Journal of Antimicrobial Chemotherapy (2005) **55**, 645–654
doi:10.1093/jac/dki088
Advance Access publication 8 April 2005

JAC

Exposure of *Candida albicans* to antifungal agents affects expression of *SAP2* and *SAP9* secreted proteinase genes

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Received 10 December 2004; returned 12 January 2005; revised 3 February 2005; accepted 7 February 2005

***Objectives*: To ascertain the effects of subinhibitory concentrations of several antifungal agents on a virulence factor: secreted proteinase (Sap) activity and expression of *SAP* genes in *Candida albicans*.**

***Methods*: Enzyme assays and growth measurements, GFP-SAP2 promoter constructs and fluorescence measurement, transcript profiling and RT–PCR.**

***Results*: For seven of eight *C. albicans* isolates tested, exposure to fluconazole gave an increase in Sap specific activity; for one isolate, resistant to azoles and flucytosine, fluconazole exposure led to a fall in Sap activity. A similar relationship between growth reduction and increased Sap activity was seen with *C. albicans* cells treated with subinhibitory concentrations of itraconazole, miconazole, flucytosine and caspofungin. Transcript profiling indicated antifungal exposure was associated with increased expression of mRNA from *SAP2* and *SAP9* genes; this was confirmed for fluconazole and caspofungin exposure by RT–PCR.**

***Conclusions*: Antifungal agents with three different mechanisms of action similarly generate a rise in expression of *SAP2* and activity of the secreted Sap2 gene product, a known virulence factor, in most isolates of *C. albicans*. One isolate of the fungus showed an opposite response.**

Keywords: fluconazole, flucytosine, caspofungin, itraconazole, amphotericin B, virulence genes, virulence

Introduction

Like many antimicrobial agents, antifungal agents have been shown to exert pharmacological effects beyond inhibition of fungal growth. Among many examples, fluconazole has been shown to inhibit adhesion of *Candida* species to acrylics[1] and buccal epithelial cells.[2] Amphotericin B and several azole antifungals have been shown to augment phagocytic effects of polymorphonuclear leucocytes[3–5] and macrophages,[6,7] and amphotericin B induces chemokine expression[8] and alters non-specific proliferative responses[9] of monocytes.

Some studies have suggested an effect of antifungal agents on expression of virulence factors by *Candida albicans*. Willis *et al.*[10] treated diabetic patients with nystatin or fluconazole and found that fluconazole treatment, but not nystatin treatment, appeared to reduce phospholipase production by *C. albicans* isolates colonizing and infecting the mouth. Lass-Florl *et al.*[11] demonstrated that sertraline, a serotonin reuptake inhibitor, activated monocyte-directed macrophages against *Candida* species and reduced levels of fungal phospholipase and proteinase activities. Wu *et al.*[12] showed that exposure of a fluconazole-susceptible *C. albicans* isolate to fluconazole at sub-MIC concentrations led to a reduction in the specific activity of secreted aspartyl proteinases (Saps), whereas the same treatment of a fluconazole-resistant isolate led to an increase in extracellular proteinase specific activity. Saps are the products of a family of 10 *SAP* genes; Sap2 is the enzyme expressed predominantly under proteinase-inducing conditions *in vitro*.[13] Ripeau *et al.*[14] showed induction of *SAP5* expression in cells treated with caspofungin.

As part of a wider ranging survey of possible links between antifungal exposure and expression of virulence factor genes in *C. albicans*, we have studied in detail the effects of antifungal agents representing four major classes of such agents on the expression of *SAP* genes and Sap activity, using a range of

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molecular biological approaches. We show that exposure to azoles, flucytosine and caspofungin all gave an increase in *SAP2* expression and in Sap specific activity under proteinase-inducing conditions.

Materials and methods

Fungal isolates

All wild-type *C. albicans* isolates tested were originally obtained from clinical sources. SC5314 and 88/025 were blood isolates; both are susceptible to amphotericin B, flucytosine, caspofungin and azoles. SC5314 is also the parent strain widely used for specific gene disruption studies in *C. albicans* and it was the progenitor of caspofungin-resistant mutant NR3.[15] J990578 and TW12-99 were oral isolates from patients with AIDS; both are resistant to azole antifungal agents; J990578 is additionally resistant to flucytosine. All isolates were maintained either on Sabouraud agar (Oxoid) or by longer-term storage at −80°C in 25% glycerol.

Strains derived from SC5314 and specifically disrupted in *SAP* genes 1 to 6[16,17] were transformed with plasmid CIp10[18] to ensure adequate expression of *URA3* at the *RPS1* locus.[19] This procedure created VC1 (*ura3*Δ::*imm434/ura3*Δ::*imm434/sap1*Δ::*hisG/sap1*Δ::*hisG/RPS1*::CIp10-*URA3*), VC2 (*ura3*Δ::*imm434/ura3*Δ::*imm434/sap2*Δ::*hisG/sap2*Δ::*hisG/RPS1*::CIp10-*URA3*), VC3 (*ura3*Δ::*imm434/ura3*Δ::*imm434/sap3*Δ::*hisG/sap3*Δ::*hisG/RPS1*::CIp10-*URA3*) and VC4-6 (*ura3*Δ::*imm434/ura3*Δ::*imm434/sap6*Δ*::hisG/sap6*Δ*::hisG sap4*Δ*::hisG/sap4*Δ*::hisG sap5*Δ*::hisG/sap5*Δ*::hisG/RPS1*::CIp10-*URA3*). Strains Δsap9 and Δsap10 were disrupted in *SAP9* and *SAP10*, respectively and had *URA3* reintegrated at the *RPS1* locus.[20] Detailed descriptions of these mutants will appear in a separate publication.

Construction of SAP2 promoter GFP fusion

To make the plasmid, pSAP2-GFP, the *CaSAP2* promoter region (−9 to −979) was PCR-amplified using the primers 5′-ACCTCGAGATCAAATGTGTTACC and 5′-TGAAGCTTGGTGATGTTTAGTGG (*Xho*I and *Hin*dIII sites underlined) and cloned between the *Xho*I and *Hin*dIII sites of pGFP.[21] The plasmid was linearized by digestion with *Bgl*II and used to transform *C. albicans* strain CAI-4. Single copy integrants were screened by PCR as previously described[20] and confirmed by Southern analysis.

Fluorescence measurements

An Axioplan 2 microscope (Carl Zeiss Ltd, UK) with filter sets XF 66, XF 67 and XF 77 (Omega Optical Inc., Brattleboro, VT, USA) was used for fluorescence microscopy. Images were generated with a Hamamatsu CCD camera, and analysed with Openlab 3.0.9 software (Improvision Ltd, Coventry, UK). Mean fluorescence intensities (±SD) were then calculated for at least 50 individual cells based on region of interest measurements.[21]

Antifungal agents

Flucytosine was purchased from Sigma. Amphotericin B was purchased as Fungizone. Other agents were gifts from Merck, Inc. (caspofungin), Pfizer UK (fluconazole) and Johnson & Johnson Pharmaceutical Research (itraconazole and miconazole). Caspofungin, fluconazole and flucytosine stock solutions were prepared in sterile distilled water. Itraconazole and miconazole were dissolved in DMSO. In all experiments, drug dilutions were prepared at 100 times final concentration in the appropriate solvent and added to 100 volumes of culture medium.

Growth under proteinase-inducing conditions

Conditions for induction of cultures expressing Sap activity were essentially the same as used by Wu *et al.*[12] Briefly, yeast carbon base/bovine serum albumin (YCB/BSA) medium containing 1.17% YCB (BD Biosciences), 1% glucose and 0.5% BSA (Sigma) was inoculated to $1-3\times10^5$ cells/mL from an overnight culture of *C. albicans* and incubated at 30°C on a rotary shaker or rotating wheel. Additions of antifungal agents or their solvents were made after 6 h of growth. Growth in the presence and absence of antifungal agents was measured as OD_{600} versus a YCB/BSA blank. Growth in the presence of antifungal agents was expressed as a percentage of control (drug-free) growth. For each isolate tested, preliminary susceptibility tests in YCB/BSA were used to determine subinhibitory concentrations of antifungal agents. MICs determined under these conditions were within one dilution of MICs determined by standard (CLSI) methodology. Because the dose–response curves for all agents tested except amphotericin B contained regions of partial growth inhibition, the results of experiments relating proteinase activity to antifungal treatments were expressed as scatterplots of relative growth (% of control) versus relative Sap specific activity (% of control). This approach reduced the impact of inter-run variability in the data. Data were excluded when growth was inhibited below 25% of control; proteinase activities in filtrates of such strongly inhibited cultures were irreproducibly low.

Proteinase assay

Proteinase-inducing cultures were set up in 5 mL volumes as described above. After 72 h of incubation, growth was measured by spectrophotometry at 600 nm. One millilitre volumes of cultures were centrifuged at 13 000 **g** for 1 min and the supernatant retained. The assay mixture comprised 0.5 mL of 1% BSA in 0.1 M citrate buffer pH 3.0 as substrate and 0.25 mL of culture supernatant. The mixture was incubated for 1 h in a 37°C water bath. The reaction was stopped with 1.25 mL of ice-cold 5% trichloroacetic acid (TCA). Precipitated material was removed by centrifugation and the OD_{280} of the supernatant was determined against a blank prepared by addition of TCA at the start of the incubation period.

RT–PCR

For measurement of the expression of *SAP2*, *SAP9* and *EFB1* by RT–PCR, cells were grown in proteinase-inducing medium in the presence or absence of antifungal agents for 72 h. *EFB1* contains an intron and was included as a control for genomic DNA contamination of cDNA. The concentrations of antifungal agents used were chosen to inhibit growth of the *C. albicans* strains tested to 50–80% of control values. RNA was extracted[22] and treated with DNase I (Invitrogen) to remove DNA. Each sample included 6 μg of RNA, 2 μL of DNase I buffer (Invitrogen), 1.5 μL of DNase I and 1.5 μL of RNase OUT (Invitrogen) made up to 20 μL with RNase-free water. The samples were incubated at room temperature for 15 min followed by the addition of 2 μL of 25 mM RNase-free EDTA (Invitrogen) and incubated at 65°C for 15 min.

cDNA was generated by addition of 2 μL of Oligo (dT)12–18 primer (500 μg/μL) (Promega) to the DNase I-treated RNA, incubated at 70°C for 10 min and snap-cooled on ice for 2 min to denature. First-strand cDNA was synthesized by addition of 8 μL of 5× First Strand Buffer, 4 μL of 0.1 M dithiothreitol (DTT), 2 μL

of 2.5 mM dNTP mixture (Invitrogen) and 2 μL of RNase OUT. The mixture was incubated at 42°C for 2 min followed by the addition of 2 μL of Superscript II reverse transcriptase (200 U/μL) (Invitrogen) and reincubation for 60 min at 42°C. The reaction was stopped by incubation at 70°C for 15 min.

Single and multiplex PCRs were done with 2× PCR Master Mix (ABgene). In single PCRs, 25 μL of 2× PCR master mix, 22 μL of water, 1 μL of SAP2-5 primer (CCTAAAGCATTCCCAGGTTAC) or SAP9-5 primer (GGTGCATATTCAACGTTCTCC), 1 μL of reverse primer SAP2-3 (CTTAGGTCAAGGCAGAAATACTG) or SAP9-3 (CTATGTCGACTGTTCTGCTG) and 1 μL of cDNA were added. In multiplex reactions of *EFB1/SAP2* or *EFB1/SAP9*, 25 μL of 2× PCR master mix, 21 μL of water, 1 μL of EFB1-5 (ATTGAACGAATTCTTGGCTGAC), 1 μL of EFB1-3 (CATCTTCTTCAACAGCAGCTTG), 1 μL of SAP2-5 or SAP9-5, 1 μL of SAP2-3 or SAP9-3 and 1 μL of cDNA were added. *SAP2* PCRs were carried out with an initial denaturing step of 94°C for 5 min, followed by 30 cycles of 1 min denaturation at 94°C, annealing for 1 min at 50°C and extension for 1 min at 72°C, with a final extension of 10 min at 72°C. *SAP9* PCR had an annealing temperature of 55°C for 1 min.

To determine gene expression in a semi-quantitative manner, we sampled every second PCR from cycle 16 to cycle 30 and estimated amounts of gene products relative to *EFB1*.

Transcript profiling with C. albicans microarrays

To measure expression of most *C. albicans* genes in cells of SC5314 grown for 72 h in proteinase-inducing conditions with and without addition of fluconazole at 2 mg/L, the cells were flash-frozen in liquid nitrogen and the RNA extracted as described above. The 72 h incubation time ensured maximum growth and proteinase induction in the medium.

From the extracted RNA, a pooled control was prepared containing equal concentrations of RNA from the cells not treated with fluconazole. cDNA was prepared from the RNA samples by reverse transcription. In brief, 2 μg of oligo (dT)12-18 primer (500 μg/μL) (Promega) was added to 50 μg of RNA and made up to 10 μL with RNase-free H_2O, incubated for 10 min at 70°C and snap-cooled on ice for 1 min. Individual lots of the pooled control sample were then labelled with the dye Cy3-dUTP by reverse transcription. To each control RNA sample, 15 μL of labelling master mix (1× RT buffer, 1 mM DTT, 500 mM dATP, 500 mM dCTP, 500 mM dGTP, 100 mM dTTP) was added followed by 3 μL of Cy3-dUTP (Amersham Biosciences). The triplicate experimental (control and fluconazole-treated) samples were individually labelled with Cy5-dUTP (Amersham Biosciences) by the same method. Following the addition of the label, 2 μL of Superscript II reverse transcriptase (Invitrogen) was added, mixed thoroughly and incubated at 42°C for 2 h. The reaction was stopped by the addition of 1.5 μL of 20 μM EDTA, followed by 1.5 μL of 500 mM NaOH and incubated for 10 min at 70°C to degrade the RNA. This reaction was neutralized by the addition of 1.5 μL of 500 mM HCl. Following reverse transcription, the samples were mixed (pooled control 1: fluconazole-treated 1; pooled control 2: fluconazole-treated 2; pooled control 3: fluconazole-treated 3) and purified in a GFX purification column (Amersham Biosciences) following the manufacturer's procedure, then concentrated in the Speed Vac to 20 μL.

Three independent hybridizations were carried out for each experimental condition against the pooled control (pooled control 1: fluconazole-treated 1; pooled control 2: fluconazole-treated 2; pooled control 3: fluconazole-treated 3). To control for intrinsic biological (experimental) variation, pooled controls labelled with Cy3 were also tested against individual Cy5-labelled control samples.

Eurogentec *C. albicans* microarray slides, representing 95% of the genome, were pre-hybridized in 5× SSC/1% SDS/1% BSA for 45 min at 42°C, washed five times in water, once in isopropanol and air-dried. The labelled samples were added to 20 μL of hybridization solution (50% formamide/10× SSC/0.2% SDS), boiled to denature for 3 min and carefully added to the printed part of the microarray slide and the lifter slip placed on the slide. The slides were incubated at 42°C overnight. Slides were washed at room temperature in 2× SSC/1% SDS for 15 min, 1× SSC/0.2% SDS for 8 min and 0.1× SSC/0.2% SDS for 5 min. The slides were centrifuged to dry, then read in the scanner. The slides were scanned at different wavelengths so that when the dual images were opened together, each spot lit up either as a yellow colour or in varying shades of green or red, depending on the relative level of expression of the individual gene in each spot.

Signals on the slides were located with a ScanArray 4000 Microarray Analysis System, and quantified with QuantArray Acquisition Software. The raw data were normalized according to the Lowess algorithm, then analysed with the program GeneSpring. GeneSpring data were further analysed statistically with Statistical Analysis of Microarray (SAM) software to identify genes that displayed statistically significant changes in their expression, and to minimize the number of false positives in the lists of genes that were up- or down-regulated. The cut-off point in this experiment was 2-fold regulation. The CandidaDB gene annotation was used to assign gene functions, and the ~6000 genes were assigned to functional categories on the basis of the MIPS functional categorization of *S. cerevisiae* genes (http://mips.gsf.de/genre/proj/yeast/index.jsp).

Results

Effects of antifungal agents on Sap enzyme activity

C. albicans isolates SC5314, J990578 and NR3 were tested extensively for Sap activity in the presence and absence of sub-MIC concentrations of several antifungal agents. For the azole-susceptible isolates SC5314 (Figure 1a) and NR3 (Figure 1b), Sap specific activity increased progressively with inhibition of growth by azole antifungals, reaching 150–200% of control values when growth was reduced by 50%. The opposite effect was seen with azole-resistant J990578 (Figure 1c), where Sap activity decreased to 50% of control values when growth was inhibited by 50%. Tests with two further *C. albicans* isolates, including one that was resistant to azoles, showed Sap specific activities increasing as growth was inhibited, in the same way as SC5314 and NR3. To date, J990578 is the sole isolate among eight we have tested with fluconazole that showed a decrease in Sap specific activity as growth was inhibited. The other seven isolates all showed an inverse relationship between Sap specific activity and growth.

Exposure of SC5314 and NR3 to flucytosine generated a similar increase in Sap specific activity proportional to the decrease in growth relative to control (Figure 2c). For J990578, we were unable to obtain flucytosine concentrations high enough to inhibit growth of this highly resistant strain, hence no relationship between growth and Sap activity could be determined (Figure 2c). Effects of amphotericin B on Sap activities in SC5314, NR3 and J990578 were difficult to determine, since partial growth inhibition by amphotericin B was rarely seen. When growth inhibition exceeded 75%, the levels of Sap enzyme activity tended to fall below the threshold of detection, probably because the fungi were killed by this cidal agent.

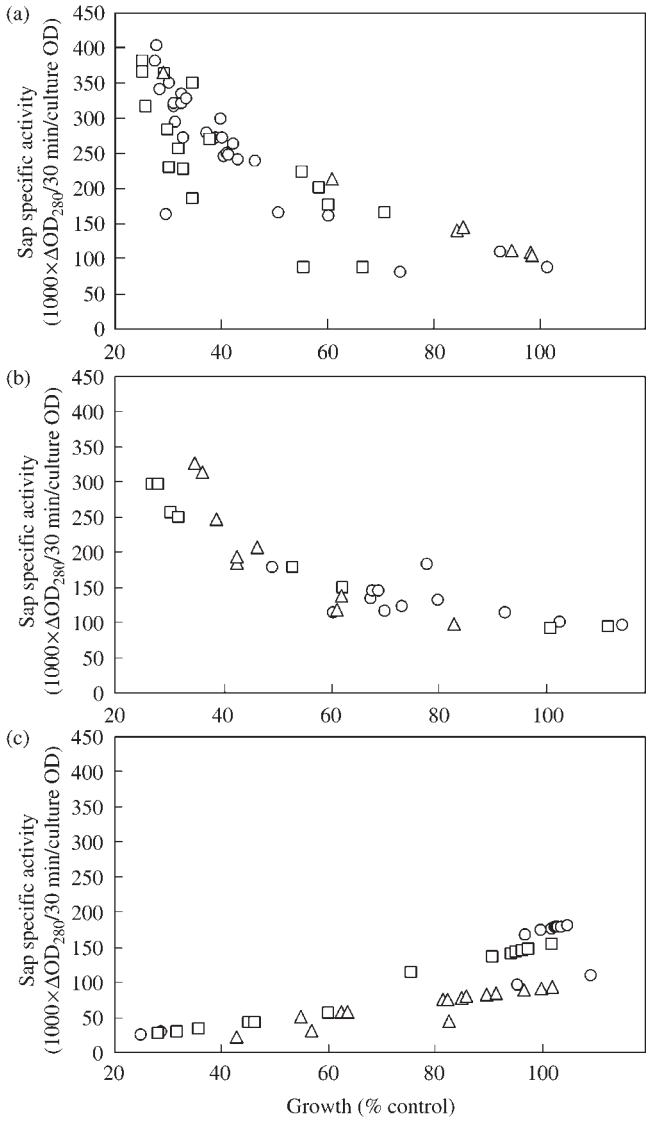

Figure 1. Scatterplots showing the effects of azole antifungals on growth and Sap specific activity in *C. albicans*. (a) Isolate SC5314; (b) isolate NR3; (c) isolate J990578. Circles, cells treated with fluconazole; squares, cells treated with itraconazole; triangles, cells treated with miconazole.

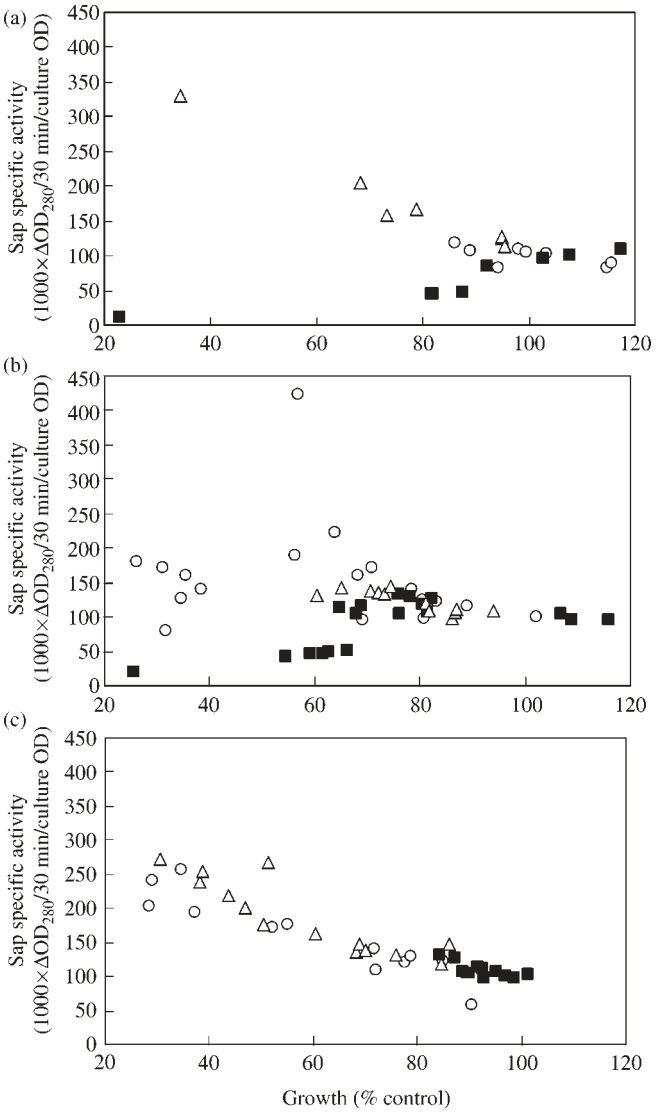

Figure 2. Scatterplots showing the effects of antifungals other than azoles on growth and Sap specific activity in *C. albicans*. (a) Cells treated with amphotericin B; (b) cells treated with caspofungin; (c) cells treated with flucytosine. Circles, isolate SC5314; triangles, isolate NR3; filled squares are used for isolate J990578 data to emphasize the opposite trend of Sap response from the other two isolates.

Where occasional partial growth inhibition with measurable growth activity did occur, exposure of SC5314 and NR3 to amphotericin B seemed to generate a rise in Sap specific activity whereas exposure of J990578 gave a fall in Sap activity (Figure 2a). These effects were similar to those seen on exposure to flucytosine and azole antifungals. However, the number of data points corroborating the relationship was small for amphotericin B.

For caspofungin (Figure 2b), the effects of the drug on Sap specific activity produced by SC5314, NR3 and J990578 were less reproducible than with the other agents tested. For drug concentrations where growth was below 50% of control, the Sap activities followed no obviously definable pattern. Above 50% of control growth, a weak inverse relation between Sap activity and growth was seen for all three strains.

As with the azole antifungals, two other *C. albicans* isolates, 88/025 and TW12-99, showed responses similar to SC5314 and NR3 when treated with antifungal concentrations that partially inhibited growth (details not shown).

Figure 3 shows the results of experiments in which *C. albicans* cells containing a pSAP2-GFP construct were grown in YCB/BSA medium with and without additions of fluconazole. After 48 h and 72 h of incubation, the mean fluorescence values for cells exposed to fluconazole at 1 mg/L were significantly higher than those of cells grown in the absence of fluconazole (Student's *t*-test, $P < 0.0001$). At 0.5 mg/L (Figure 3) and lower fluconazole concentrations (not shown), mean fluorescence was of the same order as in control cells at all three times. This indicates that the GFP-tagged *SAP2* promoter was expressed at higher levels in Sap-induced cells exposed to 1 mg/L fluconazole, a finding consistent with the increased specific enzyme activity measured in filtrates from wild-type SC5314 grown under the same conditions (Figure 1). However, the sensitivity of the cell

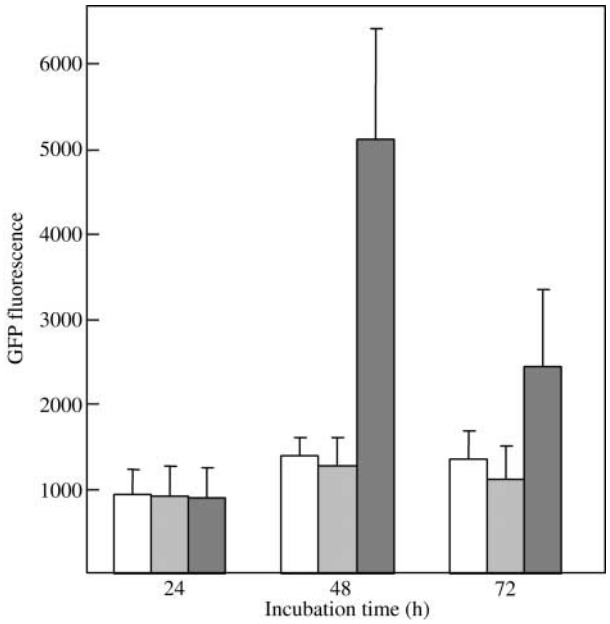

Figure 3. Fluorescence of SC5314 cells containing a *SAP2-GFP* promoter fusion. The cells were grown for the times shown and exposed to fluconazole at 0.5 mg/L (light grey bars) or 1.0 mg/L (dark grey bars). The white bars show fluorescence for control cells (no fluconazole exposure). Values are mean fluorescence per cell ± SD for at least 50 cells.

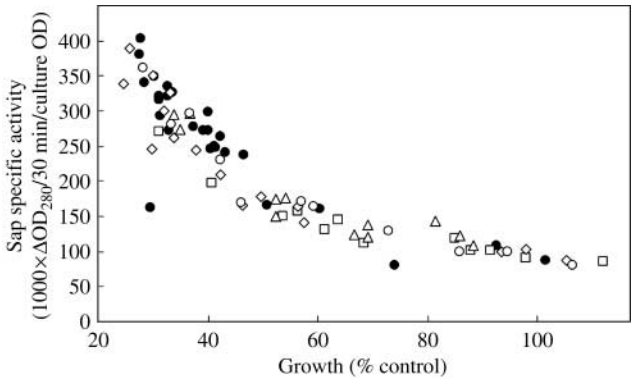

Figure 4. Growth versus Sap specific activity scatterplots for SC5314 (wild-type, filled circles), VC1 (sap1, diamonds), VC3 (sap3, open circles), Δsap9 (squares) and Δsap10 (triangles) exposed to fluconazole concentrations in the range 0.125–1.0 mg/L.

fluorescence assay was lower than that of the enzyme assay, since the latter revealed the fluconazole effect at lower concentrations than the GFP test. The enzyme assay also detects the activity of the secreted Sap2 gene product, while the fluorescence assay detects promoter activity within the cells.

Pepstatin A is a specific inhibitor of Sap activity, but does not affect other proteinases. To double-check that the increase in secreted proteinase in response to fluconazole was not the result of up-regulation of non-Sap proteinases, 2 μM pepstatin A was added at the start of a growth experiment in YCB/BSA, with and without addition of fluconazole at 2 mg/L. In the presence of fluconazole and the absence of pepstatin, growth was reduced to 73% of control, and Sap specific activity rose to 134% of control. In cultures where pepstatin was added, with or without fluconazole, no growth occurred by 72 h and no proteinase activity was detectable.

Previous studies have shown that the principal Sap expressed in YCB/BSA medium *in vitro* is the product of the *SAP2* gene.[15] We attempted to test whether *C. albicans* VC2 (*sap2/sap2*) displays a fluconazole effect by growing this strain in YCB/BSA. However, no growth was apparent in the absence of fluconazole even after 4 days of incubation, further confirming that any effects measured were attributable to levels of Sap2 secreted. For mutants VC1, VC3, Δsap9 and Δsap10, respectively bearing disruptions of both alleles of *SAP1, SAP3, SAP9* and *SAP10*, the effect of fluconazole on Sap specific activity was indistinguishable from that seen with wild-type SC5314 cells (Figure 4). Mutant VC4-6, disrupted in *SAP4, SAP5* and *SAP6*, did not grow in the YCB/BSA medium by 96 h of incubation, although growth occurred after a further 2 days of incubation. These results confirm that the elevation of specific Sap activity seen in the presence of inhibitory concentrations of fluconazole and, presumably, in the presence of the other antifungal agents that induce high Sap activities, probably results from a change in *SAP2* gene expression.

Expression profiling to investigate the effect of fluconazole under Sap-inducing conditions

We used *C. albicans* genomic microarrays to compare levels of expression of nearly all *C. albicans* genes in cells of SC5314 grown for 72 h in proteinase-inducing conditions with and without addition of fluconazole at 2 mg/L (growth was inhibited to 40% of control). Statistical analysis of the transcript profiling experiment revealed 88 genes that were significantly up-regulated more than 2-fold in fluconazole-treated cells and 26 that were down-regulated by the same factor (Table 1). Among the 88 up-regulated genes were 19 related to sugar uptake and metabolism, eight genes encoding enzymes in the ergosterol biosynthesis pathway, eight genes encoding proteins likely to be located and functioning in the cell wall, and therefore candidate factors that interact directly with the host during infection, and two SAP genes, *SAP2* and *SAP9*. Among the significantly down-regulated genes were four encoding enzymes in the glyoxylate cycle, which has been shown to play a possible role in the interaction between *C. albicans* and host phagocytic processes.[23]

RT–PCR to detect gene expression changes in response to antifungal exposure

We used semi-quantitative RT–PCR, with gels run to detect products every two cycles, to confirm the changes in *SAP* gene expression of *C. albicans* cells exposed to antifungal agents while growing under Sap-inducing conditions. We compared levels of *SAP2* and *SAP9* RNA in cells of SC5314 and J990578 exposed to concentrations of fluconazole and caspofungin that reduced growth to around 40–70% of control values by 72 h. Figure 5 shows an overview of results after 30 PCR cycles. Figure 5(a) shows that in SC5314, *SAP2* expression was substantially up-regulated in the presence of fluconazole. *SAP9* expression, though appearing similar in fluconazole-treated and -untreated cells in Figure 5(a), was also seen to be up-regulated in semi-quantitative RT–PCR (details not shown). In J990578,

Table 1. List of *C. albicans* genes that are up-regulated and down-regulated by a factor of more than 2-fold (genes are listed in decreasing order of fold change in expression)

Genes significantly up-regulated						Genes significantly down-regulated		
Gene name	putative function[a]	fold change	gene name	putative function[a]	fold change	gene name	putative function[a]	fold change
HXT62	low-affinity glucose transporter	34.0	*GAC1*	ser/thr phosphoprotein phosphatase 1, regulatory chain (by homology)	3.8	*HSP30*	heat shock protein (by homology)	16.8
IPF6518	unknown	30.7	*CPH2*	myc-type bHLH transcription factor CpH2	3.7	*FUN34.5EOC*	unknown, 5′ end	11.7
SAP2	secreted aspartyl protease	24.1	*IPF6342*	unknown	3.7	*FRP1*	member of the FRP family of proteins related to *Yarrowia lipolytica* glyoxylate pathway regulator Gpr1p and *Saccharomyces cerevisiae* Fun34p	11.6
ERG11	cytochrome P450 lanosterol 14a-demethylase	21.9	*GPH1*	glycogen phosphorylase (by homology)	3.5	*FRP2*	member of the FRP family of proteins related to *Yarrowia lipolytica* glyoxylate pathway regulator Gpr1p and *Saccharomyces cerevisiae* Fun34p	9.5
IPF3282.3EOC	putative hexose transporter (by homology)	17.7	*IPF946*	unknown	3.5	*IPF12540*	unknown	9.4
IPF3277	hexose transporter (by homology)	15.9	*IPF4959*	D-xylulose reductase (by homology)	3.4	*IPF650*	unknown	6.0
ERG6	sterol transmethylase	14.0	*FAS1*	fatty-acyl-CoA synthase, beta chain	3.4	*FRP3*	member of the FRP family of proteins related to *Yarrowia lipolytica* glyoxylate pathway regulator Gpr1p and *Saccharomyces cerevisiae* Fun34p, 3′ end	5.5
SCS7	required for hydroxylation of ceramide (by homology)	13.5	*BGL21*	endo-β-1,3-glucanase (by homology)	3.4	*ZRT2*	zinc transport protein (by homology)	4.9
ERG3	C5,6 desaturase	13.4	*OLE1*	stearoyl-CoA desaturase (by homology)	3.3	*ACS1*	acetyl-coenzyme-A synthetase (by homology)	4.7
ERG251	C-4 sterol methyl oxidase (by homology)	11.2	*AGP1*	asparagine and glutamine permease (by homology)	3.3	*CTA1*	catalase A, peroxisomal (by homology)	4.7
IFE2	unknown	9.7	*HTB1*	histone H2B (by homology)	3.3	*ALD5*	aldehyde dehydrogenase (NAD+) (by homology)	4.4
IPF19968	putative cell wall protein of the PIR family (by homology)	8.4	*TUB2*	β-tubulin	3.3	*CDR3*	ABC transporter, multidrug resistance protein	4.3
IFC1	putative peptide transporter (by homology)	7.9	*GFA1*	glutamine:fructose-6-phosphateamidotransferase	3.2	*FDH12*	formate dehydrogenase (by homology)	4.0
GPM1	phosphoglycerate mutase (by homology)	7.5	*IPF2067*	required for mannosylation of sphingolipids (by homology)	3.2	*IPF4820.5f*	putative complex I intermediate associated protein CIA30 (by homology)	3.9

Table 1. (*Continued*)

Genes significantly up-regulated						Genes significantly down-regulated		
Gene name	putative function[a]	fold change	gene name	putative function[a]	fold change	gene name	putative function[a]	fold change
IFC3	unknown	7.5	*POT14*	acetyl-CoA acetyltransferase (by homology)	3.1	*ITR2*	myo-inositol transporter (by homology)	3.8
SAH1	*S*-adenosyl-L-homocysteine hydrolase (by homology)	6.8	*ERG4*	sterol C-24 reductase (by homology)	3.1	*IPF5981*	similar to *Saccharomyces cerevisiae* Gin3p (by homology)	3.7
HXK2.3F	hexokinase II, 3′ end (by homology)	6.8	*CDR4*	multidrug resistance protein	3.0	*IPF5723*	cell surface GPI-anchored protein (by homology)	3.5
ERG1	squalene epoxidase	6.7	*PGA62*	unknown	3.0	*IPF29*	zinc finger protein (by homology	3.3
IPF19908	unknown	6.6	*IPF9552*	unknown	3.0	*IPF8762*	unknown	3.3
IPF7289	putative RNA polymerase II transcription factor (by homology)	6.4	*DAK2*	dihydroxyacetone kinase (by homology)	3.0	*PGA25*	GPI-anchored cell surface protein (by homology)	3.3
ERO1	required for protein disulphide bond formation in the ER (by homology)	6.1	*PMA1*	plasma membrane H+-transporting ATPase 1	3.0	*ACO1*	aconitate hydratase (by homology)	3.3
SAP9	secreted aspartyl protease	5.9	*GAL1*	galactokinase	2.9	*FUM12.3F*	fumarate hydratase, 3′ end (by homology)	2.7
ERG5	C-22 sterol desaturase (by homology)	5.8	*TOS1*	putative anchor subunit of α-agglutinin (by homology)	2.9	*IPF17186*	unknown	2.6
PGA24	putative cell wall protein (by homology)	5.7	*GLS21*	1,3-β-D-glucan synthase subunit	2.9	*POR1*	mitochondrial outer membrane porin (by homology)	2.6
IPF4443	unknown	5.7	*ERG7*	lanosterol synthase	2.9	*IPF7715.3eoc*	unknown	2.5
IPF11888	unknown	5.7	*MIS11*	mitochondrial C1-tetrahydrofolate synthase precursor (by homology)	2.8	*IPF8812*	unknown	1.8
HXT61	sugar transporter	5.6	*SKS1*	serine/threonine kinase (by homology)	2.8			
PGA52	unknown	5.5	*PHR2*	pH-regulated protein 2	2.7			
IFC3	unknown	5.4	*GLC3.3f*	1,4-glucan branching enzyme (by homology)	2.7			
PFK1	6-phosphofructokinase, alpha subunit	5.3	*RNR21*	ribonucleoside-diphosphate reductase (by homology)	2.7			
ECM33.3f	cell wall biogenesis, 3′ end (by homology)	5.2	*GAL10*	UDP-glucose 4-epimerase (by homology)	2.6			
FBA1	fructose-bisphosphate aldolase (by homology)	5.2	*IPF6629*	unknown	2.5			
PFK2	6-phosphofructokinase, beta subunit	5.1	*GLK1*	aldohexose specific glucokinase (by homology)	2.5			

Table 1. (*Continued*)

Genes significantly up-regulated						Genes significantly down-regulated		
Gene name	putative function[a]	fold change	gene name	putative function[a]	fold change	gene name	putative function[a]	fold change
ADH5	alcohol dehydrogenase (by homology)	5.1	*RBP1*	rapamycin-binding protein	2.4			
ADH1	alcohol dehydrogenase (by homology)	4.9	*PDA1*	pyruvate dehydrogenase alpha chain (by homology)	2.4			
HHF21	histone H4	4.8	*IPF7109*	unknown	2.3			
PDC11	pyruvate decarboxylase (by homology)	4.8	*SDS24*	similar to *Saccharomyces cerevisiae* YBR214w which presents strong similarity to hypothetical protein YGL056c	2.3			
DDR48	stress protein (by homology) heat shock	4.8	*IPF1399*	unknown	2.3			
PCK1	phosphoenolpyruvate carboxykinase	4.7	*LSC1*	succinate-CoA ligase/synthetase (by homology)	2.3			
HHF22	histone H4 (by homology)	4.6	*IPF4498*	unknown	2.3			
IPF1548	unknown	4.5	*IPF2908*	unknown	2.3			
FAS2.3F	fatty-acyl-CoA synthase, alpha chain, 3′ end	4.1	*IPF17358*	unknown	2.2			
CYB5	cytochrome b5 (by homology)	3.9	*ECM21*	involved in cell wall biogenesis (by homology)	2.1			
HXT5.3F	sugar transporter, 3′ end	3.9	*PDB1*	pyruvate dehydrogenase (by homology)	2.1			

[a] Annotations from CandidaDB (http://genolist.pasteur.fr/CandidaDB/) as of 21 January 2005.

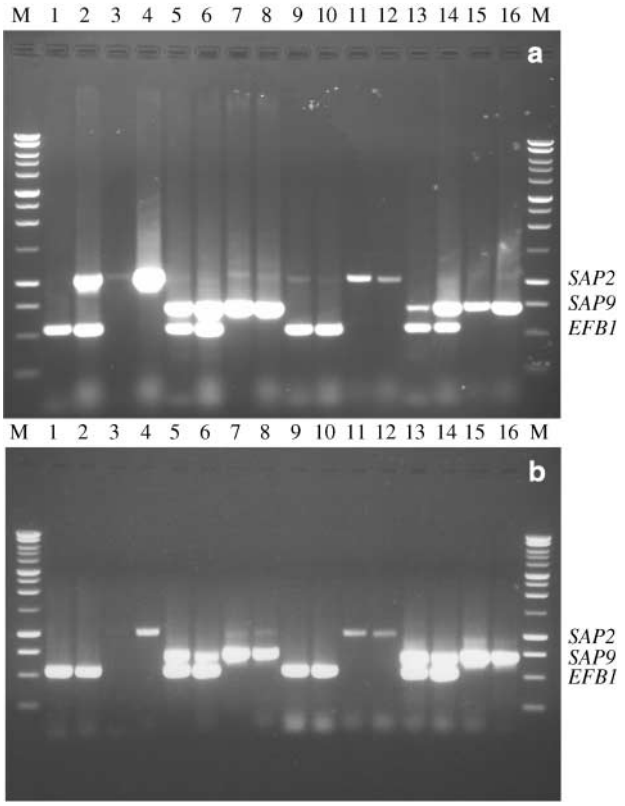

Figure 5. RT–PCR showing *SAP2* and *SAP9* RNA in cells of SC5314 (lanes 1–8) and J990578 (lanes 9–16) grown under Sap-inducing conditions with and without exposure to concentrations of fluconazole (a) and caspofungin (b) that reduced growth to ~70% of control. RT–PCR for *EFB1* served to control for the absence of genomic DNA and to provide a comparative indicator of RNA level for a constitutively expressed gene. Because inclusion of *EFB1* primers appeared to reduce the level of *SAP* PCR product, the RT–PCR was done with and without the EFB1 control in the same gel. Each pair of lanes shows the result for untreated (left) and drug-treated (right) cells. Lanes 1–4, SC5314, *SAP2* RT–PCR; lanes 5–8, SC5314, *SAP9* RT–PCR. Lanes 9–12 and 13–16 have the same layout for J990578. Lanes M are 1 kb marker ladders.

SAP2 expression was lower in cells exposed to fluconazole whereas *SAP9* expression was substantially higher. For cells exposed to caspofungin (Figure 5b), an increase in *SAP2* expression in SC5314 and a decrease in J990578 were seen similar to that in cells exposed to fluconazole, but expression of *SAP9* was lower in caspofungin-treated and -untreated cells.

Discussion

Our study has shown a clear link between expression of a known virulence factor in *C. albicans*, Sap2, and exposure of the cells to antifungal agents. Four of five *C. albicans* isolates grown under Sap-inducing conditions responded in a dose-dependent manner to partial growth inhibition by caspofungin, flucytosine and three azole antifungal agents by increasing their extracellular Sap enzyme activity. The fifth isolate responded in the opposite manner, with a decrease in Sap activity. These results partly contradict those of Wu *et al.*,[12] who found a decrease in extracellular Sap activity in susceptible strains of *C. albicans* exposed to fluconazole and an increase in resistant strains. Our isolate that showed a decrease in Sap activity when exposed to fluconazole and other agents was resistant to azoles and flucytosine. Of the four isolates that showed an increase in Sap activity, two were azole-susceptible and two were azole-resistant. We are therefore unable to confirm any general relationship between azole susceptibility and azole-related expression of Sap activity.

We studied the effects on Sap activity of a wider range of antifungal agents than have been previously investigated. Our findings indicate that enhanced Sap activity is induced not by any single class of antifungal agents, but by azoles (which target Erg11p), flucytosine (which inhibits DNA and RNA synthesis), and caspofungin (which targets β-1:3 glucan synthesis).[24] This finding suggests that at least three different classes of growth inhibitor affect a regulator of *SAP* gene expression.

Among the family of 10 *SAP* genes that encode aspartyl proteinases in *C. albicans*, most work to date has been done with *SAP1* to *SAP6*.[25,26] Little is known about *SAP7* to *SAP10*, but it appears likely that the products of *SAP9* and *SAP10* are not secreted into the culture medium.[19] The finding that *SAP9* mRNA was up-regulated in SC5314 exposed to fluconazole (Table 1) was not expected. However, our data show that the measured increase in extracellular proteinase produced by SC5314 exposed to fluconazole was the result of increased *SAP2* expression and was unrelated to expression of *SAP9*. Since Sap9 is localized on the cell surface,[19] the up-regulation of expression was unlikely to have been reflected in an increase in activity of proteinase secreted externally. The quantitative effect of fluconazole on Sap activity was the same for mutants disrupted in *SAP1, SAP3, SAP9* and *SAP10*, whereas *SAP2* and *SAP4-6* mutants did not grow in the inducing medium. The probability that genes in the *SAP4-6* series were responsible for the increase in Sap activity in fluconazole-treated cells is low; these genes are normally expressed only at neutral pH in media that favour hypha formation by *C. albicans*;[13,27] their failure to grow in YCB/BSA at the same rate as wild-type cells has been noted previously.[16] The results from the gene expression arrays (Table 1) and the RT–PCR experiments point only to *SAP2* as the proteinase-encoding gene uniquely regulated by exposure to fluconazole and caspofungin. It would seem surprising if *SAP2* were not the gene affected by exposure to other azoles and to flucytosine as well. However, others showed up-regulation of *SAP5* expression when *C. albicans* was exposed to caspofungin in non-inducing conditions,[14] so it is likely that different micro-environments influence the impact of antifungal agents on *SAP* expression.

De Backer *et al.*[28] previously reported results of expression profiling for the *URA3*$^{-}$ strain CAI-4 grown in the presence of itraconazole at 200 times its MIC and in non-proteinase-inducing conditions. Although the extreme differences in test conditions make comparison of the published data with our own partially-growth-inhibitory results difficult, it is notable that seven genes in the *ERG* family were up-regulated in both sets of data. However, in general, we found many fewer genes significantly up- or down-regulated under our milder conditions of treatment.

Placing the *GFP* gene under regulation of the *SAP2* promoter resulted in enhanced fluorescence when cells were exposed to fluconazole under Sap-inducing conditions (Figure 3). These results further confirm the role of *SAP2* expression as the mechanism underlying increased Sap secretion in response to antifungal

agents, and that assay of GFP fluorescence under a *SAP* promoter is a reasonable surrogate marker for levels of *SAP* expression.

We have established beyond doubt that several antifungal agents can influence the expression of at least one *C. albicans* virulence factor *in vitro* and are now investigating effects of antifungals on other virulence factors. Up-regulation of Sap2 secretion may represent a defence mechanism of the fungi in response to exposure to antifungal agents at subinhibitory concentrations. It is possibly a collateral effect resulting from alteration of expression of other genes as a physiological response to antifungal agents. We are currently investigating the mechanism underlying the Sap response to antifungal exposure.

Acknowledgements

We gratefully acknowledge the skilled technical assistance of Michelle Cunningham, Helen Gordon and Peter Moon. This work was supported by a programme grant from the British Society for Antimicrobial Chemotherapy. C. J. B. was supported by the Wellcome Trust.

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