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A Triple Deletion of the Secreted Aspartyl Proteinase Genes *SAP4*, *SAP5*, and *SAP6* of *Candida albicans* Causes Attenuated Virulence

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Secreted aspartyl proteinases (Saps) from *Candida albicans* are encoded by a multigene family with at least nine members (*SAP1* to *SAP9*) and are considered putative virulence factors important for the pathogenicity of this human pathogen. The role of Sap isoenzymes in the virulence of *C. albicans* has not yet been clearly established, and therefore, using recent progress in the genetics of this yeast, we have constructed a panel of isogenic yeasts, each with a disruption of one or several *SAP* genes. We focused on the construction of a *C. albicans* strain in which three related *SAP* genes (*SAP4*, *SAP5*, and *SAP6*) were disrupted. Growth of the Δ*sap4,5,6* triple homozygous null mutant DSY459 in complex medium was not affected, whereas, interestingly, growth in a medium containing protein as the sole nitrogen source was severely impaired compared to the growth of the wild-type parent strain SC5314. Since the presence of Sap2 is required for optimal growth on such medium, this suggests that Sap4, Sap5, or Sap6 plays an important role for the process of induction of *SAP2*. When guinea pigs and mice were injected intravenously with DSY459, their survival time was significantly longer than that of control animals infected with the wild-type SC5314. Attenuated virulence of DSY459 was followed by a significant reduction of yeast cells in infected organs. These data suggest that the group of Sap4, Sap5, and Sap6 isoenzymes is important for the normal progression of systemic infection by *C. albicans* in animals.

Candida albicans is the most common yeast pathogen in humans (23). Understanding of the mechanisms allowing this yeast to invade and colonize the human host has progressed in recent years through the development of appropriate molecular genetic technologies. The fitness of the host immune system is an important aspect in determining the outcome of the host-fungus interaction; however, the number of factors rendering this yeast virulent is still debated. Putative virulence factors including the yeast-to-hypha transition, the adhesion to host cells, and the ability to secrete several hydrolytic enzymes have been proposed (3, 25). Clear evidence for the role of each of these factors in virulence has not yet been provided.

C. albicans is known to produce several secreted hydrolytic enzymes, among which aspartyl proteinases and phospholipases have been characterized extensively (2, 8, 13). The proteolytic activity of secreted aspartyl proteinases (Saps) encoded by *SAP* genes has been considered to be important for the virulence of *C. albicans* in several studies. First, proteolytic activity has been associated with tissue invasion (33) and elevated levels of immunoglobulin G to Sap in serum have been observed in patients with disseminated candidiasis (18, 27, 31). Second, expression of Sap has been observed in murine macrophages after phagocytosis of *C. albicans* cells (1). Third, pepstatin, a specific inhibitor of acid proteinases, is able to block *C. albicans* adherence to human cells as well as the events occurring in early invasion of murine skin and modulates the course of experimental candidiasis in mice (26, 28, 32).

Sap isoenzymes are encoded by a gene family with at least nine members (21, 22). Individual *SAP* genes may not be regulated the same way in vitro, and some information on their regulation in vivo is available (4, 5). *SAP2* mRNA is present when *C. albicans* is grown on media containing various proteins as the sole nitrogen source (10), and Sap2, the protein encoded by *SAP2*, accounts for most of the Sap enzymes produced under these conditions (40). *SAP1* and *SAP3* are regulated during phenotypic switching (10, 40). *SAP4* to *SAP6*, which form a distinct subgroup in the *SAP* multigene family, are expressed during the yeast-to-hypha transition at neutral pH (10, 40). *SAP7* appears to be silent under all growth conditions tested so far (10), and studies of the expression of *SAP8* and *SAP9* are still in progress. Preliminary data indicate that *SAP8* is expressed preferentially at 25°C in media containing bovine serum albumin (BSA) as the sole nitrogen source (11).

With the aid of genetic tools recently developed for *C. albicans*, it is now possible to create deletions of specific genes in this yeast and to produce a lineage of isogenic strains that differ from the parent strain at only one locus (7). Using this technique, we attempted to define the precise role of Sap isoenzymes by targeted disruption of an individual *SAP* gene or several *SAP* genes. Studies of the virulence of individual Δ*sap1*, Δ*sap2*, and Δ*sap3* mutants are presented elsewhere (12). Because the *SAP4*, *SAP5*, and *SAP6* genes are close homologs whose expression patterns cannot be distinguished by North-

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TABLE 1. Genotypes of yeast strains used in this study

Strain name	Parent strain	Genotype	Reference
SC5314		*URA3/URA3*	7
CAF4-2	CAF2-1	Δ*ura3::imm434*/Δ*ura3::imm434*	7
DSY344	CAF4-2	Δ*sap6::hisG-URA3-hisG*/*SAP6*	This study
DSY345	DSY344	Δ*sap6::hisG*/*SAP6*	This study
DSY346	DSY345	Δ*sap6::hisG*/Δ*sap6::hisG-URA3-hisG*	This study
DSY349	DSY346	Δ*sap6::hisG*/Δ*sap6::hisG*	This study
DSY432	CAF4-2	Δ*sap4::hisG-URA3-hisG*/*SAP4*	This study
DSY433	DSY349	Δ*sap6::hisG*/Δ*sap6::hisG* Δ*sap4::hisG-URA3-hisG*/*SAP4*	This study
DSY434	DSY432	Δ*sap4::hisG*/*SAP4*	This study
DSY435	DSY433	Δ*sap6::hisG*/Δ*sap6::hisG* Δ*sap4::hisG*/*SAP4*	This study
DSY436	DSY434	Δ*sap4::hisG*/Δ*sap4::hisG-URA3-hisG*	This study
DSY437	DSY435	Δ*sap6::hisG*/Δ*sap6::hisG* Δ*sap4::hisG*/Δ*sap4::hisG-URA3-hisG*	This study
DSY438	DSY436	Δ*sap4::hisG*/Δ*sap4::hisG*	This study
DSY439	DSY437	Δ*sap6::hisG*/Δ*sap6::hisG* Δ*sap4::hisG*/Δ*sap4::hisG*	This study
DSY450	CAF4-2	Δ*sap5::hisG-URA3-hisG*/*SAP5*	This study
DSY451	DSY450	Δ*sap5::hisG*/*SAP5*	This study
DSY452	DSY451	Δ*sap5::hisG*/Δ*sap5::hisG-URA3-hisG*	This study
DSY457	DSY439	Δ*sap6::hisG*/Δ*sap6::hisG* Δ*sap4::hisG*/Δ*sap4::hisG* Δ*sap5::hisG-URA3-hisG*/*SAP5*	This study
DSY458	DSY457	Δ*sap6::hisG*/Δ*sap6::hisG* Δ*sap4::hisG*/Δ*sap4::hisG* Δ*sap5::hisG*/*SAP5*	This study
DSY459	DSY458	Δ*sap6::hisG*/Δ*sap6::hisG* Δ*sap4::hisG*/Δ*sap4::hisG* Δ*sap5::hisG*/Δ*sap5::hisG-URA3-hisG*	This study

ern blot analysis, we chose to create a mutant with a triple disruption of the *SAP4* to *SAP6* genes to assess the contribution of these isoenzymes to the virulence of *C. albicans.*

MATERIALS AND METHODS

Strains. The Ura$^-$ *C. albicans* strain CAF4-2 served as the parent strain for performing gene disruptions and has been described by Fonzi and Irwin (7). The parental strain of CAF4-2, SC5314, has been used as a clinical reference strain for animal experiments. Other strains and their genotypes are described in Table 1. *Escherichia coli* DH5α (9) was used for propagation of plasmids constructed in this study.

Media and growth conditions. The media and culture conditions for the *C. albicans* strains were identical to those described by Hube et al. (12). Complex medium was YPD medium with 1% yeast extract (Difco), 2% peptone (Difco), and 2% glucose.

***Candida* transformation.** Transformation of *C. albicans* with linear DNA fragments containing the gene disruption constructs was performed by a lithium acetate transformation method described by Sanglard et al. (35). Disruption of the *SAP* genes followed the conventional "Ura-blaster" protocol described elsewhere (7), and the *ura3* auxotrophy was regenerated by selection for 5-fluoroorotic acid (5-FOA)-insensitive segregants (7).

PCR. PCR buffers and *AmpliTaq* polymerase were from Perkin-Elmer (Roche Molecular Systems, Branchburg, N.J.). The buffer was composed of 10 mM Tris-HCl (pH 8.3)–50 mM KCl with 1.5 mM $MgCl_2$ containing 0.2 mM deoxynucleoside triphosphates and 2.5 U of polymerase per reaction mixture. Briefly, the PCR was carried out in a Thermal Cycler 480 (Perkin-Elmer) with a first cycle of denaturation for 4 min at 94°C followed by 30 cycles of annealing at 54°C for 2 min, elongation at 72°C for 2 min, and denaturation at 94°C for 30 s. PCR was completed by a final elongation step at 72°C for 10 min. Primers for PCR are described in the legend to Fig. 2 and were designed on the basis of the *C. albicans SAP4*, *SAP5*, and *SAP6* and *Salmonella typhimurium hisG* nucleotide sequences accessible in GenBank.

Yeast DNA templates for PCR were prepared from overnight cultures on complex medium. One milliliter of these cultures was centrifuged in Eppendorf tubes, and DNA was extracted by addition of 0.3 g of glass beads, 200 μl of a lysis buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 100 mM NaCl), and 200 μl of phenol-chloroform-isoamyl alcohol (24:24:1). After 1 min of vortexing, the tubes were centrifuged at maximum speed for 10 min in a microcentrifuge, and the supernatant was reextracted with chloroform-isoamyl alcohol (24:1). Nucleic acids were then precipitated with ethanol and resuspended in 50 μl of Tris-EDTA. Of this suspension, 1 μl was used for PCR.

Plasmid constructions. For the disruption of *SAP4*, a genomic 1.2-kb *Eco*RI fragment containing the *SAP4* gene had been previously cloned in pUC18 to give pDS235. This vector was linearized with *Spe*I and blunt ended, and the blunt-ended *Sal*I-*Bgl*II *hisG-URA3-hisG* disruption cassette from pMB7 (7) was inserted in the plasmid to give pDS237. A linear fragment obtained by digestion of pDS237 with *Sal*I (specific for *SAP4*) and *Kpn*I (from the pUC polylinker site) was isolated to transform *C. albicans*.

For the disruption of *SAP5*, a 1.8-kb *Eco*RI fragment containing the *SAP5* gene was subcloned into pBluescript SK$^+$, in which the *Hin*dIII site was destroyed by *Hin*dII and *Eco*RV digestion and religation, to give pDS209. pDS209 was cut by *Hin*dIII and *Hin*dII to remove an internal 0.4-kb fragment which was replaced by the *hisG-URA3-hisG* disruption cassette from pMB7 (7), which had been isolated previously by *Bgl*II digestion, blunt ending, and *Hin*dIII digestion, thus yielding pDS210. A linear fragment obtained by digestion of pDS210 by *Xho*I and *Sac*I (from the pBluescript polylinker site) was isolated to transform *C. albicans.*

For the disruption of *SAP6*, a 4-kb *Xho*I fragment containing the entire *SAP6* gene and flanking region from pCA6 (22) was subcloned into pBluescript KS$^+$ to give pDS194. pDS194 was cut by *Bgl*II to remove an internal 0.9-kb fragment, blunt ended, and replaced by the blunt-ended *Sal*I-*Bgl*II *hisG-URA3-hisG* disruption cassette from pMB7 (7) to give pDS198. An *Xho*I linear fragment was used to transform *C. albicans.*

Schematic representation of the disruption cassettes of the *SAP4* to *SAP6* genes is shown in Fig. 1.

Southern blotting and DNA probe hybridization. Genomic DNA was isolated as described above, and samples were then subjected to RNase A digestion. After restriction digestion, DNA was size fractionated by agarose gel electrophoresis and vacuum blotted on GeneScreen Plus membranes. Prehybridization and hybridization of the membrane with labeled DNA probe were performed at 42°C in a solution containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1% sodium dodecyl sulfate, and 100 μg of denatured salmon sperm DNA per ml. The probe was labeled with [α-^{32}P]dATP by random primer labeling (6). The membrane was washed as recommended by the manufacturer and exposed at −70°C on a Fuji X-ray film with an intensifying screen.

Proteolytic activity. Proteolytic activity was measured as described by Hube et al. (12).

Virulence tests in animals. The virulence of *C. albicans* SC5314 and the Δ*sap4,5,6* triple mutant DSY459 was tested in the guinea pig and murine models of systemic candidiasis. Pirbright guinea pigs (500 g) were injected intravenously with 4×10^4 and 8×10^4 CFU per g of body weight. Adult Swiss white mice (25 g) were injected with 4×10^3 and 8×10^3 CFU per g of body weight. Yeast cells were grown in CYGi medium (24) at 30°C with shaking at 20 rpm, and suspensions diluted in physiological saline were given intravenously to the animals. Infected organs were removed aseptically after death of the animals, weighed, and suspended in 10 ml of physiological saline. The organs were homogenized, and dilutions of the homogenates were plated on Sabouraud glucose agar for 48 h at 37°C for colony counting. Individual *C. albicans* colonies thus obtained were tested for their genotype by PCR. 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.

Statistical analysis. Statistical analyses of survival curves were performed by log-rank tests with the Kaplan-Meier estimator (15). Differences of CFU in organs of animals infected with SC5314 and DSY459 were analyzed by statistical Mann-Whitney U tests. Differences were considered statistically significant at a P of <0.05.

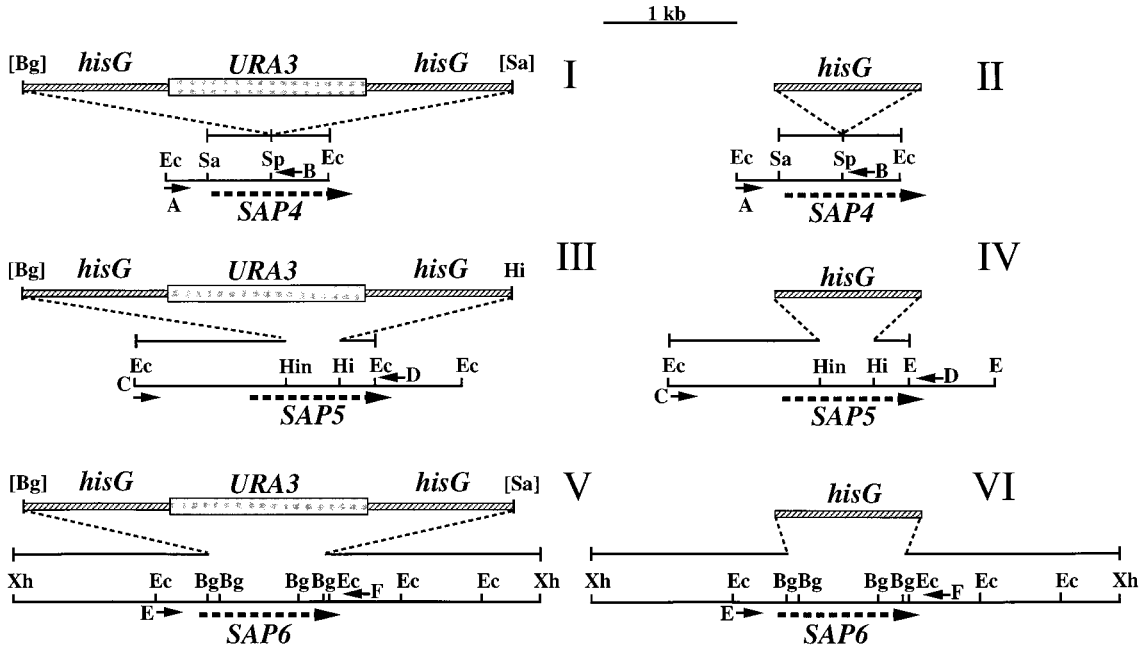

FIG. 1. Restriction maps of the *SAP4*, *SAP5*, and *SAP6* alleles in disruption experiments. Schematic representations of the disruption of the *SAP4* (I and II), *SAP5* (III and IV), and *SAP6* (V and VI) genes before (I, III, and V) and after (II, IV, and VI) regeneration of the Δ*ura3* marker by selection with 5-FOA are shown. The maps of the wild-type alleles are shown with the corresponding maps of the disrupted alleles. Maps of disrupted alleles shown in panels I, III, and V match with maps of linear fragments used for disruption experiments. The location and transcription direction of *SAP* genes are indicated (broken arrows). The locations of primers A to F used in PCR for the monitoring of the disruptions of each gene (Fig. 2) are indicated. Bg, *Bgl*II; Ec, *Eco*RI; Hi, *Hin*dIII; Hin, *Hin*dII; K, *Kpn*I; Sa, *Sal*I; Sp, *Spe*I; Xh, *Xho*I. Abbreviations in brackets indicate blunt-ended restriction sites.

RESULTS

Construction of a Δ*sap4,5,6* mutant. The *SAP1* to *SAP6* genes are expressed under specific growth conditions, and each gene product may have a specific function during host colonization and infection (10, 40). The function of each gene in vivo can be addressed by the construction of mutants with single or multiple gene deletions. Studies of the disruption of the *SAP1* to *SAP3* genes and the effect of null mutations in these genes on virulence have been performed by Hube et al. (12). Since virulence in *C. albicans* is thought to be due to the action of several individual factors, rather than constructing a single Δ*sap* null mutant, we constructed here a mutant with deletions of the closely related *SAP4*, *SAP5*, and *SAP6* genes (22). *SAP4* to *SAP6* were disrupted by the Ura-blaster protocol in the genetic background of CAF4-2, which is a Δ*ura3* strain isogenic to the clinical isolate SC5314 (7).

We first introduced the *hisG-URA3-hisG* disruption cassette of pMB7 into open reading frames of *SAP4* to *SAP6* as outlined in Fig. 1. Deletion of the three *SAP* genes was initiated by sequential disruption of *SAP6*, followed by disruption of *SAP4* in the background of a Δ*sap6* mutant, and completed by disruption of *SAP5* in the background of a Δ*sap4,6* mutant. Δ*sap* single mutants, i.e., Δ*sap4*, Δ*sap5*, and Δ*sap6*, were constructed at the same time (Table 1). Each disruption step was verified by a PCR-based approach consisting of verifying the correct genomic localization of gene replacements between the disruption cassette and the alleles of interest. To ensure the correct localization of the gene replacement in the disruption of the first allele of each gene, pairs of primers were used. One primer of each pair flanked the region of recombination (primers A, C, and E; Fig. 1), while the other was specific for *hisG*. In each case, positive PCR fragments were observed (data not shown). The positive yeasts are listed in Table 1 as DSY344, DSY432, DSY433, DSY450, and DSY457. For each Ura$^+$ strain in which the first *SAP* allele was disrupted, the Δ*ura3* genetic marker was regenerated by treatment with 5-FOA. Again, a PCR verification of the *URA3* loop-out was performed with pairs of primers, one of which was identical to the one mentioned above (primers A, C, and E; Fig. 1 and 2) and the other of which was specific for each *SAP* gene (primers B, D, and F; Fig. 1 and 2). In this case, the discrimination of 5-FOA-resistant colonies containing both wild-type alleles and disrupted alleles could be accomplished from the lengths of PCR fragments obtained from each of the wild-type alleles and the disrupted alleles. For each type of mutant detected from the analysis of the obtained results, positive strains were selected (listed in Table 1 as DSY345, DSY434, DSY435, DSY451, and DSY458).

For the disruption of the second allele of each gene, the corresponding disruption cassettes of each *SAP* gene were reutilized to transform positive 5-FOA-resistant colonies to Ura$^+$ growth. For each type of resulting mutant, PCR analysis was again performed with pairs of primers, of which one was identical to the one mentioned above (primers A, C, and E; Fig. 1 and 2) and the other was specific for each *SAP* gene (primers B, D, and F; Fig. 1 and 2). Positive colonies were identified as those from which only a single PCR fragment corresponding to the length of the disrupted alleles was detected (strains DSY346, DSY436, DSY437, DSY452, and DSY459 in Table 1). In the case of multiple disruptions, regeneration of the *ura3* marker was achieved by 5-FOA treatment. This was necessary to obtain the Δ*sap6,4* mutant and the final Δ*sap4,5,6* mutants. A PCR analysis of the final Ura$^+$ Δ*sap4,5,6* triple mutant DSY459 is shown in Fig. 2. As shown in this figure, PCR fragments of the expected sizes generated by pair of primers specific for each gene were effectively ob-

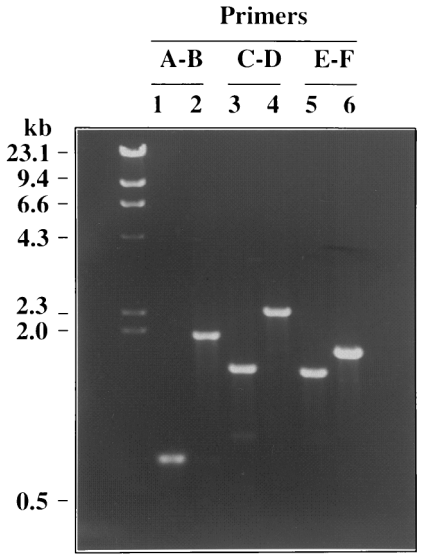

FIG. 2. PCR monitoring of *SAP4*, *SAP5*, and *SAP6* gene disruptions in the Δ*sap4,5,6* triple mutant DSY459. The pairs of PCR primers used for *SAP4*, *SAP5*, and *SAP6* amplification were as follows: A and B, 5′-TTTTC ATTAACAACCAACCATTC-3′ and 5′-GTCCTGGTGGCTTCGTTGC-3′, respectively; C and D, 5′-CATTCAAATGGCAAGCTCG-3′ and 5′-ATAATTA ATCTAAAGTCAAAGTTCTT-3′, respectively; and E and F, 5′-TTCTTCAA ACGTTTTAATTCTCT-3′ and 5′-CATAAATGACTTCAAAATATAAAT-3′, respectively. Lanes 1, 3, and 5 and lanes 2, 4, and 6, products of PCRs using as templates genomic DNA from *C. albicans* wild-type SC5314 and DSY459, respectively. Each DNA has been used with the pairs of primers indicated above the lanes. The expected lengths of PCR products with primer pairs A-B, C-D, and E-F are 0.7, 1.5, and 1.5 kb for SC5314 and 1.9, 2.3, and 1.7 kb for DSY459, respectively. These expected fragment lengths correspond approximately to those observed by ethidium bromide staining after size fractionation by agarose gel electrophoresis. For DSY459, a PCR with primer pairs C-D should yield an additional amplified fragment of 4.7 kb from the disrupted Δ*sap5::hisG-URA3-hisG* allele. This fragment was not detected due to the limit of extension of the *Taq* polymerase.

tained from the parent strain CAF4-2 and DSY459. For example, PCR with primers A and B, specific for *SAP4*, yielded only fragments of 0.7 and 1.9 kb with genomic DNA from SC5314 and DSY459, respectively. No PCR fragments corresponding to those of wild-type alleles of *SAP4*, *SAP5*, and *SAP6* were observed when DSY459 genomic DNA was used as a template. Southern analysis of single Δ*sap4*, Δ*sap5*, and Δ*sap6* mutants and DSY459 was performed with a labeled *hisG* probe to demonstrate that correct integration of the disruption cassette, and no ectopic integration of this cassette, occurred in the yeast genomes. As shown in Fig. 3, single fragments of expected lengths were observed in the Δ*sap4*, Δ*sap5*, and Δ*sap6* single mutants and a combination of the same bands was observed in the Δ*sap4,5,6* triple mutant DSY459.

Growth of DSY459 in complex and BSA-containing media. To verify that the disruption of *SAP4* to *SAP6* did not affect growth, Ura^+ strain DSY459 and parent strain SC5314 were incubated in YPD medium at 37°C and their growth was measured by optical density. As shown in Fig. 4, no significant change of growth rate could be observed, thus demonstrating that the disruption of *SAP4* to *SAP6* in *C. albicans* had no effect on growth in complex medium. The same experiment was repeated with a synthetic minimal medium, and no growth differences between the wild type and DSY459 were observed (data not shown).

However, when DSY459 was incubated in a medium containing BSA as the sole nitrogen source (a growth condition known to induce *SAP2* in most *C. albicans* strains), no increase

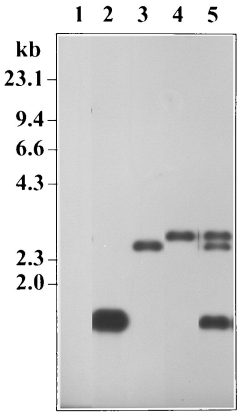

FIG. 3. Southern analysis of genomic DNA of Δ*sap* mutants. Samples (5 μg) of genomic DNA from *C. albicans* wild-type SC5314 (lane 1) and Δ*sap6* (DSY349), Δ*sap4* (DSY438), Δ*sap5* (DSY452), and Δ*sap4,5,6* (DSY459) strains (lanes 2 to 5, respectively) were digested with *Eco*RI and separated by agarose gel electrophoresis and transferred to a GeneScreen Plus membrane. The membrane was probed with a labeled *S. typhimurium hisG* probe corresponding to a 0.9-kb *Bam*HI-*Bgl*II fragment from pMB7. Expected sizes for *Eco*RI fragments hybridizing with the *hisG* probe were predicted to be 1.5 kb for DSY349 (homozygote Δ*sap6::hisG*/Δ*sap6::hisG* alleles), 2.4 kb for DSY438 (homozygote Δ*sap4::hisG*/Δ*sap4::hisG* alleles), and 2.6 kb for DSY452 (Δ*sap5::hisG*/Δ*sap5::hisG-URA3-hisG* alleles which cannot be distinguished by *Eco*RI digestion). The sizes of the three *Eco*RI bands should be combined in DSY459. These sizes correspond to those detected for the corresponding individual *C. albicans* Δ*sap* strains. As expected, no signal was detected for SC5314, since no *hisG* sequence should be present in the genome of this yeast.

of optical density was measured during 72 h of observation for this mutant. The parent strain, SC5314, grew normally under identical conditions (Fig. 5A). The growth defect of DSY459 correlated with an absence of proteolytic activity (Fig. 5B), although such activity was found in the culture supernatant of the parent strain. However, growth of DSY459 in this medium could still be observed only after a prolonged incubation of more than 5 days. Thus, although expression of *SAP4* to *SAP6* in BSA-containing medium has not been detected by others (10), the presence of these genes seemed to be necessary for normal growth in this medium.

Δ*sap4,5,6* mutant DSY459 shows attenuated virulence in animal models. The ability of DSY459 to cause systemic infection was investigated in both the murine and the guinea pig

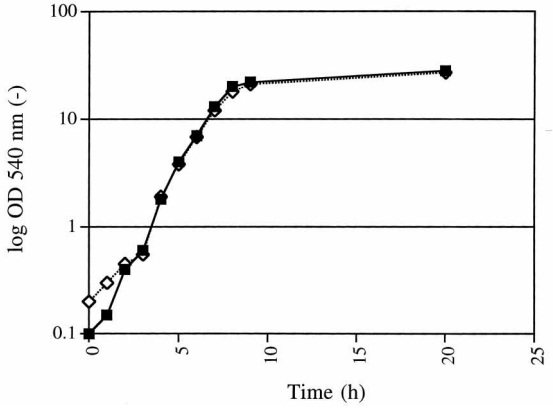

FIG. 4. Growth of Sap-deficient mutants in YPD medium. Growth was recorded under constant agitation by measurement of optical density (OD). For medium composition and growth conditions, see reference 12. ■, SC5314; ◇, DSY459.

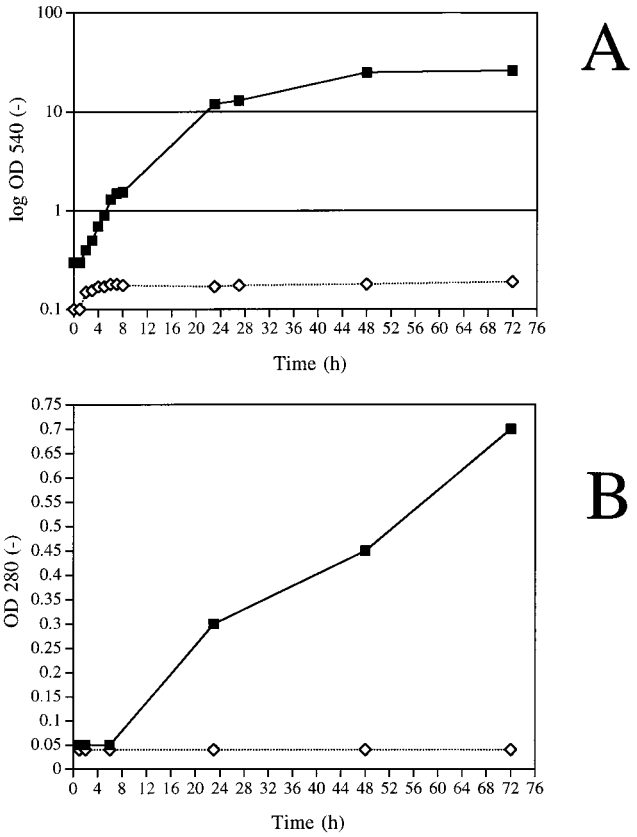

FIG. 5. Growth of Sap-deficient mutants in BSA-containing medium. Growth was recorded by measurement of optical density (OD) (A). Proteolytic activity was measured for each culture (B). For medium composition and growth conditions, see reference 12. ■, SC5314; ◇, DSY459.

models of systemic candidiasis and compared with the virulence of the wild-type strain SC5314. In both models, DSY459 exhibited a significant reduction of virulence compared to that of the isogenic parent ($P \leq 0.001$; Fig. 6). When guinea pigs were infected with 4×10^4 CFU of SC5314 per g, 96% of the animals died within 14 days of infection, and the mean survival time was 6.1 days (Table 2 and Fig. 6A). In contrast, guinea pigs infected with DSY459 had a higher mean survival time (9.4 days), and only 60% of the animals had died by 14 days after inoculation. Reduced virulence was also reflected by a significant lower CFU count for infected organs (kidneys) and samples of skin (Table 2). When mice were infected with 4×10^3 and 8×10^3 CFU of SC5314 per g, 55 and 81% of the animals died within 14 days of infection, whereas DSY459 killed only 11 and 5% of animals in the same time period, respectively (Tables 3 and 4; Fig. 6B and C). The mean CFU in kidneys decreased significantly in animals infected with 4×10^3 CFU of DSY459 compared with that for animals infected with the wild type (Table 3).

DISCUSSION

One important prerequisite for the testing of putative virulence factors in *C. albicans* is the construction of strains with single or combined defects in genes encoding these factors. Targeted gene disruption is the most reliable tool to generate mutants isogenic to a wild-type parent. The introduction of mutations in putative virulence factors by this method should not lead to growth defects in either complex or defined media. Such a requirement is important for the testing of *C. albicans* mutants in animal models, since it is known that growth defects of *C. albicans* mutants can have a great influence on virulence (16, 37). The proteinase-negative mutants generated in this study by the targeted disruption of several *SAP* genes caused no apparent growth deficiency in vitro and allowed the role(s) of these Sap isoenzymes in virulence to be properly addressed. This situation is quite different from previous studies examining the role of secreted proteinases in virulence with proteinase-negative mutants, since those strains were isolated by random mutagenesis (17, 19, 30). The mutations rendering these yeasts deficient in the production of proteinase activity in protein-rich medium were not mapped to any known genetic loci. Moreover, random mutagenesis is likely to alter other loci important for virulence. Nevertheless, the consistency of find-

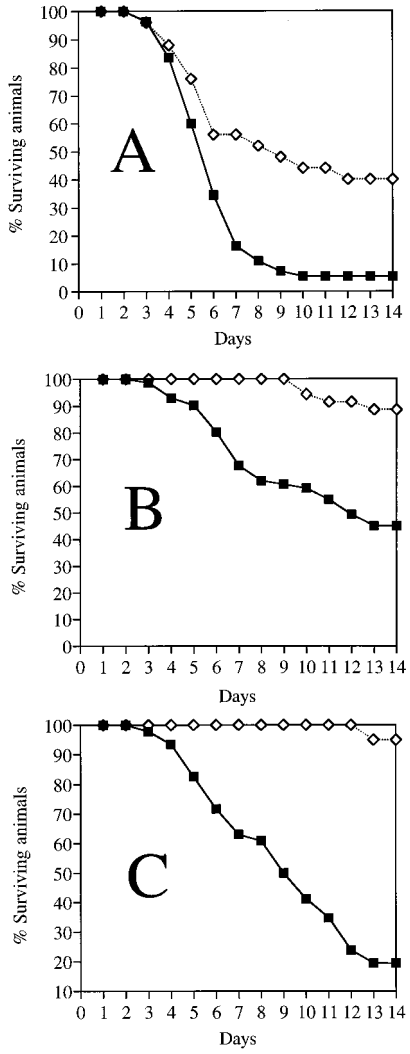

FIG. 6. Survival of animals infected with *C. albicans*. The percentages of animals surviving over a period of 14 days after intravenous infection are shown. (A) Infection of guinea pigs with 4×10^4 CFU of wild-type strain SC5314 (n = 55) and DSY459 (n = 25) per g of body weight. (B) Infection of Swiss mice with 4×10^3 CFU of wild-type SC5314 (n = 71) and DSY459 (n = 35) per g of body weight. (C) Infection of Swiss mice with 8×10^3 CFU of wild-type SC5314 (n = 46) and DSY459 (n = 20) per g of body weight. The three survival curves for DSY459 were shown to be significantly different ($P \leq 0.001$) from those obtained for SC5314. ■, SC5314; ◇, DSY459.

TABLE 2. Virulence of SC5314 and of the mutant DSY459 during systemic infection of guinea pigs with an inoculum of 4×10^4 CFU

Strain	No. of animals	Mean survival time (days)[a] ± SD	% of animals surviving at day 14	Mean log CFU in organs/g of tissue wt ± SD		
				Skin	Liver	Kidney
SC5314	55	6.1 ± 2.1	4	3.8 ± 1.3	2.2 ± 2.0	5.4 ± 1.0
DSY459	25	9.4 ± 4.0[b]	40	2.3 ± 1.8[c]	0.8 ± 1.5[d]	3.5 ± 2.2[c]

[a] Survivors at day 14 were given a survival time of 14 days.
[b] Significantly different from values for SC5314 ($P \leq 0.001$).
[c] CFU values significantly different ($P \leq 0.001$) from those obtained with SC5314.
[d] CFU values not significantly different ($P = 0.06$) from those obtained with SC5314.

ings from these studies suggested an important role for secreted acid proteinases in the virulence of *C. albicans*. The only report of a study using a proteinase-deficient mutant created by targeted gene disruption is from Togni et al. (39). This mutant was created in a *Candida tropicalis* strain in which *SAPT1*, whose product is the main proteinase secreted when this yeast grows on protein-rich medium (38), was deleted. This mutant was slightly less virulent than the wild-type parent. However, since a genetic marker (*ade2*) had been introduced previously into this yeast by UV random mutagenesis (34, 36), the genetic background was again not completely characterized, and the role of Sapt1 in virulence could not be completely elucidated. Proteinase-deficient mutants of *Aspergillus fumigatus* have also been created by targeted disruptions of corresponding genes, and thus a direct comparison of virulence between the wild-type fungus and the mutants could be made. However, neither *A. fumigatus* mutants deficient in the alkaline protease Alp and metalloprotase Mep nor mutants deficient in the secreted aspartyl proteinase Pep were less virulent than the wild-type fungus in the animal model utilized for experimental infections (14, 20, 29). These results contrast with the role of Sap isoenzymes of *C. albicans* in virulence described here.

Study of the role of secreted acid proteinases in the virulence of *C. albicans* is complicated by the fact that these isoenzymes constitute a gene family with at least nine known members. The characterization and regulation of genes *SAP1* to *SAP7* have been reported and are discussed above (see the introduction). *SAP8* and *SAP9* have been isolated very recently (21), and only preliminary data on their in vitro regulation have been available up to now (11). Phylogenetic analysis of this multigene family showed that genes *SAP1*, *SAP2*, and *SAP3* and genes *SAP4*, *SAP5*, and *SAP6* were divided into two different subgroups (22). Therefore, disruption of these genes and virulence testing were examined separately for representatives of each subgroup. The attenuated virulence of strains lacking the subgroup comprising *SAP1*, *SAP2*, and *SAP3* was reported by Hube et al. (12), while analysis of the subgroup comprising *SAP4*, *SAP5*, and *SAP6* is documented in the present study. *SAP4* to *SAP6* not only constitute a distinct gene subfamily within the *SAP* multigene family, but also are coregulated by similar growth conditions and during morphogenesis of *C. albicans*. Hube et al. (10) showed that transcription of *SAP4* to *SAP6* was stimulated during the yeast-to-hypha change of cell morphology in serum at neutral pH. More recently, White and Agabian identified *SAP5* and *SAP6* mRNAs among this group of *SAP* genes during the same morphological change, with *SAP6* mRNA being more abundant than *SAP5* mRNA. Apparently, no *SAP4* mRNA could be detected (40).

Targeted disruptions of *SAP4*, *SAP5*, and *SAP6* were verified by different techniques, including PCR analysis and Southern blotting. Within the limits of analysis of these mutants, it can be assumed that the use of the same technique for the sequential disruption of these genes did not result in ectopic integration of the gene disruption cassettes (Fig. 3). It is still possible, though, that undetected events at the level of the genome resulting from mitotic crossing-over or gene conversions took place during the construction of the mutants described in this study. However, since *C. albicans* is diploid, these events can take place at any locus during the growth of this yeast. The transformation procedure itself and 5-FOA treatment can be mutagenic as well. These events could have created alterations affecting the virulence of the yeast strains. At present, we cannot provide experimental evidence that such events have occurred in the homozygous deletion mutants constructed here, but the likelihood that the manipulations required to construct these mutants would have created uncontrolled mutations in other virulence loci can be considered low. For example, no growth defect of the mutants constructed here was observed in complex medium, and the slow growth of the Δ*sap4,5,6* triple mutant DSY459 in protein-containing medium could be reversed by the addition of ammonium salts (data not shown), which argue against the presence of undetected mutations.

The virulence of DSY459 was tested in the murine and the guinea pig models of systemic infection. This mutant showed attenuated virulence in both models compared to that of the parent, as judged by longer survival times and lower CFU counts for organs recovered from infected animals. The decreased virulence of DSY459 was more pronounced in mice than in guinea pigs and was relatively independent of the size of the inoculum injected into the animals, unlike the case for the parent strain. Attenuated virulence in the same animal

TABLE 3. Virulence of SC5314 and of the mutant DSY459 during systemic infection of mice with an inoculum of 4×10^3 CFU

Strain	No. of animals	Mean survival time (days) ± SD	% of animals surviving at day 14	Mean log CFU in kidney/g of tissue wt ± SD
SC5314	71	10.6 ± 3.8	45	5.1 ± 2.2
DSY459	35	13.6 ± 1.0[a]	89	2.6 ± 2.7[b]

[a] Significantly different from values for SC5314 ($P \leq 0.001$).
[b] CFU values significantly different ($P \leq 0.001$) from those obtained with SC5314.

TABLE 4. Virulence of SC5314 and of the mutant DSY459 during systemic infection of mice with an inoculum of 8×10^3 CFU

Strain	No. of animals	Mean survival time (days) ± SD	% of animals surviving at day 14	Mean log CFU in kidney/g of tissue weight ± SD
SC5314	46	9.4 ± 3.2	19	5.9 ± 1.0
DSY459	20	14.0 ± 0.2[a]	95	4.3 ± 2.9[b]

[a] Significantly different from values for SC5314 ($P \leq 0.001$).
[b] CFU values not significantly different ($P = 0.325$) from those obtained with SC5314.

models has been observed also with Δ*sap1*, Δ*sap2*, and Δ*sap3* single mutants (12). Parallel experiments with these mutants and DSY459 were performed using the same batch of animals and the same conditions, and DSY459 was always less virulent than the other mutants. It will be necessary in the future to use Δ*sap4*, Δ*sap5*, or Δ*sap6* single mutants in animal experiments to clarify the contribution of each isoenzyme of this subgroup to virulence. Single Δ*sap* mutants and different combinations of gene deletions (i.e., Δ*sap4,5* and Δ*sap5,6*) within this subgroup have already been constructed for that purpose (data not shown). It is also conceivable to extend the construction of deletion mutants by combining gene deletions in the *SAP1* to *SAP3* and the *SAP4* to *SAP6* subgroups. It could be expected that mutants with combinations of deletions in these two subgroups would be even less virulent than the mutants described here and by Hube et al. (12).

The contribution of each Sap isoenzyme to virulence could be assayed likewise by reintroducing into the *C. albicans* genome each of the genes encoding these proteins. Such restoration experiments could also help to exclude the possibility that other virulence loci have been altered during construction of DSY459, as mentioned above. There are, however, several technical problems behind this approach. First, each of the *SAP* genes should be reintroduced at each allele of its corresponding genome locus to restore its wild-type gene copy number. A random integration of these genes in the yeast genome could potentially result in other alterations, which could in turn affect the virulence of the strain. This is exactly the type of situation which this study attempted to avoid by establishment of a set of isogenic strains with mutations in defined loci. Second, no information on the minimal functional promoter length of the *SAP4* to *SAP6* genes is available yet, and the reintroduction of one of these genes may therefore lead to wrong interpretations. Third, the restoration of *SAP* loci is limited by the availability of only a single *ura3* genetic marker in the *C. albicans* strain used in this study.

A surprising phenotype of the Δ*sap4,5,6* triple mutant DSY459 was that it exhibited much-delayed growth compared to that of the wild type in a medium containing BSA as the sole nitrogen source. Since *SAP2* has been shown to be induced under such conditions and Sap2 has been detected under these growth conditions (40), it suggests that Sap4 to Sap6 may be required at some step in the process of induction of *SAP2*. One possible explanation for the growth defect phenotype of DSY459 in BSA-containing medium is that when *C. albicans* is switched to this medium, the absence of immediately available nitrogen sources results in a transient starvation which could induce the genes *SAP4* to *SAP6* more readily than other proteinases. Since DSY459 failed to grow on BSA-containing medium, it is tempting to correlate this phenotype in vitro with the observed attenuation of virulence in vivo. Hube et al. (12) showed that the Δ*sap2* single mutant also exhibited a severe growth defect in BSA-containing medium and that the virulence of this mutant was attenuated, although to a lesser degree than that observed for DSY459. However, such correlations may be simplistic, since Δ*sap1* and Δ*sap3* mutants showed attenuated virulence in the mouse and guinea pig infection models but could still grow on BSA-containing medium (12). It is still not clear at which stage of infection one or another Sap isoenzyme may be important for the normal progression of disseminated infection. Since some *SAP* genes are known not to be coregulated in vitro, one might expect that this might also apply in vivo during infection. To dissect the role of each Sap isoenzyme in infection, future studies should be directed to the investigation of differential expression of *SAP* genes directly from tissue sections of infected animals. Sap isoenzymes may also be important in the initial colonization of mucosal surfaces and their penetration, and thus types of infection models other than those used here (e.g., mucosal infections) may be informative.

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