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Comparison between *Candida albicans* Agglutinin-Like Sequence Gene Expression Patterns in Human Clinical Specimens and Models of Vaginal Candidiasis

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Expression of the eight genes in the *Candida albicans* agglutinin-like sequence (ALS) family was studied by reverse transcription-PCR of RNA isolated from clinical vaginal fluid specimens and vaginal candidiasis model systems. Although expression of all ALS genes was detected across the set of clinical specimens, *ALS1*, *ALS2*, *ALS3*, and *ALS9* transcripts were detected most frequently, and expression of *ALS4* and *ALS5* was detected least frequently. Laboratory strain 3153A and two *C. albicans* strains isolated from the clinical specimens were studied using two models of vaginal candidiasis to determine how closely these models mimicked the clinical specimens at the level of gene expression. ALS gene expression patterns in a murine vaginitis model were identical to those from the clinical specimens. Expression of more ALS genes was detected in specimens collected 7 days after infection compared to those collected at 4 days. Similar patterns of ALS gene expression were observed when the three *C. albicans* strains were tested in the reconstituted human vaginal epithelium model. In this model, expression of *ALS4*, *ALS5*, *ALS6*, and *ALS7* was least frequently detected. Negative or weakened signals for *ALS4* expression were observed at early time points, suggesting that *ALS4* expression, which was strong in the inoculum cells, was down-regulated upon contact of *C. albicans* with vaginal epithelial cells in this model. The data presented here support the conclusion of host-site-specific influences on ALS gene expression and validate the use of the experimental models for evaluating the phenotype of *als*/*als* mutant strains.

Approximately three-fourths of women experience an episode of vaginal candidiasis (29); *Candida albicans* is the etiological agent in over 80% of the cases (5, 29). *C. albicans* has many capabilities that aid its ability to cause disease, including phenotypic switching (30), filamentation (17), adherence (33), and secreted hydrolases (18). Some of these pathogenesis-associated factors are encoded by gene families including the agglutinin-like sequence (ALS) (10), secreted aspartyl proteinase (SAP) (20), and lipase (16) families. The eight ALS genes (*ALS1* to *ALS7* and *ALS9*) encode large, cell surface glycoproteins, some of which promote adhesion to host surfaces (6, 7, 10, 35, 36). Although they share a similar three-domain structure, sequence differences between the Als proteins can be large, suggesting that the proteins may have different functions (10). Differential expression of the ALS genes was demonstrated in vitro by Northern blotting (6, 13–15). An understanding of patterns of ALS gene expression in *C. albicans* cells from clinical specimens and disease models will provide insight into the role of the gene family in colonization and disease, provide greater knowledge about the relationships between the Als proteins, and validate models for use in phenotypic analysis of genetically altered *C. albicans* strains. Reverse transcription (RT)-PCR-based studies have been conducted to investigate similar questions for the SAP and lipase gene families and showed preferential detection of specific gene expression under different host and model conditions (20, 21, 23, 25–28, 31). An RT-PCR assay was developed for ALS genes and used to analyze *C. albicans* RNA from in vitro models of oral candidiasis and denture and catheter biofilms (9) as well as human clinical specimens and those from a hyposalivatory rat model of oral candidiasis (C. B. Green, S. M. Marretta, G. Cheng, F. F. Faddoul, E. J. Ehrhart, and L. L. Hoyer, unpublished data). In the oral specimens, expression of *ALS6* and *ALS7* was least readily detected, suggesting that those genes were transcribed at lower levels both in clinical material and in the model systems.

In this study, we use the ALS-specific RT-PCR assay to focus on human clinical vaginal specimens and two models of vaginal candidiasis. Results presented here show that although expression of each ALS gene could be detected in the clinical material and model systems, expression of *ALS4* and *ALS5* was detected least frequently. These data validate the use of the model systems for study of *als*/*als* mutants and also support the conclusion of host-site-specific effects on ALS gene expression.

MATERIALS AND METHODS

Fungal strains. *C. albicans* strains SC5314 (8) and 3153A (ATCC 36801; American Type Culture Collection, Manassas, Va.) were used in this study. Other *Candida* strains obtained from the American Type Culture Collection included *Candida stellatoidea* (ATCC 11006), *Candida tropicalis* (ATCC 13803),

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Candida glabrata (ATCC 2001), *Candida parapsilosis* (ATCC 22019), *Candida guilliermondii* (ATCC 6260), and *Candida lusitaniae* (ATCC 42720). *Candida dubliniensis* CD36 (CBS 7987) (32) and CM1 (19, 32) were a generous gift from David Coleman (Trinity College, University of Dublin).

Clinical specimen collection. This study was conducted according to the guidelines of the University of Illinois Institutional Review Board; informed consent was obtained from each patient. Women recruited for this study sought gynecologic care for symptoms of vaginal candidiasis (burning, itching, and/or dysuria with presence of a curd-like discharge) or sought routine gynecologic care. All patients had yeast, pseudohyphal, and/or hyphal cells visible by direct microscopic examination of material from a vaginal swab. Patients who had symptoms of vaginal candidiasis were classified as symptomatic and those who sought routine gynecologic care were classified as asymptomatic. There were no clinical signs of vulvovaginitis among the patients in the asymptomatic group, and microscopic fungal cell morphology was similar between the asymptomatic and symptomatic patients. Patients who were taking antibiotics or had used any antifungal treatment in the past 30 days were excluded from the study. Patients provided demographic data and information about current medications and were evaluated for general health. Patient information also included a history of previous vaginal infections, information about the menstrual cycle and pregnancy status, and hormone medication use. Within the symptomatic group, the patient's clinical signs, including the appearance of the affected area and a description of the vaginal fluid or discharge, were noted. Samples of vaginal epithelium and discharge were collected using a vaginal spatula, placed into a sterile vial, and flash frozen in liquid nitrogen within 2 min of collection. A vaginal swab was also collected and streaked onto a plate of Sabouraud agar containing 50 μg of chloramphenicol ml^{-1}. Agar plates were incubated at 37°C for at least 5 days before plates without growth were discarded.

Identification of yeasts from clinical specimens. Pure cultures were derived for fungal colonies that grew on the primary Sabouraud agar plates containing 50 μg of chloramphenicol ml^{-1}. Isolated colonies from the pure culture were used for yeast identification similar to the methods described by Naglik et al. (21). These methods included the use of API 20C AUX strips (BioMerieux), growth on CHROMagar (Paris, France) *Candida* plates, germ tube tests in brain heart infusion broth (Difco) containing 10% horse serum (Gibco BRL), and growth at 42°C. Yeasts were also grown on cornmeal-Tween agar plates to observe microscopic morphology. Samples from which isolated yeasts were identified as *C. albicans* were included in the gene expression analysis (see below). Samples that did not yield growth were eliminated from the study. One sample containing both *C. albicans* and *C. parapsilosis* was retained in the study. *C. albicans* strains isolated from the clinical specimens were grown in YPD medium (10 g of yeast extract, 10 g of peptone, and 20 g dextrose [per liter]) and frozen at −80°C in 15% glycerol. *C. albicans* clinical isolates were designated by the letters GC followed by the patient or sample number. Of the 47 women enrolled in the study, specimens collected from 23 patients contained viable fungal cells that were identified as *C. albicans*.

ALS gene profile of clinical *C. albicans* isolates. Since each *C. albicans* strain does not encode all ALS genes (11, 12), genomic DNA was isolated from each clinical isolate and tested by PCR with ALS-specific primers as described previously (9), using 200 ng of genomic DNA as a template. Resulting PCR products were resolved on 2% agarose–Tris-acetate-EDTA gels and visualized by ethidium bromide staining. All ALS genes were detected in each clinical isolate except for *ALS5* in strains GC6 and GC23.

RT-PCR assay. RT-PCR analysis was done according to a previously published assay (9). RNA was isolated from specimens by using a hot phenol method (2). Assay tubes for all PCRs in this study were prepared in large batches that were quality controlled using positive and negative control templates (9). PCR tubes were frozen at −20°C until use. This method reduced assay variability and increased the reproducibility of the analysis (9). Initial results from the RT-PCR analysis suggested that for some samples, PCR products were present but barely visible on a gel. Signals from these products were far weaker than signals obtained from amplification of genomic DNA (9). Since these products did not fit either positive or negative categories that were in use, a "weak" category was established for these results. The running of all ALS PCR products from a single specimen next to each other on the same gel, and with a fixed amount of 1-kb ladder markers (Invitrogen) as an internal visualization control, facilitated identification of such weak reaction products. All RT-PCRs were run by the same person and evaluated in a blinded fashion by another observer.

The RT-PCR assay was used in a pilot study of total RNA from two clinical specimens (numbers 29 and 33) and detected expression of several ALS genes (see Table 2). In order to determine whether expression of more ALS genes could be detected, poly(A) RNA was selected from the remaining total RNA for these two specimens by using a MicroPoly(A) Purist kit (Ambion). RT-PCR analysis detected expression of more ALS genes from poly(A) RNA than from total RNA, suggesting that poly(A) selection increased the sensitivity of the method. For most specimens, therefore, both types of RNA samples were analyzed, with the analysis of poly(A) RNA conducted first. For four specimens (numbers 28, 30, 32, and 45) where RNA quantities were limiting, RT-PCR was run only on poly(A) RNA. Up to 2 μg of RNA was used for cDNA synthesis and subsequent PCR. Previous work addressed the sensitivity of this assay (9) and showed that ALS gene expression could be detected with the addition of as little as 0.2 ng of cDNA per reaction. Titration of genomic DNA showed that all ALS-specific reactions yielded a product when 20 pg was used as template, and all reactions were negative at the 2-pg dilution (data not shown). Statistical analysis of the frequency of gene expression detection was conducted by using the mixed procedure PROC MIXED in SAS (24).

Nested PCR analysis. Previous RT-PCR analysis of ALS gene expression utilized in vitro systems containing either pure *C. albicans* RNA or a mixture of *C. albicans* and human epithelial RNA (9). Because yeast other than *C. albicans* may be present in the vaginal microflora, we tested the RT-PCR primers to see whether they amplified DNA from other *Candida* species. This control was important since genomic DNA from other *Candida* species could be present in vaginal fluids even if fungal colonies were not recovered on agar plates. Genomic DNA was extracted from the organisms listed above and added to PCRs specific for each of the ALS genes. PCR products of the expected size (9) were generated for both *C. albicans* strains. For *C. stellatoidea*, each ALS-specific PCR product was observed, except for *ALS5*, suggesting that the gene was absent from the strain tested or had sequence polymorphisms within at least one of the priming sites. No PCR products were observed for genomic DNA from *C. dubliniensis* CD36, *C. guilliermondii*, *C. lusitaniae*, and *C. glabrata*. However, some sporadic positive reactions were noted: an *ALS7* product was amplified from *C. dubliniensis* CM1, an *ALS2* product was amplified from *C. tropicalis*, and *ALS6* products were amplified from *C. tropicalis* and *C. parapsilosis* (data not shown). Since these PCR products were of similar size compared to those amplified from *C. albicans*, it was possible that they represented cross-reactive bands that could complicate interpretation of the clinical isolate analysis. Nested PCR primers were designed to distinguish *C. albicans* ALS-derived PCR products from those that arose from other sources (Table 1). While each *C. albicans* ALS PCR product could be amplified with the correct nested primer pair, products that arose from the other *Candida* species could not be amplified. Control reactions demonstrated that the nested primer set could amplify the products of the expected size from each of the clinical isolates collected in this project. The nested primers are not specific for the individual ALS genes and are intended only to amplify products from the initial set of RT-PCRs.

PCRs with the nested primers were used to validate positive reactions from each RT-PCR. PCR conditions were identical to those for the RT-PCR primer pairs (9), except that an annealing temperature of 57°C was used. A one-tenth volume of the total RT-PCR was purified by using the Wizard PCR Preps DNA purification system (Promega) and resuspended in a volume of 50 μl. One microliter of this preparation was used as a template for the nested PCR primers. Ten microliters of each nested PCR product was run on a 2% agarose–Tris-acetate-EDTA gel and ethidium bromide stained to visualize the products. To resolve the smaller PCR products and accurately determine their sizes, nested PCR products and *ALS6* products from the initial RT-PCRs were also separated on 8% polyacrylamide–Tris-Borate-EDTA gels and stained with ethidium bromide. Negative nested PCRs were considered to indicate false positives in the initial RT-PCR and were recorded as negatives in data tables.

Mouse model. The murine model of vaginal candidiasis was described previously (3, 4). In this model, mice were injected with 0.2 mg of estradiol valerate in 0.1 ml of sesame oil 72 h prior to vaginal inoculation with washed *C. albicans* yeast forms. *C. albicans* cells were grown in 1% phytone-peptone (Becton Dickinson) plus 0.1% glucose for 18 h at 25°C with orbital shaking at 200 rpm. At 4 and 7 days postinoculation, *C. albicans* cells were collected by vaginal lavage with phosphate-buffered saline. Mice were used for only one time point to avoid sampling the same animal twice. The lavage fluid was flash frozen and stored at −80°C until it was analyzed for gene expression. RNA extraction and RT-PCR analysis were done according to the methods described above. RNA was also extracted from inoculum cells and analyzed by RT-PCR to determine which ALS genes were expressed in *C. albicans* cells before inoculation into the mice.

RHVE model. Reconstituted human vaginal epithelium (RHVE) is a product of SkinEthic Laboratories (Nice, France). The product consists of human epithelial cell lines cultured on polycarbonate filters in vitro at the air-liquid interface in a serum-free chemically defined medium. RHVE is derived from the A431 cell line. *C. albicans* inoculum cultures were prepared as described previously (9). RHVE was inoculated by pipetting 50 μl of *C. albicans*-phosphate-buffered saline suspension (2×10^6 cells total) onto the surface of the tissue.

TABLE 1. Nested primers used for validation of RT-PCR products

Gene	Primer name	Sequence (5′ → 3′)	PCR product size (bp)
ALS1	RTALS1ChF	CCATCACTGAAGATATCACCACA	239
	RTALS1ChR	TGGAGCTTCTGTAGGACTGGTT	
ALS2	RTALS2ChF	GGTGCAATGGGGTTCATAGT	223
	RTALS2ChR	TCTGGCCTGACACCAGAGTA	
ALS3	RTALS3ChF	CCAAGTGTTCCAACAACTGAAA	292
	RTALS3ChR	GAACCGGTTGTTGCTATGGT	
ALS4	RTALS4ChF	TGGGATTTTCAACTGCTGGT	308
	RTALS4ChR	GCTGACTCGTGTTGCAGAAA	
ALS5	RTALS5ChF	TGGCACCACAATGTGAAAAC	263
	RTALS5ChR	CCGGCAGGAACATTTTGATA	
ALS6	RTALS6ChF	CGCAAGGGTTATTCCAAGT	98
	RTALS6ChR	TGAAAATCCGAGCACACC	
ALS7	RTALS7ChF	TTCCATGTCGCTCGATACAA	117
	RTALS7ChR	GAACAATCAATTTCAAAATCATCATC	
ALS9	RTALS9ChF	ACAGCTAATTCACAAGTTTCTCAAA	141
	RTALS9ChR	TGAAGTTTGATATTGAGTGACAGC	

Samples were incubated in maintenance medium, which is based on Clonetics MCDB-153, contains 5 μg of insulin ml^{-1}, and is free of antimicrobials. RHVE was placed into a 37°C incubator with 5% CO_2 and saturated humidity. Maintenance medium was changed every 24 h. At specified time points, tissues were harvested and processed for microscopy as described previously (9) or flash frozen and stored at −80°C for RT-PCR analysis. Samples were run in duplicate.

RESULTS

ALS gene expression in clinical vaginal specimens. RT-PCR analysis of RNA isolated from clinical vaginal specimens showed that, in general, expression of more ALS genes was detected from poly(A)-selected RNA than from total RNA (Table 2) ($P = 0.01$). Expression of *ALS1*, *ALS2*, *ALS3*, and *ALS9* was detected in approximately half of the total RNA specimens. *ALS4* and *ALS5* expression was detected least frequently in total RNA, but the ability to detect *ALS4*- and *ALS5*-specific transcripts increased for poly(A)-selected RNA. Expression of *ALS4* and *ALS5* was observed most readily in asymptomatic women who were not pregnant. Expression of *ALS6* and *ALS7* was detected less frequently overall than expression of *ALS1*, *ALS2*, *ALS3* or *ALS9*. Contrary to the pattern established for the other genes, analysis of poly(A)-selected RNA decreased the ability to detect *ALS6* and *ALS7* transcripts. This result might be explained by the fact that the PCR primers for *ALS6* and *ALS7* are located within the 5′ domain of each gene, thereby requiring a long, intact RNA molecule to derive a positive signal. However, the PCR primers for *ALS2* and *ALS4* are also located within this region of each gene (9), and detection of expression for these genes improved with poly(A)-selected RNA. It is possible that the *ALS6* and *ALS7* mRNA molecules are less stable than the others. A single break in the molecule that dissociates the 5′-end sequences from the poly(A) tail would be sufficient to render the molecule nondetectable in our assay.

A second round of PCRs using the set of nested primers (Table 1) was performed to confirm results from the RT-PCR analysis. In nearly all cases, positive reactions observed with RT-PCR were verified with the nested primer set, indicating that the initial RT-PCR product was due to amplification of *C. albicans* ALS sequences. In a very limited number of cases, positive reactions from the initial RT-PCR could not be reproduced with the nested primer set. These results were presumed to be due to cross-reactivity with other microbial sequences and were reported as negative reactions in the data tables. Interestingly, nested primer amplification of presumably negative RT-PCR results from the clinical isolates yielded the correct-sized ALS-specific products in nearly all cases. These additional data points were not recorded in Table 2 since the secondary PCR amplification changed the detection limit of the initial assay that was used in all previous studies (9; Green et al., unpublished; C. B. Green, X. Zhao, and L. L. Hoyer, unpublished data). Control reactions were performed with RNA that had not been reverse transcribed to ensure that the increased nested-primer-based limit of detection was not due to low levels of genomic DNA that were undetectable in the initial controls. Additional controls used specific ALS amplification products as templates with the nested primer set for a different ALS gene. Negative results for these reactions showed that the nested primers did not produce false positives by amplification of other sequences in the initial substrate. These results and controls demonstrated the ability of the nested primers to extend the sensitivity of the RT-PCR assay should a lower limit of detection be required.

Data from analysis of the clinical specimens was sorted according to several patient history variables such as pregnancy status and the presence of clinical symptoms (Table 2). The frequency of detection of gene-specific transcripts was not affected by pregnancy status ($P = 0.39$). Comparison of frequency of transcript detection between symptomatic and

TABLE 2. Patient demographic information and ALS RT-PCR results for human clinical specimens[a]

Sample no.[b] or % detection	Age (yr)	Pregnant	Symptomatic	Estrogen supplement[d]	RT-PCR of 2 μg of total RNA								Poly(A) RNA used in RT-PCR analysis (μg)	RT-PCR of poly(A) RNA							
					ALS1	*ALS2*	*ALS3*	*ALS4*	*ALS5*	*ALS6*	*ALS7*	*ALS9*		*ALS1*	*ALS2*	*ALS3*	*ALS4*	*ALS5*	*ALS6*	*ALS7*	*ALS9*
Sample no.																					
23	16	No	No										1.0	+	+	+					+
45	18	No	No	Alesse	NR	NR	NR	NR	NR	NR	NR	NR	2.0	+	+	+	+	+		+	+
30	23	No	No		NR	NR	NR	NR	NR	NR	NR	NR	1.8	+	+	+	W				W
8	47	No	No		+	+	+		+	+		+	2.0	+	+	+	W	+			+
36	18	Yes	No			+	+						1.3	+	+						+
28	22	Yes	No		NR	NR	NR	NR	NR	NR	NR	NR	1.1			+					
32	24	Yes	No		NR	NR	NR	NR	NR	NR	NR	NR	2.0	+	+	+					
33	24	Yes	No	Ortho-Tri-Cyclen	+	+	+			W		+	2.0	+	+	+			+	+	+
22	27	Yes	No		+	+				+	+	+	2.0	+	+	+	+	+		+	+
2	21	No	Yes	Ortho-Tri-Cyclen			+						2.0								
6	22	No	Yes									+	1.2	+	+					+	+
24	25	No	Yes									+	1.5	+	+	+					+
31	26	No	Yes	Loestrin 1/20						W		+	1.3	+							
29	29	No	Yes	Clomid	+		+						2.0	+	+	+			+	W	+
27	34	No	Yes	Nordette	+					+	W	+	1.7	+	+	+	+	+			+
41	40	No	Yes	Premarin	+	+	+			+	+	+	1.2	W	W						W
7	46	No	Yes		+		+						2.0	+	+	+	+	+		+	+
5[c]	57	No	Yes	Prempro									2.0	+							
42	18	Yes	Yes			+	+				+	+	1.1	+	+	+					+
15	22	Yes	Yes		+	+	+			+	+	+	2.0	+	+	+					+
40	22	Yes	Yes										2.0								
21	23	Yes	Yes		+	+				W	W	+	2.0	+	+	+	W	W			+
39	26	Yes	Yes			+	+			W			0.8	+	+	+			+		
% Detection					47	47	53	0	5	47	32	58		87	78	70	30	26	13	26	70
Pregnant														80	80	80	20	20	20	20	60
Nonpregnant														92	77	62	38	31	8	31	77
Symptomatic														86	71	57	21	21	14	21	64
Asymptomatic														89	89	89	44	33	11	33	78

[a] Results are reported as + for a strong positive signal and W for a weak positive signal. A blank space designates a sample that fell below the detection limit of the assay (9). Samples that were not run are designated as NR.

[b] *C. albicans* strains isolated from these clinical samples used the sample number preceeded by GC.

[c] *C. albicans* and *C. parapsilosis* were recovered from this specimen.

[d] Generic names for the estrogen supplements are as follows: for Alesse and Nordette, ethinyl estradiol and levonorgestrel; for Ortho Tri-Cyclen, ethinyl estradiol and norgestimate; for Loestrin 1/20, ethinyl estradiol and norethindrone; for Clomid, clomiphene citrate; for Premarin, conjugated estrogens; and for Prempro, conjugated estrogens and medroxyprogesterone acetate.

TABLE 3. ALS RT-PCR results for murine model specimens[a]

Time or % detection	Strain	Mouse no.	Total RNA (ng)	RT-PCR result							
				ALS1	*ALS2*	*ALS3*	*ALS4*	*ALS5*	*ALS6*	*ALS7*	*ALS9*
Time											
Inoculum	3153A			+	+	+	+	+	+		+
	GC2			+	+	+	+	+	+	W	+
	GC8			+	+	+	+	+	+		+
Day 4	3153A	1	400	+	+				W		+
		2	60	+	+						
		3	700								
	GC2	1	130						+		
		2	270	+	+	+					+
		3	800	+		+					
		4	150	+	+	W				W	+
	GC8	1	220	+	+	+					+
		2	210								
		3	290	W	+	W					+
Day 7	3153A	1	220	+	+	+		+	W	+	+
		2	500	+	+	W					+
	GC2	1	220	+	+	+	W				
		2	280	+	+	+					
	GC8	1	590	+	+	+					
% Detection											
Overall				80	73	67	7	7	20	13	47
Day 4				70	60	50	0	0	20	10	50
Day 7				100	100	100	20	20	20	20	40

[a] Results are reported as + for a strong positive signal and W for a weak positive signal. A blank space designates a sample that fell below the detection limit of the assay (9).

asymptomatic patients suggested that it was easier to detect ALS expression among asymptomatic individuals, although this difference was not statistically significant ($P = 0.07$). Further subdivision of the data set using these patient history variables and also considering use of hormonal therapies did not provide a large enough sample size for meaningful statistical analyses and consequently was not pursued.

ALS gene expression in a murine model of vaginitis. A major goal of this work was to compare ALS gene expression in clinical vaginal isolates to that observed in disease models. We studied two of the clinical strains described above and one laboratory strain (3153A) in mice using an established model of murine vaginitis. In this model, pseudoestrus is induced by treating mice with estradiol valerate (3, 4). The two clinical strains selected were GC2 and GC8 because they represented extremes in the analysis of the human vaginal fluids. The GC2 clinical specimen yielded a low amount of RNA, and expression of only one ALS gene was detected by RT-PCR analysis (Table 2). GC8 was chosen because the most RNA was recovered from the corresponding clinical specimen and expression of nearly all ALS genes was detected. GC2 and GC8 were also attractive choices for further analysis because one was isolated from an asymptomatic patient and the other was isolated from a patient with clinical disease. *C. albicans* cells were collected from mice by vaginal lavage at two time points (4 and 7 days) following inoculation of the animals. Total RNA was isolated from the lavage fluid, quantitated, and subjected to RT-PCR analysis. Positive results were confirmed by the nested primer analysis described above.

Similar to results obtained from the clinical specimens, expression of *ALS1*, *ALS2*, *ALS3*, and *ALS9* was detected most readily, while expression of *ALS4* and *ALS5* was least frequent (Table 3). Results were comparable for each strain tested, with similar frequencies and patterns of gene expression over the course of the experiment. Detection of ALS-specific transcripts was more frequent for samples collected on day 7 than for those collected on day 4 ($P = 0.04$). Assay of a larger quantity of total RNA did not necessarily provide evidence of expression from a greater number of ALS genes. The lack of a positive correlation between these variables was most likely due to the presence of RNA from organisms other than *C. albicans* in the vaginal lavage fluid. For strain GC2, expression of more ALS genes was observed in the murine model than in the clinical specimen; the converse was true for analysis of strain GC8.

ALS gene expression in the RHVE model. RHVE was inoculated with strain GC2, GC8, or 3153A, and samples were collected every 12 h for a total of 36 h. Temporal destruction of the model epithelial layer was similar to results observed previously (1, 9, 25). The three *C. albicans* strains produced similar epithelial damage. An uninoculated control remained unchanged over the course of the experiment. By 12 h follow-

TABLE 4. Results from RT-PCR detection of ALS gene expression in *C. albicans*-inoculated RHVE specimens[a]

Time (h) or % positive signal	Strain	RT-PCR result							
		ALS1	*ALS2*	*ALS3*	*ALS4*	*ALS5*	*ALS6*	*ALS7*	*ALS9*
Inoculum culture	3153A	+	+	+	+	+	+	W	+
	GC2	+	+	+	+	+	−	+	+
	GC8	+	+	+	+	+	+	+	+
12	3153A	+/+	+/+	+/+	+/−	+/+	−/+	−/−	+/+
24		+/+	+/+	+/+	W/+	W/+	W/+	W/+	W/+
36		+/+	+/+	+/+	+/+	+/+	+/+	−/W	+/+
12	GC2	+/+	+/+	+/+	−/−	W/W	W/W	+/+	+/+
24		+/+	+/+	+/+	−/+	+/+	+/W	−/−	+/+
36		+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
12	GC8	+/+	+/+	+/+	W/W	+/+	W/W	+/W	+/+
24		+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
36		+/+	+/+	+/+	+/+	+/+	−/W	+/+	+/+
% Positive signal		100	100	100	61	83	50	61	94

[a] Results are reported as + for a strong positive signal, W for a weak signal, and − for samples that fell below the detection limit of the assay (9). Duplicate samples are indicated for each time point in the RHE analysis.

ing inoculation, vacuolation and edema were apparent as well as penetration of the epithelial layers by fungal elements. The depth of the epithelial layer affected and degree of epithelial detachment and splitting increased over the period of incubation. Fungal abundance and organization into a biofilm-like structure also increased over time, as noted previously for inoculation of the buccal reconstituted human epithelium (RHE) model (9).

RT-PCR analysis with the ALS primer set was conducted on 2 μg of total RNA extracted from uninoculated and inoculated RHVE as well as the *C. albicans* culture used to inoculate the model (Table 4). Results were similar for all three strains tested. RT-PCR analysis of total RNA extracted from uninoculated RHVE failed to detect any ALS-specific messages. All ALS messages, with the exception of *ALS6* in strain GC2, were detected in the inoculum cultures for each of the three strains. Similar to the analysis of the human clinical and mouse model specimens, expression of *ALS1*, *ALS2*, *ALS3*, and *ALS9* provided consistently strong, positive RT-PCR signals in the RHVE model. The remaining ALS genes showed a greater frequency of negative or weakened signals. Although not a quantitative designation, we separated weakened signals from positive signals because the weakened bands were so obviously reduced in intensity and barely visible compared to standard bands on a gel (see Materials and Methods). *ALS4*, *ALS6*, and *ALS7* showed negative or weakened signals most frequently, while weakened signal strength was also observed for *ALS5*. Negative and weak signals for *ALS4* tended to be detected for the earlier time points in the analysis, suggesting that *ALS4* expression, which was strong in the inoculum cells, was down-regulated upon contact of *C. albicans* with vaginal epithelial cells in this model.

DISCUSSION

RT-PCR analysis was used to evaluate ALS gene expression in human clinical vaginal specimens and two models of vaginal candidiasis. Cross-reactivity of the RT-PCR primer pairs with a limited number of sequences from non-*C. albicans Candida* species was overcome by design of nested primers that distinguish true positive signals from non-*C. albicans* ALS-derived products. The RT-PCR analysis used in this work provides information (positive, negative, and weak reaction products) that is instructive for ranking genes with respect to the relative abundance of specific RNA in the specimen studied. Quantitative RT-PCR methods could be used to provide precise estimates of RNA abundance but would require redesign of the PCR primers to amplify smaller products. Other aspects of the assay contribute to whether the PCR product is detectable. We found that some PCR products were not visible when run on agarose gels but could be seen when the same reaction was run on an acrylamide gel. We also demonstrated the difference in results when total RNA was analyzed and compared to poly(A)-selected RNA from the same specimen (Table 2). In general, the association of PCR product detection with fungal cell numbers is difficult because it is common for microscopically visible fungal cells in clinical specimens not to grow in culture (S. R. Trupin, unpublished observation). Therefore, viable cell counts from a clinical specimen are not likely to indicate the total number of *C. albicans* cells that contribute RNA to the RT-PCR. Despite these factors that affect detection limit of the assay, definite trends emerge from the overall results.

The overall results indicate that expression of *ALS1*, *ALS2*, *ALS3*, and *ALS9* is most commonly observed. Detection of expression of *ALS6* and *ALS7* is the next most difficult, while expression of *ALS4* and *ALS5* is least readily detected in clinical vaginal specimens. Despite the overall difficulty in detection of their expression, *ALS4* and *ALS5* are more obvious in specimens from asymptomatic, nonpregnant women. These results suggest that pregnancy or symptomatic conditions may down-regulate transcription of *ALS4* and *ALS5* and that these genes are more likely to be transcribed under commensal con-

ditions. These conclusions could be refined further by increasing the number of clinical specimens studied. Such a study was reported for *C. albicans* SAP genes (22). Analysis of 137 clinical samples showed significant differential expression of SAP and phospholipase gene expression between symptomatic and asymptomatic individuals and also between oral and vaginal specimens.

One striking feature of the ALS gene expression data presented here is that the rank order of gene expression in the clinical specimens (Table 2) was the same for strains tested in the murine vaginitis model (Table 3). This trend carried forward into the RHVE model, where expression of the same four genes (*ALS4*, *ALS5*, *ALS6*, and *ALS7*) was less readily detected (Table 4). The apparent down-regulation of *ALS4* during the early time points when *C. albicans* initially contacts RHVE is particularly obvious, as is the return of signal strength as incubation progresses. These results suggest an effect of vaginal epithelial cell contact on *ALS4* expression in this model. Increased *ALS4* transcription during longer incubation with RHVE could be triggered by epithelial damage or by formation of the biofilm layer that develops over the epithelial surface in this model (9). However, concluding that *ALS4* increases transcription during epithelial damage would contradict results from analysis of the human clinical specimens that showed that *ALS4* expression is detected more frequently in specimens from asymptomatic patients. The close concurrence of ALS gene expression patterns between clinical specimens and model systems validates the use of these models for testing the phenotypic effects of *als*/*als* mutant strains.

In previous work, we used the same RT-PCR assay to evaluate ALS gene expression in oral clinical specimens and model systems (9; Green et al., unpublished). In oral clinical specimens from human immunodeficiency virus-infected patients, *ALS6* and *ALS7* expression is least readily detected (Green et al., unpublished). Expression of the same genes is also least readily detected when *C. albicans* is inoculated onto buccal RHE (9) and in the hyposalivatory rat model of oral candidiasis (Green et al., unpublished). These results may indicate that transcription of *ALS6* and *ALS7* is at lower levels than that of the other ALS genes and that low levels of transcript are sufficient for adequate cellular representation of Als6p and Als7p. The low level of *ALS7* transcript in cultured cells was noted initially in other work (34). However, *ALS4*- and *ALS5*-specific transcripts are detected easily in oral specimens and oral models, suggesting that the results from analysis of vaginal specimens and models are unique. These data support the conclusion of host-site-specific influences on ALS gene expression.

The experiments described here provide a larger view of ALS gene expression by defining trends from the analysis of clinical vaginal specimens. Demonstration of the concurrence of results from clinical specimens with those from vaginal candidiasis models validates the use of these models to assess the effects of Als proteins in vaginal disease. The further demonstration of host-site-specific influences on ALS gene expression suggests that *C. albicans* uses the ALS genes in a niche-specific manner. Assessment of the phenotype of *als*/*als* mutant strains in the vaginal model systems will provide additional insight into the role of the Als proteins in vaginal colonization and pathogenesis.

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REFERENCES

1. **Bernhardt, J., D. Herman, M. Sheridan, and R. Calderone.** 2001. Adherence and invasion studies of *Candida albicans* strains using in vitro models of esophageal candidiasis. J. Infect. Dis. **184:**1170–1175.
2. **Collart, M. A., and S. Oliviero.** 1993. Preparation of yeast RNA, p. 13.12.1–13.12.5. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology, vol. 2. John Wiley & Sons, Inc., New York, N.Y.
3. **Fidel, P. L., Jr., J. Cutright, and C. Steele.** 2000. Effects of reproductive hormones on experimental vaginal candidiasis. Infect. Immun. **68:**651–657.
4. **Fidel, P. L., Jr., M. E. Lynch, and J. D. Sobel.** 1993. *Candida*-specific cell-mediated immunity is demonstrable in mice with experimental vaginal candidiasis. Infect. Immun. **61:**1990–1995.
5. **Fidel, P. L., Jr., J. A. Vazquez, and J. D. Sobel.** 1999. *Candida glabrata*: review of epidemiology, pathogenesis, and clinical disease with comparison to *C. albicans*. Clin. Microbiol. Rev. **12:**80–96.
6. **Fu, Y., A. S. Ibrahim, D. C. Sheppard, Y. C. Chen, S. W. French, J. E. Cutler, S. G. Filler, and J. E. Edwards, Jr.** 2002. *Candida albicans* Als1p: an adhesin that is a downstream effector of the *EFG1* filamentation pathway. Mol. Microbiol. **44:**61–72.
7. **Gaur, N. K., and S. A. Klotz.** 1997. Expression, cloning, and characterization of a *Candida albicans* gene, *ALA1*, that confers adherence properties upon *Saccharomyces cerevisiae* for extracellular matrix proteins. Infect. Immun. **65:**5289–5294.
8. **Gillum, A. M., E. Y. H. Tsay, and D. R. Kirsch.** 1984. Isolation of the *Candida albicans* genes for orotidine-5′-phosphate decarboxylase by complementation *of S. cerevisiae ura3* and *E. coli pyrF* mutations. Mol. Gen. Genet. **198:**179–182.
9. **Green, C. B., G. Cheng, J. Chandra, M. A. Ghannoum, and L. L. Hoyer.** 2004. RT-PCR detection of *Candida albicans ALS* gene expression in the reconstituted human epithelium (RHE) model of oral candidiasis and in model biofilms. Microbiology **150:**267–275.
10. **Hoyer, L. L.** 2001. The ALS gene family of *Candida albicans*. Trends Microbiol. **9:**176–180.
11. **Hoyer, L. L., and J. E. Hecht.** 2000 The *ALS6* and *ALS7* genes of *Candida albicans*. Yeast **16:**847–855.
12. **Hoyer, L. L., and J. E. Hecht.** 2001. The *ALS5* gene of *Candida albicans* and analysis of the Als5p N-terminal domain. Yeast **18:**49–60.
13. **Hoyer, L. L., T. L. Payne, M. Bell, A. M. Myers, and S. Scherer.** 1998. *Candida albicans ALS3* and insights into the nature of the ALS gene family. Curr. Genet. **33:**451–459.
14. **Hoyer, L. L., T. L. Payne, and J. E. Hecht.** 1998. Identification of *Candida albicans ALS2* and *ALS4* and localization of Als proteins to the fungal cell surface. J. Bacteriol. **180:**5334–5343.
15. **Hoyer, L. L., S. Scherer, A. R. Shatzman, and G. P. Livi.** 1995. *Candida albicans* ALS1: domains related to a *Saccharomyces cerevisiae* sexual agglutinin separated by a repeating motif. Mol. Microbiol. **15:**39–54.
16. **Hube, B., F. Stehr, M. Bossenz, A. Mazur, M. Kretschmar, and W. Schafer.** 2000. Secreted lipases of *Candida albicans*: cloning, characterization and expression analysis of a new gene family with at least ten members. Arch. Microbiol. **174:**362–374.
17. **Mitchell, A. P.** 1998. Dimorphism and virulence in *Candida albicans*. Curr. Opin. Microbiol. **6:**687–692.
18. **Monod, M., and M. Borg-von Zepelin.** 2002. Secreted aspartic proteases as virulence factors of *Candida* species. Biol. Chem. **383:**1087–1093.
19. **Moran, G. P., D. J. Sullivan, M. C. Henman, C. E. McCreary, B. J. Harrington, D. B. Shanley, and D. C. Coleman.** 1997. Antifungal drug susceptibilities of oral *Candida dubliniensis* isolates from human immunodeficiency virus (HIV)-infected and non-HIV-infected subjects and generation of stable fluconazole-resistant derivatives in vitro. Antimicrob. Agents Chemother. **41:**617–623.
20. **Naglik, J. R., S. J. Challacombe, and B. Hube.** 2003. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. Microbiol. Mol. Biol. Rev. **67:**400–428.
21. **Naglik, J. R., G. Newport, T. C. White, L. L. Fernandes-Naglik, J. S. Greenspan, D. Greenspan, S. P. Sweet, S. J. Challacombe, and N. Agabian.** 1999. In vivo analysis of secreted aspartyl proteinase expression in human oral candidiasis. Infect. Immun. **67:**2482–2490.
22. **Naglik, J. R., C. A. Rodgers, P. J. Shirlaw, J. L. Dobbie, L. L. Fernandes-Naglik, D. Greenspan, N. Agabian, and S. J. Challacombe.** 2003. Differential expression of *Candida albicans* secreted aspartyl proteinase and phospho-

lipase B genes in human correlates with active oral and vaginal infections. J. Infect. Dis. **188:**469–479.
23. **Ripeau, J. S., M. Fiorillo, F. Aumont, P. Belhumeur, and L. de Repentigny.** 2002. Evidence for differential expression of *Candida albicans* virulence genes during oral infection in intact and human immunodeficiency virus type 1-transgenic mice. J. Infect. Dis. **185:**1094–1102.
24. **SAS Institute Inc.** 2000. SAS/STAT software version 8. SAS Institute Inc., Cary, N.C.
25. **Schaller, M., M. Bein, H. C. Korting, S. Bauer, G. Hamm, M. Monod, S. Beinhauer, and B. Hube.** 2003. The secreted aspartyl proteinases Sap1 and Sap2 cause tissue damage in an in vitro model of vaginal candidiasis based on reconstituted human vaginal epithelium. Infect. Immun. **71:**3227–3234.
26. **Schaller, M., H. C. Korting, W. Schafer, J. Bastert, W. Chen, and B. Hube.** 1999. Secreted aspartic proteinase (Sap) activity contributes to tissue damage in a model of human oral candidosis. Mol. Microbiol. **34:**169–180.
27. **Schaller, M., C. Schackert, H. C. Korting, E. Januschke, and B. Hube.** 2000. Invasion of *Candida albicans* correlates with expression of secreted aspartic proteinases during experimental infection of human epidermis. J. Investig. Dermatol. **114:**712–717.
28. **Schaller, M., W. Schafer, 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.
29. **Sobel, J. D., S. Faro, R. W. Force, B. Foxman, W. J. Ledger, P. R. Nyirjesy, B. D. Reed, and P. R. Summers.** 1998. Vulvovaginal candidiasis: epidemiologic, diagnostic, and therapeutic considerations. Am. J. Obstet. Gynecol. **178:**203–211.
30. **Soll, D. R.** 2002. Phenotypic switching, p. 123–142. *In* R. A. Calderone (ed.), *Candida* and candidiasis. ASM Press, Washington, D.C.
31. **Stehr, F., A. Felk, A. Gacser, M. Kretschmar, B. Mahnss, K. Neuber, B. Hube, and W. Schafer.** 2004. Expression analysis of the *Candida albicans* lipase gene family during experimental infections and in patient samples. FEMS Yeast Res. **4:**401–408.
32. **Sullivan, D. J., T. J. Westerneng, K. A. Haynes, D. E. Bennett, and D. C. Coleman.** 1995. *Candida dubliniensis* sp. nov.: phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals. Microbiology **141:**1507–1521.
33. **Sundstrom, P.** 2002. Adhesion in *Candida* spp. Cell. Microbiol. **8:**461–469.
34. **Zhang, N., A. L. Harrex, B. R. Holland, L. E. Fenton, R. Cannon, and J. Schmid.** 2003. Sixty alleles of the *ALS7* open reading frame in *Candida albicans*: *ALS7* is a hypermutable contingency locus. Genome Res. **13:**2005–2017.
35. **Zhao, X., C. Pujol, D. R. Soll, and L. L. Hoyer.** 2003. Allelic variation in the contiguous loci encoding *Candida albicans ALS5*, *ALS1* and *ALS9*. Microbiology **149:**2947–2960.
36. **Zhao, X., S.-H. Oh, G. Cheng, C. B. Green, J. A. Nuessen, K. Yeater, R. P. Leng, A. J. P. Brown, and L. L. Hoyer.** 2004. *ALS3* and *ALS8* represent a single locus that encodes a *Candida albicans* adhesin; functional comparisons between Als3p and Als1p. Microbiology **150:**2415–2428.

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