Functional Genomics of Adhesion, Invasion, and Mycelial Formation in Schizosaccharomyces pombe

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Investigation into the switch between single-celled and filamentous forms of fungi may provide insights into cell polarity, differentiation, and fungal pathogenicity. At the molecular level, much of this investigation has fallen on two closely related budding yeasts, Candida albicans and Saccharomyces cerevisiae. Recently, the much more distant fission yeast Schizosaccharomyces pombe was shown to form invasive filaments after nitrogen limitation (E. Amoah-Buahin, N. Bone, and J. Armstrong, Eukaryot. Cell 4:1287–1297, 2005) and this genetically tractable organism provides an alternative system for the study of dimorphic growth. Here we describe a second mode of mycelial formation of S. pombe, on rich media. Screening of an S. pombe haploid deletion library identified 12 genes required for mycelial development which encode potential transcription factors, orthologues of S. cerevisiae Sec14p and Tlg2p, and the formin For3, among others. These were further grouped into two phenotypic classes representing different stages of the process. We show that galactose-dependent cell adhesion and actin assembly are both required for mycelial formation and mutants lacking a range of genes controlling cell polarity all produce mycelia but with radically altered morphology.

Many fungi are capable of growth either as single-celled yeasts or in a variety of multicellular and invasive forms. The ability to switch between these forms may be related to the ability to undertake different phases of infection (28). Thus, the study of this switching process may provide not only an understanding of a basic process of eukaryotic differentiation but also an insight into mechanisms of pathogenicity.

The fission yeast Schizosaccharomyces pombe is well-established as a model eukaryote to study the cell cycle and control of polarized growth. It had widely been considered to exist only in a single-celled form. However, we discovered that under conditions of nitrogen limitation, S. pombe can form elaborate branched multicellular structures which deeply invade the growth medium, for which the cyclic AMP (cAMP) signaling pathway is required (2). Subsequently, it was shown that, as in Saccharomyces cerevisiae, a putative ammonium transporter is also required, presumably to sense the nitrogen concentration (18). In contrast to budding yeasts (25), invasion appeared to occur only within a very narrow range of conditions (2).

Here we describe a second form of invasive growth by S. pombe which occurs on rich media. This allowed us to devise a simple screening of a collection of approximately 2,600 haploid deletion mutants for strains unable to invade the medium. This identified 12 genes associated with a range of functions, including transcriptional regulation, membrane trafficking, and control of actin assembly. Their roles in the invasion process could be further ordered into phases of adhesion to the surface and subsequent invasion of the medium. We investigated the role of specific sugars in this adhesion process and of the actin cytoskeleton in the formation of mycelia, whose normal development requires several proteins which regulate cell polarity.

MATERIALS AND METHODS

The strains used in this study are described in Table 1. The YES and LNB media used were described previously (2, 20). Latrunculin A (Sigma-Aldrich) was added to YES medium at the concentrations described from a 1 mM stock solution in dimethyl sulfoxide. Inoculation of strains at high cell density for induction of mycelial growth was done as described previously (2).

Preparation of mycelial colonies for high-magnification microscopy (63× objective) was done as described previously (2). Low-magnification microscopy (10× and 20× objectives) was performed by excising a small section of agar onto the top of a glass microscope slide and then imaging through the glass. Images were obtained with a Zeiss LSM510 microscope and further processed in Zeiss LSM Image Browser and Adobe Photoshop CS2 9.0.

A library of 2,638 haploid S. pombe strains, each with one gene deleted as described elsewhere (http://pombe.bioneer.co.kr/), was screened for lack of invasive growth (see Fig. 4). Candidate strains selected in the initial screening were further tested individually a minimum of four times. Only strains exhibiting a complete absence of invasion were included in the final table of results. Linkage of the invasion phenotype to the gene deletion was tested by random spore analysis (20) after backcrossing to strain 972. Haploid progeny were replicated from each cross, eight G418-resistant and eight G418-sensitive colonies were tested for complete absence of invasion were included in the final table of results. Linkage of the invasion phenotype to the gene deletion was tested by random spore analysis (20) after backcrossing to strain 972. Haploid progeny were replicated onto YES medium supplemented with 300 mg/liter G418 (Invitrogen). From each cross, eight G418-resistant and eight G418-sensitive colonies were tested for invasion on YES medium. One strain (Δaur2) was found to be sterile. Therefore, the heterozygous diploid, from which this strain was originally produced, was obtained from Bioneer and restored to meiotic competence by transformation with plasmid PON177 (26, 35). The resulting diploid was induced for meiosis, and linkage to G418 resistance and the invasion phenotype was tested as described above.

Small-scale DNA isolation from S. pombe cells was done as described previously (20). PCR amplification was performed with the Taq polymerase, buffer, and protocol supplied by Invitrogen. Oligonucleotides were 21 nucleotides in
TABLE 1. Strains used in this study

| Strain   | Genotype                                | Source           |
|----------|-----------------------------------------|------------------|
| 972      | h<sup>-</sup> wild type                | Our stock        |
| JZ633    | h<sup>+</sup> pka1::ura4<sup>+</sup> ade6<sup>-</sup>-m216 leu1<sup>-</sup>-32 ura4-D18 | A. Carr          |
| No. 10   | h<sup>+</sup> cyr1::leu2<sup>+</sup> leu1<sup>-</sup>-32 ura4-D18 | A. Carr          |
| SLP47    | h<sup>+</sup> git11::kan<sup>+</sup> ura4::fbp1-lacZ leu1<sup>-</sup>-32 his<sup>+</sup>-366 | C. Hoffman       |
| RWP29    | h<sup>+</sup> git3::kan<sup>+</sup> ura4::fbp1-lacZ leu1<sup>-</sup>-32 his<sup>+</sup>-366 | C. Hoffman       |
| CHP460   | h<sup>+</sup> gpa2::ura4<sup>+</sup> ura4::fbp1-lacZ leu1<sup>-</sup>-32 his<sup>+</sup>-366 | C. Hoffman       |
| CHP463   | h<sup>+</sup> git5-1::his7<sup>-</sup>-366 ura4::fbp1-lacZ leu1<sup>-</sup>-32 his<sup>+</sup>-366 | C. Hoffman       |

length and were supplied by MWG-Biotech AG (Ebersberg, Germany). DNA sequencing was performed by Cogenics (Takeley, Essex, United Kingdom).

RESULTS

Mycelial formation on rich media by *S. pombe*. Previously we described invasive growth by common laboratory strain 972 of *S. pombe* under a narrow range of conditions involving nitrogen starvation (2). In the course of investigating genetic factors involved in this phenomenon, we observed invasion by some strains on rich (YES) medium. These included an older stock of strain 972 (Fig. 1A). Further tests revealed that the strain used previously had acquired a single recessive mutation, in a gene which remains to be identified, preventing invasion on rich media (data not shown). Strains lacking this mutation were also found to invade nitrogen-limited medium (LNB) more rapidly (Fig. 1B) and over a wider temperature range (Fig. 1C). The mutation was not evident in any of a range of strains obtained from other sources (data not shown). Therefore, wild-type *S. pombe* can invade the medium and form mycelia under quite a wide range of growth conditions.

Mycelia formed more slowly and less efficiently on rich YES medium than on LNB medium. However, after prolonged incubation, characteristic elaborate and branched structures appeared (Fig. 2A), composed of long chains of separated cells (Fig. 2B). At early stages of mycelial growth, elongated filaments were seen to emerge from the invasion foci (Fig. 2C). On YES medium, a second type of invasive structure was also observed, namely, lenticular colonies below the surface of the agar but composed of single cells packed closely together (Fig. 2D). These structures could result from a short period of invasive growth followed by reversion to the conventional yeast growth pattern.

The genetic requirements for invasion of YES medium were investigated. To compare with mycelial development on LNB medium, strains with deletions of genes for the activating components of the cAMP-Pka1 pathway (Δcyr1<sup>+</sup>, Δpka1<sup>+</sup>, Δgit11<sup>+</sup>, Δgit3<sup>+</sup>, Δgpa2<sup>+</sup>, and Δgit5<sup>+</sup>) were examined. The Δpka1<sup>+</sup> strain formed invasive structures on YES medium (Fig. 3B). However, mycelial growth of this strain was slower than that of the wild type (Fig. 3A) and its morphology was distinct, comprising invasive filaments composed of elongated cells with no detectable branching (Fig. 3C). The strains with deletions in each of the other five genes in this group behaved similarly (data not shown).

Screening of an *S. pombe* deletion library for genes required for invasion. A collection of *S. pombe* strains with deletions of individual genes (http://pombe.bioneer.co.kr/) was made in a genetic background including conventional adenine, leucine, and uracil auxotrophies. Addition of any of these supplements to the medium represents a significant nitrogen source and therefore alleviates the nitrogen limitation required for inva-

FIG. 1. Deficient mycelial growth in a laboratory strain of *S. pombe* 972. (A) A standard strain of 972 (left) formed invasive structures after incubation at 30°C for 14 days on rich YES medium, but the laboratory strain (right) did not. (B) Standard 972 (left) formed invasive structures on nitrogen-limited LNB medium more rapidly and vigorously than did the laboratory strain (right) after 7 days at 30°C. (C) Standard 972 (left panel) formed invasive structures on LNB after incubation for 14 days at a range of temperatures, while the laboratory strain (right panel) did so only at 30°C. All colonies are shown before (above) and after (below) vigorous washing to reveal cells that invaded the medium.
sive growth on LNB medium (2). The discovery of invasion on rich medium allowed us to develop a relatively simple screening of the library for lack of invasive growth (Fig. 4). A total of 2,638 strains were screened. Candidate strains from the initial screening were further individually tested a minimum of four times. Twelve strains were thereby identified as unable to invade (Table 2). Eleven of these were backcrossed to the wild type, and random spores were tested for linkage between the invasion phenotype and G418 resistance, the marker for the deleted gene. The 12th, the \texttt{ral2}/H9004 mutant, was sterile. Therefore, the heterozygous diploid strain originally made by Bioneer was sporulated and haploid progeny were tested for linkage between G418 resistance and the invasion defect. These tests showed complete coincidence between the gene deletion and the lack of invasive growth for all 12 genes.

These 12 genes are implicated in a range of cellular processes, from transcription to cytoskeletal regulation and membrane trafficking. One of them, \texttt{spo20}, is the orthologue of \texttt{SEC14} in \textit{S. cerevisiae} and has been reported to be essential (21). Therefore, the identity and extent of the gene deletion in each strain were checked by amplification of sequences on either side of the integrated \texttt{kanMX4} cassette. This showed that 11 of the strains had precise gene deletions, as expected. However, in the \texttt{spo20} mutant strain, the cassette was found to have been integrated 474 bp downstream of the translation start site, leaving 143 of the 286 codons intact. Presumably, the resulting protein retains sufficient function for viability but not for invasive growth.

We observed that, following a short incubation but before invasion had clearly occurred, cells on the surface of the medium became resistant to removal by gentle washing. This process of surface adhesion presumably represents the first stage of mycelial formation, before invasion of the medium. The 12 mutant strains were then tested in this way for adhesion. This showed that five of the strains failed to adhere, while the remaining seven produced adherent cells but failed to invade (Fig. 5 and Table 2).

Inhibition of cell-to-surface adhesion by galactose. Genes SPBC1289.10c and SPCC1494.10, both required for adhesion, encode similar proteins which include LUFS domains (http://www.genedb.org/genedb/pombe/), found also in Flo8p of \textit{S. cerevisiae} and its \textit{Candida albicans} orthologue (4, 15). These are each required for filamentous growth, and Flo8p is also required for flocculation. This raised the possibility that the processes of flocculation and adhesion might be connected in \textit{S. pombe}. Flocculation has not been intensively investigated in \textit{S. pombe}, but it is known that surface galactose, which is not produced by \textit{S. cerevisiae}, is involved (16, 31). We therefore

![FIG. 2. Morphology of invasive structures formed on rich YES medium. (A) After 11 weeks of incubation, threadlike filaments form large and complex structures (10× objective; scale bar, 100 μm). (B) High magnification shows that the filaments are composed of chains of cells (63× objective; scale bar, 10 μm). (C) After 3 days of incubation, individual foci produce short filaments (arrow) that are elongated and bent (20× objective; scale bar, 10 μm). (D) After 7 days of incubation on YES medium, numerous lenticular colonies embedded in the agar are observed that are composed of nonfilamentous cells (2.5× objective; scale bar, 100 μm).](image)
investigated the effects of various sugars on the process of adhesion to the surface.

Addition of 1 M fructose, glucose, or mannose to the medium had no effect on adhesion or invasion. In contrast, 1 M galactose completely blocked adhesion (Fig. 6). This suggests that flocculation and adhesion may both require recognition of surface galactose (but see the Discussion).

Role of the \textit{S. pombe} cytoskeleton in invasion and mycelial development. The strain with a deletion of \textit{for3} was found to be noninvasive, although still exhibiting normal adhesion (Table 2 and Fig. 5). \textit{For3} is a formin implicated in F-actin polymerization and in particular the formation of actin cables at the cell tips under the control of \textit{Tea1} and \textit{Tea4} (7, 17). This led us to examine the role of the cytoskeleton in \textit{S. pombe} invasion and mycelial development.

First, the effect of latrunculin A, which destabilizes actin filaments, was investigated. Addition of 1 \textmu M latrunculin A completely blocked invasion, although adhesion was still observed (Fig. 7), indicating a role for actin cables in invasion. To investigate the role of the interphase microtubule cytoskeleton in invasion and mycelial development, strains with deletions of genes involved in interphase microtubule organization or en-
coding microtubule-based cortical markers and effectors (Δtea2, Δmal3, Δtip1, Δtea1, Δtea4) (5) were selected from the library and examined. Under conditions that induce invasion, all of these strains formed a substantial number of invasive structures but exhibited only a very small number of morphologically distorted filaments that were frequently truncated, swollen, and twisted (Fig. 8). These define a third phenotypic group of strains that adhere to and invade the medium but are defective in mycelial formation, suggesting that although it is not required for invasion, regulation of microtubule-dependent polarized growth is required for the formation of normal mycelia.

**DISCUSSION**

The ability to switch between yeast and mycelial growth forms is widespread among fungi. Previously we described this process in *S. pombe* as a response to nitrogen limitation in the presence of abundant carbon. We described the resulting structures as hyphae (2). However, this term is generally defined as involving parallel-sided growth (24), which is a useful distinction for budding yeasts but not for *S. pombe*, whose single cells grow in a cylindrical shape. Therefore, we have adopted the more general term of mycelia to describe these structures.

Here we have shown that, as for other fungi, invasive growth and mycelial formation can occur under different conditions. What are the signals which induce these responses? The features common to both conditions are a good carbon source and high cell density. On rich medium, the latter could produce local deficiencies in nutrients such as nitrogen, but if so, the cAMP signaling pathway (Fig. 3) is not required. Possibly, a quorum-sensing mechanism, as implicated in other yeasts (33), could be involved, but as yet we have found no evidence for this, so the means of detecting high cell density is still not clear.

The results reported here also clarify the surprisingly narrow range of conditions under which mycelial growth was previously observed (2). The earlier work was, ironically, carried out with a strain deficient in invasion (Fig. 1). This trait segregates as a single Mendelian factor, but we have not yet identified the gene involved. Thus, *S. pombe* is clearly quite competent at invasive mycelial growth, despite the long history of a general assumption that it is not. There is a very early report of mycelial formation (“mycelialen Gestalten”) (13), but since this predates the development of solid media, is not illustrated, and was not carried out with the 972 strain commonly used today, it is difficult to determine its relevance to the phenomenon described here.

The observation of mycelial growth on rich medium allowed us to use a simple screening of a library of 2,600 strains with deletions of individual genes for failure to invade, based on the resistance to washing of cells that had invaded the medium. This identified 12 genes required for the process. This does not represent a complete list of genes involved in mycelial growth for at least four reasons. First, essential genes are not expected to be represented in the library. Second, the collection represents only approximately 70% of the nonessential genes (6). Third, some functions may well be redundant. Fourth, many genes may be involved in normal formation of mycelia but not required for invasion, as described below. Nevertheless, it represents a significant step in describing the genetic basis of mycelial growth. More detailed examination of the phenotypes allowed us to dissect the process into stages of adhesion and invasion.

![FIG. 5. Distinction between adhesive and nonadhesive phenotypes of noninvasive strains. Strains were plated at high cell density on YES medium, incubated at 30°C for 14 days, gently rinsed, and then vigorously washed. Gentle rinsing (center) removed all of the cells of the strain with a deletion of *snf5*, but some surface cells of the strain with a deletion of *for3* and wild-type 972 remained attached to the agar. Vigorous washing (right) removed all of the remaining surface cells, revealing invasive structures formed by 972 but none formed by the mutant strains.](image-url)
invasion, with five mutants blocked at the first stage and seven at the second.

Of the five genes implicated in adhesion, SPBC1289.10c and SPCC1494.10 encode related proteins predicted to be transcription factors containing LUFS domains (http://www.genedb.org/genedb/pombe/). These are found in Flo8p of S. cerevisiae, which is required for flocculation and invasive growth, and its orthologue in C. albicans (4, 15). SPBC30B4.03c may also be involved in transcription, as its product has weak homology to the SEUSS protein of Arabidopsis thaliana, a transcription factor involved in plant development (34). This protein contains a domain which may interact with LUFS domains (3). However, this part is not present in the S. pombe protein, suggesting that any interaction with the SPBC1289.10c and SPCC1494.10 products would be indirect.

Nevertheless, the suggestion of a link to flocculation led us to investigate this possibility and to find that adhesion is specifically blocked by high concentrations of galactose, which also block flocculation in S. pombe (16, 31). This suggests a role for galactose-binding adhesin proteins. Although none of these emerged from the screening, this group of proteins may have redundant functions (14). Thus, cell-cell interactions may be required for surface adhesion, although it should be noted that galactose is also a major constituent of agar (22). Surface adhesion had not previously been studied in S. pombe, but during the preparation of our manuscript, it was reported that

FIG. 6. Galactose-specific inhibition of cell-to-surface adhesion. Strain 972 was plated at high cell density on LNB medium with supplements as shown. After 7 days at 30°C, the colonies were first gently rinsed with water (middle panels) to show adherent cells and then washed vigorously (lower panels) to show invasive structures. Only the addition of 1 M galactose prevented adhesion to the surface.

FIG. 7. Effect of latrunculin (Lat) A on mycelial growth. Strain 972 was plated at high density on YES medium supplemented as shown and incubated at 30°C for 7 days. At all concentrations of latrunculin A, normal adhesion to the surface (middle panels) was observed. Vigorous washing (lower panels) showed no invasive structures with 1 μM latrunculin A and reduced invasion at lower concentrations.
FIG. 8. Strains with deletions of genes involved in microtubule organization and as microtubule-based cortical markers and effectors (Δtea1, Δtea2, Δtea4, Δtip1, and Δmal3) were invasive but showed aberrant filament formation. Only a few filaments were formed, and these frequently appeared twisted (arrow A), swollen (arrow B), and truncated (arrow C). Scale bar, 40 μm.
adhesion and invasion can be stimulated by iron (23), suggesting that mycelial growth may also be influenced by a range of environmental factors. An earlier report also described flocculation and invasion as consequences of loss of the kinase Lkh1 (11). This phenotype may have been an enhancement of the wild-type response described here and again emphasizes the potential range of conditions which may influence it.

Of the remaining two genes required for adhesion, tlg2 encodes a SNARE protein homologous to Tlg1p and Tlg2p of S. cerevisiae, involved in various steps of membrane trafficking in the endocytic and vacuolar system (1), while snf5 encodes a component of the SWI/SNF complex involved in chromatin remodeling (19).

The seven genes implicated in invasion after adhesion have a diverse range of proposed functions. A study of filamentous growth in S. cerevisiae identified 487 genes by either disruption or overexpression (10). The only gene of those identified here which has a clear ortholog in this extensive collection is SPBC11B10.07c, which corresponds to YNR045W. These encode membrane proteins with cdc50 domains, but functional information on either is otherwise lacking.

The remaining six genes include arp42, whose product is part of both the RSC and SWI/SNF complexes (19). ral2 has been implicated in control of cell morphology (8). spo20 is the ortholog of sec14 from S. cerevisiae, which encodes a phosphatidylinositol transfer protein required for trafficking through the secretory pathway (27). Some spo20 mutants are defective in sporulation, but complete deletion is lethal (21). However, the strain identified here fortuitously retains part of the gene. By analogy to studies with S. cerevisiae, the resulting N-terminal portion should be sufficient to target to the Golgi complex (32) but should be unable to bind phosphatidylinositol (30), suggesting that lipid transfer is required for invasive growth, while the N-terminal portion is competent for an as-yet-unknown essential function. ubr1 encodes a ubiquitin ligase (12), and sgf73 is a component of the SAGA complex (9).

Finally, for3 encodes a formin implicated in the organization of actin filaments and establishment of cell polarity (29). We found that disruption of actin assembly with latrunculin A also blocked invasion but not adhesion (Fig. 7). This led us to investigate other proteins involved in cytoskeletal interactions and control of cell polarity whose genes were represented in the library. Although none of these strains were identified in the initial screening, several showed distinct disruption of normal mycelial formation (Fig. 8). Thus, a more detailed morphological analysis of mycelia formed by all of the strains in the collection might implicate a significant number of additional genes and identify further aspects of the process.

One characteristic of the list of genes in Table 2 is its absence. Numerous genes are clearly implicated in the same processes as the genes identified, but their deletion still allowed adhesion and invasion. In some cases, there appears to be a related phenotype; for example, deletion of gen5, which encodes another subunit of the SAGA complex (9), caused a greatly reduced extent of invasion (data not shown), indicating again that a detailed morphological screening should reveal many more genes involved in mycelial formation. Another possible interpretation of these absences from the screening is that a particular subset of proteins has specific functions in mycelial growth in addition to those already known. Now that the first stage of genetic analysis has been accomplished with S. pombe, an organism very diverged from budding yeasts, it should be possible to investigate the process in more detail and provide a broader phylogenetic basis for the molecular description of these important phenomena for fungi in general.

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