INFECTION AND IMMUNITY, May 1999, p. 2482–2490 Vol. 67, No. 5
0019-9567/99/$04.00+0


# In Vivo Analysis of Secreted Aspartyl Proteinase Expression in Human Oral Candidiasis

JULIAN R. NAGLIK,[1] GEORGE NEWPORT,[2] THEODORE C. WHITE,[2]† LYNETTE L. FERNANDES-NAGLIK,[1] JOHN S. GREENSPAN,[2] DEBORAH GREENSPAN,[2] SIMON P. SWEET,[1] STEPHEN J. CHALLACOMBE,[1] AND NINA AGABIAN[2,3]*

*Oral AIDS Research Unit, Department of Oral Medicine and Pathology, GKT Guy's Hospital, London SE1 9RT, United Kingdom,[1] and Department of Stomatology[2] and Departments of Microbiology and Immunology and of Pharmaceutical Chemistry,[3] University of California at San Francisco, San Francisco, California 94143*

Received 23 November 1998/Returned for modification 13 January 1999/Accepted 23 February 1999

**Secreted aspartyl proteinases are putative virulence factors in *Candida* infections. *Candida albicans* possesses at least nine members of a *SAP* gene family, all of which have been sequenced. Although the expression of the *SAP* genes has been extensively characterized under laboratory growth conditions, no studies have analyzed in detail the in vivo expression of these proteinases in human oral colonization and infection. We have developed a reliable and sensitive procedure to detect *C. albicans* mRNA from whole saliva of patients with oral *C. albicans* infection and those with asymptomatic *Candida* carriage. The reverse transcription-PCR protocol was used to determine which of the *SAP1* to *SAP7* genes are expressed by *C. albicans* during colonization and infection of the oral cavity. *SAP2* and the *SAP4* to *SAP6* subfamily were the predominant proteinase genes expressed in the oral cavities of both *Candida* carriers and patients with oral candidiasis; *SAP4*, *SAP5*, or *SAP6* mRNA was detected in all subjects. *SAP1* and *SAP3* transcripts were observed only in patients with oral candidiasis. *SAP7* mRNA expression, which has never been demonstrated under laboratory conditions, was detected in several of the patient samples. All seven *SAP* genes were simultaneously expressed in some patients with oral candidiasis. This is the first detailed study showing that the *SAP* gene family is expressed by *C. albicans* during colonization and infection in humans and that *C. albicans* infection is associated with the differential expression of individual *SAP* genes which may be involved in the pathogenesis of oral candidiasis.**

*Candida albicans* is a commensal fungus commonly colonizing human mucosal surfaces. Under conditions of immune dysfunction, *C. albicans* can become an opportunistic pathogen causing recurrent chronic oral and vaginal candidiasis and life-threatening disseminated infections (38). Putative attributes which contribute to *C. albicans* virulence include adhesion, formation of hyphae, phenotypic switching, and proteinase secretion (1, 15, 39, 48). Secreted aspartyl proteinases (Saps) exhibit broad substrate specificity since they are able to degrade many human proteins found at lesion sites, such as albumin, hemoglobin, keratin, collagen, mucin, and secretory immunoglobulin A (sIgA) (13, 22, 41).

To date, nine different *SAP* genes have been identified in *C. albicans*. Each *SAP* gene in *C. albicans* is regulated at the transcriptional level and processed by a signal peptidase and a Kex2-like proteinase (37, 50, 53). Recently, the *KEX2* gene of *C. albicans* has been disrupted, resulting in impaired Sap secretion and hyphal growth (37). The presence of nine *SAP* genes points to their importance in *Candida* pathogenesis and cell biology. Many studies have explored the regulation of *SAP* expression under laboratory conditions, primarily through the analysis of mRNA and protein synthesis under various nutritional and ionic conditions and in different strains. *SAP2* is the most frequently expressed *SAP* gene; it is expressed in nearly all strains of *C. albicans* during log-phase growth in proteinase-inducing media (23, 53), and most studies which predate the recognition of the complexity of this gene family have most likely described the properties and characteristics of this proteinase. *SAP1* and *SAP3* are regulated during phenotypic switching (35, 36, 52), but *SAP3* has also been detected in some strains when *SAP2* is expressed (23, 46, 52). In vitro studies suggest that *SAP1*, *SAP2*, and *SAP3* are expressed by yeast cells only. *SAP4*, *SAP5*, and *SAP6* expression has been detected in *C. albicans* undergoing a transition from yeast to hyphae at neutral pH (23, 52), although S1 nuclease assays indicated that *SAP6* is the predominant transcript (52). *SAP7* expression has never been detected under any laboratory growth conditions (23). While *SAP1*, *SAP2*, and *SAP3* comprise a subfamily distinct from that of *SAP4*, *SAP5*, and *SAP6* by sequence homology, *SAP7* appears to be the most divergent, showing only 20.4% similarity to *SAP1* and about 26% homology to *SAP4*, *SAP5*, and *SAP6* (34). *SAP8* transcripts have been detected in yeast cells grown at 25°C in a defined medium with bovine serum albumin as the sole source of protein, and *SAP9* is preferentially expressed in later growth phases, when the expression of other *SAP* genes has decreased (33).

The importance of the secreted proteinases as a virulence factor has been investigated in model systems of candidiasis. In the rat vaginitis model, only proteinase-producing *Candida* species were shown to be pathogenic (2), and in *C. albicans* infection, *SAP1* and *SAP2* expression has been demonstrated by Northern analysis in vivo (17). Sap2 was shown to be actively secreted during experimental vaginitis in the rat (47), and anti-Sap2 polyclonal antibodies appeared to confer partial protection against infection (8). In mouse and guinea pig models of disseminated candidiasis, mutant strains of *C. albicans* in which individual *SAP* genes had been disrupted exhibited attenuated virulence and reduced accumulation in host organs (24, 44). More recently, *SAP* expression was investigated in vitro by us-

* Corresponding author. Mailing address: Department of Stomatology, University of California, San Francisco, Box 0422, 521 Parnassus St., San Francisco, CA 94143-0422. Phone: (415) 476-6844. Fax: (415) 476-0664. E-mail: agabian@itsa.ucsf.edu.

† Present address: Seattle Biomedical Research Institute, Department of Pathobiology, University of Seattle, Seattle, WA 98109-1651.

ing reconstituted human epithelium as a model for human oral candidiasis (45). *SAP1* and *SAP3* were the first proteinases to be expressed, followed by *SAP6*, *SAP8*, and finally, *SAP2*. However, it is difficult to know whether this expression profile is representative of the initial stages of human oral *C. albicans* infection in vivo.

The expression pattern of members of the *SAP* gene family, as established under laboratory growth conditions, is under complex regulatory control. Due to the range of environmental conditions which regulate *SAP* gene expression in vitro, the collective and individual roles of Saps during infection are incompletely understood. In the present paper, we describe methods that allow direct assay of the expression of individual *SAP* genes in the oral cavity. The aims of this study were to develop a reliable and sensitive method for the detection of *C. albicans SAP1* to *SAP7* mRNA from whole saliva by using reverse transcription (RT)-PCR. Using this protocol, we compared the in vivo expression of genes *SAP1* to *SAP7* in oral candidiasis patients with that in asymptomatic *Candida* carriers.

## MATERIALS AND METHODS

**Strains, media, and culture conditions.** The *Candida* reference strains used in this study were *C. albicans* NCPF 3156, *C. stellatoidea* ATCC 11006 (type I), *C. dubliniensis* NCPF 3949, *C. tropicalis* ATCC 750, *C. parapsilosis* ATCC 22019, and *C. guilliermondii* NCPF 3099. Strains were maintained on Sabouraud (SAB) dextrose agar plates (Oxoid Ltd., Basingstoke, England). *C. albicans* NCPF 3156 was used for all optimization experiments and as a positive control for *SAP2* expression throughout the study. For total RNA isolation from *C. albicans* NCPF 3156, cultures were grown in YCB-BSA medium (1.17% [wt/vol] yeast carbon base, 1% bovine serum albumin) for 24 h at 27°C. For DNA isolation, yeast cultures were grown in SAB broth (Oxoid) for 2 days at 27°C. For all cultures, 10 ml of test medium was inoculated with 10 μl of a 2-day culture of *Candida* grown in SAB broth at 27°C and shaken on an orbital incubator.

**Study populations and sample collection.** The following three study populations were used: (i) a *Candida*-negative group, consisting of seven subjects whose saliva samples were consistently *Candida* negative by culture six times over a period of 3 months (four males and three females; mean age ± standard deviation, 31.3 ± 3.9 years); (ii) an asymptomatic *Candida* carrier group, consisting of eight subjects without any clinical signs or symptoms of candidiasis but harboring 50 to 800 *C. albicans* CFU/ml of saliva on six occasions over a period of 3 months (seven males and one female; mean age, 37.1 ± 10.9 years); and (iii) patients with oral *Candida* infection, consisting of 10 patients with clinical signs and symptoms of candidiasis and salivary counts of $>2 \times 10^3$ *C. albicans* CFU/ml (14) (six males and four females; mean age, 45.5 ± 12.5 years). All the *Candida* carriers were volunteers that were not tested for human immunodeficiency virus (HIV) infection. Six of the oral candidiasis patients were HIV seropositive, and four were HIV seronegative. No individual in our patient group had been treated with anti-HIV proteinase drugs prior to or at the time of sample collection. Although a number of these patients had previously been treated with antifungal drugs, none were being treated with either antibiotic drugs or antimycotic drugs at the time of sampling.

Two unstimulated saliva samples were collected from each subject; one was immediately frozen on dry ice at −70°C to preserve the integrity of RNA for *SAP* analysis, and the other was used to determine the number of *Candida* CFU per milliliter of saliva. Yeast isolates were identified as *C. albicans* by using the API 32C AUX system (bioMerieux, Lyon, France), CHROMagar *Candida* (CHROMagar, Paris, France), germ tube formation, and growth at 42°C.

**Germ tube formation and growth at 42°C.** One milliliter of 10% horse serum in brain heart infusion broth was inoculated with each strain and incubated at 35°C for 3 h. Production of germ tubes was observed microscopically under magnification at ×40. Each strain was observed for growth on SAB plates after incubation at 42°C for 48 h.

**RNA and DNA isolation.** The use of glass beads and the length of vortexing were critical for optimal RNA isolation from *Candida* cells. All experiments were performed in 1.5-ml microcentrifuge tubes. Glass beads of different sizes (400 to 600 μm or 700 to 1,100 μm) and volumes (0.1 to 0.5 ml) were added to 0.5- or 1-ml *C. albicans* suspensions containing $10^4$ cells/ml. Lysis of cells was measured at intervals during 30 min of vortexing by plating 100-μl samples onto SAB agar to evaluate percent survival. Optimal RNA recovery was obtained by vortexing a 1-ml volume of *Candida* cell culture with 0.5 ml of glass beads (size, 400 to 600 μm) for 30 min. This resulted in 100% killing of *Candida* cells.

Total RNA was prepared by a modified version of the procedure described by Chomczynski and Sacchi (11). *C. albicans* cells grown in culture or whole saliva samples were centrifuged, and 1 ml of Tri-reagent (Sigma, Poole, United Kingdom) and 0.5 ml of glass beads (size, 400 to 600 μM; Sigma) were added to the pellet prior to vortexing for 30 min. After the addition of 0.2 ml of chloroform, samples were centrifuged at 12,000 × *g* for 15 min at 4°C. Total RNA was precipitated from the aqueous fraction with isopropanol and centrifuged at 12,000 × *g* for 30 min at 4°C. Total RNA was treated with DNase (40 mM Tris-Cl [pH 7.9], 1 mM NaCl, 6 mM $MgCl_2$, 10 mM $CaCl_2$, 0.5 U of RNasin [Promega, Southampton, United Kingdom], 20 to 50 U of RQ-1 DNase [Promega]), reextracted twice with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) at pH 4.3 and twice with chloroform-isoamyl alcohol (24:1, vol/vol), and finally precipitated with 2.5 volumes of ethanol. Total RNA concentrations were determined by using a GeneQuant II spectrophotometer (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom). Purified RNA from each sample was confirmed to be DNA free by the absence of an amplified product after PCR performed by using fungus-specific primers complementary to the 5.8S rRNA gene (rDNA) plus intergenic sequence regions (54).

For DNA isolation, yeasts grown in SAB broth were harvested after 2 days, washed in sterile water, and resuspended in 0.4 ml of lysis buffer (0.2 M NaCl, 0.4% sodium dodecyl sulfate, 0.1 M Tris-Cl [pH 7.5], 5 mM EDTA [pH 8]). Equal volumes of phenol (pH 8) and glass beads were added, and the mixture was vortexed for 30 min. DNA was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) at pH 8 and twice with chloroform-isoamyl alcohol (24:1, vol/vol) and precipitated with 2.5 volumes of ethanol. DNA concentrations were determined on a GeneQuant II spectrophotometer.

**Selection of *SAP*-, actin-, and fungus-specific primers.** One primer pair each for *SAP1*, *SAP2*, *SAP3*, and *SAP7* and one primer pair for *SAP4*, *SAP5*, and *SAP6*, which have near-identical sequence homology, were chosen (Table 1). Two primer pairs were also selected to detect the *C. albicans* actin gene (*ACT1*); the pairs consisted of two primers that recognize separate sequences on the plus strand and one common primer that recognizes the minus strand (Table 1). Primers detecting the 5.8S rDNA plus intergenic spacer regions have been described previously (54) (Table 1). None of the primer sets used in this study amplified regions containing introns. The *SAP1* to *SAP7* and *ACT1* primers were tested against *C. albicans* DNA to check for accurate amplification of the correct genes and against a panel of genomic DNAs isolated from different *Candida* species to test for cross-reactivity. The two actin primer sets reacted with all of the *Candida* species tested, yielding PCR products of the same size as for *C. albicans*, and thus served as a positive control for *Candida* colonization. Two sets of actin primers were used as a rigorous positive control; our criteria required that the results obtained with both actin primer pairs concurred to confirm the presence or absence of *Candida*. This study did not analyze the expression of *SAP8* and *SAP9* because these sequences became available only toward the completion of the work.

**RT-PCR *SAP1* to *SAP7* mRNA expression in clinical whole saliva samples.** Ten RT-PCRs were performed for each sample of RNA isolated from each patient saliva specimen. These included the five experimental reactions for *SAP* detection (*SAP1*, *SAP2*, *SAP3*, *SAP4* to *SAP6*, and *SAP7*) and five different control reactions, as follows: two actin control reactions to demonstrate the presence or absence of *Candida* species, a negative (water) control reaction, a positive control reaction carried out by using total RNA isolated from *SAP2* mRNA-expressing *C. albicans* NCPF 3156 cells (*SAP2* RNA), and a salivary inhibitor control reaction whereby *SAP2* RNA was added to the saliva RNA preparation to check if the latter was inhibitory to the RT-PCR.

RT-PCR experiments with the actin and *SAP* primers were performed at an annealing temperature of 62°C by using *Access* RT-PCR reagents (Promega). RT-PCR conditions were optimized by modified Taguchi methods (12) and included a touchdown protocol. Template RNA was added to an RT-PCR mixture containing 1× avian myeloblastosis virus-*Tfl* buffer (Promega), 1 mM $MgSO_4$, 0.1 mM deoxynucleoside triphosphates, 0.6 μM primers, 3.75 U of avian myeloblastosis virus reverse transcriptase, and 1 μCi of $^{32}$P-labelled dCTP (ICN, Thame, United Kingdom). Radioactive labelling was used to maximize sensitivity. After RT (48°C for 45 min), the cDNA-RNA hybrid was denatured at 94°C for 3 min and 2.5 U of *Tfl* DNA polymerase was added to the reaction mixture. Annealing temperatures used for touchdown cycling were as follows: 66°C for 2 cycles, 65°C for 2 cycles, 64°C for 2 cycles, and 63°C for 2 cycles, followed by 62°C for 35 cycles. Cycling times were as follows: denaturation at 94°C, annealing at 62°C, and extension at 72°C, each for 30 s. A final extension step of 72°C for 10 min followed the cycling.

For control experiments detecting the 5.8S rDNA plus intergenic spacer regions, RT-PCRs and PCRs were carried out at an annealing temperature of 55°C. For PCRs testing the cross-reactivity of the actin and *SAP* primers against genomic DNAs isolated from different *Candida* species, an annealing temperature of 62°C was used. PCR was performed by using *Taq* polymerase (Boehringer Mannheim, Lewes, United Kingdom). PCR conditions were optimized by modified Taguchi methods (12). Template DNA was added to a PCR mixture containing 2.5 mM $MgCl_2$, 0.1 mM deoxynucleoside triphosphates, 0.6 μM primers, 1 U of *Taq* DNA polymerase, and 1 μCi of $^{32}$P-labelled dCTP. DNA was denatured at 94°C for 3 min and amplified for 35 cycles at the following cycling times: denaturation at 94°C, annealing at 62 or 55°C, depending on the primers used, and extension at 72°C, each for 30 s. A final extension step of 72°C for 10 min followed the cycling.

All radiolabelled RT-PCR and PCR products were mixed with formamide loading buffer (80% formamide, 50 mM Tris-borate buffer [pH 8.3], 1 mM EDTA, 0.1% [wt/vol] xylene cyanol, 0.1% [wt/vol] bromophenol blue), incubated at 95°C for 5 min, and cooled on ice. The products were electrophoresed through

TABLE 1. Oligonucleotide primer sets detecting *ACT1* and *SAP1* to *SAP7* gene mRNAs and 5.8S rRNA

| Gene (reference) | Accession no. | Primer | Sequence | RT-PCR product size (bp) | Primer positions |
|---|---|---|---|---|---|
| *SAP1* (25) | X56867 | Upstream | 5′-TCAATCAATTTACTCTTCCATTTCTAACA-3′ | | 96–124 |
| | | Downstream | 5′-CCAGTAGCATTAACAGGAGTTTTAATGACA-3′ | 161 | 237–256 |
| *SAP2* (55) | M83663 | Upstream | 5′-AACAACAACCCACTAGACATCACC-3′ | | 94–117 |
| | | Downstream | 5′-TGACCATTAGTAACTGGGAATGCTTTAGGA-3′ | 178 | 242–271 |
| *SAP3* (53) | L22358 | Upstream | 5′-CCTTCTCTAAAATTATGGATTGGAAC-3′ | | 151–176 |
| | | Downstream | 5′-TTGATTTCACCTTGGGGACCAGTAACATTT-3′ | 231 | 352–381 |
| *SAP4* (32) | L22388 | Upstream | 5′-CATTCATTCCTTTAATACCGACTATC-3′ | | 69–94 |
| | | Downstream | 5′-GGTAACAAACCCTGTAGATCTTTTAAC-3′ | 156 | 198–224 |
| *SAP5* (34) | 30191 | Upstream | Same as *SAP4* | | 149–174, 174–199, 199–224 |
| | | Downstream | Same as *SAP4* | 156, 181, 206 | 328–354 |
| *SAP6* (34) | 30192 | Upstream | Same as *SAP4* | | 105–130, 130–155, 155–180 |
| | | Downstream | Same as *SAP4* | 156, 181, 206 | 284–310 |
| *SAP7* (34) | 30193 | Upstream | 5′-GAAATGCAAAGAGTATTAGAGTTATTAC-3′ | | 280–307 |
| | | Downstream | 5′-GAATGATTTGGTTTACATCATCTTCAACTG-3′ | 196 | 446–475 |
| *ACT1* (29) | XI6377 | Upstream A | 5′-GGCTGGTAGAGACTTGACCAACCATTTG-3′ | 304 | 2224–2251 |
| | | Upstream B | 5′-GATTTTGTCTGAACGTGGTAACAG-3′ | 271 | 2257–2280 |
| | | Downstream | 5′-GGAGTTGAAAGTGGTTTGGTCAATAC-3′ | | 2502–2527 |
| 5.8S rRNA (30) | L07796 | Upstream | 5′-TCCGTAGGTGAACCTGCGG-3′ | | 196–214 |
| | | Downstream | 5′-TCCTCCGCTTATTGATATGC-3′ | 534 | 710–729 |

a denaturing 7% polyacrylamide gel (7 M urea), exposed to autoradiography film at −70°C, and developed.

**Sensitivity and specificity of RT-PCR.** *SAP2*-expressing *C. albicans* cells grown as described above were added to *Candida* culture-negative saliva at concentrations of $10^0$ to $10^6$ cells/ml. Total RNA was isolated from the spiked saliva, and *SAP2* mRNA expression was assayed by RT-PCR. In a separate experiment, the sensitivity of the RT-PCR method was assessed by adding decreasing amounts (from $10^5$ to $10^0$ pg) of *SAP2* RNA from cells grown as described above.

Selected PCR products were sequenced to assess the specificity of the reaction. After electrophoresis through a 2% agarose gel, PCR products were purified by the QIAquick gel extraction protocol as described by the manufacturer (Qiagen, Crawley, United Kingdom). Each PCR product was sequenced by using the automated ABI Prism 377 sequencing system at the Advanced Biotechnology Centre, London, United Kingdom.

## RESULTS

*SAP* gene expression is extremely sensitive and responsive to changes in environmental growth conditions, pH, and metal ion concentrations. *Candida* is also a commensal organism and is present at relatively low concentrations in asymptomatic *Candida* carriers. Therefore, we needed to differentiate between individuals who were carriers and those individuals who were truly *Candida* negative. Thus, assessments of the sensitivity, as well as the specificity, of *SAP* gene expression in vivo from the oral cavity were necessary.

**Sensitivity of the RNA isolation and RT-PCR protocols.** The sensitivity of the *SAP* mRNA detection protocol was tested in spiking experiments whereby saliva (*Candida* negative by culture) was spiked with $10^1$ to $10^6$ *SAP2*-expressing *C. albicans* cells/ml of saliva; this concentration range is representative of the range of *C. albicans* cells present in asymptomatic *Candida* carriers and candidiasis patients. *SAP2* mRNA from samples containing between 10 and 100 *SAP2* mRNA-expressing *C. albicans* cells was consistently amplified by RT-PCR (Fig. 1A) by using the cell lysis and RNA isolation protocols described above. The RT-PCR method, optimized by modified Taguchi methods (12), could detect as little as 1 pg of *SAP2* RNA (Fig. 1B), although it is difficult to relate this to *Candida* cell numbers in vivo.

**Specificity of the *SAP1* to *SAP7* primers.** PCR amplification primers were chosen from upstream transcribed but untranslated regions of each of the *C. albicans SAP1* to *SAP7* gene sequences and resulted in products ranging in size from 156 to 231 bp (Table 1). *SAP4*, *SAP5*, and *SAP6* are nearly identical in the sequences of both translated and untranslated regions of their respective mRNAs. Therefore, a single set of PCR prim-

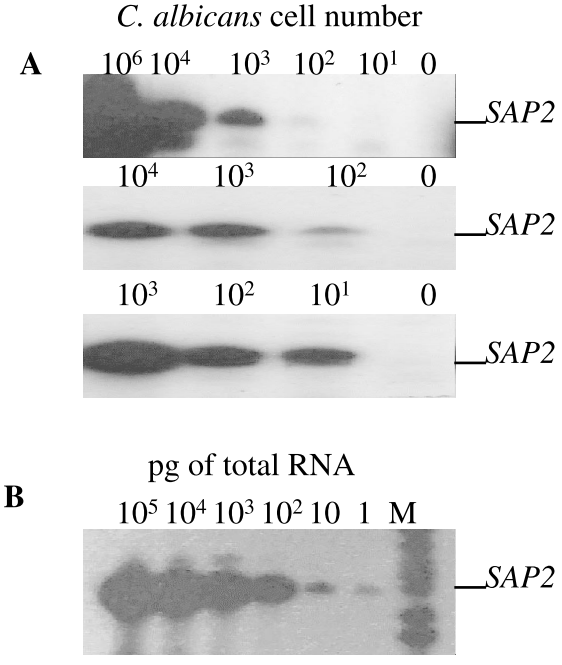


FIG. 1. Sensitivity of the RNA isolation protocol and RT-PCR system. (A) *SAP2* mRNA amplification from total RNA isolated from *Candida* culture-negative saliva spiked with different amounts of *SAP2*-expressing *C. albicans* NCPF 3156 cells ($10^1$ to $10^6$). The autoradiographs from triplicate experiments are shown. (B) *SAP2* mRNA amplification from RT-PCR mixtures containing between $10^0$ and $10^5$ pg of total RNA isolated from *SAP2*-expressing *C. albicans* NCPF 3156 cells. M denotes radiolabelled pBR322 *Msp*I-digested molecular size markers (from the bottom, 147, 160, 180, 190, 201, 217, and 240 bp).

ers which produces a 156-bp product derived from any one member or all three members of the *SAP4*, *SAP5*, and *SAP6* gene subfamily was chosen. While *SAP4* contains only a single target DNA sequence complementary to the 5′ PCR primer, upstream of both *SAP5* and *SAP6*, this sequence is repeated twice in tandem, resulting in two additional PCR products of 181 and 206 bp. Thus, the absence of these two bands in the presence of the common 156-bp signal indicates that *SAP4* is uniquely expressed. Two primer sets designed to detect the *Candida* actin gene (*ACT1*) served as a positive control for the presence of *Candida* species in the absence of *SAP* gene expression. We used two sets of actin primers which produced PCR products of 304 and 271 bp. Using RT-PCR, both actin primer sets were consistently positive or negative, confirming the presence or absence of *Candida* in the saliva samples.

Each of the five different *SAP* primer sets was specific for each of the *SAP* genes (Fig. 2A), excluding the possibility that any one *SAP* primer set could elicit a false-positive result. Cross-reactivity with other *SAP* genes was assayed by PCR by using the specific *SAP1* to *SAP7* primer sets and *C. albicans* genomic DNA. The specificity and identity of the *SAP1*, *SAP2*, *SAP3*, and *SAP7* PCR products were confirmed by DNA sequence analysis (data not shown). Inefficient separation of the *SAP4*, *SAP5*, and *SAP6* gene products complicated sequence analysis, but the derived sizes of the PCR products were consistent with the amplification of the correct gene fragments. In occasional saliva samples obtained from *Candida* carriers and *Candida*-infected individuals, the *SAP2* primer set amplified a 165-bp fragment in conjunction with the expected 178-bp *SAP2* mRNA product. The 165-bp fragment was sequenced and found to correspond to mRNA from human cytochrome *b*, a common, highly expressed mitochondrial protein (data not shown).

In addition to the ability to recognize the appropriate *C. albicans SAP* genes, each of the *SAP1* to *SAP7* primer sets was also checked for specificity by attempting to amplify each of the *SAP* genes from genomic DNAs isolated from the following five other pathogenic *Candida* species: *C. stellatoidea*, *C. dubliniensis* (a new *Candida* species closely related to *C. albicans*), *C. tropicalis*, *C. parapsilosis*, and *C. guilliermondii*. The *SAP1* to *SAP7* primer pairs did not react with any of these species except *C. stellatoidea* (Fig. 2). All of the *C. albicans SAP1* to *SAP7* primer sets produced the appropriate amplification products from *C. stellatoidea* (Fig. 2B), although this was expected since it is generally accepted that *C. stellatoidea* is a variant of *C. albicans* (27, 38).

**Sample collection.** For assay of *SAP* mRNA expression in vivo, whole saliva was chosen, as it is a quickly and conveniently collected sample and provided reliable and consistent *SAP* mRNA detection. The sample volumes and their concentrations of *Candida* CFU for saliva collected from all subjects who participated in this study are shown in Table 2. Between 0.7 and 8 ml of whole saliva was collected for total RNA isolation; salivary *Candida* counts varied from 70 to $>10^4$ CFU/ml (excluding the *Candida* culture-negative group). Consequently, the minimal number of yeast cells present for total RNA isolation was 420, indicating that all experimental samples were above the detection limit determined for this assay (Fig. 1A). The absence of fungal DNA in the isolated total RNA preparations was confirmed by PCR by using generic fungus-specific primers which failed to detect the 5.8S rDNA plus intergenic sequence regions (54) (Fig. 3).

**In vivo expression of *C. albicans SAP1* to *SAP7* genes in saliva.** All seven *Candida* culture-negative subjects were negative for actin mRNA and all seven *SAP* genes, confirming

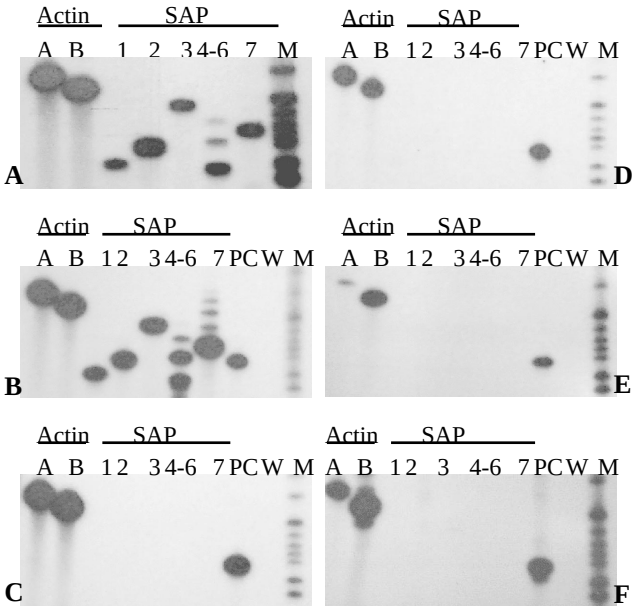


FIG. 2. Reactivity of *SAP1* to *SAP7* primer sets with genomic DNAs isolated from different *Candida* species. (A) *C. albicans* NCPF 3153; (B) *C. stellatoidea* ATCC 11006 (type I); (C) *C. dubliniensis* NCPF 3949; (D) *C. tropicalis* ATCC 750; (E) *C. parapsilosis* ATCC 22019; (F) *C. guilliermondii* NCPF 3099. In autoradiographs B to F, *SAP2* detection from *C. albicans* NCPF 3156 DNA was included as a positive control (PC) and water was used as a negative control (W). M denotes radiolabelled pBR322 *Msp*I-digested molecular size markers (from the bottom, 147, 160, 180, 190, 201, 217, 240, and 307 bp). The expected *SAP1* to *SAP7* gene products are amplified from DNAs isolated from *C. albicans* (A) and *C. stellatoidea* (B). The autoradiographs for the other four species (C to F) indicate a lack of any *SAP* gene products, suggesting that the *SAP1* to *SAP7* primer pairs are specific for *C. albicans* species.

that *Candida* species were absent from the saliva samples (Fig. 4A and Table 3). The absence of either specific or nonspecific RT-PCR amplification products also demonstrated that the primer sets were not cross-reactive with other unknown RNA sequences present in the saliva samples. We also observed that occasional saliva RNA preparations were inhibitory to the RT-PCR. To control for this eventuality, *SAP2* RNA was added to each saliva RNA sample; synthesis of a *SAP2* amplification product indicated that these saliva RNA preparations did not inhibit the RT-PCR.

The eight specimens obtained from asymptomatic *Candida* carriers were each positive for both actin primer sets, confirming the presence of *Candida* mRNA in the saliva RNA samples. The yeast species was previously identified as *C. albicans*. The presence of RT-PCR products from *Candida* carriers with low CFU counts (Table 2) confirmed the sensitivity of the protocol, which was capable of detecting fewer than 100 *C. albicans* cells in saliva (Fig. 1A). Two of the eight asymptomatic carriers were positive for *SAP4*, *SAP5*, or *SAP6* mRNA only; four were positive for *SAP2* and *SAP4*, *SAP5*, or *SAP6* transcripts; two were positive for *SAP2*, *SAP4*, *SAP5*, or *SAP6*, and *SAP7* mRNA. All *Candida* carriers were positive for *SAP4*, *SAP5*, or *SAP6* transcripts, but none were positive for *SAP1* or *SAP3* mRNA (Fig. 4B and Table 3).

The primer sets designed to detect *SAP4*, *SAP5*, or *SAP6* mRNA produced three RT-PCR fragments of 156, 181, and 206 bp. However, it was noted that samples from some *Candida* carriers gave rise only to the 156-bp fragment (Fig. 4B). If *SAP5* and *SAP6* transcripts were present, the primers might be expected to amplify the 181- and 206-bp fragments in addition

TABLE 2. Salivary volumes and *Candida* counts in the three study populations

| Group | Saliva vol (ml) | *Candida* count (CFU/ml) | Total *Candida* count in extraction (CFU) |
|---|---|---|---|
| *Candida* culture-negative controls | | | |
| A | 2.5 | 0 | 0 |
| B | 5 | 0 | 0 |
| C | 5 | 0 | 0 |
| D | 2.5 | 0 | 0 |
| E | 5 | 0 | 0 |
| F | 2.5 | 0 | 0 |
| G | 2.5 | 0 | 0 |
| *Candida* carriers[a] | | | |
| A | 6 | 300 | 1,800 |
| B | 6.5 | 800 | 5,200 |
| C | 8 | 300 | 2,400 |
| D | 4.5 | 200 | 900 |
| E | 5.5 | 200 | 1,100 |
| F | 7 | 780 | 5,460 |
| G | 7 | 680 | 4,760 |
| H | 6 | 70 | 420 |
| Oral candidiasis patients[a] | | | |
| A | 1.5 | (1,720)[b] | (2,580)[b] |
| B | 2 | 4,800 | 9,600 |
| C | 1 | 5,360 | 5,360 |
| D | 4 | >10,000 | >40,000 |
| E | 1 | (1,620)[b] | (1,620)[b] |
| F | 2 | 2,880 | 5,760 |
| G | 2.3 | >10,000 | >23,000 |
| H | 1 | 4,000 | 4,000 |
| I | 0.7 | >10,000 | >7,000 |
| J | 2.5 | >10,000 | >25,000 |

[a] Subjects harbored only *C. albicans*.
[b] Counts were not performed at time the saliva sample was taken. However, post-thawed counts (in parentheses) indicated high initial *C. albicans* counts.

to the 156-bp fragment. Their absence suggests that only *SAP4* mRNA is expressed in these *Candida* carriers. Whether *SAP4* mRNA alone or a combination of *SAP4*, *SAP5*, and *SAP6* transcripts is present in these RNA preparations, the results clearly demonstrate that the *SAP4* to *SAP6* gene subfamily was expressed in all of the saliva samples containing *C. albicans*. As *SAP4* mRNA has never been detected under laboratory conditions (23, 52), the expression of this proteinase alone, or in conjunction with *SAP5* and *SAP6*, may be different in vivo.

All 10 patients with oral *C. albicans* infection were positive for actin, confirming the presence of *Candida* mRNA in the saliva RNA sample. Two patients expressed the full repertoire of all seven *SAP* genes, and three patients were positive for *SAP2*, *SAP3*, *SAP4*, -5, or -6, and *SAP7* mRNA but not *SAP1* mRNA. One patient was positive for *SAP2*, *SAP3*, and *SAP4*, *SAP5*, or *SAP6* transcripts, and one other patient expressed *SAP4*, *SAP5*, or *SAP6* and *SAP2* and *SAP7* transcripts. Three patients expressed *SAP2* and *SAP4*, *SAP5*, or *SAP6* transcripts only. All 10 patients were positive for *SAP2* and *SAP4*, *SAP5*, or *SAP6* mRNA transcripts (Fig. 4C and Table 3).

## DISCUSSION

This study has established a convenient, reliable, and highly sensitive method for the detection of *C. albicans SAP* mRNA expression directly in patient samples. Specific primers constructed for seven *SAP* genes (*SAP1* to *SAP7*) showed that all seven are expressed in vivo and provided preliminary data indicating which of the seven proteinases are commonly expressed in individuals with *C. albicans* oral colonization and infection. *SAP* mRNA expression patterns observed in vivo appear to be different from those previously demonstrated under laboratory culture conditions.

The *SAP1* to *SAP7* primers were shown to be *C. albicans* specific against a panel of genomic DNAs isolated from various *Candida* species. The *SAP* primers reacted only with DNAs isolated from *C. albicans* and *C. stellatoidea* (Fig. 2A and B, respectively), the latter of which is considered to be a variant of *C. albicans* (27, 38). The *SAP* primers did not react with DNA isolated from *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*, or *C. guilliermondii* (Fig. 2C, D, E, and F, respectively). *C. dubliniensis* has recently been shown to have its own *SAP* family comprised of at least seven genes (20), and the lack of cross-reactivity of the *C. dubliniensis* DNA with the *C. albicans SAP1* to *SAP7* primers supports the species distinction between *C. dubliniensis* and *C. albicans*.

Analysis of the differential expression of genes *SAP1* to *SAP7* in vivo indicated that *SAP2* and *SAP4*, *SAP5*, and *SAP6* appear to be the predominant genes expressed in the oral cavities of both asymptomatic *Candida* carriers and patients with oral candidiasis, with *SAP4*, *SAP5*, or *SAP6* mRNA being found in all of the subjects examined so far (Table 3). This study also demonstrated the expression of *SAP1* and *SAP3* transcripts in oral candidiasis patients but not in *Candida* carriers (Table 3). Expression of *SAP1* and *SAP3* has been associated with phenotypic switching in vitro, and this association with oral infection may implicate phenotypic switching in the virulence of *C. albicans*. A further observation was the expression of *SAP7* mRNA, which has never before been demonstrated (Table 3). This study suggests that progression from colonization to symptomatic infection with *C. albicans* is reflected by the expression pattern of certain proteinases, most likely due to

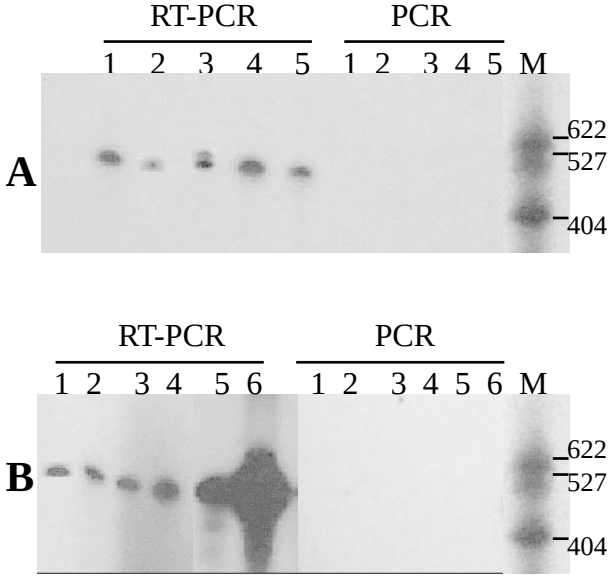


FIG. 3. Detection of DNA and RNA in DNase-treated total RNA preparations in five *Candida* carrier subjects (A) and six patients with oral candidiasis (B). An absence of PCR-generated products confirmed that the preparations were DNA free, while RT-PCR generated the expected products from RNA. RT-PCR and PCR used primers specific for the 5.8S rDNA plus intergenic spacer regions. All of the total RNA preparations were DNA free. Differences in mobility were due to a gel artifact. M denotes radiolabelled pBR322 *Msp*I-digested molecular size markers. Molecular sizes are indicated on the right in base pairs.

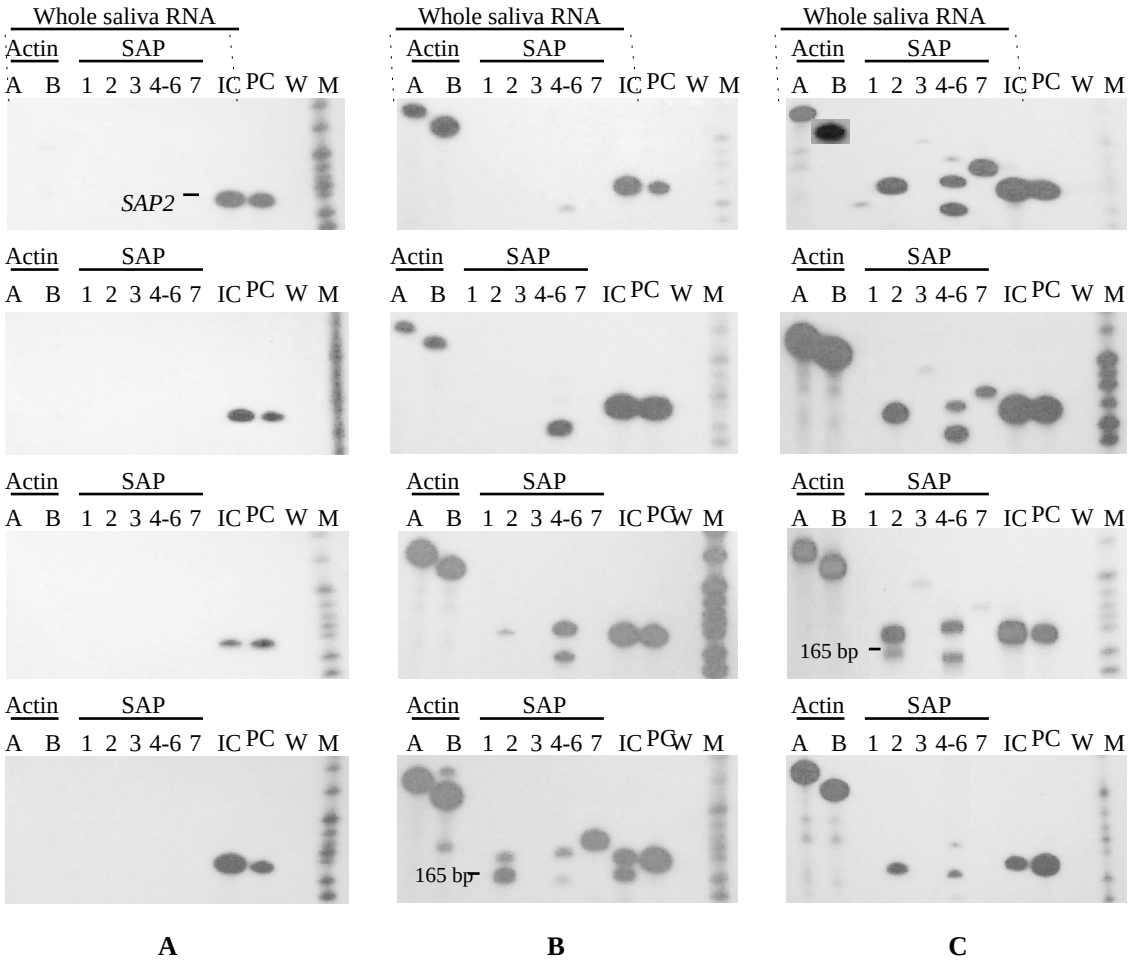


FIG. 4. Detection of *C. albicans ACT1* and *SAP1* to *SAP7* mRNAs from clinical saliva samples. Four representative autoradiographs are presented for each of the following study groups: *Candida* culture-negative controls (A), asymptomatic *Candida* carriers (B), and oral candidiasis patients (C). Lanes A and B show the two primer pairs used for the detection of actin. The presence or absence of actin signals correlated with the presence (B and C) or absence (A) of *C. albicans* in the saliva samples. Lanes 3 to 7 show the five primer pairs used to detect *SAP1* to *SAP7* transcripts (*SAP1*, *SAP2*, *SAP3*, *SAP4* to *SAP6*, and *SAP7*). Expression of *SAP1* to *SAP7* mRNA was absent in group A and variable in groups B and C. The following three controls detecting *SAP2* mRNA were included in each assay: an inhibition control (lane 8) whereby *SAP2* RNA was added to the saliva RNA to determine if the latter was inhibitory to the RT-PCR (lane IC), a positive control (lane 9) with *SAP2* RNA alone (lane PC), and a negative water control (lane W). M denotes radiolabelled pBR322 *Msp*I-digested molecular size markers (from the bottom, 147, 160, 180, 190, 201, 217, 240, 307, and 404 bp). The 165-bp fragment (B, bottom panel) was sequenced and found to correspond to mRNA from human cytochrome *b*, a common, highly expressed mitochondrial protein.

different environmental conditions in the oral cavities of carriers and those of diseased individuals.

Data acquired in vitro suggest that Sap2 is the main proteinase secreted in protein-containing media (22, 52, 55). Our results are consistent with the premise that Sap2 is a predominant proteinase of *C. albicans*, as *SAP2* expression was observed in all patients with oral candidiasis and all but two *Candida* carrier individuals (Table 3). Under laboratory conditions, Sap2 is capable of degrading numerous substrates, including extracellular matrix proteins, mucin, and sIgA (13, 22, 41), all of which constitute host proteins in the oral cavity. Digestion of these (and other) nutrients in vivo may help *C. albicans* to acquire essential nitrogen for growth and/or to attach to and penetrate oral mucosa. In addition, digestion of sIgA (which is normally resistant to proteolysis) may assist *C. albicans* to evade the immune response.

The samples from the two *Candida* carriers that did not express *SAP2* were positive for *SAP4*, *SAP5*, or *SAP6* transcripts, suggesting that this subfamily of proteinases may have functional properties similar to those of, and may be able to substitute for, Sap2 in the oral cavity. Studies of a rat model have demonstrated *SAP1* and *SAP2* expression in *C. albicans* experimental vaginitis (17) and indicated that Sap2, but not Sap4, Sap5, or Sap6, may be important in vaginal candidiasis in rats (16). In our study, we observed *SAP4*, *SAP5*, or *SAP6* expression in samples collected from the oral cavities of all *C. albicans*-positive individuals, indicating that differential expression of this proteinase family in the human oral cavity may be very different from that in the rat vagina. This is supported by a recent study suggesting that pH-dependent genes are differentially regulated in systemic and vaginal infections (19).

The expression of *SAP4*, *SAP5*, or *SAP6* transcripts in all *Candida* carriers and patients with oral candidiasis suggests that this proteinase subfamily may have an important role in oral *C. albicans* colonization and infection. This is supported by a study of animal models of disseminated candidiasis in which a *sap4 sap5 sap6* triple null mutant showed marked attenuation of virulence (44). The Sap4, Sap5, and Sap6 subfamily may also play a role in immune evasion, as their production appears to partially protect *C. albicans* from phago-

TABLE 3. Summary of in vivo expression of *C. albicans ACT1* and *SAP1* to *SAP7* mRNAs in *Candida* culture-negative control, *Candida* carrier, and oral candidiasis study populations

| Study population | HIV status[a] | Actin[b] | *SAP* gene expression[b] | | | | |
|---|---|---|---|---|---|---|---|
| | | | *SAP1* | *SAP2* | *SAP3* | *SAP4–SAP6* | *SAP7* |
| Total no. of controls positive[c] | ND[d] | 0 | 0 | 0 | 0 | 0 | 0 |
| *Candida* carriers | | | | | | | |
| A | ND | + | − | − | − | + | − |
| B | ND | + | − | − | − | + | − |
| C | ND | + | − | + | − | + | + |
| D | ND | + | − | + | − | + | − |
| E | ND | + | − | + | − | + | − |
| F | ND | + | − | + | − | + | − |
| G | ND | + | − | + | − | + | − |
| H | ND | + | − | + | − | + | + |
| Total no. positive | ND | 8 | 0 | 6 | 0 | 8 | 2 |
| Oral candidiasis patients | | | | | | | |
| A | + | + | − | + | + | + | + |
| B | − | + | − | + | − | + | − |
| C | + | + | − | + | − | + | − |
| D | + | + | + | + | + | + | + |
| E | + | + | − | + | − | + | − |
| F | + | + | − | + | + | + | − |
| G | − | + | − | + | + | + | + |
| H | − | + | − | + | − | + | + |
| I | + | + | + | + | + | + | + |
| J | − | + | − | + | + | + | + |
| Total no. positive | 6 | 10 | 2 | 10 | 6 | 10 | 6 |

[a] +, HIV-seropositive patient; −, HIV-seronegative patient.
[b] +, presence of mRNA detected by RT-PCR; −, absence of mRNA determined by RT-PCR.
[c] Seven *Candida* culture-negative controls were studied.
[d] ND, not determined.

cytic killing by murine macrophages (6). Sanglard et al. (44) also speculated that *SAP4*, *SAP5*, or *SAP6* expression may be required in the process of *SAP2* induction. In patient samples, we did not observe *SAP2* mRNA without the expression of *SAP4*, *SAP5*, or *SAP6*, suggesting that this regulation may possibly occur in vivo.

There is evidence acquired in vitro that Sap antigens are expressed on the surface of *C. albicans* following attachment to epithelial cells (5) and on the surface of hyphal forms (42), which are known to be more adherent than yeast forms (43). White and Agabian (52) and Hube et al. (23) also showed that only *SAP4*, *SAP5*, and *SAP6* transcripts were expressed during the transition from yeast to hyphae at neutral pH in vitro. This suggests that the secretion of Sap4, Sap5, and Sap6 in the oral cavity may facilitate attachment of *C. albicans* to the oral mucosa. However, a recent study showed that a *sap4 sap5 sap6* null mutant exhibited increased adherence to buccal epithelial cells in vitro (51). The presence of hyphae, and hence the expression of *SAP4*, *SAP5*, or *SAP6* mRNA, may be expected in oral candidiasis patients. However, the association of hyphae with the carrier state has not been thoroughly investigated; therefore, the expression of these three proteinases in all of the *Candida* carriers is suggestive of the presence of hyphae. Alternatively, in vivo, yeast cells may express *SAP4*, *SAP5*, and *SAP6*. Although there is no in vitro evidence that *SAP4*, *SAP5*, and *SAP6* genes are expressed by yeast cells, the expression of the three proteinases they encode in *Candida* carriers and the first demonstration of *SAP7* expression in vivo (vide infra) confirm that the oral milieu is quite different from laboratory culture conditions used to study *SAP* mRNA expression.

Similar patterns of specific gene expression have been observed in studies of other organisms. For example, during infection with the spirochete *Borrelia burgdorferi*, several genes have been shown to be selectively induced in vivo, and different *Borrelia* gene products have been detected in different sites of the body (3, 10). The extended repertoire of *SAP* genes in *C. albicans* and their differential expression in vivo indicate that different *SAP* genes may be selectively expressed at different stages and in different clinical forms of candidiasis, such as pseudomembranous, erythematous, atrophic, and hyperplastic forms (4, 28). Further studies using the techniques developed in this study will address this issue.

This is the first study to detect expression of the *SAP7* gene. It also indicates that the proteinase encoded by this gene, if translated, may be associated with *C. albicans* infection, as *SAP7* transcripts were detected in 60% of oral candidiasis patients (6 of 10 patients), as opposed to 25% of *Candida* carriers (2 of 8 carriers) (Table 3). The function of Sap7 is unknown, but its apparent differential expression in oral candidiasis patients is noteworthy and clearly warrants further investigation.

Interestingly, *SAP1* and *SAP3* mRNA transcripts were detected only in patients with oral *C. albicans* infections and not in *Candida* carriers (Table 3). Under laboratory conditions, the expression of *SAP1* and *SAP3* is coordinately regulated during phenotypic switching (23, 35, 36, 52). Therefore, the detection of *SAP1* and *SAP3* transcripts in oral candidiasis suggests that phenotypic switching occurs in vivo, and furthermore, that it may be associated with the development of infection. This is supported by the observation that *C. albicans* isolated from infected patients exhibits, on average, higher rates of phenotypic switching than commensal strains from the oral cavity (21). Recently, Schaller et al. (45) investigated the temporal expression of *SAP* genes in an in vitro model of oral candidiasis using reconstituted human epithelium. They showed that *SAP1* and *SAP3* were the first *SAP* genes to be expressed by *C. albicans*. In addition, they noted that the expression of these two genes coincided with the development of lesions and suggested that their model reflects the infectious state. This is supported by our findings, since we did not detect *SAP1* or *SAP3* mRNA expression in any of the *Candida* carriers tested, only in the oral candidiasis group.

In this study, we have found differences in the expression profiles of *SAP1* through *SAP7* mRNA between patients with oral *C. albicans* infections and asymptomatic *Candida* carrier subjects. We do not believe these differences are due to the greater numbers of *C. albicans* cells present in the samples obtained from patients with oral candidiasis, because in some cases, the total numbers of *C. albicans* cells present in the RNA extraction were similar in the two study groups (Table 2). In addition, two *Candida* carriers with lower total *C. albicans* cell numbers expressed more *SAP* genes than three other *Candida* carriers who had greater total *C. albicans* cell counts (compare Table 2 with Table 3). Furthermore, the detection of *C. albicans* actin mRNA in all of the *Candida* carrier saliva samples indicated that the methods employed were sufficiently sensitive to detect the presence of *SAP* mRNA. In terms of *SAP* mRNA expression, we realize that the assay used does not quantify exact levels of individual mRNAs and that comparative levels of different *SAP* mRNAs may vary.

Two additional elements that may possibly affect *SAP* gene expression in *C. albicans* in vivo are HIV infection and anti-

fungal drug therapy, which may select for more pathogenic *C. albicans* strains (48). Selection of *C. albicans* strains is thought to occur early in HIV infection and may be associated with increased Sap production (18, 40), increased adherence to oral epithelial cells (49), and genotypic alterations (9). Antifungal drug therapy, which has been correlated with colonial morphology changes (31) and phenotypic differences (7, 26), may also be a major cause of *Candida* strain selection. A number of the patients had previously been treated with antifungal drugs, although none were being treated with either antibiotic or antimycotic drugs at the time of sampling; 6 of 10 patients with oral candidiasis were HIV seropositive. However, no correlation between HIV infection or antimicrobial treatment history and *SAP* expression was evident.

In summary, this is the first study to show that genes *SAP1* to *SAP7* are expressed in vivo, providing evidence of a role for this proteinase family in human oral candidiasis. It further indicates that the pathogenesis of oral *C. albicans* infections may be associated with the differential expression of individual *SAP* genes. In addition, *SAP* mRNA expression patterns observed in vivo appear to be different from those first demonstrated under laboratory culture conditions, indicating that the involvement of secreted aspartyl proteinases in *C. albicans* pathogenesis in vivo may not be necessarily inferred from conventional laboratory growth and analysis.

## ACKNOWLEDGMENTS

We thank Sue Howell for kindly supplying the *Candida* reference strains used in this study.

This work was supported by the Dunhill Medical Trust and NIH grants AI33317 and POI-DE-07946.

## REFERENCES

1. **Agabian, N., F. C. Odds, D. Poulain, D. R. Soll, and T. C. White.** 1994. Pathogenesis of invasive candidiasis. J. Med. Vet. Mycol. **32:**229–237.
2. **Agatensi, L., F. Franchi, F. Mondello, R. L. Bevilacqua, T. Ceddia, F. De Bernardis, A. Cassone, R. Ruchel, F. Zimmermann, B. Boning-Stutzer, and U. Helmchen.** 1991. Vaginopathic and proteolytic *Candida* species in outpatients attending a gynaecology clinic. J. Clin. Pathol. **419:**199–202.
3. **Akins, D. R., S. F. Porcella, T. G. Popova, D. Shevchenko, S. I. Baker, M. Li, M. V. Norgard, and J. D. Radolf.** 1995. Evidence for *in vivo* but not *in vitro* expression of a *Borrelia burgdorferi* outer surface protein F (OspF) homologue. Mol. Microbiol. **18:**507–520.
4. **Axell, T., A. Baert, C. Brocheriou, S. J. Challacombe, D. Greenspan, W. Ten Kate, G. Laskaris, A. Mano Azul, J. J. Pindborg, P. Reichart, E. A. J. M. Schulten, C. Scully, S. Syrjanen, I. Van der Wall, and D. Williams.** 1991. An update of the classification and diagnostic criteria of oral lesions in HIV infection. J. Oral Pathol. Med. **20:**97–100.
5. **Borg, M., D. Watters, B. Reich, and R. Rüchel.** 1988. Production and characterization of monoclonal antibodies against secretory proteinase of *Candida albicans* CBS 2730. Zentbl. Bakteriol. Mikrobiol. Hyg. Ser. A **268:**62–73.
6. **Borg-von Zepelin, M., S. Beggah, K. Boggian, D. Sanglard, and M. Monod.** 1998. The expression of the secreted aspartyl proteinases Sap4 to Sap6 from *Candida albicans* in murine macrophages. Mol. Microbiol. **28:**543–554.
7. **Bruatto, M., V. Vidotto, G. Marinuzzi, R. Raiteri, and A. Sinicco.** 1991. *Candida albicans* biotypes in human immunodeficiency virus type 1-infected patients with oral candidiasis before and after antifungal therapy. J. Clin. Microbiol. **29:**726–730.
8. **Cassone, A., M. Boccanera, D. Adriani, G. Santoni, and F. De Bernardis.** 1995. Rats clearing a vaginal infection by *Candida albicans* acquire specific, antibody-mediated resistance to vaginal reinfection. Infect. Immun. **63:**2619–2624.
9. **Challacombe, S. J., J. Muir, S. A. Howell, and S. P. Sweet.** 1995. Genetic variability of *Candida albicans* in HIV infection. Microb. Ecol. Health Dis. **8:** 63–70.
10. **Champion, C. I., D. R. Blanco, J. T. Skare, D. A. Haake, M. Giladi, D. Foley, J. N. Miller, and J. A. Lovett.** 1994. A 9.0-kilobase-pair circular plasmid of *Borrelia burgdorferi* encodes an exported protein: evidence for expression only during infection. Infect. Immun. **62:**2653–2661.
11. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
12. **Cobb, B. D., and J. M. Clarkson.** 1994. A simple procedure for optimising the polymerase chain reaction (PCR) using modified Taguchi methods. Nucleic Acids Res. **22:**3801–3805.
13. **Colina, A. R., F. Aumont, N. Deslauriers, P. Belhumeur, and L. de Repentigny.** 1996. Evidence for degradation of gastrointestinal mucin by *Candida albicans* secretory aspartyl proteinase. Infect. Immun. **64:**4514–4519.
14. **Coogan, M. M., S. P. Sweet, and S. J. Challacombe.** 1994. Immunoglobulin A (IgA), IgA1, and IgA2 antibodies to *Candida albicans* in whole and parotid saliva in human immunodeficiency virus infection and AIDS. Infect. Immun. **62:**892–896.
15. **Cutler, J. E.** 1991. Putative virulence factors of *Candida albicans*. Annu. Rev. Microbiol. **45:**187–218.
16. **De Bernardis, F., S. Arancia, L. Morelli, B. Hube, D. Sanglard, W. Schäfer, and A. Cassone.** 1999. Evidence that members of the secretory aspartyl proteinases gene family (SAP), in particular SAP2, are virulence factors for *Candida* vaginitis. J. Infect. Dis. **179:**201–208.
17. **De Bernardis, F., A. Cassone, J. Sturtevant, and R. Calderone.** 1995. Expression of *Candida albicans* SAP1 and SAP2 in experimental vaginitis. Infect. Immun. **63:**1887–1892.
18. **De Bernardis, F., P. Chiani, M. Ciccozzi, G. Pellegrini, T. Ceddia, G. D'Offizzi, I. Quinti, P. A. Sullivan, and A. Cassone.** 1996. Elevated aspartic proteinase secretion and experimental pathogenicity of *Candida albicans* isolates from oral cavities of subjects infected with human immunodeficiency virus. Infect. Immun. **64:**466–471.
19. **De Bernardis, F., F. A. Muhlschlegel, A. Cassone, and W. A. Fonzi.** 1998. The pH of the host niche controls gene expression in and virulence of *Candida albicans*. Infect. Immun. **66:**3317–3325.
20. **Gilfillan, G. D., D. J. Sullivan, K. Haynes, T. Parkinson, D. C. Coleman, and N. A. R. Gow.** 1998. *Candida dubliniensis*: phylogeny and putative virulence factors. Microbiology **144:**829–838.
21. **Hellstein, J., H. Vawter-Hugart, P. Fotos, J. Schmid, and D. R. Soll.** 1993. Genetic similarity and phenotypic diversity of commensal and pathogenic strains of *Candida albicans* isolated from the oral cavity. J. Clin. Microbiol. **31:**3190–3199.
22. **Hube, B.** 1996. *Candida albicans* secreted aspartyl proteinases. Curr. Top. Med. Mycol. **7:**55–69.
23. **Hube, B., M. Monod, D. A. Schofield, A. J. P. Brown, and N. A. R. Gow.** 1994. Expression of seven members of the gene family encoding secretory aspartyl proteinases in *Candida albicans*. Mol. Microbiol. **14:**87–99.
24. **Hube, B., D. Sanglard, F. C. Odds, D. Hess, M. Monod, W. Schafer, A. J. Brown, and N. A. Gow.** 1997. Disruption of each of the secreted aspartyl proteinase genes SAP1, SAP2, and SAP3 of *Candida albicans* attenuates virulence. Infect. Immun. **65:**3529–3538.
25. **Hube, B., C. J. Turver, F. C. Odds, H. Eiffert, G. J. Boulnois, H. Kochel, and R. Ruchel.** 1991. Sequence of the *Candida albicans* gene encoding the secretory aspartate proteinase. J. Med. Vet. Mycol. **29:**129–132.
26. **Imbert-Bernard, C., A. Valentin, and M. Mallie.** 1995. Involvement of *Candida albicans* cell wall proteins in the adherence of blastospores to human buccal epithelial cells. Exp. Mycol. **19:**247–253.
27. **Kwon-Chung, K. J., W. S. Riggsby, R. A. Uphoff, J. B. Hicks, W. L. Whelan, E. Reiss, B. B. Magee, and B. L. Wickes.** 1989. Genetic differences between type I and type II *Candida stellatoidea*. Infect. Immun. **57:**527–532.
28. **Lehner, T.** 1967. Oral candidosis. Dent. Pract. **17:**209–216.
29. **Losberger, C., and J. F. Ernst.** 1989. Sequence of the *Candida albicans* gene encoding actin. Nucleic Acids Res. **17:**9488.
30. **Lott, T. J., R. J. Kuykendall, and E. Reiss.** 1993. Nucleotide sequence analysis of the 5.8S rDNA and adjacent ITS2 region of *Candida albicans* and related species. Yeast **9:**1199–1206.
31. **McCullough, M. J., B. C. Ross, B. D. Dwyer, and P. C. Reade.** 1994. Genotype and phenotype of oral *Candida albicans* from patients infected with the human immunodeficiency virus. Microbiology **140:**1195–1202.
32. **Miyasaki, S. H., T. C. White, and N. Agabian.** 1994. A fourth secreted aspartyl proteinase gene (*SAP4*) and a *CARE2* repetitive element are located upstream of the *SAP1* gene in *Candida albicans*. J. Bacteriol. **176:**1702–1710.
33. **Monod, M., B. Hube, D. Hess, and D. Sanglard.** 1998. Differential regulation of *SAP8* and *SAP9*, which encode two new members of the secreted aspartic proteinase family in *Candida albicans*. Microbiology **144:**2731–2737.
34. **Monod, M., G. Togni, B. Hube, and D. Sanglard.** 1994. Multiplicity of genes encoding secreted aspartic proteinases in *Candida* species. Mol. Microbiol. **13:**357–368.
35. **Morrow, B., H. Ramsey, and D. R. Soll.** 1994. Regulation of phase-specific genes in the more general switching system of *Candida albicans* strain 3153A. J. Med. Vet. Mycol. **32:**287–294.
36. **Morrow, B., T. Srikantha, and D. R. Soll.** 1992. Transcription of the gene for a pepsinogen, PEP1, is regulated by white-opaque switching in *Candida albicans*. Mol. Cell. Biol. **12:**2997–3005.
37. **Newport, G., and N. Agabian.** 1997. KEX2 influences *Candida albicans* proteinase secretion and hyphal formation. J. Biol. Chem. **272:**28954–28961.
38. **Odds, F. C.** 1988. *Candida* and candidosis. Bailliere Tindall, Philadelphia, Pa.
39. **Odds, F. C.** 1994. *Candida* species and virulence. ASM News **60:**313–318.
40. **Ollert, M. W., C. Wende, M. Gorlich, C. G. McMullan-Vogel, M. Borg-von Zepelin, C. W. Vogel, and H. C. Korting.** 1995. Increased expression of *Candida albicans* secretory proteinase, a putative virulence factor, in isolates

from human immunodeficiency virus-positive patients. J. Clin. Microbiol. **33:** 2543–2549.
41. **Rüchel, R.** 1984. A variety of *Candida* proteinases and their possible targets of proteolytic attack in the host. Zentbl. Bakteriol. Hyg. A **257:**266–274.
42. **Rüchel, R., F. Zimmermann, B. Boning-Stutzer, and U. Helmchen.** 1991. Candidiasis visualised by proteinase-directed immunofluorescence. Virchow's Arch. Abt. A Pathol. Anat. **419:**199–202.
43. **Sandin, R. L., A. L. Rogers, R. J. Patterson, and E. S. Beneke.** 1982. Evidence for mannose-mediated adherence of *Candida albicans* to human buccal cells in vitro. Infect. Immun. **35:**79–85.
44. **Sanglard, D., B. Hube, M. Monod, F. C. Odds, and N. A. Gow.** 1997. A triple deletion of the secreted aspartyl proteinase genes *SAP4*, *SAP5*, and *SAP6* of *Candida albicans* causes attenuated virulence. Infect. Immun. **65:**3539–3546.
45. **Schaller, M., W. Schäfer, H. C. Korting, and B. Hube.** 1998. Differential expression of secreted aspartyl proteinases in a model of human oral candidosis and in patient samples from the oral cavity. Mol. Microbiol. **29:** 605–615.
46. **Smolenski, G., P. A. Sullivan, S. M. Cutfield, and J. F. Cutfield.** 1997. Analysis of secreted aspartic proteinases from *Candida albicans*: purification and characterization of individual Sap1, Sap2 and Sap3 isoenzymes. Microbiology **143:**349–356.
47. **Stringaro, A., P. Crateri, G. Pellegrini, G. Arancia, A. Cassone, and F. De Bernardis.** 1997. Ultrastructural localization of the secretory aspartyl proteinase in *Candida albicans* cell wall *in vitro* and in experimentally infected rat vagina. Mycopathologia **137:**95–105.
48. **Sweet, S. P.** 1997. Selection and pathogenicity of *Candida albicans* in HIV infection. Oral Dis. **3:**S88–S95.
49. **Sweet, S. P., S. Cookson, and S. J. Challacombe.** 1995. *Candida albicans* isolates from HIV-infected and AIDS patients exhibit enhanced adherence to epithelial cells. J. Med. Microbiol. **43:**452–457.
50. **Togni, G., D. Sanglard, M. Quadroni, S. I. Foundling, and M. Monod.** 1996. Acid proteinase secreted by *Candida tropicalis*: functional analysis of preproregion cleavages in *C. tropicalis* and *Saccharomyces cerevisiae*. Microbiology **142:**493–503.
51. **Watts, H. J., F. S. Cheah, B. Hube, D. Sanglard, and N. A. R. Gow.** 1998. Altered adherence in strains of *Candida albicans* harbouring null mutations in secreted aspartic proteinase genes. FEMS Microbiol. Lett. **159:**129–135.
52. **White, T. C., and N. Agabian.** 1995. *Candida albicans* secreted aspartyl proteinases: isoenzyme pattern is determined by cell type, and levels are determined by environmental factors. J. Bacteriol. **177:**5215–5221.
53. **White, T. C., S. H. Miyasaki, and N. Agabian.** 1993. Three distinct secreted aspartyl proteinases in *Candida albicans*. J. Bacteriol. **175:**2997–3005.
54. **White, T. J., T. Bruns, S. Lee, and J. Taylor.** 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315–322. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, Inc., New York, N.Y.
55. **Wright, R. J., A. Carne, A. D. Hieber, I. L. Lamont, G. W. Emerson, and P. A. Sullivan.** 1992. A second gene for a secreted aspartate proteinase in *Candida albicans*. J. Bacteriol. **174:**7848–7853.

*Editor:* T. R. Kozel