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Disruption of Each of the Secreted Aspartyl Proteinase Genes *SAP1*, *SAP2*, and *SAP3* of *Candida albicans* Attenuates Virulence

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Secreted aspartyl proteinases (Saps), encoded by a gene family with at least nine members (*SAP1* to *SAP9*), are one of the most discussed virulence factors produced by the human pathogen *Candida albicans*. In order to study the role of each Sap isoenzyme in pathogenicity, we have constructed strains which harbor mutations at selected *SAP* genes. *SAP1*, *SAP2*, and *SAP3*, which are regulated differentially in vitro, were mutated by targeted gene disruption. The growth rates of all homozygous null mutants were similar to those of the isogenic wild-type parental strain (SC5314) in complex and defined media. In medium with protein as the sole source of nitrogen, *sap1* and *sap3* mutants grew with reduced growth rates but reached optical densities similar to those measured for SC5314. In contrast, *sap2* null mutants tended to clump, grew poorly in this medium, and produced the lowest proteolytic activity. Addition of ammonium ions reversed such growth defects. These results support the view that Sap2 is the dominant isoenzyme. When *sap1*, *sap2*, and *sap3* mutants were injected intravenously in guinea pigs and mice, the animals had increased survival rates compared to those of control animals infected with SC5314. However, reduction of proteolytic activity in vitro did not correlate directly with the extent of attenuation of virulence observed for all Sap-deficient mutants. These data suggest that *SAP1*, *SAP2*, and *SAP3* all contribute to the overall virulence of *C. albicans* and presumably all play important roles during disseminated infections.

The number of patients predisposed to infections with opportunistic microorganisms such as the human fungal pathogen *Candida albicans* has increased significantly over the last decade (2, 43). Patients at risk include those being treated for cancer, organ transplantation, or AIDS. Although putative virulence factors such as the yeast-to-hypha transition, adhesion factors, phenotypic switching, and secreted hydrolytic enzymes have long been recognized for *C. albicans*, unequivocal evidence for the role of these attributes in pathogenicity is still lacking (7, 36, 45). Investigations to clarify the role of each putative virulence factor are complicated, because it is likely that several of these features act cooperatively to determine the virulence properties of the organism (7, 45, 47) and because molecular techniques to disrupt specific cellular functions in an isogenic background have only recently become available (13, 16).

C. albicans is known to produce secreted aspartyl proteinases (Saps) when grown in medium with protein as the sole source of nitrogen (15, 18, 52). Such in vitro proteolytic activity has been correlated with virulence by several investigators (5, 9, 14, 24, 25, 32, 54, 61, 62). The substrate specificity of *C. albicans* Sap enzymes is very broad (52). Thus, host proteins such as keratin, collagen, mucin, immunoglobulins, proteinase inhibitors, enzymes from macrophages, and enzymes of the blood-clotting cascade and the kallikrein-kinin system are all possible targets for Saps during infection (6, 15, 18, 52). Antigen-antibody studies provided evidence that Saps are expressed in vivo (1, 8, 22, 31, 49, 53). Since adhesion of *C. albicans* to human mucosal or epidermal cells was reduced by the addition of the proteinase inhibitor pepstatin A (4, 10, 46), it has also been suggested that Saps may have an influence on adhesion. Pretreatment of neutropenic mice with the same inhibitor afforded a strong dose-dependent protection against a subsequent lethal intranasal dose of *C. albicans*, indicating an important role of proteinases during early dissemination (11).

Secreted proteinases in *C. albicans* are encoded by a gene family with at least nine members (*SAP1* to *SAP9*) (21, 34, 37, 38, 40, 64, 65). *SAP* genes were shown to be regulated differentially in vitro at the transcriptional level. *SAP1* and *SAP3* were regulated during phenotypic switching (19, 41, 42, 63). *SAP3* was also expressed in yeast cells under conditions in which *SAP2* was highly expressed (19, 58). *SAP2* mRNA was the main transcript in log-phase yeast cells and was induced by peptides (19, 63). The expression pattern of *SAP2* and comparison of amino-terminal amino acid sequences of purified Saps (19, 64) suggest that *SAP2* is principally responsible for the proteolytic activity observed in most strains growing in the yeast form in vitro. *SAP4* to *SAP6* were expressed during the yeast-to-hypha transition at neutral pH (19, 63), while *SAP7* was transcriptionally silent under all conditions tested in vitro (19). Expression of *SAP8* was increased at 25°C compared to levels of expression at physiological temperatures (20). This differential expression of *SAP* genes suggests that Sap isoenzymes may play different roles in the invasion of the host.

The most direct way of studying the role of candidate virulence factors is to generate mutants that harbor defined mutations in genes necessary for their production. However, due

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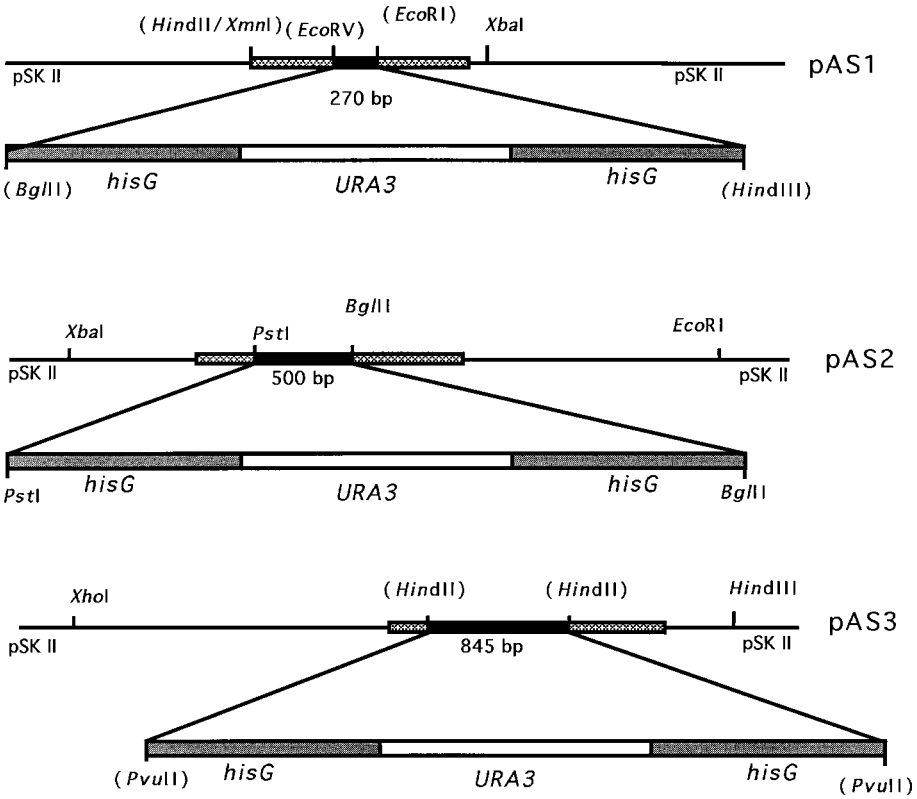


FIG. 1. Disruption of *SAP1*, *SAP2*, and *SAP3* by deletion and insertion of the *hisG*::*URA3*::*hisG* blaster cassette into the *SAP* ORFs (hatched boxes). Gene sequences that were displaced by the *hisG*::*URA3*::*hisG* cassettes (black boxes) and restriction sites used for the construction are indicated. Restriction sites in parentheses were deleted during the cloning procedure. pSK II, pBluescript II SK^+.

to the permanent diploid status of the asexual yeast *C. albicans*, the construction of such mutants in an isogenic background is technically difficult. The Ura-blaster protocol (13, 16) employed in this study provides a method which allows the sequential disruption of targeted alleles by using $Ura3^-$ auxotrophy as a recyclable, selectable marker and therefore enables the construction of genetically defined mutants.

Evidence for the function of specific *SAP* genes in pathogenicity may be elucidated by comparison of strains harboring mutations in these genes. In the present report, we show that the disruption of each of the proteinase genes *SAP1*, *SAP2*, and *SAP3* attenuated virulence of strains in disseminated infections.

The disruption of genes *SAP4* to *SAP6*, which may also act as virulence factors, and the phenotypes of the resulting mutants are described elsewhere (56).

MATERIALS AND METHODS

***Candida* strains.** The Ura^- strain CAI4 of *C. albicans* (Robin) Berkhout was provided by W. A. Fonzi, Georgetown University, and used for disruption of *SAP* genes. The growth rates, proteolytic activity, and virulence of *SAP*::*hisG* Ura^+ mutants were compared with those of the parental Ura^+ clinical isolate SC5314.

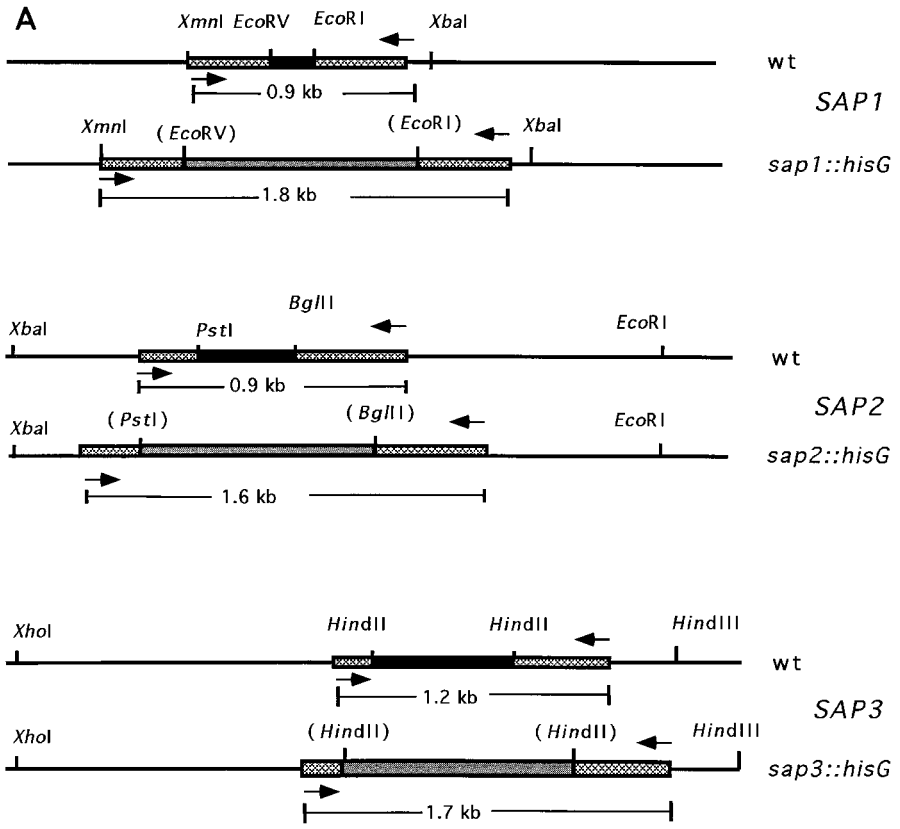
Media and growth conditions. In order to test the growth rates and proteolytic activity of *sap* mutants, the following liquid media were used: YCB/$(NH_4)_2SO_4$ medium, consisting of 1.17% yeast carbon base (YCB) (Difco, West Molesey, United Kingdom), 1% glucose, and 0.2% $(NH_4)_2SO_4$; YCB/BSA medium, consisting of 1.17% YCB, 1% glucose, and 0.5% bovine serum albumin (BSA) (Sigma, Poole, United Kingdom); and YPG medium, consisting of 1% yeast extract, 2% peptone, and 2% glucose. For all cultures, 100-ml volumes of test media were inoculated with 2 ml of an overnight culture of *C. albicans*. The cultures were shaken in an orbital incubator at 37°C. Growth was measured by optical density at 550 nm or by counting cells, and the proteolytic activity of culture media was determined at the same time points. To count cells of mutant strains that clumped in liquid media, samples were sonicated in the presence of 1% sodium dodecyl sulfate (SDS). Transformants generated during the Ura-blaster protocol were grown in synthetic dextrose (SD) medium, which contained 0.17% yeast nitrogen base (Difco) and 2% glucose.

***Candida* transformation and gene disruption.** Protoplasts were prepared by a modification of the procedure of Kurtz et al. (28). Late-log-phase *C. albicans* cells were pelleted and resuspended in 5 ml of 1 M sorbitol containing 50 mM dithiothreitol and 25 mM EDTA (pH 8.0) for 5 min at 30°C. Pelleted cells were resuspended in 5 ml of 0.1 M sodium citrate (pH 5.8)–1 M sorbitol–25 mM EDTA, to which 0.05 ml of β-glucuronidase (type HP-2S; Sigma) (114,000 U/ml) was added. The cells were incubated for 5 min at 30°C and harvested. The spheroplasts were then resuspended in 0.5 ml of 1 M sorbitol–10 mM $CaCl_2$–10 mM Tris-Cl (pH 7.5) and used for transformation.

Plasmid DNA (10 μg) containing *SAP* disruption cassettes was linearized, mixed with 10 μl of carrier DNA (3 mg/ml) (herring sperm DNA; Clontech, Heidelberg, Germany), and added to 100-μl preparations of protoplasts, and the mixtures were incubated for 15 min at room temperature. The cells were incubated for a further 15 min after addition of 10 volumes of polyethylene glycol 4000 (20%, wt/vol)–10 mM $CaCl_2$–10 mM Tris-Cl, pH 7.5. The protoplasts were then resuspended in 100 μl of SD medium supplemented with 1 M sorbitol (SD/sorbitol) and plated on SD/sorbitol agar. For 5-fluoro-orotic acid (5-FOA) selection of Ura^+ recombinants (3, 13), cloned transformants were resuspended in 1 ml of water and plated on SD agar containing 5-FOA (1 mg/ml) and uridine (25 μg/ml).

Hybridization probes and radiolabelling. A 1.3-kb *Xmn*I/*Xba*I fragment containing 1.2 kb of *SAP1* (21), a 3.0-kb *Eco*RI/*Xba*I fragment containing 1.2 kb of *SAP2* (65), and a 1.0-kb *Eco*RI fragment containing 0.8 kb of *SAP3* (64) were used as hybridization probes in this study. DNA fragments were size fractionated and then purified by using a Prep-a-Gene kit (Bio-Rad, Hemel Hempstead, United Kingdom). DNA fragments were labelled with [α-^{32}P]dCTP (~3,000 Ci/mmol) (ICN Flow, Thame, United Kingdom) by means of a random-primed DNA labelling kit (Boehringer, Mannheim, Germany) (12).

PCR. PCR was used as a primary screen for 5-FOA-treated segregants. Primers which amplified the parts of the genes that were removed during the construction of the cassettes were designed (see Fig. 2). The pairs of primers were as follows: 5′-CTCTTCCATTTCTAACAAAC-3′ and 5′-CAGTTTCAATTCA GCTTGG-3′ for *SAP1*, 5′-TGATTGTCAAGTCACTTATAGT-3′ and 5′-CTT AGGTCAAGGCAGAAATACTG-3′ for *SAP2*, and 5′-TGGATTGGAACAT



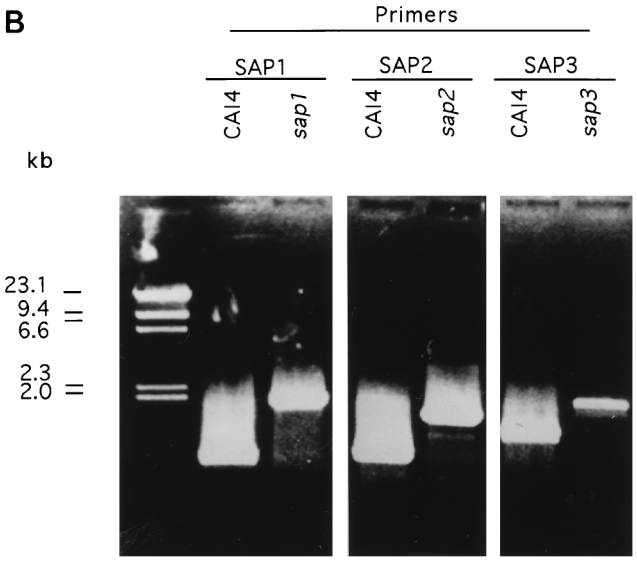


FIG. 2. Screening of Sap-deficient mutants by PCR showing structure of the wild-type (wt) and disrupted (*sap*::*hisG*) alleles. Primers which amplified the parts of the ORFs that were removed during the construction of the cassettes were designed. The primer sites for PCR amplification are indicated (arrows). (A) Fragment size of each PCR product. (B) Fragments resulting from PCR amplification of genomic DNA from the wild-type CAI4 and double mutants (*sap1*, Δ*sap1*::*hisG*/Δ*sap1*::*hisG*::*URA3*::*hisG*; *sap2*, Δ*sap2*::*hisG*/Δ*sap2*::*hisG*::*URA3*::*hisG*; *sap3*, Δ*sap3*::*hisG*/Δ*sap3*::*hisG*::*URA3*::*hisG*).

TTCTA-3′ and 5′-GTTATAATCACTAGTTCCA-3′ for *SAP3*. Thus, amplified DNA fragments changed in size after disruption of each wild-type copy.

Southern blotting. Genomic DNA was isolated from lyticase-digested cells essentially as described by Magee (33). For Southern blot analysis, DNA was digested with restriction endonuclease, size fractionated by gel electrophoresis, blotted onto a nylon membrane, and cross-linked to the membrane with UV light (55).

Hybridization and autoradiography. Southern blots were hybridized as described by Hube et al. (19). The nylon membranes were rinsed with 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and prehybridized at 42°C for at least 5 h with 40% formamide–5× Denhardt's reagent–6× SSC–0.5% SDS–100 μg of denatured, degraded herring sperm DNA per ml. Denatured radiolabelled DNA probes were added, and the mixtures were incubated under the same conditions for 12 to 18 h. The membranes were washed for 15 min with 5× SSC–0.1% SDS at 42°C, 15 min with 1× SSC–0.5% SDS at 42°C, and 30 min with 0.1× SSC–1% SDS at 65°C. The membranes were exposed at −70°C to X-ray film with an intensifying screen.

Proteolytic activity. The enzyme activity of proteinases secreted by *C. albicans* strains into culture medium was measured by a modification of the procedure of Rüchel (51). Culture samples from various stages of growth were centrifuged for 3 min at 3,000 × *g*, and the supernatant was frozen and tested for proteolytic activity. Aliquots (50 μl) of the supernatants were mixed with 150 μl of 0.1 M sodium citrate (pH 2.8) and 150 μl of 1% (wt/vol) hemoglobin and incubated for 1 h at 37°C. Trichloroacetic acid (20%) was used to stop proteinase digestion of the hemoglobin and to precipitate protein. After centrifugation for 2 min at 8,000 × *g*, the peptide concentration of the supernatant was measured by absorbance at 280 nm.

Pathogenicity in vivo. Proteinase mutants were tested in a guinea pig model and a murine model of disseminated candidosis. For the guinea pig model, young normal adult Pirbright guinea pigs (500 g) were injected intravenously with 40,000 or 80,000 *C. albicans* cells/g of body weight. For the mouse model, young normal adult Swiss white mice (25 g) were injected intravenously with 2,000, 4,000, or 8,000 *C. albicans* cells/g of body weight. Before the animals were injected, yeast cells were grown in CYGi medium (44) at 30°C, with shaking at 20 rpm, and suspensions were diluted in physiological saline. Following death, infected organs were removed aseptically, weighed, and suspended in 10 ml of physiological saline. The organs were homogenized in saline, and 10-fold dilutions of the homogenates were plated on Sabouraud glucose agar and incubated

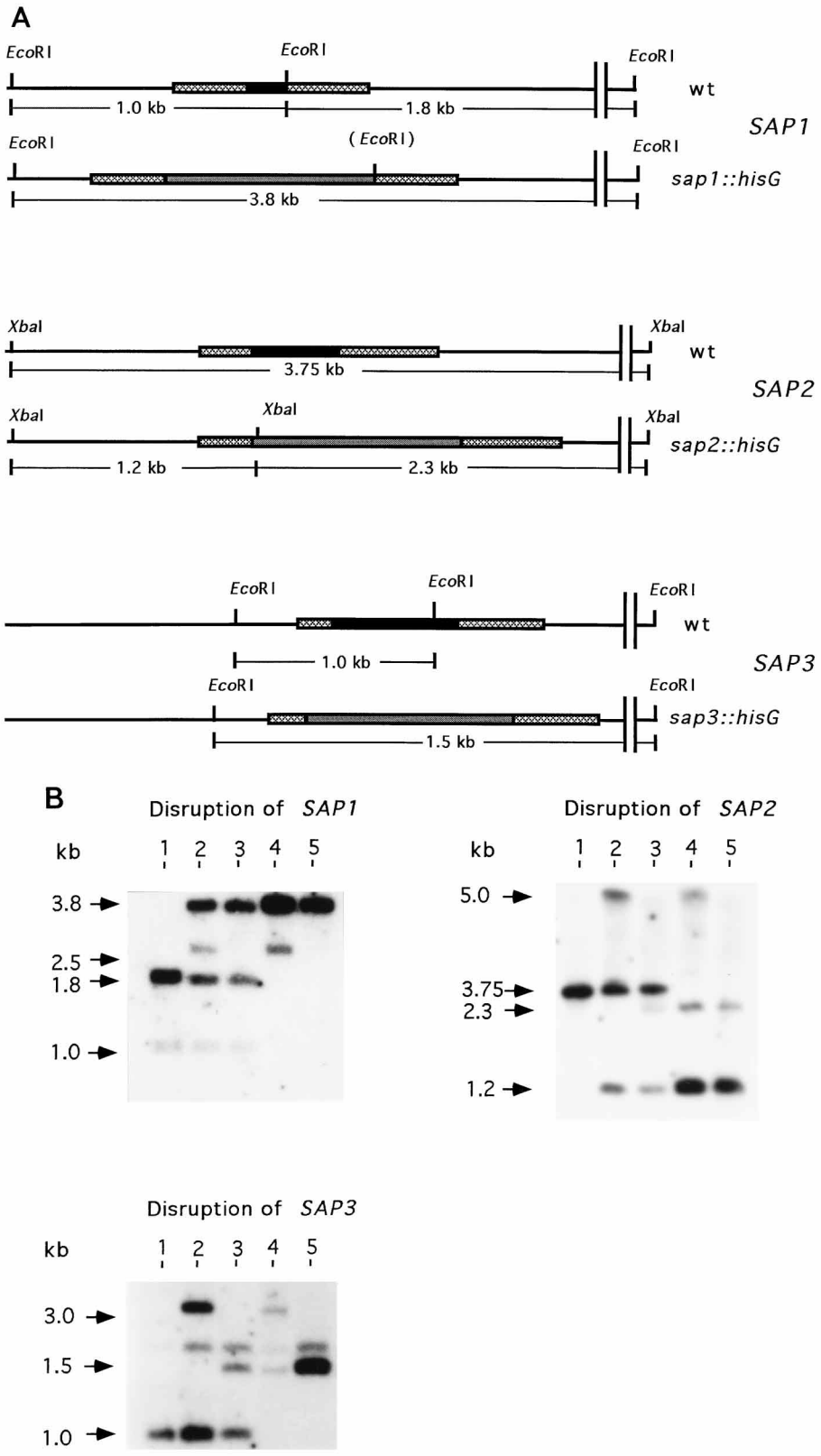


FIG. 3. Diagnostic Southern analysis of Sap-deficient mutants. Genomic DNA of each strain was digested with *Eco*RI (*SAP1* and *SAP3*) or *Xba*I (*SAP2*), blotted, and hybridized to *SAP1*, *SAP2*, and *SAP3* probes. (A) Sizes of *Eco*RI or *Xba*I fragments for the wild type (wt) and the disruptant (*sap*::*hisG*). (B) Southern analysis of segregants. Lanes: 1, parental strain CAI4; 2, heterozygote mutant containing one *sap*::*hisG*::*URA3*::*hisG*::*sap* allele (first-round transformants: BH24, BH52, and BH1); 3, heterozygote mutant containing one *sap*::*hisG* allele (BH24-15, BH52-1, and BH1-1); 4, double mutant containing one *sap*::*hisG*::*URA3*::*hisG*::*sap* and one *sap*::*hisG* allele (second-round transformants: BH24-15-1, BH52-1-17, and BH1-1-1); 5, homozygote mutant containing two *sap*::*hisG* alleles (BH24-15-1-3, BH52-1-17-1, and BH1-1-1-1). The expected fragments as shown in panel A are indicated (arrows). In addition to a 3.8-kb band due to *sap1*::*hisG* alleles, a 4.1-kb band, which could not be discriminated from the 3.8-kb band, was generated by *sap1*::*hisG*::*URA3*::*hisG*::*sap1* alleles (lanes 2 and 4). During the disruption procedure, additional bands of 2.5 (*SAP1*), 5.0 (*SAP2*), and 3.0 (*SAP3*) kb in lanes 2 and 4 are due to *Eco*RI (*SAP1* and *SAP3*) or *Xba*I (*SAP2*) sites in the *URA3* gene. When disruption of *SAP3* was analyzed, an extra band between 1.5 and 3.0 kb was frequently detected. This extra band was due to cross-hybridization with an *Eco*RI fragment corresponding to *SAP5*.

TABLE 1. Genotypes of *sap* mutants produced in this study

Strain type and no.	Genotype
Clinical isolate, SC5314 CAI4	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*; congenic to SC5314
sap1	
BH24	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*/*SAP1*/Δ*sap1*::*hisG*::*URA3*::*hisG*
BH24-15	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*/*SAP1*/Δ*sap1*::*hisG*
BH24-15-1	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*/Δ*sap1*::*hisG*/Δ*sap1*::*hisG*::*URA3*::*hisG*
BH24-15-1-3	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*/Δ*sap1*::*hisG*/Δ*sap1*::*hisG*
sap2	
BH52	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*/*SAP2*/Δ*sap2*::*hisG*::*URA3*::*hisG*
BH52-1	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*/*SAP2*/Δ*sap2*::*hisG*
BH52-1-17	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*/Δ*sap2*::*hisG*/Δ*sap2*::*hisG*::*URA3*::*hisG*
BH52-1-17-1	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*/Δ*sap2*::*hisG*/Δ*sap2*::*hisG*
sap3	
BH1	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*/*SAP3*/Δ*sap3*::*hisG*::*URA3*::*hisG*
BH1-1	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*/*SAP3*/Δ*sap2*::*hisG*
BH1-1-1	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*/Δ*sap3*::*hisG*/Δ*sap3*::*hisG*::*URA3*::*hisG*
BH1-1-1-1	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*/Δ*sap3*::*hisG*/Δ*sap3*::*hisG*

for 48 h at 37°C. Reisolated *Candida* cells were tested for the genotype at the relevant *SAP* locus by PCR and Southern analysis.

Statistical analyses of survival curves were performed by log-rank tests with the Kaplan-Meier estimator. Differences of CFU in organs of animals infected with SC5314 and *sap1*, *sap2*, and *sap3* double mutants were analyzed by statistical Mann-Whitney U tests. Differences were considered statistically significant at a P of <0.05.

Virulence tests in animals were carried out over a period of 14 days. Animals surviving the time of experimentation were sacrificed, and organs were obtained as described above for *C. albicans* colony counting.

RESULTS

Disruption of *SAP1* to *SAP3*. It has been demonstrated that *SAP1* to *SAP6* are expressed differentially in vitro. This suggests that each gene may have a distinct role during colonization and infection. Evidence for the function of the different proteinase isoenzymes in vivo can be resolved by the disruption of single or multiple *SAP* genes and comparison of the resulting *sap* mutants with the parental strain. Consequently, we disrupted the *SAP1* to *SAP3* genes in the Ura$^-$ strain CAI4, using the Ura-blaster protocol (13, 16). The first step in the Ura-blaster protocol is the in vitro disruption of the cloned target genes by introducing the *hisG*::*URA3*::*hisG* cassette into the open reading frames (ORFs). The *hisG*::*URA3*::*hisG* cassette was inserted into the ORFs of *SAP1* to *SAP3* as follows (Fig. 1). For *SAP1*, the *Xmn*I/*Xba*I fragment of *SAP1* containing the ORF was subcloned into the *Hin*dII/*Xba*I site of pBluescript II SK$^+$ to give pAS1. A 270-bp *Eco*RI/*Eco*RV fragment of pAS1 was removed, and a blunt-ended *Bgl*II/*Hin*dIII *hisG*::*URA3*::*hisG* fragment was inserted. For *SAP2*, the *Eco*RI/*Xba*I fragment containing the ORF was subcloned into the *Eco*RI/*Xba*I site of pBluescript II SK$^+$ to give pAS2. A *Pst*I/*Bgl*II-digested *hisG*::*URA3*::*hisG* fragment was inserted into the *Pst*I/*Bgl*II site of pAS2. For *SAP3*, an *Xho*I/*Hin*dIII fragment containing the ORF of *SAP3* was subcloned into pBluescript II SK$^+$ to give pAS3. A *Pvu*II/*Pvu*II-digested *hisG*::*URA3*::*hisG* fragment was blunt-end ligated into the *Hin*dII/*Hin*dII sites of pAS3.

The *SAP*::*hisG*::*URA3*::*hisG*::*SAP* cassettes (Fig. 1) were used to disrupt wild-type *SAP* genes in the Ura$^-$ strain CAI4 by integrative transformation. Ura$^+$ transformants and 5-FOA-resistant segregants of these transformants were analyzed by PCR and Southern analysis. For PCR screening, primers which amplified the parts of the ORFs that were removed during the construction of the cassettes were designed. For Southern analysis, genomic DNA was digested with *Eco*RI or *Xba*I and band patterns of the predicted sizes were observed. *SAP1*, *SAP2*, and *SAP3* were disrupted in both alleles of CAI4, as shown by PCR (Fig. 2) and Southern analysis (Fig. 3). For all growth and virulence tests, Δ*sap*::*hisG*/Δ*sap*::*hisG*::*URA3*::*hisG* mutants (Ura$^+$) were used (Table 1).

Growth of *sap1* to *sap3* null mutants in complex and defined media. To assess whether treatment used to produce *sap* mutants or the disruptions themselves affected the growth rate and viability of each strain, cells were grown in YPG and YCB/$(NH_4)_2SO_4$ media at 37°C for several days. The specific growth rate and the yield of each *sap* mutant in the yeast form were identical to those for the parental strain, SC5314 (not shown). This demonstrates that disruption of *SAP1*, *SAP2*, and *SAP3* did not affect any factor necessary for growth of the yeast form in complex and defined media.

Growth and proteolytic activity of *sap1* to *sap3* null mutants in protein medium. *SAP2* was shown previously to be the main transcript during growth in YCB/BSA medium for all strains tested (19, 63). In contrast, expression of *SAP3* was observed only in the opaque form of strain WO-1 and in other strains under conditions in which *SAP2* was highly expressed (19, 58). *SAP1* was detected only in the opaque form of WO-1 or the basic smooth phenotype of strain 3153A (19, 41, 42, 63). Therefore, it was predicted that the growth of the *sap2* null mutant may be more significantly affected in YCB/BSA medium than that of null mutants of *sap1* and *sap3*. When *sap2* mutants were grown in YCB/BSA medium, the cells tended to clump and grew poorly compared to SC5314 (Fig. 4A). To determine whether this was due to the *SAP2* disruption, the proteolytic activity of the culture supernatant was measured. The slow growth of the *sap2* null mutant correlated with low proteinase activity in the culture supernatant (Fig. 4B). When $(NH_4)_2SO_4$ (0.2%) was added to such cultures, *sap2* null mutants grew at rates similar to that of SC5314 (not shown), supporting the view that the disruption of *SAP2* caused the observed phenotype. Surprisingly, both the *sap1* and the *sap3* null mutants had reduced growth in YCB/BSA medium over 2 days, suggesting reduced proteinase secretion. This was confirmed by measurement of reduced proteolytic activity detected in the cultures (Fig. 4). Still, after 4 days, *sap1* and *sap3* null mutant cultures reached the same optical density and the same supernatant proteolytic activity as the wild-type SC5314

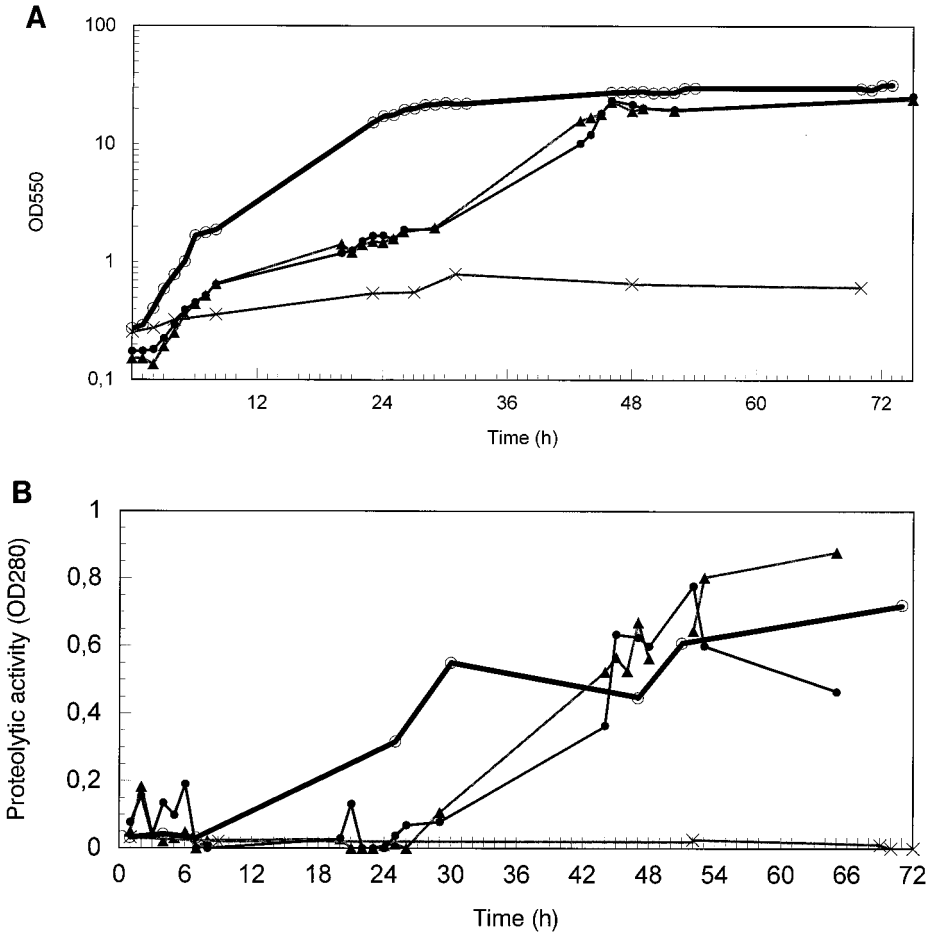


FIG. 4. Growth (A) and extracellular proteolytic activity (B) of Sap-deficient mutants in protein medium (YCB/BSA medium). Cells were grown at 37°C, and growth was recorded for 3 days. *sap1*, Δ*sap1*::*hisG*/Δ*sap1*::*hisG*::*URA3*::*hisG*; *sap2*, Δ*sap2*::*hisG*/Δ*sap2*::*hisG*::*URA3*::*hisG*; *sap3*, Δ*sap3*::*hisG*/Δ*sap3*::*hisG*::*URA3*::*hisG*. OD550, optical density at 550 nm. Symbols: ○, SC5314; ●, *sap1*; ×, *sap2*; ▲, *sap3*.

strain. Thus, proteinase production was only delayed in *sap1* and *sap3* null mutants compared to that in SC5314.

Virulence of *sap1* to *sap3* null mutants. Since all *sap* null mutants were shown to have a reduced proteolytic activity in vitro, we tested the ability of these strains to cause infections in animal models. The ability of Sap-deficient mutants to cause disseminated infections was investigated in both the murine and the guinea pig models of disseminated candidosis and compared to that of the isogenic parental strain, SC5314.

All *sap* null mutants showed attenuated virulence compared to SC5314 in both animal models. However, the observed reduction of virulence for the Sap-deficient strains did not correlate directly with the proteolytic activity in vitro, and the relative virulence of the strains varied between the two animal models. For example, the growth rate and proteolytic activity of the Sap2-deficient disruptant in vitro were reduced significantly compared to those of *sap1* and/or *sap3* null mutants, but attenuation of the *sap1* and *sap3* null mutants was similar to or greater than that for *sap2* (Fig. 4 to 6; Tables 2 to 4). The extent of infection varied with the number of yeast cells in the inoculum. Only slight differences in virulence were found between the wild type and each Sap-deficient mutant at low or high inocula (not shown). For instance, at a low inoculum of 2,000 CFU per g of body weight in the mouse model, more than 92% of the animals survived until day 14, while at a high inoculum of 80,000 CFU/g in the guinea pig model, 94% of the animals died by day 7. When guinea pigs were inoculated with 40,000 CFU of *Candida* strains per g, SC5314 killed 96% of the animals by 14 days and the mean survival time was 6.1 days (Fig. 5; Table 2). In contrast, guinea pigs infected with *sap2*, *sap1*, and *sap3* null mutants had significantly ($P < 0.001$ as determined by Kaplan-Meier survival analysis) longer mean survival times (9.3, 8.6, and 8.1 days, respectively) (Fig. 5 and Table 2). Attenuated virulence was also evident in the number of animals surviving at day 14 after infection. The mean numbers of CFU per gram recovered from the skin, livers, and kidneys of infected animals were lowest for the *sap2* double mutant but did not differ significantly between all mutants and the wild type (except for numbers of cells recovered from kidneys of *sap2* mutants [Table 2]). However, not all organs of infected guinea pigs were colonized by *Candida* cells, and animals infected with the Sap2-deficient disruptant had the highest number of culture-negative liver (60%) and kidney (20%) samples (not shown).

The observed hierarchy of attenuated virulence for *sap* null mutants (*sap2* > *sap1* > *sap3*) in the guinea pig model was not seen in the murine model of systemic candidosis. However, when mice were challenged with 4,000 CFU/g, all Sap-deficient mutants showed significantly ($P < 0.001$) reduced virulence in terms of mean survival time and the percentage of mice alive

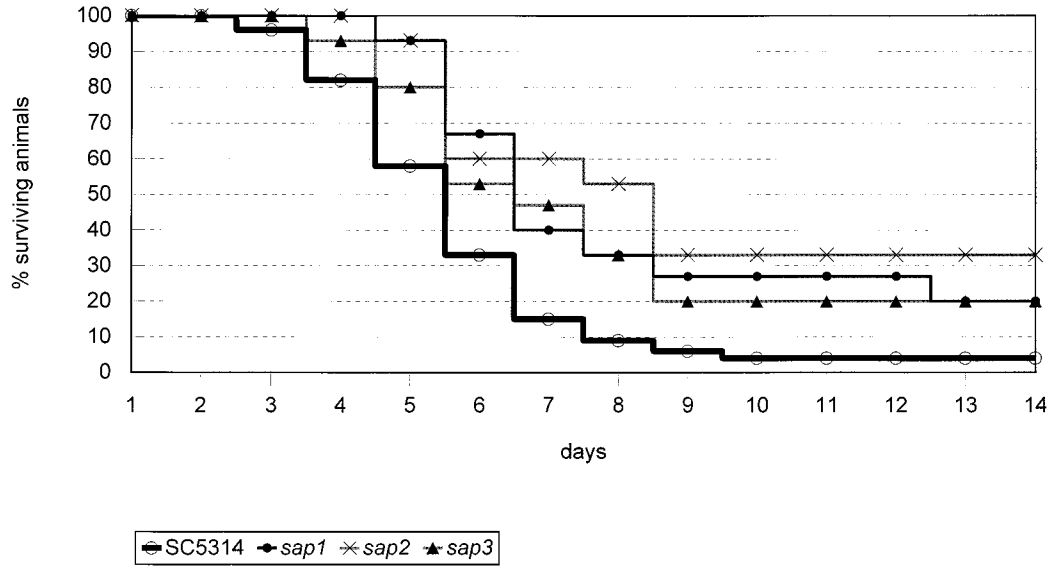


FIG. 5. Intravenous infection of guinea pigs with 40,000 CFU of Sap-deficient mutants per g of body weight. The percentages of animals surviving over a period of 14 days after infection with SC5314 ($n = 55$) and *sap1* (Δ*sap1*::*hisG*/Δ*sap1*::*hisG*::*URA3*::*hisG*) ($n = 15$), *sap2* (Δ*sap2*::*hisG*/Δ*sap2*::*hisG*::*URA3*::*hisG*) ($n = 15$), and *sap3* (Δ*sap3*::*hisG*/Δ*sap3*::*hisG*::*URA3*::*hisG*) ($n = 15$) mutants are shown.

on day 14 (Fig. 6; Table 3). Attenuated virulence for all *sap* null mutants was also observed with larger inoculum sizes (8,000 CFU/g) in the mouse model (Table 4). Thus, while the extent of attenuation varied with the animal model used, all *sap* mutants clearly showed reduced virulence in both animal models of disseminated infection.

DISCUSSION

To date, the strongest evidence for a role of secreted aspartyl proteinases in the virulence of *C. albicans* still comes from studies with spontaneous or induced mutants that are affected in proteinase production (8, 29, 32, 50). However, such proteinase-deficient mutants were obtained by chemical or UV treatment and perhaps harbor multiple mutations at loci that may influence the growth and virulence of the fungus. For example, auxotrophic mutants are known to be significantly less virulent than wild-type strains (16, 26, 35, 48, 57). The construction of mutants by targeted gene disruption, using the Ura-blaster protocol, overcomes the problem of generating unforeseen pleiotropic mutations (13, 16). In order to investigate the role of each Sap isoenzyme, we individually disrupted *SAP1*, *SAP2*, and *SAP3* using this method. Successful targeted disruption of the *SAP1* to *SAP3* genes was confirmed by PCR screening and Southern analysis (Fig. 2 and 3).

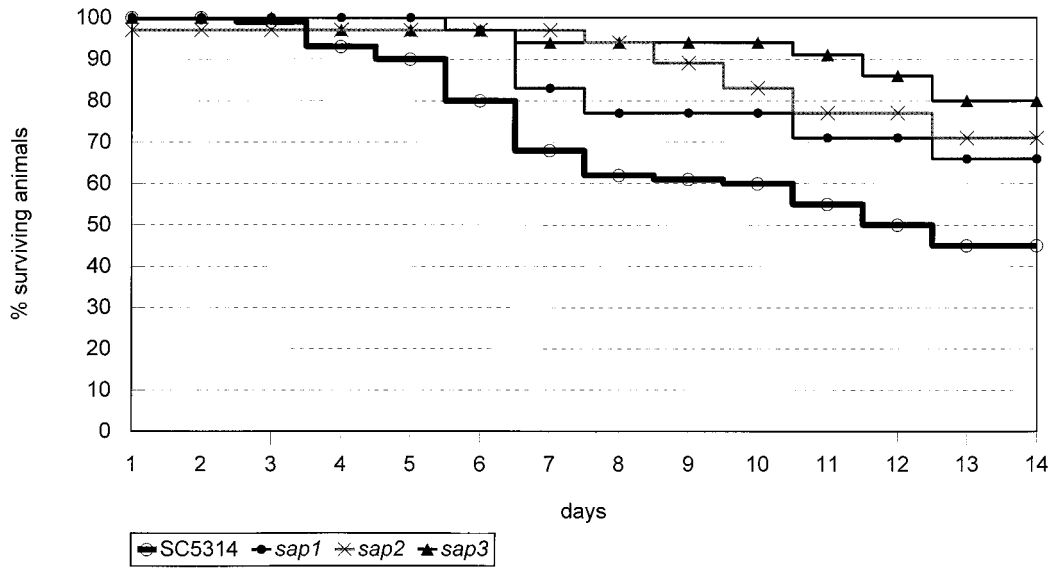


FIG. 6. Intravenous infection of Swiss mice with 4,000 CFU of Sap-deficient mutants per g of body weight. The percentages of animals surviving over a period of 14 days after infection with SC5314 ($n = 71$) and *sap1* (Δ*sap1*::*hisG*/Δ*sap1*::*hisG*::*URA3*::*hisG*) ($n = 35$), *sap2* (Δ*sap2*::*hisG*/Δ*sap2*::*hisG*::*URA3*::*hisG*) ($n = 35$), and *sap3* (Δ*sap3*::*hisG*/Δ*sap3*::*hisG*::*URA3*::*hisG*) ($n = 35$) mutants are shown.

TABLE 2. Virulence of *sap* null mutants during disseminated infection of guinea pigs with an inoculum of 40,000 CFU of *C. albicans* per g

Strain[a]	No. of animals	Mean survival time[b] (days) ± SD	% Survivors at day 14	Mean log CFU in organs/g of tissue ± SD		
				Skin	Liver	Kidney
SC5314	55	6.1 ± 2.1	3.6	3.8 ± 1.3	2.2 ± 2.0	5.4 ± 1.0
sap1	15	8.6 ± 3.2*	20.0	3.9 ± 0.6	2.6 ± 1.7	5.0 ± 1.7
sap2	15	9.3 ± 3.5*	33.3	3.6 ± 1.3	1.7 ± 2.2	3.9 ± 2.3[c]
sap3	15	8.1 ± 3.4*	20.0	4.1 ± 0.5	1.9 ± 2.0	4.5 ± 1.8

[a] *sap1*, Δ*sap1*::*hisG*/Δ*sap1*::*hisG*::*URA3*::*hisG*; *sap2*, Δ*sap2*::*hisG*/Δ*sap2*::*hisG*::*URA3*::*hisG*; *sap3*, Δ*sap3*::*hisG*/Δ*sap3*::*hisG*::*URA3*::*hisG*.
[b] Survivors at day 14 were given a survival time of 14 days. *, significantly different from values for SC5314 ($P < 0.001$) as determined by Kaplan-Meier survival analysis (log-rank test).
[c] Significantly different from value for SC5314 ($P = 0.0145$) as determined by Mann-Whitney U tests.

It cannot be excluded completely that transformation of *Candida* cells and treatment with 5-FOA altered the properties of transformants; still, all mutants appeared to grow normally in complex and defined media compared to the parental wild-type strain, SC5314 (not shown). Moreover, the reduced growth of *sap* mutants in medium with protein as the sole source of nitrogen could be reversed by addition of ammonium ions (not shown), thus making it unlikely that the construction of *sap* mutants created undetected mutations.

A desirable control to test whether the Ura-blaster procedure caused unforeseen alterations in the genetic background of the host strain would be to reintroduce a copy of the wild-type allele, in single copy or multicopy, to the null mutant and to compare the phenotypes of these strains with that of the original parental strain. For instance, reintroduction of the wild-type alleles of the *Candida CST20* and *HST7* genes was shown recently to be able to restore a hypha-positive phenotype to the respective null strains, which were defective in hyphal development in some media (27, 30). Interpretation of such experiments in the context of genes encoding putative virulence functions is, however, less explicit and suffers from a range of potential difficulties. The principal phenotype of our *SAP1* to *SAP3* disruptions is virulence attenuation in the double null mutants. In our case, it would therefore be necessary to restore both copies of each allele by single integration events at the original loci, with the genes under the control of their native promoters, so as to ensure that the transcription of the restored wild-type locus was at the original gene dosage and was subject to the same regulatory conditions. For example, it has been demonstrated for genes (such as *SAP1* and *SAP3*) which are subject to regulation by phenotypic switching that the transformability of specific loci depended on the switch state of the cell (59). This may be due to changes in the density of heterochromatin packing in different switch states, which in turn perhaps regulates transcription. Finally, it is also possible that the gene restoration experiments may themselves lead to attenuation of virulence by spurious alteration of other virulence-enhancing genes. This concern is even more significant in the context of the accompanying paper, which analyzes a triple *sap4* to *sap6* mutant (56), for which six additional transformations would be necessary to reestablish the synthetic wild type. In the absence of available information on the nature of the promoter regions of the *SAP* genes and in the light of the concerns raised here about the interpretation of such gene restoration experiments, the interpretation of the phenotypes generated was therefore based on face value analysis of the null-mutant data. It is conceded that there is an unlikely but definite possibility that targeted gene disruptions generated by the Ura-blaster protocol could generate unforeseen changes due to mitotic recombination and gene conversion, etc., and strain isogenicity may not always be sustained. Such events may also occur during the normal growth and maintenance of *Candida* cultures in the absence of genetic manipulation.

In vitro expression studies and sequencing data suggested that Sap2 was the main extracellular proteolytic enzyme of *C. albicans* yeast cells in protein-containing media (19, 63, 65), and hence it is likely that one function of Sap2 is the production of peptides for cell growth. This view is supported by the observation that the *sap2* null mutant grew poorly in such media (Fig. 4A) and had markedly reduced proteolytic activity compared to those of SC5314 and *sap1* and *sap3* mutants (Fig. 4B). This exhibited in vitro phenotype was similar to that described for proteinase-deficient mutants obtained by chemical or UV treatment (8, 29, 31, 50).

Although *SAP1* transcription had been observed previously

TABLE 3. Virulence of *sap* null mutants during disseminated infection of mice with an inoculum of 4,000 CFU of *C. albicans* per g

Strain[a]	No. of animals	Mean survival time[b] (days) ± SD	% Survivors at day 14	Mean log CFU in kidney/g of tissue ± SD
SC5314	71	10.6 ± 3.8	45.1	5.1 ± 2.2
sap1	35	12.2 ± 2.9*	65.7	5.0 ± 2.4
sap2	35	12.7 ± 2.8*	71.4	4.7 ± 2.2
sap3	35	13.2 ± 2.1*	80.0	4.9 ± 2.3

[a] *sap1*, Δ*sap1*::*hisG*/Δ*sap1*::*hisG*::*URA3*::*hisG*; *sap2*, Δ*sap2*::*hisG*/Δ*sap2*::*hisG*::*URA3*::*hisG*; *sap3*, Δ*sap3*::*hisG*/Δ*sap3*::*hisG*::*URA3*::*hisG*.
[b] Survivors at day 14 were given a survival time of 14 days. *, significantly different from values for SC5314 ($P < 0.001$) as determined by Kaplan-Meier survival analysis (log-rank test).

TABLE 4. Virulence of *sap* null mutants during disseminated infection of mice with an inoculum of 8,000 CFU of *C. albicans* per g

Strain[a]	No. of animals	Mean survival time[b] (days) ± SD	% Survivors at day 14	Mean log CFU in kidney/g of tissue ± SD
SC5314	46	9.4 ± 3.5	19.6	5.9 ± 1.0
sap1	10	12.5 ± 4.2*	80.0	4.9 ± 2.6
sap2	10	10.8 ± 2.3*	30.0	5.8 ± 0.4
sap3	10	11.1 ± 2.4*	30.0	5.1 ± 2.0

[a] *sap1*, Δ*sap1*::*hisG*/Δ*sap1*::*hisG*::*URA3*::*hisG*; *sap2*, Δ*sap2*::*hisG*/Δ*sap2*::*hisG*::*URA3*::*hisG*; *sap3*, Δ*sap3*::*hisG*/Δ*sap3*::*hisG*::*URA3*::*hisG*.
[b] Survivors at day 14 were given a survival time of 14 days. *, significantly different from values for SC5314 ($P < 0.001$) as determined by Kaplan-Meier survival analysis (log-rank test).

only in the opaque form of strain WO-1 and *SAP3* transcription was detected only under conditions in which *SAP2* was highly expressed (19, 58), both Sap1- and Sap3-deficient mutants showed reduced growth rates in protein medium (Fig. 4A). Hence, it can be concluded that *SAP1* and *SAP3* genes are also important for optimal growth for strains other than WO-1 during yeast-like growth when protein is provided as the sole source of nitrogen. Furthermore, since the *sap2* mutant grew poorly, it is unlikely that *SAP1*, *SAP3*, or *SAP4* to *SAP6* were induced in this mutant background. Nonetheless, it is possible that expression of *SAP1*, *SAP3*, or *SAP4* to *SAP6* in vitro depends on Sap2 activity and that *SAP2* disruption in turn causes a reduced expression of other *SAP* genes.

The differential expression of the *SAP* gene family in vitro leads one to speculate that different Sap isoenzymes may be active during different phases of infection and/or in different tissues (34). It is also conceivable that one Sap or a few Saps act as dominant virulence factors. Numerous correlations between the proteolytic activity of *Candida* species in vitro and virulence have been reported (5, 9, 14, 17, 53, 66). In this case, one would expect that a gene such as *SAP2*, which is principally responsible for proteolytic activity in vitro, might have a dominant role in virulence. After all, although disruption of *SAP2* attenuated virulence in our models of disseminated infections (Fig. 5 and 6; Tables 2 to 4), the effect on pathogenicity was less than that of proteinase-deficient mutants obtained by chemical or UV treatment (8, 29, 32, 50). This indicates that the previously described mutants are likely to harbor mutations at multiple *SAP* loci or at loci for pathogenicity determinants distinct from the *SAP* loci. In addition, our data show that the extent of in vitro proteolytic activity need not correlate directly with the potential to cause systemic infections in vivo. For example, the disruption of *SAP1* and *SAP3* produced the strains with the longest mean survival time in the mouse model, but the proteolytic activity in vitro was only slightly reduced (Tables 3 and 4; Fig. 4B).

Although the level of *SAP1* mRNA may be low under nonswitching conditions in vitro (see above), the *sap1* mutant had significantly ($P < 0.001$) reduced virulence compared to that of the wild type (Fig. 5 and 6; Tables 2 and 3). Thus, expression of *SAP1* in wild-type strains may be up-regulated in vivo. For instance, it is possible that the growth of *C. albicans* in vivo selects phenotypically switched variants that express *SAP1* or other *SAP* genes that are not normally expressed in vitro.

We tested the virulence of Sap-deficient mutants in murine and guinea pig models of disseminated infection. In both models, all mutants showed attenuated virulence assessed by longer mean survival times. However, the extents of attenuation for each mutant were not the same in the two models (Fig. 5 and 6; Tables 2 and 3), confirming that virulence of *C. albicans* is a complex property that depends on the host species and inoculum size as well as the expression of specific genes. Furthermore, extended survival time of mice did not correlate with organ burden in the kidneys. It can be hypothesized that although the numbers of fungal cells in the organs were the same, the virulence properties of the *sap* disruptants must be different from those of the wild-type cells, thus causing reduced virulence.

The proteinase genes *SAP4* to *SAP6*, which may play a critical role during morphological changes and in environments with neutral pH values (19, 63), also seem to be important during pathogenesis, since the disruption of these genes also led to marked attenuation of virulence in models of disseminated infections (56).

The importance of Sap isoenzymes is presumed to depend also on the prevailing immune status of the host, and the role of proteolytic enzymes in the initial colonization of mucosal surfaces and/or penetration into mucosal tissues may be influenced by other immunological properties of the host (11). Such a role for Sap isoenzymes remains to be tested in a mucosal infection model.

In conclusion, none of the three *SAP* genes investigated here is a single dominant virulence factor in disseminated infections. Virulence of *C. albicans* is clearly the result of the concerted action of a multiplicity of genes encoding virulence and growth factors depending on the tissue and the stage of infection (7, 45, 47). However, all *sap* mutants were reduced significantly in virulence, which is in contrast to proteinase-deficient mutants of another human fungal pathogen, *Aspergillus fumigatus* (23, 39, 60). These gene disruption experiments therefore underline the status of secreted aspartyl proteinases as important aspects of the pathogenic properties of *C. albicans.*

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