High environmental iron concentrations stimulate adhesion and invasive growth of *Schizosaccharomyces pombe*

Martin Převorovský, Jana Staňurová, František Púta & Petr Folk

Department of Cell Biology, Faculty of Science, Charles University in Prague, Prague, Czech Republic

**Abstract**

We have found that a high iron concentration in solid complete cultivation medium potentiates cell–cell and cell–surface adhesion of the fission yeast *Schizosaccharomyces pombe*. Spotted giant colonies grown on iron-rich media were found to be more compact and more resistant to washing than those grown on plates with a standard iron content. Furthermore, we have documented that excess environmental iron stimulates the invasive growth of *S. pombe* (and *Saccharomyces cerevisiae*). Three-dimensional, branched, washing-resistant structures composed mostly of elongated, but separate fission yeast cells, were formed within the solid agar medium. The degree of both adhesion and invasion displayed a specific, iron concentration-dependent response. Our results suggest a novel link between iron availability and the intensively studied and important fungal virulence factors, adhesion and invasion.

**Introduction**

Iron is an essential element required for numerous redox processes in virtually all organisms. The availability of iron in the environment is a limiting factor for both intra- and extracellular pathogens. As a result, their hosts have evolved ways to sequester iron from them, and many pathogenic microorganisms possess elaborate uptake systems to cope with this iron scarcity (for a review, see Sutak et al., 2008). Perturbations of the iron homeostasis can exacerbate, for example, the severity of *Cryptococcus neoformans* infections, where iron availability was shown to regulate the major virulence traits of this organism (capsule formation, melanin production, growth at 37 °C; Jung et al., 2006).

The ability of cells to adhere to various substrates, such as, for example, the host extracellular matrix, is considered a critical factor for many unicellular pathogenic organisms to establish infection. A number of adhesive proteins have been identified in several fungal pathogens, including *Candida albicans* and *Aspergillus fumigatus*, and their expression has been shown to represent an important virulence trait (for a review, see Mendes-Giannini et al., 2005).

Another such virulence factor is the cell’s ability to invade host tissues. Several pathogenic fungal species are able to switch between yeast and invasive hyphal forms of growth, and infections by strains in which this switching is perturbed display significantly lower mortality rates (Saville et al., 2003; Nemecek et al., 2006).

In this article, we analyzed the relationship between the above-mentioned virulence-associated phenomena using a nonpathogenic model organism, the fission yeast *Schizosaccharomyces pombe*.

**Materials and methods**

The fission and budding yeast strains used in this study are listed in Table 1. Cells were subjected to adhesivity/invasivity assays based on Guldal & Broach (2006). Exponentially growing liquid cultures were harvested by centrifugation, washed once in sterile deionized water and the cell suspension (1 x 10⁵ cells in 5 µL) was plated on various solid media. For most experiments, the YES complex medium (0.5% yeast extract, 3% glucose) with SP supplements (a standard mixture of adenine, leucine, uracil, histidine and lysine; Formedium) solidified with 2% agar was used as a basis. FeCl₃ (1–4 mM), CuSO₄ (0.5–2 mM), ZnSO₄ (0.25–1 mM) or the iron chelator ferrozine (0.5–1 mM) were further added to YES where indicated. The formation...
of hyphae on the low-nitrogen LNB medium (See Amoah-Buahin et al., 2005) and the effect of 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP) on adhesion/invasion were tested as described (Amoah-Buahin et al., 2005). The plates were then incubated at 30 °C for up to 19 days in a wet chamber, and sample plates were subjected to adhesivity/invasivity assays as required.

The adhesivity assay consisted of washing the plates evenly with a stream of water for 1 min. The spot cell mass remaining attached to the plate was then documented by photography, and the plate was subjected to the invasivity assay, in which the residual cells were washed off the surface completely by rubbing the plate carefully under running water. Under these conditions, only cells residing deep within the agar resist the washing. The spot location was photographed again to score for macroscopically visible invasive growth, and finally the invasive structures formed were investigated using an Olympus CK2 light microscope with an Olympus SP-350 digital camera attached to it.

Results

While conducting adhesivity tests with some of our adhesion-deficient fission yeast mutant strains, we noticed that the wild-type control cells displayed markedly increased adhesion when cultured on a solid rich medium to which millimolar FeCl₂ was added. Furthermore, the cells seemed to have grown deep into the agar, a behavior that is normally not observed with S. pombe. As this could have had implications for a number of important pathogenic microorganisms, we decided to examine these observations further. We also wished to compare our findings with a recent work describing the ability of S. pombe to form invasive filamentous structures within the agar under certain cultivation conditions (Amoah-Buahin et al., 2005).

Fission yeast colonies were grown in the presence of various concentrations of iron and their adhesive and invasive properties were analyzed using the washing assays described under Materials and methods. Representative spots are shown in Fig. 1. There was a gradual increase in the adherent cells’ mass from nearly none, on plates to which no extra iron was added, up to almost the entire colony, which resisted washing on plates containing 4 mM FeCl₂ (Fig. 1b, wild-type strains PN559 and FY435). In order to

Table 1. Yeast strains used in this study

<table>
<thead>
<tr>
<th>Designation</th>
<th>Species</th>
<th>Genotype</th>
<th>Source</th>
</tr>
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<tr>
<td>PN559</td>
<td>S. pombe</td>
<td>h⁻ leu1-32 ura4-A10 ade6-M216</td>
<td>Decottignies et al. (2003)</td>
</tr>
<tr>
<td>FY435</td>
<td>S. pombe</td>
<td>h⁺ leu1-32 ura4-A10 ade6-M210 his7-366</td>
<td>Pelletier et al. (2002)</td>
</tr>
<tr>
<td>Δfep1</td>
<td>S. pombe</td>
<td>h⁻ leu1-32 ura4-A10 ade6-M210 his7-366 fep1Δ:ura4⁻</td>
<td>Pelletier et al. (2002)</td>
</tr>
<tr>
<td>L972</td>
<td>S. pombe</td>
<td>h⁺</td>
<td>Grallert et al. (1999)</td>
</tr>
<tr>
<td>EGY48</td>
<td>S. cerevisiae</td>
<td>MATα ura3 his3 trp1 LexAop/C26-L6C2-LEU2</td>
<td>Clontech</td>
</tr>
</tbody>
</table>

![Fig. 1. Effect of iron on the adhesion and invasiveness of yeast. (a) Fission yeast (Schizosaccharomyces pombe (S. p.)) and budding yeast (Saccharomyces cerevisiae (S. c.)) cells of the indicated strains were spotted on YES agar plates to which either the iron chelator ferrozine or FeCl₂ was added to the final concentrations indicated. After 7 days at 30 °C, the spots were washed with a stream of water to score for adhesion (b), and then rubbed carefully to test for invasive growth (c). The top left spot is about 1 cm in diameter; all spots were photographed at the same scale.](image-url)
assess the specificity of the observed phenomenon, we also assayed cells grown on plates containing the iron chelator ferrozine (Fig. 1b, left column). As expected, there was a notable decrease in adhesion when iron was scarce. Furthermore, no increase in adhesion was detected when either copper (up to 2 mM) or zinc (up to 1 mM) was supplemented to the growth media instead of iron (data not shown). In *S. pombe*, the uptake of iron is negatively regulated by the Fep1 transcription factor and Δfep1 cells are more sensitive to iron (Pelletier *et al*., 2002). Strikingly, we found that the Δfep1 strain displayed decreased adhesion as compared with its parental strain FY435 (Fig. 1b). The Δfep1 growth was compromised on media with high iron (or copper; data not shown) content; however, the decrease in adhesion was evident even on the standard YES plates. Thus, the Δfep1 mutant strain somewhat resembles wild-type cells treated with ferrozine. We conclude from these results that both cell–surface and cell–cell adhesion is specifically potentiated by the presence of a high iron concentration in the environment.

A similar iron-dose dependency was documented for both the number and the size of invasive structures formed within the agar (Fig. 1c). On plates with 2 mM FeCl₂, invasive bodies could be observed microscopically as soon as on day 7 postplating. Invasive growth occurred mostly at the periphery of the spot, forming a ring-like pattern. Under a microscope, the invasive bodies appeared as elaborately branched three-dimensional structures, composed of rather elongated, but seemingly separate, fission yeast cells (Fig. 2a) that grew in all directions within the solid agar medium. We classify these structures as invasive pseudomyelia. Again, this effect was specific for iron as no invasion was triggered by zinc and only a small number of miniature invasion bodies was formed in the presence of 1 mM copper after 14 days postplating. Invasion was also severely impaired in the Δfep1 strain (Fig. 1c and data not shown).

We next tested whether iron influences adhesion or invasion in other yeast species as well. We subjected EGY48, a widely used budding yeast strain, to the same series of treatments as described above for *S. pombe*. The adhesion of EGY48 on YES medium was found to be rather low and we have not observed any increase under any of the conditions used. Interestingly, the EGY48 cells displayed a low degree of invasion even on YES plates. Importantly, there was a marked potentiation in both the number and the size of invasion bodies formed when FeCl₂ was supplemented to the medium (Fig. 1b and c). Branched structures were formed by EGY48 cells deep within the agar (Fig. 2b); thus, the stimulatory effect of iron on invasion is not confined to *S. pombe*.

It has been recently described for *S. pombe* that under the conditions of low nitrogen and abundant carbon source at 30 °C, the cells can form invasive mycelia consisting of hyphae (Amoah-Buahin *et al*., 2005). The invasion was found to be potentiated by the addition of 8-Br-cAMP, a cell-permeable analog of the nutrient-sensing second messenger, to the cultivation medium. We wanted to compare this phenomenon with the iron-stimulated pseudohyphal invasive growth observed in this study. First, we plated the *S. pombe* L972 strain, which does not require any nitrogen-containing supplements, on either YES plates supplemented with 0, 2 or 4 mM FeCl₂ or the low-nitrogen LNB plates, and cultivated it for 14 days at 30 °C. Both adhesion and invasion were stimulated on iron-enriched YES plates and the invasive bodies formed were indistinguishable from the other fission yeast strains we have used so far (data not shown). However, we did not observe any invasion on the LNB plates. Therefore, we decided to test directly the effect of 8-Br-cAMP instead. YES plates supplemented with 2 or 4 mM FeCl₂ were prepared, filter disks soaked with 40 μL of
either water or 0.1 M 8-Br-cAMP were placed in the center of the plates and the PN559, FY435 and Δfep1 fission yeast strains were spotted on the plates 1 cm away from the disk. Adhesion and invasion was scored after 7 and 14 days. We found no stimulatory effect of 8-Br-cAMP on either adhesion or invasion. However, the presence of the cAMP analog had a profound impact on the morphology of the invading structures (Fig. 3). Under low magnification, the invasive bodies formed on 8-Br-cAMP plates were similar to those induced by iron alone; however, when observed under a higher magnification, these structures consisted of long branched hyphae, very similar in appearance to the mycelia reported by Amoah-Buahin et al. (2005). Thus, in our hands, iron seems to be the chief regulatory stimulus for invasion, with cAMP modulating the morphology of the invading structures.

**Discussion**

We have presented evidence that high iron concentrations in the cultivation media stimulate the adhesion and invasion of fission yeast cells. This effect seems to be specific for iron for several reasons. First, other metal ions (redox-active copper and redox-inactive zinc) influence adhesion/invasion either negligibly or not at all. Second, when iron is depleted from the medium by the ferrozine chelator, adhesion is lowered. Third, S. pombe cells lacking the key iron-uptake regulator Fep1 (Pelletier et al., 2002) also show lowered adhesion and invasion. The iron-uptake systems are upregulated in these cells and the intracellular iron concentration is actually higher than in the wild-type controls. However, the Δfep1 transcriptome was found to resemble closely that of cells treated with ferrozine, and in terms of gene expression, Δfep1 cells may be considered constitutively iron starved (Rustici et al., 2007). Possibly, Δfep1 cells are unable to properly sense intracellular iron and this signaling defect may be responsible for the decreased adhesion/invasion we observed.

Recently, invasive filamentous (mycelial) growth has been described for fission yeast. Under the conditions of low nitrogen and high glucose as a carbon source (LN5 medium), S. pombe can start to grow as long and branched invasive hyphae (Amoah-Buahin et al., 2005). The mycelia also form mostly at the colony periphery (a ring-like pattern) and their three-dimensional structure is very similar to the iron-induced pseudomycelia we observed. However, these mycelia are formed by long, septated hyphae, and, significantly, their formation was shown to be inhibited by the addition of nitrogen-containing supplements (lysine, adenine and uracil) to the cultivation media (Amoah-Buahin et al., 2005). In contrast to this, we observed invasive

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**Fig. 3.** The influence of cAMP on the mode of invasive growth. *Schizosaccharomyces pombe* PN559 cells were grown on YES + 2 mM FeCl2 plates in the presence (cAMP) or absence (mock) of 8-Br-cAMP. After 14 days, the plates were subjected to the invasion assay and photographed. 8-Br-cAMP had no stimulatory effect on the degree of adhesion; however, most invasive bodies formed in its presence consisted of long branched hyphae. White and black bars represent 500 and 50 μm, respectively.
pseudomycelia formation on a rich medium with a standard nitrogen content and with the SP nutritional supplements added, the trigger being solely the abundant iron. Furthermore, we were able to induce the formation of long branched hyphae on high-iron complete medium by supplementing it with 8-Br-cAMP, which was previously shown to potentiate the invasive hyphal growth on low-nitrogen media (Amoah-Buahin et al., 2005). Under high-iron conditions, we did not observe any such potentiation, but instead found that 8-Br-cAMP had a strong effect on cell morphology. This finding suggests that signals elicited by iron overload and by cAMP affect distinct cellular effector systems, which then induce distinct changes in cell shape, growth and division. Nevertheless, high-iron-induced pseudohyphae seem to be fully sufficient for invasive growth.

Remarkably, the effect of iron seems not to be limited to the fission yeast. We found that iron also stimulates invasion in the distantly related yeast *Saccharomyces cerevisiae* and it is likely that studies in other species will yield similar results.

In summary, we have described in this study an unexpected link between the extracellular iron concentration and the fission yeast’s adhesive properties and its ability to invade solid media. We have shown that high iron strongly potentiates adhesion both to the agar surface and within the yeast colony. Furthermore, abundant environmental iron stimulates cells to invade solid substrates. *Schizosaccharomyces pombe* and *S. cerevisiae* are nonpathogenic species, but because adhesion and invasive growth are important virulence traits of numerous pathogenic microorganisms (Saville et al., 2003; Mendes-Giannini et al., 2005), we believe that our results set the stage for studying this novel role of iron in the models of infection as well.

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**References**


