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Tetracycline-Inducible Expression of Individual Secreted Aspartic Proteases in *Candida albicans* Allows Isoenzyme-Specific Inhibitor Screening▽

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The yeast *Candida albicans* possesses a gene family that encodes secreted aspartic proteases (Saps), which are important for the virulence of this human fungal pathogen. Inhibitors of the Saps could therefore be used as novel antimycotic agents for the treatment of *C. albicans* infections. In the present study, we established a bioassay which allows testing of the activity of potential protease inhibitors against specific Sap isoenzymes by their ability to inhibit protease-dependent growth of *C. albicans*. In a medium containing bovine serum albumin (BSA) as the sole source of nitrogen, *C. albicans* specifically expresses the Sap2p isoenzyme, which degrades the BSA and thereby enables the fungus to grow. As the other *SAP* genes are not significantly expressed under these conditions, mutants lacking *SAP2* are unable to utilize BSA as a nitrogen source and cannot grow in such a medium. To investigate whether forced expression of *SAP* genes other than *SAP2* would also allow growth on BSA, we constructed a set of strains expressing each of the 10 *SAP* genes from a tetracycline-inducible promoter in a *sap2*Δ mutant background. Expression of Sap1p, Sap2p, Sap3p, Sap4p, Sap5p, Sap6p, Sap8p, and a C-terminally truncated, secreted Sap9p restored the growth of the *sap2*Δ mutant with different efficiencies. This set of strains was then used to test the activities of various aspartic protease inhibitors against specific Sap isoenzymes by monitoring growth on BSA in the presence of the inhibitors. While pepstatin blocked the activity of all of the Saps tested, the human immunodeficiency virus protease inhibitors ritonavir and saquinavir inhibited growth of the strains expressing Sap1p to Sap3p and Sap1p, respectively, but not that of strains expressing other Saps. Therefore, the strain set can be used to test the activity of new protease inhibitors against individual *C. albicans* Sap isoenzymes by their ability to block the growth of the pathogen.

Candida albicans is a major human fungal pathogen which can cause superficial, as well as life-threatening systemic, mycoses in immunocompromised patients (35). Potent drugs for the treatment of *C. albicans* infections are available; however, problems with the toxicity of amphotericin B and the development of resistance to the other drugs have stimulated the search for new pharmaceuticals with different drug targets (8). An alternative approach to cure *C. albicans* infections could be the inhibition of specific pathogenicity-related factors of the fungal cells, which should decrease their virulence and help the remaining host defense mechanisms to successfully combat the pathogen (38). Secreted aspartic proteases (Saps) are known virulence factors of *C. albicans*, and many investigations performed in the last decades have revealed that these enzymes contribute to the pathogenicity of *C. albicans* in different ways. The Saps can provide nutrients by degrading host proteins but also support adherence to host surfaces and invasion of tissue barriers (12, 32, 46, 52). They are encoded by a family of 10 homologous genes which are differentially regulated during infection, indicating that the individual isoenzymes fulfill specific functions (33, 34, 43, 47). The hypothesis that *C. albicans* infections can be attenuated by inhibition of the Saps was supported to some extent in animal models by treatment with the aspartic protease inhibitor pepstatin. Whereas a protective role in mucosal and peritoneal infections was demonstrated (13, 27), results obtained in systemic-infection models were contradictory, a finding which was partly attributed to the inappropriate pharmacokinetics of this compound (16, 18, 42, 56). Nevertheless, the idea of using protease inhibitors in the treatment of candidiasis has received new attention in recent years. It was observed that highly active antiretroviral therapy, which includes human immunodeficiency virus (HIV) aspartic protease inhibitors, coincided with decreasing numbers of *C. albicans* infections in HIV and AIDS patients (10, 20, 21, 36, 55). A direct inhibitory effect of HIV protease inhibitors on *C. albicans* was supported by experimental in vitro and in vivo infection models. Using concentrations which are nontoxic for the fungal cells, some of the HIV protease inhibitors decreased *C. albicans* adherence and also attenuated mucosal infection (3, 7, 9, 26). However, the limited specificity of these inhibitors

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TABLE 1. *C. albicans* strains used in this study

Strain(s)	Parent	Relevant genotype or characteristics[a]	Reference
SC5314		Wild type	19
SAP2MS1A	SC5314	*sap2-1*::*SAT1-FLIP*/*SAP2-2*	This study
SAP2MS1B	SC5314	*SAP2-1*/*sap2-2*::*SAT1-FLIP*	This study
SAP2MS2A	SAP2MS1A	*sap2-1*::*FRT*/*SAP2-2*	This study
SAP2MS2B	SAP2MS1B	*SAP2-1*/*sap2-2*::*FRT*	This study
SAP2MS3A	SAP2MS2A	*sap2-1*::*FRT*/*sap2-2*::*SAT1-FLIP*	This study
SAP2MS3B	SAP2MS2B	*sap2-1*::*SAT1-FLIP*/*sap2-2*::*FRT*	This study
SAP2MS4A	SAP2MS3A	*sap2-1*::*FRT*/*sap2-2*::*FRT*	This study
SAP2MS4B	SAP2MS3B	*sap2-1*::*FRT*/*sap2-2*::*FRT*	This study
SAP2KS3A	SAP2MS4A	*sap2-1*::*FRT*/*sap2-2*::P_{SAP2-2}-*SAP2-2*-T_{ACT1}-*SAT1-FLIP*	This study
SAP2KS3B	SAP2MS4B	*sap2-1*::*FRT*/*sap2-2*::P_{SAP2-2}-*SAP2-2*-T_{ACT1}-*SAT1-FLIP*	This study
SAP2KS4A	SAP2KS3A	*sap2-1*::*FRT*/*sap2-2*::P_{SAP2-2}-*SAP2-2*-T_{ACT1}-*FRT*	This study
SAP2KS4B	SAP2KS3B	*sap2-1*::*FRT*/*sap2-2*::P_{SAP2-2}-*SAP2-2*-T_{ACT1}-*FRT*	This study
SAP1ex4A and -B	SAP2MS4B	*ADH1*/*adh1*::P_{tet}-*SAP1*	This study
SAP2ex4A and -B	SAP2MS4B	*ADH1*/*adh1*::P_{tet}-*SAP2-1*	This study
SAP3ex4A and -B	SAP2MS4B	*ADH1*/*adh1*::P_{tet}-*SAP3*	This study
SAP4ex4A and -B	SAP2MS4B	*ADH1*/*adh1*::P_{tet}-*SAP4*	This study
SAP5ex4A and -B	SAP2MS4B	*ADH1*/*adh1*::P_{tet}-*SAP5*	This study
SAP6ex4A and -B	SAP2MS4B	*ADH1*/*adh1*::P_{tet}-*SAP6-2*	This study
SAP7ex4A and -B	SAP2MS4B	*ADH1*/*adh1*::P_{tet}-*SAP7*	This study
SAP8ex4A and -B	SAP2MS4B	*ADH1*/*adh1*::P_{tet}-*SAP8*	This study
SAP9ex4A and -B	SAP2MS4B	*ADH1*/*adh1*::P_{tet}-*SAP9*	This study
SAP10ex4A and -B	SAP2MS4B	*ADH1*/*adh1*::P_{tet}-*SAP10*	This study
SAP9ΔC1ex4A and -B	SAP2MS4B	*ADH1*/*adh1*::P_{tet}-$SAP9^{\Delta C520}$	This study
SAP9ΔC2ex4A and -B	SAP2MS4B	*ADH1*/*adh1*::P_{tet}-$SAP9^{\Delta C493}$	This study
SAP10ΔC1ex4A and -B	SAP2MS4B	*ADH1*/*adh1*::P_{tet}-$SAP10^{\Delta C427}$	This study
SAP10ΔC2ex4A and -B	SAP2MS4B	*ADH1*/*adh1*::P_{tet}-$SAP10^{\Delta C387}$	This study

[a] *SAT1-FLIP* denotes the *SAT1* flipper cassette.

for the Saps and the finding that they act on only some of the different isoenzymes are expected to prevent their application against disseminated disease (7). Since different Sap isoenzymes contribute to the progression of *C. albicans* infections, new Sap inhibitors should block the action of as many of the Saps as possible in order to paralyze the fungus most efficiently. Analysis of the inhibitory effect of protease inhibitors on individual Saps requires the expression of these enzymes under in vitro conditions. Some of the Saps have been expressed as recombinant proteins in the heterologous hosts *Escherichia coli* (24), *Saccharomyces cerevisiae* (45), and *Pichia pastoris* (6), but most of the Saps cannot easily be expressed in the native host under laboratory conditions. It has long been known that *C. albicans* secretes protease during growth in a medium containing a protein, e.g., bovine serum albumin (BSA), as the sole source of nitrogen, and growth in such media can be blocked by the addition of pepstatin (41, 46). It was later found that of all of the members of the Sap family, only the Sap2p isoenzyme is significantly expressed under these conditions and inactivation of the *SAP2* gene rendered the mutants unable to grow on BSA (22, 23, 48). Therefore, it seemed possible that forced expression of other members of the *SAP* gene family in a *sap2*Δ mutant background might bypass the requirement for *SAP2* and enable the cells to grow under these conditions. This, in turn, would allow testing of the activity of protease inhibitors against specific *C. albicans* Sap isoenzymes by assessment of their ability to block the growth of strains expressing the corresponding *SAP* gene. In the present work, we generated a set of *C. albicans* reporter strains expressing individual *SAP* genes from a tetracycline-inducible promoter and demonstrated the feasibility of this approach.

MATERIALS AND METHODS

Strains and growth conditions. The *C. albicans* strains used in this study are listed in Table 1. All strains were stored as frozen stocks with 15% glycerol at −80°C. The strains were routinely grown in YPD medium (10 g yeast extract, 20 g peptone [BBL Trypticase Peptone; Becton Dickinson, Sparks, MD], and 20 g glucose per liter) at 30°C. For solid medium, 1.5% agar was added before autoclaving. To select nourseothricin-resistant (Nou^r) transformants, 200 μg ml^{-1} of nourseothricin (Werner Bioagents, Jena, Germany) was added to YPD agar. To obtain nourseothricin-sensitive (Nou^s) derivatives in which the *SAT1* flipper was excised by FLP-mediated recombination, transformants were cultivated for 6 h in YPM medium (10 g yeast extract, 20 g peptone, and 20 g maltose per liter) without selective pressure to induce the *MAL2* promoter. One hundred to 200 cells were spread on YPD plates containing 20 μg ml^{-1} nourseothricin and grown for 2 days at 30°C. Nou^s clones were identified by their small colony size and confirmed by restreaking on YPD plates containing 100 μg ml^{-1} nourseothricin as described previously (40).

To test *C. albicans* transformants expressing the different *SAP* genes for growth on BSA as the sole source of nitrogen, strains were pregrown overnight in SD medium (6.7 g yeast nitrogen base without amino acids [YNB; Bio 101, Vista, CA] and 20 g glucose per liter) at 30°C and then diluted 1:100 in YCB-BSA medium (23.4 g yeast carbon base and 4 g BSA per liter). Depending on the expressed Sap, the pH of YCB-BSA medium was adjusted to 4.0 or 5.0 and the culture was incubated at 30°C or 37°C (pH 4.0 and 30°C for expression of *SAP1*, *SAP2*, *SAP3*, and *SAP8* and pH 5.0 and 37°C for expression of *SAP4*, *SAP5*, *SAP6*, and *SAP9*). To induce the expression of *SAP* genes from the Tet promoter, 50 μg ml^{-1} doxycycline was added to cultures incubated at pH 4.0 and 30°C and 25 μg ml^{-1} was added to cultures incubated at pH 5.0 and 37°C because 50 μg ml^{-1} doxycycline had a slight adverse effect on growth of the cells under the latter conditions. The aspartic protease inhibitor pepstatin A (Sigma, Deisenhofen, Germany) was added at a concentration of 7.3 μM, and the HIV protease inhibitors ritonavir (Abbott, Chicago, IL) and saquinavir (Roche, Welwyn Gar-

TABLE 2. Primers used in this study

Primer	Sequence[a]
SAP2M	5′-CTGCCTTGACCGCGGATGAAGGGGTG-3′
SAP2N	5′-GGGCCCATGACGAGCTCTAGTTTGAGC-3′
SAP2P1	5′-TTGTTGGGCCCGTTGTCAATTTATGGGCCGATCTG-3′
SAP2P5	5′-ATATAGGGCCCGCATTTGAATAAACGGCATC-3′
SAP2E	5′-CCACCCCTTCATCTGCAGTCAAGGCAGAAATAC-3′
SAP1ex1	5′-TCTAGTCGACA**ATG**TTTTTAAAGAATATTTTCAT-3′
SAP1ex2	5′-AGTAGGGATCC**CTA**GGTAAGAGCAGCAATGTTTGAAGC-3′
SAP2ex1	5′-ACCAGTCGACA**ATG**TTTTTAAAGAATATTTTCAT-3′
SAP2ex2	5′-ACCCCGGATCC**TTA**GGTCAAGGCAGAAATACTGGAAGC-3′
SAP3ex1	5′-ATATGTCGACA**ATG**TTTTTAAAAAATATCTTTAT-3′
SAP3ex2	5′-TGTACGGATCC**CTA**AGTAAGAGCAGCAATGTTAGAAGC-3′
SAP4ex1	5′-CTCACTCGAGA**ATG**TTCTTACAAAATATCTTGAG-3′
SAP4ex2	5′-ACCAAGGATCC**CTA**ATTAATAGCAACAATGTTAGACTG-3′
SAP56ex1	5′-CTCAGTCGACA**ATG**TTCTTGAAAAATATCTTGAG-3′
SAP5ex2	5′-GTCAAGGATCC**TTA**ATTAATAGCAACAATGTCAGACTCGG-3′
SAP6ex2	5′-ACCAAGGATCC**CTA**ATTAATAGCACCAATGTTAGACTC-3′
SAP7ex1	5′-GAAGGTCGACA**ATG**CAAAGAGTATTAGAGTTATT-3′
SAP7ex2	5′-TTCTAGGATCC**TTA**CTCGATAGGAACAACGGCATGGTT-3′
SAP8ex1	5′-AACCGTCGACA**ATG**GTCTCCATTATTACTTTTAC-3′
SAP8ex2	5′-GAACAGGATCC**CTA**TAAAGTAGAAATACTTGAAGAAGT-3′
SAP9ex1	5′-TTTCGTCGACA**ATG**AGACTCAATTCTGTTGCGTT-3′
SAP9ex2	5′-TCTCTGGATCC**TTA**AACCAAAACATAGTAGGATATCAA-3′
SAP9ex3	5′-TATAGGATCC**TTA**TGAATGACGTGTGCTGGTAC-3′
SAP9ex4	5′-TATAGGATCC**TTA**ACCAATGACTTCAATCGATTCC-3′
SAP10ex1	5′-TATAGTCGACA**ATG**GACCTAGTAATAATGAATTT-3′
SAP10ex2	5′-ATTCTGGATCC**CTA**TATAATATGTATATACACGAGGAA-3′
SAP10ex3	5′-TATAGGATCC**TTA**GCTAGTAGTGTTCTTGTCCATGTTC-3′
SAP10ex4	5′-TATAGGATCC**TTA**GTTTAAAATCTCTTCAATATCTTCGTCTTC-3′
SAP5CF	5′-CGGGGATCCATGGTTAACTGTCGGTTTAAG-3′
SAP5CR	5′-CGCTCGAGATTAATAGCAACAATGT-3′

[a] Restriction sites introduced into the primers are underlined; the start and stop (reverse sequence) codons of the *SAP* genes are in bold.

den City, United Kingdom) were used at a concentration of 100 μM (0.125% dimethyl sulfoxide end concentration).

Plasmid constructions. To generate a *SAP2* deletion construct, an ApaI-SalI fragment from plasmid pSFL213 containing 1.1 kb of *SAP2* upstream sequences (47) was used to replace the *OPT1* upstream region in ApaI/XhoI-digested plasmid pOPT1M3 (40), resulting in plasmid pSAP2MS1. A *SAP2* downstream fragment was obtained by PCR with primers SAP2M and SAP2N (Table 2 contains the sequences of the primers used in this study), digested at the introduced SacII and SacI sites, and substituted for the *OPT1* downstream region in pSAP2MS1, yielding plasmid pSAP2MS2, in which the *SAT1* flipper cassette is flanked by *SAP2* upstream and downstream sequences (Fig. 1A). For reinsertion of a functional *SAP2* copy at its original locus in *sap2*Δ mutants, we first ligated a KpnI-SalI fragment from plasmid pMEP1M4 (4), which contained the transcription termination sequence of the *ACT1* gene, together with an XhoI-PstI fragment from pSFS2 (40) containing part of the *SAT1* flipper cassette into KpnI/PstI-digested pSAP2MS2 to generate pSAP2KS1. An ApaI-BamHI fragment containing the coding region and 0.3 kb of upstream sequences of the *SAP2-1* allele was then amplified with primers SAP2P5 and SAP2ex2 and cloned into ApaI/BglII-digested pSAP2KS1 to result in pSAP2KS2. Finally, the complete coding region and 2 kb of upstream sequences of the *SAP2-2* allele were PCR amplified with primers SAP2P1 and SAP2E by using genomic DNA from the heterozygous *sap2-1*Δ/*SAP2-2* mutant SAP2MS2A as a template. The PCR product was digested with ApaI/XbaI and with XbaI/BglII, and the two fragments were ligated together into ApaI/BglII-digested pSAP2KS2 to result in pSAP2KS3 (Fig. 1B).

To express the individual *SAP* genes from a Tet-inducible promoter (P_{tet}) (37), the coding region of each of the *SAP* genes was amplified from genomic DNA of strain SC5314 by PCR with the following primer pairs: SAP1ex1 and SAP1ex2 for *SAP1*, SAP2ex1 and SAP2ex2 for *SAP2* (allele 1), SAP3ex1 and SAP3ex2 for *SAP3*, SAP4ex1 and SAP4ex2 for *SAP4*, SAP56ex1 and SAP5ex2 for *SAP5*, SAP56ex1 and SAP6ex2 for *SAP6* (allele 2), SAP7ex1 and SAP7ex2 for *SAP7*, SAP8ex1 and SAP8ex2 for *SAP8*, SAP9ex1 and SAP9ex2 for *SAP9*, and SAP10ex1 and SAP10ex2 for *SAP10*. Truncated *SAP9* and *SAP10* alleles were generated by the introduction of a stop codon within the coding region, in front of the putative cleavage sites for addition of the glycosylphosphatidylinositol (GPI) anchor. Two different truncations (ΔC1 and ΔC2) were produced for each of the *SAP9* ($SAP9^{\Delta C520}$ and $SAP9^{\Delta C493}$) and *SAP10* ($SAP10^{\Delta C427}$ and $SAP10^{\Delta C387}$) genes by PCR with the primer pairs SAP9ex1/SAP9ex3, SAP9ex1/SAP9ex4, SAP10ex1/SAP10ex3, and SAP10ex1/SAP10ex4, respectively. The PCR products were digested at the restriction sites introduced in front of the start codon and behind the stop codon and cloned between P_{tet} and T_{ACT1} in a SalI/BglII-digested derivative of plasmid pNIM1 (37), resulting in plasmids pSAP1ex4, pSAP2ex4, pSAP3ex4, pSAP4ex4, pSAP5ex4, pSAP6ex4, pSAP7ex4, pSAP8ex4, pSAP9ex4, pSAP10ex4, pSAP9ΔC1ex4, pSAP9ΔC2ex4, pSAP10ΔC1ex4, and pSAP10ΔC2ex4.

***C. albicans* transformation.** *C. albicans* strains were transformed by electroporation (25) with the following gel-purified, linear DNA fragments: the ApaI-SacI fragment from pSAP2MS2 to inactivate the *SAP2* gene in strain SC5314, the ApaI-SacI fragment from pSAP2KS3 to reinsert the *SAP2-2* allele in the *sap2*Δ deletion mutants, and the ApaI-SacII fragments from plasmids pSAP1ex4, pSAP2ex4, pSAP3ex4, pSAP4ex4, pSAP5ex4, pSAP6ex4, pSAP7ex4, pSAP8ex4, pSAP9ex4, pSAP10ex4, pSAP9ΔC1ex4, pSAP9ΔC2ex4, pSAP10ΔC1ex4, and pSAP10ΔC2ex4 to integrate the individual P_{tet}-*SAP* fusions into the *ADH1* locus of the *sap2*Δ mutant SAP2MS4B. Single-copy integration of all constructs was confirmed by Southern hybridization.

Isolation of genomic DNA and Southern hybridization. Genomic DNA from *C. albicans* strains was isolated as described previously (28). DNA (15 μg) was digested with appropriate restriction enzymes, separated in 1% (wt/vol) agarose gels and, after ethidium bromide staining, transferred by vacuum blotting onto nylon membranes and fixed by UV cross-linking. Southern hybridization with enhanced-chemiluminescence (ECL)-labeled probes was performed with the Amersham ECL Direct Nucleic Acid Labeling and Detection System (GE Healthcare, Braunschweig, Germany) according to the instructions of the manufacturer.

Generation of antibodies for Western blot experiments. A fusion protein consisting of glutathione *S*-transferase (GST) and the C-terminal 148 amino acids of Sap5p (GST-Sap5F) was prepared by using the GST gene fusion system from Amersham. A *SAP5* DNA fragment that encodes the C-terminal part of the Sap5 protein from Leu^{271} to Asn^{418} was obtained by PCR with primers SAP5CF and SAP5CR. Plasmid pCA5 (31) was used as the DNA template for the PCR. The amplified fragment was cloned via the introduced BamHI and XhoI sites into BamHI/XhoI-digested vector pGEX-4T (Pharmacia, Uppsala, Sweden) to obtain an in-frame fusion of GST and the C-terminal part of Sap5p. The fusion protein was produced and purified by affinity chromatography according to the recommendations of the manufacturer. Rabbit antisera were made by Eurogentec (Liège, Belgium). Antiserum to Sap2p was produced by using a 15-amino-acid peptide (H_2N-DPSGSSASQDLNTPF-$CONH_2$) as an antigen. Antiserum to Sap5p was produced by using GST-Sap5F as an antigen.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot experiments. Fifteen microliters of the culture supernatant of *C. albicans* strains grown in YCB-BSA was analyzed on an SDS-12% polyacrylamide gel. Protein bands were visualized by staining with colloidal Coomassie dye. The molecular weights of the proteins were determined by use of the Precision Plus protein standards all blue size marker (Bio-Rad, Munich, Germany). Proteins were transferred onto nitrocellulose membranes with a Semi-Dry-Trans-Blot SD blot apparatus (Bio-Rad). Sap bands were detected with the antibodies raised against Sap2p and Sap5p; as a second antibody, an anti-rabbit antibody was used. Signals were detected with ECL detection solutions on ECL films (Amersham, Braunschweig, Germany).

RESULTS

Construction of *sap2*Δ mutants of *C. albicans* wild-type strain SC5314. As we wanted to use wild-type *C. albicans* model strain SC5314 for our experiments, we first created a *sap2*Δ mutant of this strain by using the *SAT1*-flipping strategy (40) for sequential deletion of both *SAP2* alleles. Strain SC5314 was transformed with a DNA fragment in which the *SAT1* flipper cassette was flanked by upstream and downstream sequences of the *SAP2* gene (Fig. 1A). Two different

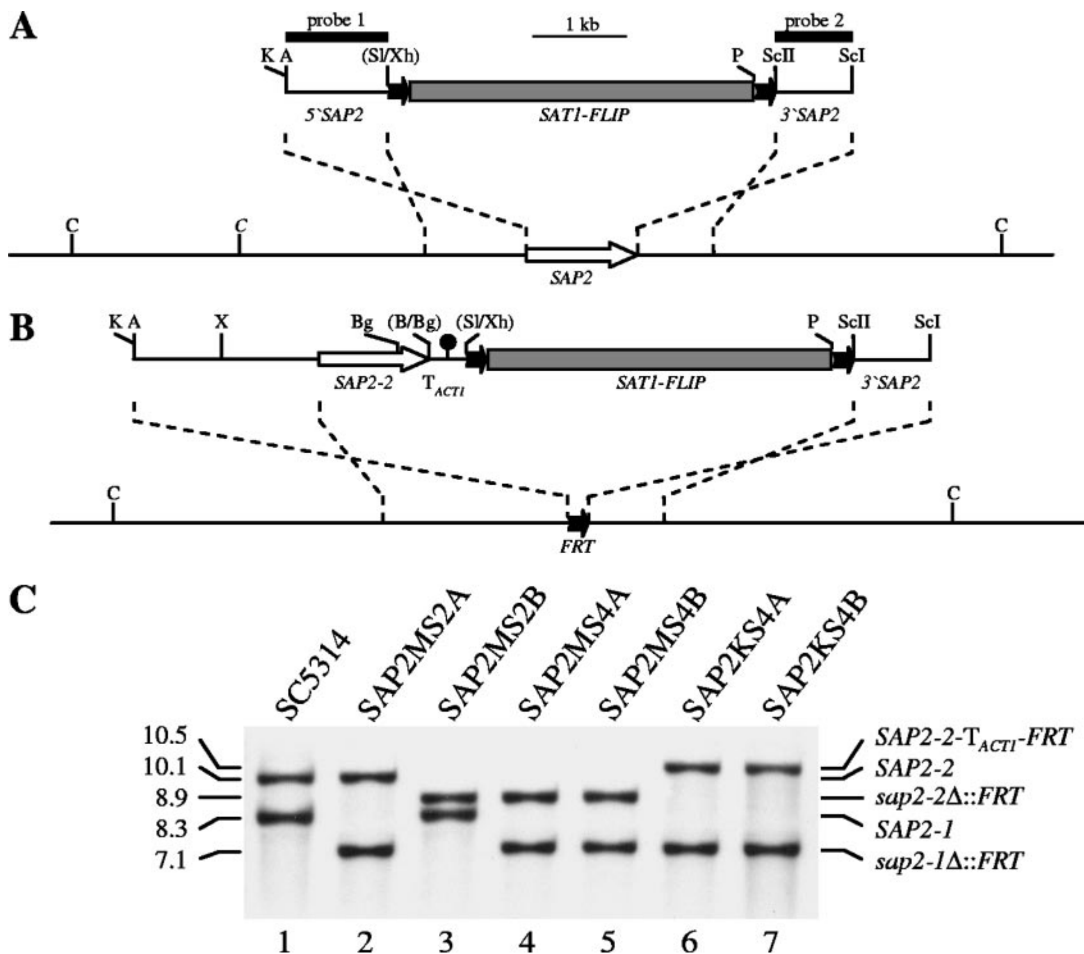

FIG. 1. Construction of *sap2*Δ mutants and complemented strains. (A) Structure of the deletion cassette from plasmid pSAP2MS2 (top), which was used to delete both *SAP2* alleles, and genomic structure of the *SAP2* locus in strain SC5314 (bottom). The *SAP2* coding region is represented by the white arrow, and the upstream and downstream regions are represented by the solid lines. Details of the *SAT1* flipper cassette (gray rectangle bordered by *FRT* sites [black arrows]) have been presented elsewhere (40). The 34-bp *FRT* sites are not drawn to scale. The probes used for Southern hybridization analysis of the mutants are indicated by the black bars. (B) Structure of the DNA fragment from pSAP2KS3 (top), which was used for reinsertion of the *SAP2-2* allele into its original site in the *sap2*Δ mutants (bottom). The transcription termination sequence of the *ACT1* gene (T_{ACT1}) is indicated by the filled circle. Only the following relevant restriction sites are given in panels A and B: A, ApaI; B, BamHI; Bg, BglII; C, ClaI; K, KpnI; P, PstI; ScI, SacI; ScII, SacII; Sl, SalI; X, XbaI; Xh, XhoI. Sites in parentheses were destroyed by the cloning procedure. The ClaI site in italics is present only in the *SAP2-1* allele. (C) Southern hybridization of ClaI-digested genomic DNA of parental strain SC5314 (lane 1), heterozygous *sap2* mutants SAP2MS2A (lane 2) and SAP2MS2B (lane 3), homozygous *sap2*Δ mutants SAP2MS4A (lane 4) and SAP2MS4B (lane 5), and reconstituted strains SAP2KS4A (lane 6) and SAP2KS4B (lane 7) with *SAP2*-specific probe 1. The sizes of the hybridizing fragments (in kilobases) are on the left side of the blot, and their identities are on the right.

heterozygous mutants in which the deletion cassette had been inserted into the *SAP2-1* or the *SAP2-2* allele, followed by FLP-mediated excision of the cassette (strains SAP2MS2A and SAP2MS2B, Fig. 1C, lanes 2 and 3), were selected. These strains were transformed again with the same deletion cassette to inactivate the remaining wild-type *SAP2* allele, generating homozygous *sap2*Δ mutants SAP2MS4A and SAP2MS4B (Fig. 1C, lanes 4 and 5). Reinsertion of the *SAP2-2* allele at its original locus in both independently generated *sap2*Δ mutants with the help of the *SAT1* flipper cassette (Fig. 1B), which was subsequently excised again, resulted in complemented strains SAP2KS4A and SAP2KS4B (Fig. 1C, lanes 6 and 7).

The heterozygous and homozygous *sap2*Δ mutants were tested for the ability to grow in YCB-BSA medium. In contrast to wild-type strain SC5314, homozygous *sap2*Δ mutants SAP2MS4A and SAP2MS4B were unable to grow under these conditions, as expected from previous work (23, 48). After 48 h of growth, the optical densities of the corresponding cultures were similar to that of the wild type incubated in the presence of the protease inhibitor pepstatin (Fig. 2A). We had previously demonstrated that the two *SAP2* alleles in strain CAI4, a derivative of strain SC5314, are differentially regulated and that inactivation of the *SAP2-2* allele abolished growth in YCB-BSA, while inactivation of the *SAP2-1* allele had no effect (48). The same result was obtained in the present study with the heterozygous *sap2* mutants of wild-type strain SC5314. Strain SAP2MS2A, in which the *SAP2-1* allele was deleted, grew like the wild type in YCB-BSA medium, while strain SAP2MS2B, which lacked the *SAP2-2* allele, was unable to grow under these conditions, like the homozygous *sap2*Δ mu-

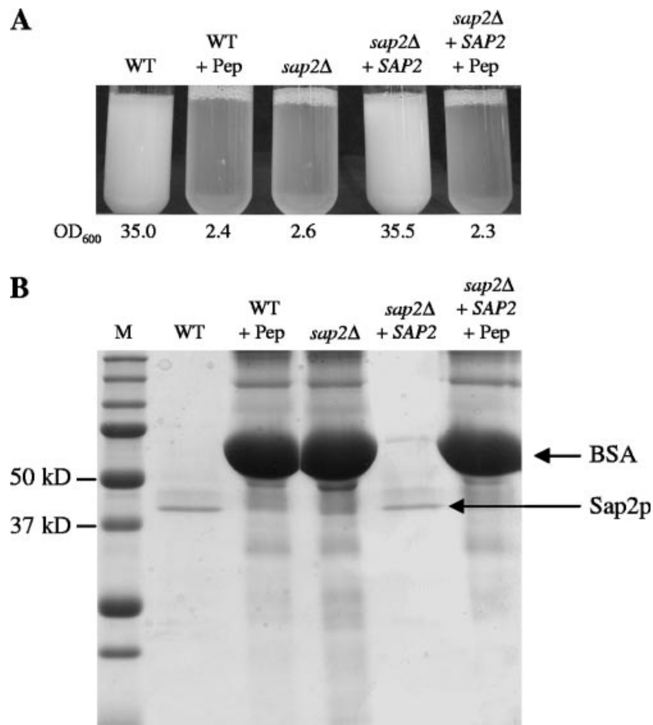

FIG. 2. The protease Sap2p is required for growth of *C. albicans* strain SC5314 in a medium with BSA as the sole source of nitrogen. (A) Growth of wild-type strain SC5314 (WT), *sap2*Δ mutant SAP2MS4B (*sap2*Δ), and complemented strain SAP2KS4B (*sap2*Δ + *SAP2*) in YCB-BSA. Overnight cultures of each strain in SD medium were diluted 1:100 in YCB-BSA, incubated for 48 h at 30°C, and photographed. Strain SC5314 and complemented strain SAP2KS4B were also incubated in the presence the protease inhibitor pepstatin (+ Pep). The optical densities at 600 nm (OD_{600}) of the cultures are given below each tube. The two independently constructed *sap2*Δ mutants and complemented strains behaved identically, and only one is shown. (B) Expression of Sap2p in wild-type strain SC5314, the *sap2*Δ mutants, and the complemented strains. Supernatants of the cultures shown in panel A were analyzed by SDS-PAGE. The bands corresponding to BSA and Sap2p are indicated. Lane M, molecular size markers.

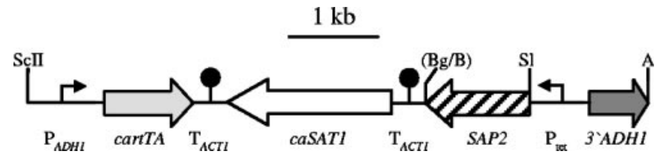

FIG. 3. Structure of the DNA cassettes used to integrate the Tet-inducible *SAP* genes into the *ADH1* locus of *C. albicans* strain SAP2MS4B (in this example shown for *SAP2* expression). Bent arrows symbolize promoters (P); filled circles represent T_{ACT1}, which serves for proper transcription termination of the *Candida*-adapted, reverse tetracycline-dependent transactivator (*cartTA*) and the target genes in this cassette (37). Only the following relevant restriction sites used to construct the plasmids or to excise the whole cassette from the vector backbone are shown: A, ApaI; B, BamHI; Bg, BglII; S, SalI; ScII, SacII.

tants (data not shown). Reinsertion of the *SAP2-2* allele at its native locus in homozygous *sap2*Δ mutants SAP2MS4A and SAP2MS4B restored the growth of resulting strains SAP2KS4A and SAP2KS4B to wild-type levels (Fig. 2A). Accordingly, the BSA in the culture medium was almost completely degraded during growth and the Sap2p band could be detected in the culture supernatants of the wild-type and complemented strains (Fig. 2B).

Tetracycline-inducible expression of individual *SAP* genes in a *sap2*Δ mutant. The inability of *C. albicans sap2*Δ mutants to grow in YCB-BSA medium allowed us to test whether inducible expression of individual members of the *SAP* gene family would restore the growth of the mutants under these conditions. Therefore, we cloned the coding regions of the *SAP1* to *SAP10* genes and placed them under the control of a tetracycline-inducible promoter (37). The resulting expression cassettes (Fig. 3) were used to transform the *sap2*Δ mutant SAP2MS4B, and correct integration into the *ADH1* locus was confirmed by Southern hybridization with *ADH1* upstream and downstream probes, as well as *SAP*-specific probes (data not shown). In each case, two independent transformants were kept for further analysis (Table 1).

In order to test whether expression of individual *SAP* genes would result in BSA degradation and growth of the *sap2*Δ mutants, precultures of strains SAP1ex4A/B to SAP10ex4A/B grown overnight in liquid SD medium were diluted in YCB-BSA medium with and without doxycycline and incubated under different conditions, as the parameters that allow optimal proteolytic activity vary for the different Saps (33). Doxycycline-induced expression of most of the *SAP* genes allowed the growth of the *sap2*Δ mutants. Dense growth of transformants expressing *SAP1*, *SAP2*, *SAP3*, and *SAP8* was reached after 48 h at pH 4.0 and 30°C, whereas growth of strains expressing *SAP4*, *SAP5*, and *SAP6* was strongest after 90 to 120 h at pH 5.0 and 37°C (Fig. 4), although the latter, especially the strains expressing *SAP4*, grew more slowly in YCB-BSA and the corresponding cultures did not reach as high densities as those expressing *SAP1*, *SAP2*, *SAP3*, or *SAP8*. Therefore, the various Sap isoenzymes differ in the ability to mediate growth on BSA as a nitrogen source. After prolonged incubation at pH 5.0 and 37°C, a slight increase in the optical density of the *sap2*Δ mutant (and transformants grown in the absence of doxycycline) was also observed. However, this growth was significantly weaker than the growth of the cells in which expression of the analyzed *SAP* genes was induced by doxycycline.

The transformants expressing *SAP7*, *SAP9*, or *SAP10* did not grow in YCB-BSA medium under any of the conditions tested (data not shown). The inability of the strains expressing *SAP9* and *SAP10* from the Tet-inducible promoter to grow in YCB-BSA might be due to the fact that Sap9p and Sap10p are located in the cell membrane and/or the cell wall and, unlike the other Sap isoenzymes, are not released into the culture medium (1). Therefore, we expressed truncated *SAP9* and *SAP10* genes lacking the sequences required for GPI anchor addition. Because different putative GPI anchorage sites have been proposed for Sap9p and Sap10p (1, 14, 17, 30), two different C-terminally truncated alleles were generated for each of these two *SAP* genes (see Materials and Methods and Table 1). Strains expressing *SAP9* alleles lacking the region that encodes the last 24 ($SAP9^{\Delta C520}$) or 51 ($SAP9^{\Delta C493}$) amino acids were able to grow in YCB-BSA medium at a pH of 5.0 and 37°C, indicating that Sap9p, when secreted into the culture medium, could mediate the degradation of BSA for use as a

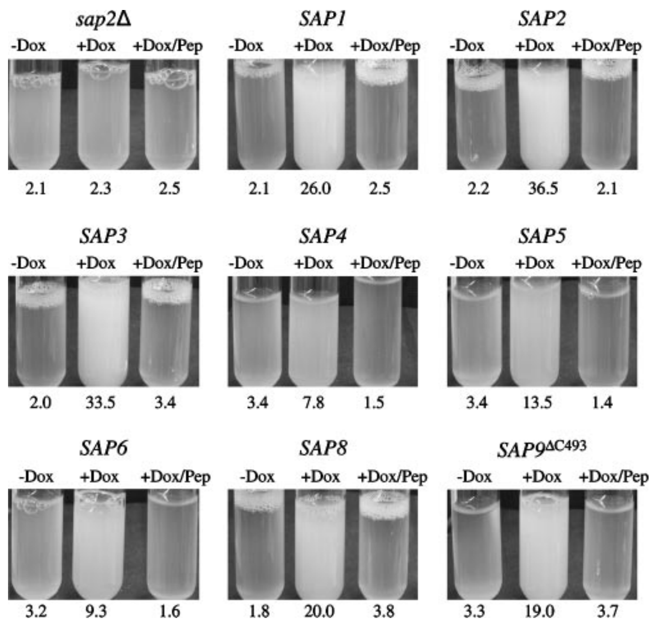

FIG. 4. Tet-inducible expression of *SAP1*, *SAP2*, *SAP3*, *SAP4*, *SAP5*, *SAP6*, *SAP8*, and *SAP9*$^{\Delta C493}$ allows growth of a *sap2*Δ mutant in YCB-BSA. Overnight cultures of strains SAP1ex4A, SAP2ex4A, SAP3ex4A, SAP4ex4A, SAP5ex4A, SAP6ex4A, SAP8ex4A, and SAP9ΔC2ex4A were diluted 1:100 in YCB-BSA without (−Dox) or with (+Dox) doxycycline. In addition, the strains were also incubated in the presence of both doxycycline and pepstatin (+Dox/Pep). Untransformed *sap2*Δ mutant SAP2MS4B served as a control. The tubes were photographed, and the optical densities at 600 nm of the cultures were measured after 48 h (for SAP2MS4B and strains expressing *SAP1*, *SAP2*, *SAP3*, and *SAP8*) or 96 h (for strains expressing *SAP4*, *SAP5*, *SAP6*, and *SAP9*$^{\Delta C493}$). The OD_{600} values are given below each tube. The two independently constructed transformants for Tet-inducible expression of the individual *SAP* genes behaved identically, and only one of them is shown in each case.

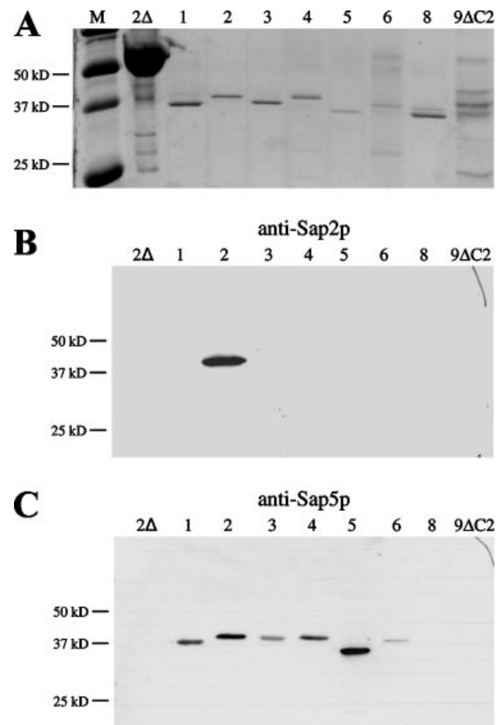

FIG. 5. Detection of individual Saps in the culture supernatants of *SAP*-expressing transformants. *sap2*Δ mutant SAP2MS4B and strains expressing specific *SAP* genes, as indicated by the numbers above the lanes, were cultivated in YCB-BSA medium with doxycycline (for details, see Materials and Methods). (A) Culture supernatants of the strains were analyzed on an SDS-polyacrylamide gel stained with colloidal Coomassie dye. Lane M, molecular size markers. (B and C) Detection of the Saps in the supernatants of the analyzed strains by Western immunoblotting with antibodies raised against Sap2p (B) and Sap5p (C).

nitrogen source (Fig. 4 and data not shown). In contrast, doxycycline-induced expression of neither of the truncated *SAP10* alleles restored the growth of the *sap2*Δ mutant (data not shown).

The culture supernatants of strains expressing *SAP1*, *SAP2*, *SAP3*, *SAP4*, *SAP5*, *SAP6*, *SAP8*, and *SAP9*$^{\Delta C493}$ were analyzed by SDS-PAGE. In all cases, the BSA was almost completely degraded, and specific protein bands in the size range of approximately 35 kDa to 45 kDa could be detected for Sap1p, Sap2p, Sap3p, Sap4p, Sap5p, Sap6p, and Sap8p (Fig. 5A). In contrast, several bands of different sizes were observed in the culture supernatant of the strain expressing *SAP9*$^{\Delta C493}$. Sap9p has been reported to be cleaved into two subfragments when heterologously expressed and secreted in *P. pastoris* (1, 11), but we were unable to decide which of the proteins present in our culture supernatants corresponded to mature Sap9p. To confirm the identities of the different Sap isoenzymes in the culture supernatants, we performed Western immunoblotting experiments with antibodies raised against Sap2p and Sap5p. The anti-Sap2p antibody specifically recognized only Sap2p (Fig. 5B), whereas the anti-Sap5p antibody showed cross-reactivity with all of the Sap1p-Sap6p proteins (Fig. 5C). Neither of the two antibodies reacted with the proteins secreted by the strains expressing *SAP8* or *SAP9*$^{\Delta C493}$. The proteins recognized by the antibodies in the Western blot experiments exhibited the same sizes as the protease bands in the corresponding Coomassie-stained gel (compare Fig. 5A and C), confirming that no other Sap isoenzyme than the one expressed from the Tet promoter was secreted at detectable levels by these strains.

In summary, doxycycline-inducible expression of 8 of the 10 members of the *SAP* gene family enabled *C. albicans sap2*Δ mutants to grow in a medium containing BSA as the sole nitrogen source. Therefore, this phenotype could be used in a simple bioassay to test the activities of various protease inhibitors against individual Sap isoenzymes.

Inhibition of Sap-dependent growth by different protease inhibitors. We then tested the abilities of various aspartic protease inhibitors to block the growth of strains expressing specific Sap isoenzymes under control of the Tet promoter in YCB-BSA medium. The strains were incubated under conditions allowing optimal activity of the corresponding Sap isoenzyme, and growth was monitored by measuring the optical densities of the cultures over time in the absence or presence of the inhibitors. All experiments were performed in duplicate with two independent transformants, which yielded essentially identical results. As demonstrated in Fig. 6A and B, the aspartic protease inhibitor pepstatin was active against all of the eight Saps under the conditions tested; i.e., none of the *SAP*-

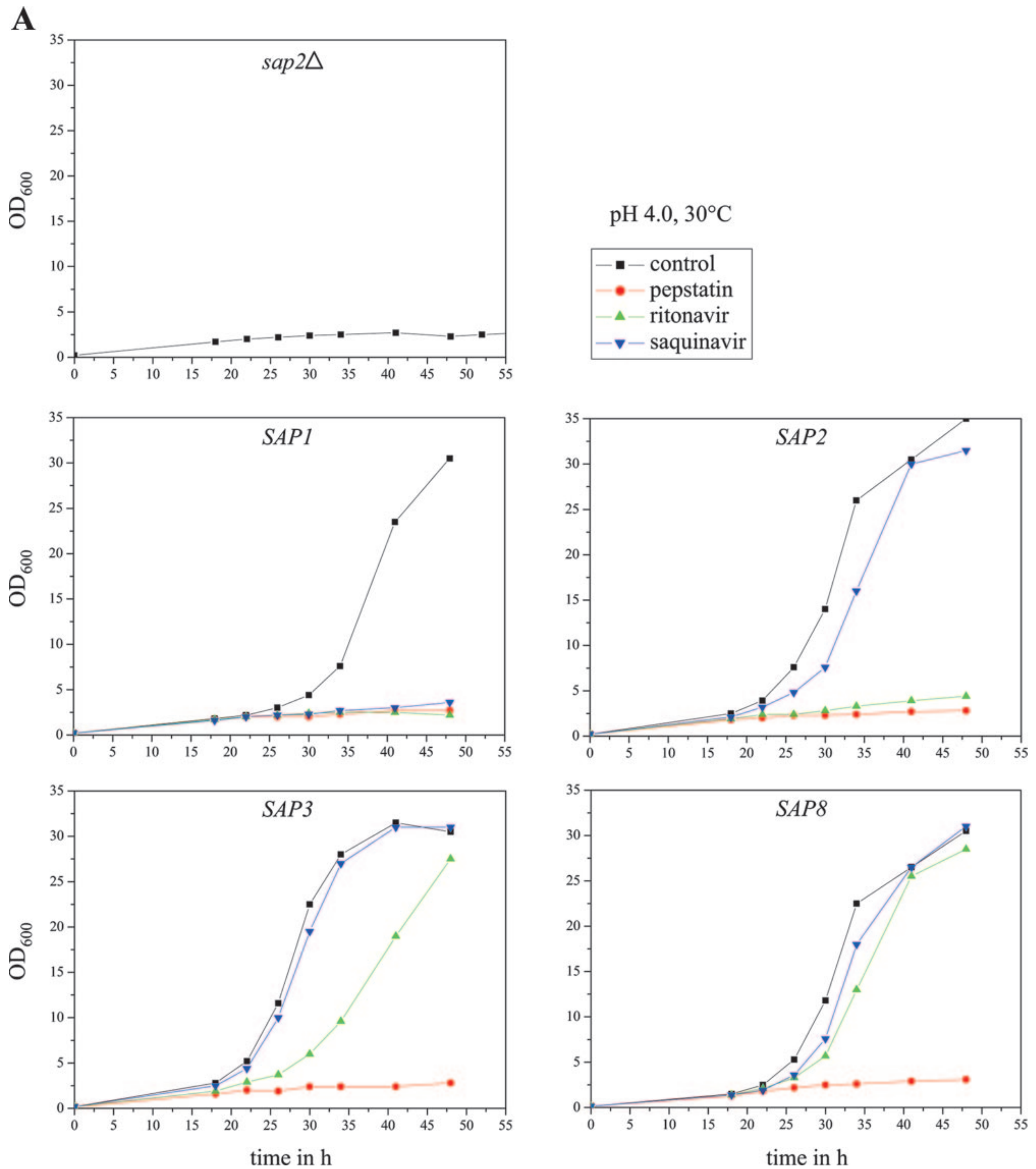

FIG. 6. Inhibition of Sap-dependent growth of *C. albicans* by different protease inhibitors. Overnight cultures of *sap2*Δ mutant SAP2MS4B and strains SAP1ex4A, SAP2ex4A, SAP3ex4A, SAP4ex4A, SAP5ex4A, SAP6ex4A, SAP8ex4A, and SAP9ΔC2ex4A were diluted 1:100 in YCB-BSA medium with doxycycline in the absence (control, containing dimethyl sulfoxide only) or presence of the protease inhibitor pepstatin, ritonavir, or saquinavir. The optical densities at 600 nm (OD_{600}) of the cultures were measured at the indicated times. (A) Cultures incubated in YCB-BSA, pH 4.0, at 30°C. (B) Cultures incubated in YCB-BSA, pH 5.0, at 37°C. The two independently constructed transformants for Tet-inducible expression of the individual *SAP* genes yielded essentially identical results, and for better clarity, only one of them is shown in each case.

expressing strains was able to grow on BSA in the presence of this inhibitor. In contrast, the HIV protease inhibitors ritonavir and saquinavir displayed a differential activity against the individual Saps at the tested concentration of 100 μM. Ritonavir blocked the growth of the strains expressing *SAP1* and *SAP2* and slightly delayed the growth of the strains expressing *SAP3*. In contrast, saquinavir only blocked the growth of the strains expressing *SAP1*. Neither of the two HIV protease inhibitors

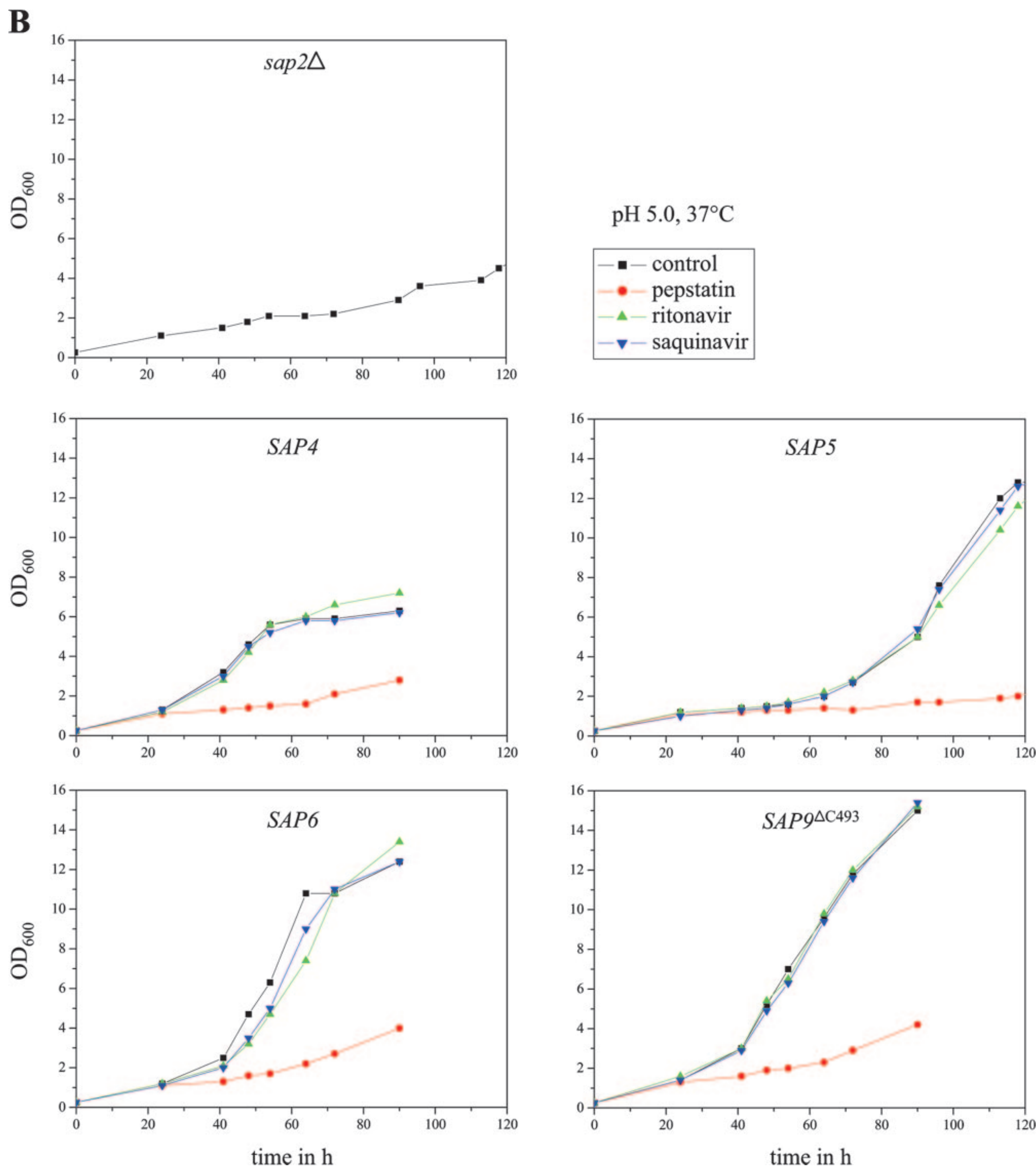

FIG. 6—*Continued*.

was able to inhibit the growth of the strains expressing *SAP4*, *SAP5*, *SAP6*, *SAP8*, or *SAP9*$^{\Delta C493}$ under the conditions tested, in agreement with previously reported activities of the same protease inhibitors on recombinantly expressed, purified Sap isoenzymes (7, 29), demonstrating that protease-dependent growth of strains expressing specific *SAP* genes can be used as a simple assay to test the activities of novel protease inhibitors against almost all of the members of the secreted aspartic protease family of *C. albicans*.

DISCUSSION

Since proteolytic activity was discovered in *C. albicans*, the secreted aspartic proteases have been intensively studied vir-

ulence attributes of this human-pathogenic yeast. Because of their importance for the virulence of *C. albicans*, the Saps have also been considered as potential drug targets for a long time (49). As *C. albicans* possesses 10 *SAP* genes, which are differentially expressed during infection and have different roles in colonization, invasion, and proliferation of the fungus in host tissues, potent inhibitors should block the activities of as many of the Sap isoenzymes as possible. In this work, we established a bioassay which allows the screening of potential protease inhibitors for activity against 8 of the 10 Saps by their ability to inhibit protease-dependent growth of *C. albicans*. In contrast to approaches that are based on the expression of Saps in a heterologous host and in vitro testing of inhibitors against the purified proteases, this assay enables for the first time inhibitor testing against Saps other than Sap2p in their native host. The assay relies on the fact that *C. albicans* can grow in YCB-BSA medium by secreting Sap2p, which is the only Sap isoenzyme expressed by most *C. albicans* strains under these conditions (22, 53). The expression of *SAP* genes under the control of an inducible promoter in a *sap2Δ* mutant therefore rendered the growth of the so-generated strains dependent on the activity of the corresponding Sap isoenzyme. As a first step, we had to evaluate whether the expression of the various *SAP* genes from the Tet promoter would indeed allow the growth of *sap2Δ* mutants in YCB-BSA. Expression of all of the *SAP* genes except *SAP7* and *SAP10* resulted in BSA degradation and growth of the strains, albeit with various efficiencies even after optimization of the culture conditions to allow maximum growth of the different strains. Under our standard conditions for culturing *C. albicans* in YCB-BSA (30°C, pH 4.0), expression of *SAP1*, *SAP2*, *SAP3*, and *SAP8* resulted in dense growth of the strains. In contrast, significant growth of strains expressing *SAP4*, *SAP5*, *SAP6*, or $SAP9^{\Delta C493}$ was only observed at an elevated temperature (37°C) and pH (5.0). After prolonged incubation at 37°C, the *sap2Δ* mutant also exhibited some residual growth, which may have been caused by some self-degradation of BSA over time under these conditions. However, the growth of the strains expressing the various *SAP* genes from the Tet promoter was significantly higher in all cases, and without doxycycline induction the strains behaved like the *sap2Δ* mutant, confirming that growth was indeed mediated by the protease expressed from the Tet promoter.

SAP9 could mediate growth in YCB-BSA only after removal of the GPI-anchoring sequence, supporting the view that release of the secreted proteases from the cells is necessary for efficient extracellular proteolysis. When Sap9p was expressed in the heterologous host *P. pastoris*, secretion also occurred only after deletion of the GPI anchor sequence (1). However, recombinantly expressed Sap9p was not able to hydrolyze serum albumin in that study. Cell surface-anchored Sap9p and Sap10p, which display sequence homology to the *S. cerevisiae* yapsins, have functions other than the degradation of extracellular proteins and are involved in cell surface integrity, cell separation, and adherence of the fungal cells to host tissue (1, 30). Nevertheless, our results indicate that forced secretion of Sap9p can also mediate the growth of *C. albicans* on BSA and that this phenotype can be used to search for Sap9p inhibitors.

Unlike the other *SAP* genes, Tet-induced expression of *SAP7* and *SAP10* did not allow growth of the *sap2Δ* mutant. These two genes, together with *SAP9*, are the most divergent genes within the *SAP* gene family. It is possible that BSA is not a good substrate for Sap7p and Sap10p or that the enzymes are not active or not functionally expressed under the growth conditions used in our experiments.

Most of the studies on *C. albicans* Sap inhibition have been carried out with the aspartic protease inhibitor pepstatin (51). Its high potential to block Sap activity, which was demonstrated before for heterologously expressed enzymes Sap1p to Sap6p (7), was confirmed in our experiments, as none of the *SAP*-expressing strains could grow in BSA medium in the presence of pepstatin. In contrast, the HIV protease inhibitors ritonavir and saquinavir were active only against some of the Saps. The potential of ritonavir, but not saquinavir, to inhibit the growth of a *C. albicans* wild-type strain in YCB-BSA has been demonstrated before (5). As *C. albicans* usually expresses only *SAP2* under these conditions, the growth inhibition presumably reflected the ability of ritonavir to inhibit Sap2p. Using our set of test strains, we could demonstrate that ritonavir also inhibits Sap1p-dependent and (partially) Sap3p-dependent growth and that saquinavir could inhibit Sap1p-dependent, but not Sap2p- or Sap3p-dependent, growth of *C. albicans*. These findings are consistent with recent observations that ritonavir had a stronger inhibitory effect on these three Saps than did saquinavir (2). Until now, HIV protease inhibitors have only once been tested against Sap4p-Sap6p expressed in *P. pastoris* (7). The data of this former study were confirmed by our results. Ritonavir and saquinavir had no detectable inhibitory effect on Sap4p to Sap6p. In addition to Sap1p to Sap6p, we could now also test protease inhibitors against Sap8p and Sap9p. Neither ritonavir nor saquinavir could inhibit the growth of *C. albicans* mediated by these enzymes, at least at the concentration tested.

The Tet-inducible expression of individual Sap enzymes in their native host, *C. albicans*, not only offers the possibility to test the abilities of potential Sap inhibitors to block the growth of *C. albicans* but also allows the purification of Saps from culture supernatant for further detailed characterization. Expression of Saps in their native host should circumvent possible caveats related to their expression in a heterologous host, like missing or inappropriate posttranslational modifications. Except for Sap9p, the individual Saps in the culture supernatants of the Sap-expressing strains were easily detectable in Coomassie-stained gels. Their presence and size were also confirmed in Western blotting experiments using Sap-specific antibodies. As demonstrated before, antibodies raised against protease Sap4p, Sap5p, or Sap6p displayed cross-reactivity with other Saps (6). However, Sap8p and Sap9p were not recognized by the antibodies we used.

Until now, none of the few Sap inhibitors tested was able to reproducibly inhibit or cure an experimental systemic infection (18, 49). Efforts have thus been made to identify new Sap inhibitors by different approaches; however, promising candidates for a therapeutic application have not been identified so far. As Sap2p is the only isoenzyme that can be easily expressed in *C. albicans* under laboratory conditions in large amounts, the applied screening assays were mostly based on this isoenzyme (15, 39, 44, 50, 54) or on Sap1p to Sap3p (2). However, because deep *C. albicans* mycoses are associated with the expression of many different Sap isoenzymes, including Sap4p to Sap6p, new inhibitors are needed which act against as many of

the Saps as possible and at the same time are applicable in vivo. The set of reporter strains created in the present work should be highly useful for the identification and analysis of newly synthesized protease inhibitors. Compounds which specifically act on the *C. albicans* Saps would be promising candidates in the treatment of *C. albicans* infections in the future.

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