. efflux pumps removing preservatives. Metabolism of acetic acid by *Z. bailii* in the presence of glucose has been demonstrated (Guerriero et al., 2012; Rodrigues et al., 2012; Sousa et al., 1996, 1998) as have degradation of benzoic acid and sorbic acid (Ingram, 1960; Mollapour and Piper, 2001). However, removal of sufficient acids to affect resistance has not been confirmed and earlier studies (Warth, 1977) concluded that weak-acid metabolism was insufficient to explain resistance in *Z. bailii*. Efficient decarboxylation of weak-acid preservatives using the fungal Pad decarboxylation system was shown not to occur in *Z. bailii* (Stratford et al., 2007). Efflux of preservatives due to a "sorbate pump" was proposed by Warth (1977, 1988). It has been shown that lipophilic weak acids enter the cell rapidly by simple diffusion (Stratford and Rose, 1986; Warth, 1989a) but are concentrated because of the higher pH of the cytoplasm causing acid dissociation into their respective anions. This concentration effect led to early claims that uptake was an active transport process (Macris, 1975). At higher pH, there is evidence of mediated uptake of low concentrations of acetate (Sousa et al., 1996). Pre-growth of *Z. bailii* cells in benzoic or propionic acids, however, resulted in a 40% slower uptake of preservatives, which was proposed to be the result of active acid efflux from adapted cells (Warth, 1977, 1989a). Preservative resistance in 23 other yeast species was also correlated with uptake rate of propionic acid (Warth, 1989b). A similar sorbate efflux system has been reported in *S. cerevisiae*, encoded by the *PDR12* gene (Piper et al., 1998). However, it has been shown that such a system is not induced in *Z. bailii* in response to preservatives (Piper et al., 2001). Therefore, the causes of extreme preservative resistance in *Z. bailii* remain unresolved.

In this paper, we set out to investigate the causes of weak-acid preservative resistance in *Z. bailii*. Population heterogeneity to weak acids was also examined in light of an earlier study showing that only a very small proportion of the population of *Z. bailii* cells were resistant to sorbic acid (Steels et al., 2000).

2. Materials and methods

2.1. Strain variation in Z. bailii

The yeast strains used in this study are listed in Table 1 together with their source of isolation. The identity of all strains was confirmed by sequencing the D1/D2 region of the 26S rDNA using the methods described by Kurtzman (2003). Yeast strains were stored in glycerol on ceramic beads at −80 °C (Microbank™), and maintained short term on MEA (malt extract agar, Oxoid) slopes at 4 °C.

The growth medium used to assess strain variation was YEPD, glucose 20 g/l, bacteriological peptone (Oxoid) 20 g/l, and yeast extract (Oxoid) 10 g/l, adjusted to pH 4.0 with 10 M HCl prior to heat sterilisation. Starter cultures comprised 10 ml YEPD pH 4.0 in 28 ml McCartney bottles, inoculated and incubated for 48 h at 25 °C.

Table 1
Strains of *Zygosaccharomyces bailii* and *Saccharomyces cerevisiae* used in this study and their origins. NCYC strains are available from the National Collection of Yeast Cultures, Norwich UK. Others were collected (strain numbers) over several years from the food industry. All strains were confirmed in identity by D1/D2 rDNA sequencing. Weak-acid preservative resistance, sorbic acid, benzoic acid and acetic acid, was measured in YEPD pH 4.0 at 10^3 cells/ml and incubated at 25 °C for 2 weeks at pH 4.0. Numbers provided in the columns headed by sorbic, benzoic and acetic are the lowest concentration of weak acids (mM) to completely inhibit growth.

Strain	Species	Origin	Sorbic	Benzoic	Acetic
BY4741	*S. cerevisiae*	Euroscarf	3	2.9	120
NCYC 3253	*S. cerevisiae*	Spoilage, soft drink UK	3.5	3.4	145
4	*Z. bailii*	Spoilage, canned ice tea USA	6.54	8.5	550
5	*Z. bailii*	Spoilage, canned fruit USA	6.55	8	533
6	*Z. bailii*	Spoilage, bottled ice tea USA	7.46	9.12	545
7	*Z. bailii*	Spoilage, preserved fruit punch USA	6.67	8.13	475
8	*Z. bailii*	Spoilage, soft drink USA	6.68	8.5	467
9	*Z. bailii*	Spoilage, carbonated orange drink USA	8.04	8.13	468
10	*Z. bailii*	Spoilage, soft drink USA	6.35	8.33	483
11	*Z. bailii*	Spoilage, soft drink USA	7	9.13	466
12	*Z. bailii*	Spoilage, carbonated orange drink USA	8.09	9.75	468
13	*Z. bailii*	Spoilage, soft drink USA	7.06	10.12	467
15	*Z. bailii*	Spoilage, salad dressing Netherlands	7.44	8.88	444
16	*Z. bailii*	Spoilage, salad dressing Netherlands	7.13	7.75	400
17	*Z. bailii*	Spoilage, salad dressing UK	6.69	8.87	517
21	*Z. bailii*	Spoilage, herring in tomato sauce UK	4.55	7.65	275
52	*Z. bailii*	Spoilage, salad dressing Netherlands	5.83	9.13	567
80	*Z. bailii*	Spoilage, Mexican Topping sauce UK	6.2	9.75	450
105	*Z. bailii*	Spoilage, tomato sauce UK	7.97	8.37	475
106	*Z. bailii*	Spoilage, tomato sauce UK	7.75	8.11	470
107	*Z. bailii*	Spoilage, tomato sauce UK	7.34	8.2	400
108	*Z. bailii*	Spoilage, tomato sauce UK	7.83	8.14	466
112	*Z. bailii*	Spoilage, ice tea Belgium	6.6	8.25	450
114	*Z. bailii*	Spoilage, ice tea Belgium	6.3	9.25	450
119	*Z. bailii*	Spoilage, soft drink Netherlands	8.75	9.75	500
280	*Z. bailii*	Spoilage, soft drink South Africa	8.4	9	400
362	*Z. bailii*	Factory isolate Turkey	6.8	8.3	440
475	*Z. bailii*	Factory isolate Brazil	7	8	450
503	*Z. bailii*	Kombucha, fermented tea UK	8.5	9.5	530
505	*Z. bailii*	Kombucha, fermented tea UK	8.8	10	450
593	*Z. bailii*	Factory isolate Philippines	4.5	8.25	450
595	*Z. bailii*	Spoilage, dried fruit Spain	7.9	10.1	500
DBVPG 6924	*Z. bailii*	Anne Vaughn-Martini, USA	8.5	9.5	580
NCYC 1766	*Z. bailii*	Spoilage, Blackcurrant & Grape UK	7.62	8.65	467
NCYC 563	*Z. bailii*	Spoilage, sorghum brandy	5.75	7.75	375
NCYC 3378	*Z. bailii*	Factory isolate Philippines	7.65	9.15	550
NCYC 3407	*Z. bailii*	Spoilage, lemon tea UK	6.19	9.12	484
NCYC 3410	*Z. bailii*	Spoilage, herring in tomato sauce UK	6.13	8.12	383
NCYC 3414	*Z. bailii*	Spoilage, orange concentrate UK	5.85	6.25	450
NCYC 3590	*Z. bailii*	Spoilage, jam Sweden	9.45	11	390
		Z. bailii Mean	7.10	8.75	465.39
		Z. bailii S.D.	1.11	0.89	59.57

Resistance to weak-acid preservatives was determined by the minimum inhibitory concentration (MIC) of each acid to completely inhibit growth. Series of McCartney bottles were prepared with 10 ml aliquots of YEPD, each containing a progressively higher concentration of preservative. The pH of all media was back-titrated to