RESEARCH LETTER

Effect of trace iron levels and iron withdrawal by chelation on the growth of *Candida albicans* and *Candida vini*

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Abstract

The iron requirements of the opportunistic pathogenic yeast, *Candida albicans*, and the related nonpathogenic spoilage yeast *Candida vini* were investigated along with their responses to various exogenous iron chelators. The influence of iron as well as the exogenous chelating agents lactoferrin, EDTA, deferiprone, desferrioxamine, bathophenanthroline sulphonate and a novel carried chelator with a hydroxypyridinone-like Fe-ligand functionality, DIBI, on fungal growth was studied in a chemically defined medium deferrated to trace iron levels ($< 1.2\,\mu g\,L^{-1}$ or 0.02 μM of Fe). *Candida albicans* competed better at low iron levels compared with *C. vini*, which was also more susceptible to most added chelators. *Candida albicans* was resistant to lactoferrin at physiologically relevant concentrations, but was inhibited by low concentrations of DIBI. *Candida vini* was sensitive to lactoferrin as well as to DIBI, whose inhibitory activity was shown to be Fe reversible. The pathogenic potential of *C. albicans* and the nonpathogenic nature of *C. vini* were consistent with their differing abilities to grow under iron-limiting conditions and in the presence of exogenous iron chelators. Both yeasts could be controlled by appropriately strong chelators. This work provides the first evidence of the iron requirements of the spoilage organism *C. vini* and its response to exogenous chelators. Efficient iron withdrawal has the potential to provide the basis for new fungal growth control strategies.

Introduction

Microbial spoilage of foods, beverages and other aqueous consumer products, such as personal care cosmetics or ophthalmic solutions, presents significant challenges for product preservation and may lead to health implications. Traditional techniques involving chemical preservatives to suppress microorganisms can have the limitation of the development of microbial resistances (Russell, 1991; Chapman, 2003) and may not generally be compatible with product formulations or may lead to undesirable reactions among sensitive consumers (Jong *et al.*, 2007). Fungal spoilage is particularly important, given their propensity for growth at low pH values, as often used to inhibit bacterial growth. Combinations of chemical agents within a so-called hurdle approach to preservation have yielded some improvements (Leistner, 2000). For example, EDTA, which is known to chelate Fe, Ca and various other essential cations (Ueno *et al.*, 1992), has been shown to increase the sensitivities of preservative-tolerant isolates, such as *Pseudomonas* (Chapman *et al.*, 1998).

The underlying iron requirement of microbial growth could provide the basis for a general approach to increasing microbial stability of products. Iron is known to be irreplaceably required for a multitude of essential physiological functions in all microorganisms (Neilands, 1974), except for lactic acid bacteria, which do not seem to require or incorporate iron (Bruyneel *et al.*, 1989; Pandey *et al.*, 1994). Accordingly, both nonpathogenic as well as pathogenic bacteria (Ratledge & Dover, 2000) and fungi (Howard, 2004) require Fe for growth in the various environments in which they proliferate. Previous work has demonstrated the potential effectiveness of iron and other trace metal withdrawal for the inhibition of *Saccharomyces cerevisiae* growth (Feng *et al.*, 1997a).

In this work, a trace iron methodology was developed and applied in order to study the effect of iron removal on

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microbial growth in a chemically defined medium. In addition to using media with trace iron concentrations, microbial inhibition by the natural host defence Fe chelator, lactoferrin, clinically used chelators, such as desferrioxamine and deferiprone, and other strong chelators, such as bathophenanthroline sulphonate (BPS), EDTA and a novel carried chelator with hydroxypyridinone-like Fe-ligand functionality, DIBI, was also studied. The organisms chosen for this study were the well-known opportunistic pathogen *Candida albicans* (McCullough *et al*., 1996) and *Candida vini* (Barnett *et al*., 1983), a related, but lesser-known nonpathogenic spoilage yeast.

Materials and methods

Microorganisms and chelating agents

Candida albicans (ATCC 10231) and *C. vini* (ATCC 20217) were obtained from the Microbiology Laboratory Culture Collection at the Department of Food Science, University of Guelph, Canada. Desferrioxamine (Desferal) was donated by Ciba Geigy, now Novartis, Basel, Switzerland. Deferiprone, EDTA, BPS and bovine lactoferrin were obtained from Sigma-Aldrich. The developmental compounds DIBI and FEC-1 were donated by Chelation Partners. Apolactoferrin (i.e. Fe depleted) was prepared according to Holbein (1981). The other chelators were dissolved directly in the medium. The iron-binding capacity of the DIBI was determined to be 800 μmol dry weight g^{-1} DIBI by adding varying amounts of Fe-citrate (1 : 3 molar ratio) to aqueous DIBI samples of known mass and then reading the Fe complex $A_{530\,nm}$, the main visible range absorption peak for the DIBI chelate as determined by an absorption scan.

Media, preparation of inocula and culture conditions

Throughout the work, the aerobic growth version of the chemically defined glucose-phosphate-proline (GPP) medium (pH 4.5) of Dumitru *et al*. (2004) was used with one modification: the mineral concentrate was prepared without the inclusion of $FeSO_4$. Trace iron GPP was prepared by removing iron contaminations with the Fe-specific resin, FEC-1 (Feng *et al*., 1997b). For this, 5 g of hydrated and washed FEC-1 resin were batch contacted by shaking overnight with 1 L of complete GPP medium in a flask. After removal of the resin by filtration, the Fe-extracted medium was filter sterilized (0.22-μm nylon filter, Millipore) and stored in sterile plastic bottles at 4 °C. Typical trace iron concentrations attained using this method were 1.2 μg L^{-1}. Different known iron concentrations were adjusted in the trace iron GPP by addition of appropriate amounts of a 0.5 mM Fe-citrate stock solution containing a 3 M excess of sodium citrate to $FeSO_4$.

Inoculations were carried out from precultures grown for 24 h in trace iron GPP at inoculation rates of 0.1% v/v to minimize carryover of iron. The total initial cell counts of cultures thus inoculated typically were 5×10^4 mL^{-1} and 3×10^3 mL^{-1} for *C. albicans* and *C. vini*, respectively. Incubation of flask cultures was carried out aerobically in a temperature-regulated shaker at 30 °C and 200 r.p.m.

Trace iron methodology and analytical methods

Media and stock solutions were kept in sterile plastic ware (polypropylene, Nalgene) for this work. Glassware used for incubations was first washed with a conventional detergent (Alconox, Fisher), followed by 24-h soaking in a 3% v/v solution of a commercial trace metal removal detergent (Citronox, Fisher) and nine rinses in deionized water. The growth of microorganisms was measured by following the $OD_{600\,nm}$ of cultures in 1-cm light path cuvettes. For dry weight determinations, cells were harvested by centrifugation at 1200 ***g*** for 10 min and washed twice with deionized water. Then, the cell mass was determined after drying at 100 °C for 24 h, with cooling in a vacuum dessicator containing a granular desiccant (Drierite, Xenia, OH) on preweighed aluminium dishes to a constant weight. The total cell counts were carried out using a 0.1-mm depth haemocytometer with improved Neubauer ruling (Brightline, Hausser Scientific, Horsham, PA). Trace iron and other trace metal concentrations in the media before and after extraction were determined in quadruplicate by high-resolution magnetic-sector ICP-MS at the Environmental Chemistry & Technology and Wisconsin State Laboratory of Hygiene, University of Wisconsin-Madison.

Results

Medium iron extraction and effect of iron on growth

Table 1 shows the concentrations of iron and several other metals in the chemically defined medium prepared without any Fe addition before and after Fe extraction. Using an insoluble resin in a batch-contacting process, it was possible

Table 1. Concentration of iron and several other metals in the chemically defined medium (GPP) before and after Fe batch extraction with an FEC-1 chelating resin

	μg L^{-1} (μM)							
Metal	Ca	Co	Cu	Fe	Ni	Mn	Mg	Zn
Non extracted GPP	91.7	0.03	320.5 (5.1)	7.0 (0.125)	1.3	682.2	94 911	301.1
Extracted GPP	141.6	0.05	5.7 (0.09)	1.2 (0.021)	1.7	756.5	108 209	302.4

to reduce iron concentrations by $>80\%$ to $1.2\,\mu g\,L^{-1}$ ($0.021\,\mu M$) in the chemically defined medium used. The residual Fe content in the Fe-extracted medium was found to result in Fe-restricted growth for both *C. albicans* and *C. vini* with increased lag phases and lower specific growth rates as compared with cultivations with added iron (Fig. 1a and b, respectively). *Candida vini* appeared to be more affected by low Fe concentrations than *C. albicans*. Accordingly, the maximum growth yields (Y_{max}) determined after 44-h growth exhibited a stronger dose dependence for added iron in the case of *C. vini* (Fig. 2). At the lowest iron concentration tested ($0.02\,\mu M$), the maximum growth yield attained by *C. vini* was less than half the Y_{max} value obtained for *C. albicans*.

Influence of exogenous chelators

The comparison of the effects of several iron chelators including the clinically relevant desferrioxamine and deferiprone at relatively low concentrations ($0.25\,g\,L^{-1}$) showed that the growth of *C. albicans* was not inhibited by desferrioxamine in comparison with the control treatment with no added iron chelator (Fig. 3). Both lactoferrin and deferiprone provided a short-term inhibition of *C. albicans* growth that extended the lag phase for approximately 12 h, followed by growth at rates that were comparable to the control without an added chelator and the treatment with desferrioxamine. The growth of *C. albicans* was inhibited in the presence of $0.25\,g\,L^{-1}$ DIBI for 24 h and displayed very weak growth thereafter (Fig. 3a). After 4 days, the maximum specific growth yield in the presence of $0.25\,g\,L^{-1}$ of DIBI reached 4% of the Y_{max} obtained in the control culture. *Candida vini* responded differently to the presence of the same chelators (Fig. 3b). Both lactoferrin and DIBI provided complete inhibition over the 4-day incubation period. In contrast, desferrioxamine and deferiprone led to similar growth kinetics in *C. vini* as compared with the control with no added chelator (Fig. 3b). Compared with control incubations with no added chelator, a slight, but statistically not

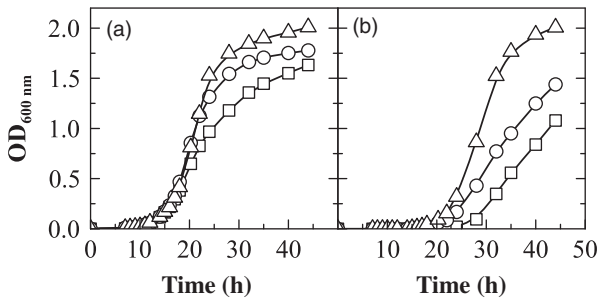

Fig. 1. Effect of iron addition to deferrated GPP medium on the growth of *Candida albicans* (a) and *Candida vini* (b). □, no added Fe (0.02 μM Fe residue); ○, 0.1 μM iron added; △, 0.5 μM iron added. The graphs show the typical results from three replicated experiments.

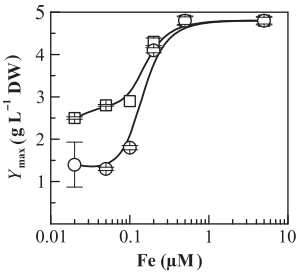

Fig. 2. Effect of addition of Fe to deferrated GPP medium (0.02 μM residual Fe) on the specific growth yield (Y_{max} at 44 h, hollow symbols) of *Candida albicans* and *Candida vini*. □, *C. albicans*; ○, *C. vini*. Data present the average results of three replicates ± SD.

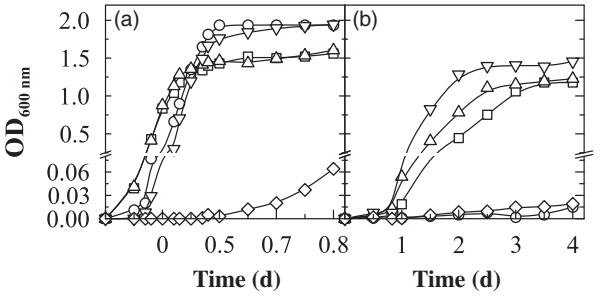

Fig. 3. Effect of $0.25\,g\,L^{-1}$ of lactoferrin, the clinically used chelators desferrioxamine and deferiprone and DIBI on the growth of *Candida albicans* (a) and *Candida vini* (b) in Fe-extracted chemically defined medium. □, control, no added chelator; △, desferrioxamine B; ▽, deferiprone; ○, lactoferrin; ◇, DIBI. Data show the average results of two replicated experiments.

significant increase ($P=0.05$) of the maximum specific growth yields could be observed for incubations with added deferiprone and lactoferrin (*C. albicans*) and deferiprone (*C. vini*).

Growth inhibition of the two yeasts by DIBI was investigated further at a lower chelator concentration ($0.17\,g\,L^{-1}$), over a longer incubation course (15 days) and in comparison with the well-characterized synthetic chelators EDTA and BPS (Fig. 4). Both EDTA and DIBI inhibited the growth of *C. albicans* leading to prolonged lag phases (3 days) and lower growth rates compared with the control, but the maximum specific growth yields observed after 15 days were comparable to those obtained for the control (Fig. 4a). BPS addition led to longer lag phases, lower growth rates and a Y_{max} that only reached approximately 30% of the control growth over the experimental period.

Candida vini displayed a similar inhibition response to BPS (Fig. 4b). However, the effect of DIBI on *C. vini* was stronger and led to a growth inhibition that was comparable to that of BPS until day 10. *Candida vini* also differed in its response to EDTA. Specifically, the lag phase was shorter (approximately 3 days) and the growth kinetics were similar

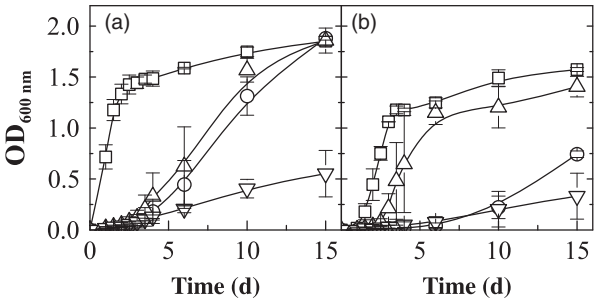

Fig. 4. Effect of low concentrations (0.17 g L^{-1}) of the iron chelators EDTA, BPS and DIBI on the growth of *Candida albicans* (a) and *Candida vini* (b) over a period of 15 days in Fe-extracted chemically defined medium: □, control with no added chelator; △, EDTA; ▽, BPS; ○, DIBI. Data present the average results of two replicates ± SD.

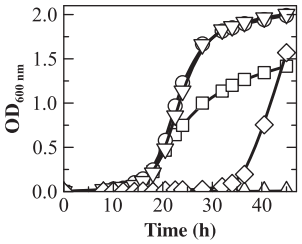

Fig. 5. Effect of the addition of the iron chelator DIBI and Fe on the growth of *Candida albicans* in Fe-extracted chemically defined medium (0.02 μM residual Fe). DIBI (adjusted to 0.25 g L^{-1}) and/or iron (adjusted to 300 μM Fe) were added at different cultivation times: □, control with no added iron or DIBI; ○, control with 300 μM iron added at $t = 0$; △, DIBI added at $t = 0$; ▽, DIBI and excess iron added at $t = 0$ h; ◇, DIBI added at $t = 0$ and excess iron added at $t = 20.5$ h. Data show the typical results of two replicated experiments.

to the control with regard to the growth rate and yield (Fig. 4b).

Nature of DIBI inhibition

The nature of the inhibition caused by DIBI was further investigated. The inhibitory activity of *C. albicans* could be characterized as being both fungistatic and Fe specific because it could be prevented or reversed by adding iron to levels sufficient to saturate the added DIBI iron-binding capacity (Fig. 5) by adding iron together with DIBI at the time of inoculation or adding Fe after 20.5 h, respectively.

Discussion

Candida albicans is prevalent in human vaginal infections, but is also the most common opportunistic pathogen associated with human immunodeficiency syndrome (Kullberg & Filler, 2002) as well as the third most common cause of nosocomial bloodstream infections (Walsh *et al.*, 2004). In contrast, *C. vini* is not known to be a human pathogen, but is frequently encountered as a film-forming spoilage yeast of various foods and beverages (Deak & Beuchat, 1996; Deak, 2004). This study investigated the effect of trace iron conditions on the growth of these two species, as well as their response to iron sequestration by chelators.

Metal analysis by ICP-MS revealed high residual concentrations (0.12 μM) of iron in the chemically defined medium used in spite of the absence of added iron and demonstrated the requirement for deferration and confirmatory trace Fe analysis. The iron contamination from other medium constituents could be successfully reduced to < 0.02 μM in batch processes using an insoluble chelating resin. Cu concentrations were also significantly reduced in the extracted chemically defined medium, but significant recovery of growth could be achieved by supplementation of the extracted medium with Fe only. Both *C. albicans* and *C. vini* were found to require approximately 0.5 μM added iron for complete unrestricted growth in the extracted chemically defined medium, but differed in their abilities to grow at reduced iron concentrations.

The observed differences between *C. albicans* and *C. vini* were consistent with the different environments these respective yeast species typically colonize or invade. Grape musts and wines, in which *C. vini* typically appears as a spoilage yeast, generally have high Fe concentrations of between 30 and 200 μM (Ough *et al.*, 1982). The predominantly reducing environment and the low pH of grape musts and wines also favour the formation of the more soluble free ferrous species, and this Fe would be expected to have a higher bioavailability (Howard, 1999). In sharp contrast, the ecological niches that *C. albicans* can colonize or invade in relation to human pathogenesis are highly limiting for Fe (Weinberg, 1999).

Desferrioxamine and deferiprone are two chelators used clinically to relieve the Fe overload associated with certain human haematological disorders such as thalassaemia (Chaston & Richardson, 2003; Franchini, 2006). Desferrioxamine failed to inhibit both *C. albicans* and *C. vini*. Deferiprone did not inhibit *C. vini* while leading to a slightly increased lag phase in *C. albicans*. However, the observed differences between *C. albicans* and *C. vini* persisted in their growth response in the presence of lactoferrin. Lactoferrin is a major component of the mammalian innate immune system (Actor *et al.*, 2009) and one of the vertebrate host defence Fe chelators, which is present in mucosal secretions (Gonzalez-Chavez *et al.*, 2009). Lactoferrin, at the physiologically relevant concentration of 0.25 mg mL^{-1} and at pH 4.5, and thus, representative of the vaginal environment (Novak *et al.*, 2007), only led to a transient inhibition of *C. albicans*, but inhibited the growth of *C. vini* over the incubation period. The results are in agreement with the lack of observed pathogenicity of *C. vini* and its greater susceptibility to iron restriction, while the pathogenicity of

C. albicans is consistent with its resistance to lactoferrin. In contrast to lactoferrin, desferrioxamine and deferiprone, DIBI provided almost complete inhibition of the growth of both *C. albicans* and *C. vini* over a 4-day incubation period. *Candida albicans* has been reported to use iron from the ferriproteins haemin, haemoglobin and myoglobin (Han, 2005), and to acquire iron from transferrin (Knight *et al.*, 2005). However, the slight increase of the maximum specific growth yields observed in the presence of some chelators in this study was not significant enough to support chelator-assisted iron acquisition.

In a long-term study with reduced, subinhibitory concentrations ($0.17\,g\,L^{-1}$), DIBI did allow delayed and gradual growth of both yeasts, which was comparable to inhibition by EDTA for *C. albicans* and to BPS in *C. vini*. In contrast to EDTA and BPS, which are known to readily chelate other transition metals (Ueno *et al.*, 1992), DIBI was shown to be iron-selective and its inhibitory activity was shown to be Fe reversible. Accordingly, DIBI appeared to be a more potent iron scavenger than any of the other clinically relevant chelators examined.

This work presents the first evidence of the iron requirements of *C. vini*, a nonpathogenic food spoilage organism, and the inhibition of *C. vini* and the opportunistic pathogen *C. albicans* by several strong chelators. The differences observed with respect to the ability of *C. vini* and *C. albicans* to grow under iron-restricted conditions were consistent with the respective environmental niches and pathogenicity. The present work provides a foundation for future studies that may investigate the possible synergistic effects of iron withdrawal in combination with antifungal preservative addition.

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