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Deletion of the *SSK1* Response Regulator Gene in *Candida albicans* Contributes to Enhanced Killing by Human Polymorphonuclear Neutrophils

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The isolation and partial functional characterization of the two-component response regulator *SSK1* gene of *Candida albicans* was previously reported. Compared to wild-type (CAF2-1) and gene-reconstituted (SSK23) strains, the *ssk1* null strain (SSK21) was avirulent in a murine model of hematogenously disseminated candidiasis and less able to adhere to human esophageal cells. More recent data indicate that SSK21 is sensitive to 4 to 8 mM H_2O_2 in vitro than CAF2-1 and SSK23. Furthermore, microarray studies indicate that the regulation of two classes of genes, those encoding cell wall functions and stress adaptation, are altered in the *ssk1* mutant. In the present study, the susceptibility of strains CAF2-1, SSK21, and SSK23 to killing by human polymorphonuclear neutrophils (PMNs) was assessed. Results are also described for a newly constructed *ssk1* mutant (SSK24) in which the *URA3* gene is integrated into its native locus. Our results indicate that killing of SSK21 and SSK24 was significantly greater than that of CAF2-1 and SSK23 ($P < 0.01$). In order to determine why Ssk1p at least partially protects the organism against the killing activity of human PMNs, we compared the signal transduction activity and the inflammatory response gene profiles of PMNs infected with either the wild type or the *ssk1* mutant. Phosphorylation of the mitogen-activated protein kinases p42/44 and p38 from neutrophils infected with either CAF2-1 (wild type) or SSK21 (*ssk1*/*ssk1*) was similar, while expression and phosphorylation of the JNK mitogen-activated protein kinase was not observed following infection with either strain. On the other hand, we observed an upregulation of seven inflammatory response genes in PMNs infected with the SSK21 mutant only, while an increase in interleukin-10 expression was measured in PMNs infected with either strain. Downregulation of interleukin-2 was observed in PMNs infected with either strain. Verification of the transcriptional profiling was obtained by reverse transcription-PCR for three of the genes that were upregulated in neutrophils infected with the *ssk1* mutant. Also, the sensitivity of strain SSK21 to human defensin-1, one of the nonoxidative, antimicrobial peptides of PMNs, was greater than that of CAF2-1, demonstrating that nonoxidative killing in PMNs may contribute to the increased susceptibility of the *ssk1* mutant. Our results indicate that the Ssk1p response regulator protein may provide at least partial adaptive functions for the survival of *C. albicans* following its encounter with human neutrophils.

Candida albicans is the most common opportunistic fungal pathogen of humans, causing cutaneous, mucocutaneous, and invasive disease in the setting of congenital, induced, or disease-related immune dysfunction. Invasive disease carries a high attributable mortality because of insensitive methods of detection and because of treatment failures (51). In patients with invasive disease, most often a reduction in the number or function of circulating neutrophils is observed (5, 22, 51). Thus, neutrophils represent the most prominent phagocytic leukocyte of the acute inflammatory response that protects against invasive candidiasis. However, an aggressive innate immune response by human polymorphonuclear neutrophils (PMNs) may be responsible for acute and recurrent vulvovaginal candidiasis in humans (19).

Previous studies of neutrophil interactions with *C. albicans* have demonstrated that killing requires adherence, phagocytosis, mitogen-activated protein (MAP) kinase signal transduction, and the coordinated stimulation of a respiratory burst and lysosomal degranulation (9, 10, 14–16, 33, 47–50, 53, 54, 56). MAP kinase signaling is a complex of several pathways that are critical to controlling microbial growth in human leukocytes. For example, the upstream GTPases Rac and Cdc42 are linked to MAP kinase pathways that regulate the level of lytic granule movement and phagocytosis within PMNs (9). More recently, Zhong et al. probed the intracellular signaling pathways which regulate neutrophil responses to *C. albicans* and found elevated levels of mitogen-activated protein kinase/extracellular regulatory kinase (MAP kinase/ERK) in human neutrophils incubated for as little as 5 min after infection with the organism (56). This pathway is independent of Ras/Rho activation but instead requires activation through a Rac/Cdc42-dependent p44/42 MAP kinase/ERK. Nonoxidative killing of *C. albicans* by neutrophil defensins is also well studied (21, 25, 29). The defensins comprise two groups of 29- to 42-amino-acid cationic peptides that are found in high concentrations within neutrophils.

Two-component signal transduction in fungi and bacteria regulates adaptation of cells to stress conditions, in some cases through a quorum-sensing mechanism (3, 28, 41). The two-component signal pathways include sensor, histidine kinase and response regulator proteins (41). In *C. albicans* there are three histidine kinase proteins (Sln1p, Chk1p, and Nik1p) and

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

Strain	Relevant genotype	Reference
CAF2-1	*ura3*Δ::*imm434*/*URA3*	20
SSK21	*ura3*Δ::*imm434*/*ura3*Δ::*imm434* *ssk1*Δ::*hisG*/*ssk1*Δ::*hisG-URA3-hisG*	6
SSK23	*ura3*Δ::*imm434*/*ura3*Δ::*imm434* *ssk1*Δ::*hisG*/*SSK1*::*URA3-hisG*	6
SSK22	*ura3*Δ::*imm434*/*ura3*Δ::*imm434* *ssk1*Δ::*hisG*/*ssk1*Δ::*hisG*	6
SSK24	*IRO1-URA3*/*iro1-ura3*Δ::*imm434* *ssk1*Δ::*hisG*/*ssk1*Δ::*hisG*	This study

two response regulator proteins (Ssk1p and Skn7p) (1, 6–8, 35, 41, 44, 46, 55). A two-component histidine kinase (Fos1p) has also been identified in *Aspergillus fumigatus*, and strains deleted of the encoding gene are reduced in virulence (13, 37).

Previous studies with the *C. albicans* response regulator mutant strain SSK21 have demonstrated its avirulence and its sensitivity in vitro to H_2O_2, menadione, KO_2, and *t*-butyl hydroperoxide (6, 11). Microarray transcriptional profiling of the *ssk1* mutant revealed that the regulation of stress-related and cell wall-associated gene-encoded proteins was altered in the mutant (11). Als1p, a known adherence protein of *C. albicans*, was downregulated in the mutant, which may explain its reduced adherence to human esophageal tissues (4, 32).

To determine if oxidant sensitivity in vitro reflects its ability to survive following phagocytosis, the interaction of the *ssk1* deletion mutant (SSK21) with human PMNs was examined.

MATERIALS AND METHODS

Strains and growth conditions. *C. albicans* strains CAF2-1, SSK21, and SSK23 have been described previously and are listed in Table 1 (6, 20). The *ssk1* deletion mutant (strain SSK21) and gene-reconstituted strain (SSK23) were constructed following the Urablaster protocol (20). In addition, strain SSK24, an *ssk1*/*ssk1* mutant but with *URA3* integrated into the *URA3* locus, was used in some experiments. All strains were maintained by subculture for 2 to 3 weeks on YPD agar medium (1% yeast extract, 2% peptone [BD, Bacto], 2% glucose, 1.5% agar) from frozen stocks and propagated as yeast cells in YPD broth medium at room temperature overnight with shaking.

Reagents. Human defensin-1 (HNP-1), obtained from the American Peptide Co, Inc., Sunnyvale, Calif., was dissolved in water prior to experiments. The anti-MAP kinase rabbit polyclonal antibodies anti-p44/42 and anti-phospho-p42/44 MAP kinase antibody (Thr202/Tyr204); anti-p38 MAP kinase antibody; and anti-phospho-p38 MAP kinase, anti-SAPK/JNK, and a secondary antibody (donkey anti-rabbit immunoglobulin G-alkaline phosphatase conjugate) were obtained from Cell Signaling Tech, Inc., Beverly, Mass.

Construction of SSK24. A PstI/BglII fragment was isolated from plasmid pLUBP. This fragment includes both the *C. albicans URA3* and *IRO1* genes. Transformation of *C. albicans* strain SSK22 (*ssk1*/*ssk1 ura3*/*ura3*) with the PstI/BglII fragment was carried out with lithium acetate as described previously (6). *URA3*-positive clones were selected on YNB medium lacking uridine, and integration of *URA3* was confirmed by Southern hybridization (Fig. 1). The probe that was used in the Southern analyses was a 592-bp PCR product (including the 5′ *URA3* sequence) that was amplified with primer set ura5′ TATCCCAGC TACTTCGATTG and ura3′ CATCAGTGGGATCATTAG. Genomic DNA from positive clones was digested with EcoRI or BclI and, following electrophoresis, hybridized with the PCR probe mentioned above. Hybridizing bands of

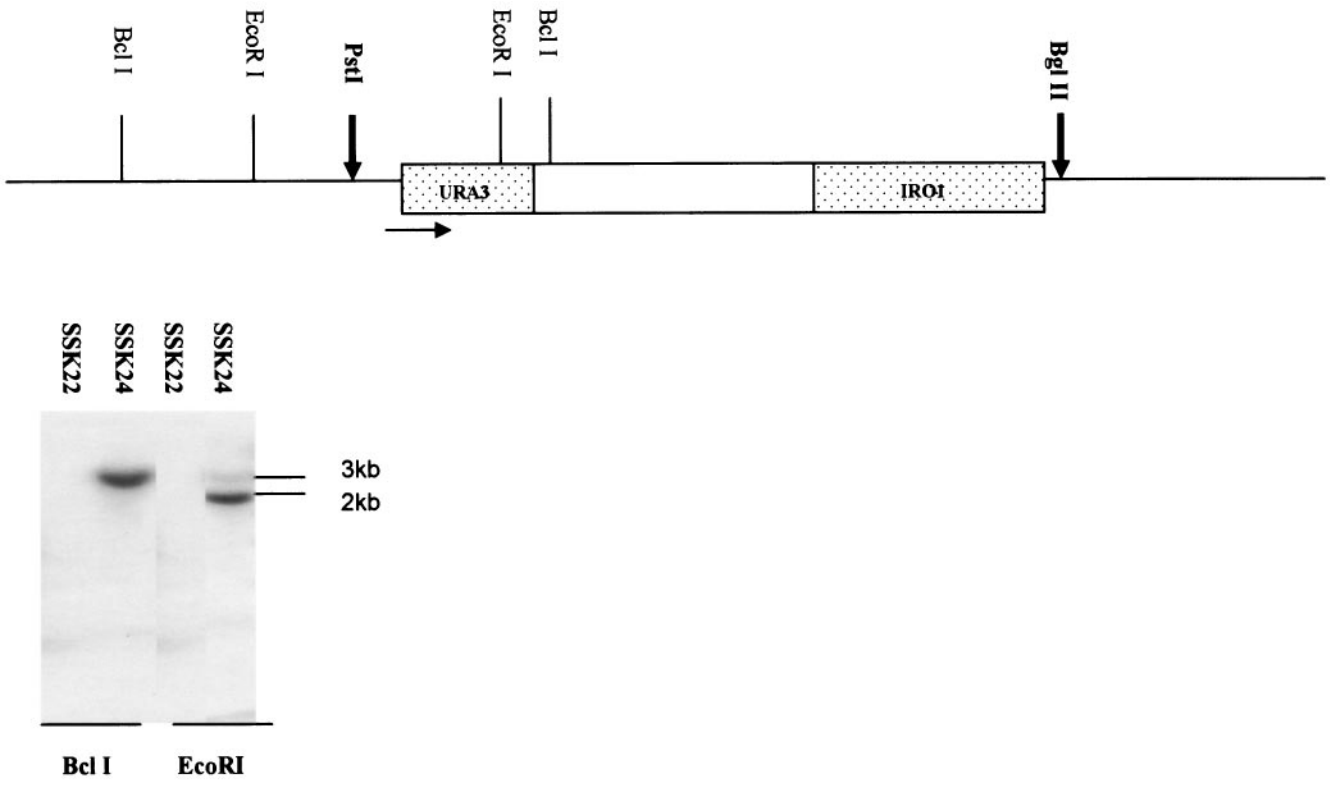

FIG. 1. Construction of strain SSK24. Strain SSK22 (*ssk1*/*ssk1 ura3*/*ura3*) was transformed with the *IRO1-URA3* cassette. Ura$^+$ transformants (*ssk1*Δ/*ssk1*Δ, *IRO1-URA3*/*iro1-ura3*Δ) were isolated and confirmed by Southern blot hybridization. Top. A restriction map of the *URA3-IRO1* gene cassette. The vertical arrows indicate the full-length *URA3-IRO1* gene cassette, while the probe used for the Southern hybridizations is indicated by a horizontal arrow beneath the *URA3* gene. Bottom. Southern hybridization of SSK22 and SSK24 restricted with either BclI or EcoRI. The probe for the Southerns is as indicated above. The 3-kb (BclI) and 2-kb (EcoRI) fragments are observed in strain SSK24 but not in strain SSK22.

2.0 kb and 3.1 kb were observed as expected with EcoRI and BclI, respectively (Fig. 1).

Isolation of human PMNs. Human PMNs were isolated from the peripheral blood of healthy volunteers by dextran sedimentation and centrifugation through lymphocyte separation medium (1.078 ± 0.002 g/ml; Mediatech Cellgro). PMNs were enriched in number by a brief hypotonic lysis of erythrocytes (0.2% NaCl, 30 s). Cells were suspended in RPMI 1640 medium plus 10% fetal bovine serum. These cells were >99% viable as judged by trypan blue dye exclusion.

Fungicidal assays with PMNs. All assays were performed in 14-ml round-bottomed tubes. Strains CAF2-1, SSK21, SSK23, and SSK24 were grown in YPD broth, as described above, and washed twice with phosphate-buffered saline (pH 7.4). For neutrophil killing assays, yeast cells were opsonized with 50% normal human serum (37°C, 30 min). Freshly isolated human PMNs and opsonized *Candida* cells suspended in RPMI 1640 medium plus 10% fetal bovine serum were mixed at an effector-to-target cell ratio of 50:1 (PMNs/*Candida*). All cultures were incubated for 2 h at 37°C with gentle shaking. Cell suspensions were then centrifuged, water was added to lyse the neutrophils, and serial dilutions of all strains were performed; 100 μl of each dilution was added to YPD agar plates, and samples were spread over the agar for determinations of CFU after incubation for 24 h at 30°C. The fungicidal assays were done a total of three times, and the percent killing of each strain was calculated by the formula [(CFU without PMNs) − (CFU with PMNs)/(CFU without PMNs)] × 100.

To determine the phagocytosis of each strain, we followed published methods (40). Briefly, microscopic smears of each mixture of PMNs and *Candida* cells were prepared and stained, and 100 neutrophils were counted per strain. The phagocytosed yeasts of each strain were determined. A total of three phagocytosis experiments were done.

Western blots. CAF2-1 and SSK21 strain *C. albicans* yeast cells were mixed with human PMNs at an effector-to-target cell ratio of 1:10 (56). As a control, a suspension of *C. albicans* yeast cells was also prepared and treated as described below. The cell mixtures were pelleted rapidly at 1000 rpm in a microcentrifuge followed by incubation for 5 min, then suspended in 1X sodium dodecyl sulfate sample buffer (0.125 M Tris-HCl, 10% glycerol, 1% sodium dodecyl sulfate, 0.1 M dithiothreitol) at 100°C for 10 min. Cell lysates were centrifuged at 12,000 rpm for 5 min to remove cell debris. Denatured proteins were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and transferred to nitrocellulose (100 V; 1 h). Immediately following transfer, the membranes were stained with Ponceau S (0.1% in 5% acetic acid) to confirm equal loading of all lanes. The membranes were blocked with 5% nonfat milk in Tris-buffered saline–0.01% Tween 20 for 1 h, blotted with the specific primary antibody (phosphorylated or nonphosphorylated MAP kinases, p44/42, p38, and JNK) for 2 h, and then reacted with an alkaline phosphatase-conjugated secondary antibody for 1 h. Bands were visualized by incubation with CDP-Star substrate (Bio-Rad) and exposed to Kodak film. These experiments were performed a total of three times each with different cell suspensions and extracts.

Inflammatory response gene expression profiles in infected PMNs. At 30 min after coculture at an effector-to-target cell ratio of 1:10, suspensions of PMNs and *C. albicans* CAF2-1 or SSK21 were centrifuged and processed as described below. The gene profile of uninfected PMNs was also evaluated to compare responses with and without the fungal strains. Tri-reagent (Sigma) was used to extract total RNA from cells. Microarray analysis of cytokine gene expression was performed according to the manufacturer's instructions (Superarray, Fredrick, Md., S-series). Briefly, 2 μg of total RNA was used as the template for biotin-labeled cDNA probe synthesis. The denatured cDNA probe was used to hybridize a GEArray S series membrane overnight with continuous agitation at 60°C. After washing, the membrane was incubated with alkaline phosphatase-conjugated streptavidin, and then the CDP-Star chemiluminescent substrate was added. Finally, the membrane was exposed to Kodak X-ray film to visualize signals.

To perform data analysis, we used the GE Array Analyzer software, developed by SuperArray Biosciences, to perform pairwise comparisons between the processed arrays. The data from replicate spots per array were subtracted from background (blanks) and normalized with the *ACT1* housekeeping gene for all combinations of cell mixtures. Array experiments were done two times, each with different RNA preparations. Data are presented as the increase or decrease in arrays compared to PMNs incubated without *C. albicans*. Upregulation was considered to be at a ratio of 1.5, while downregulation was ≤0.5.

Reverse transcription-PCR was performed with the reverse transcription-PCR kits (Superarray Inc., Frederick, Md.). RNA was isolated as described above for each cell mixture. First-strand cDNA synthesis was accomplished with 2 μg of total RNA. PCR was performed according to the manufacturer's recommendations (SingleGene PCR kit, Superarray Inc.) in order to determine mRNA levels of interleukin (IL)-8, CDC25A, and CX3CR1. The optical density of each amplicon was determined for semiquantitative analysis and compared to β-actin, which was used as an internal control. Data are expressed relative to uninfected PMNs.

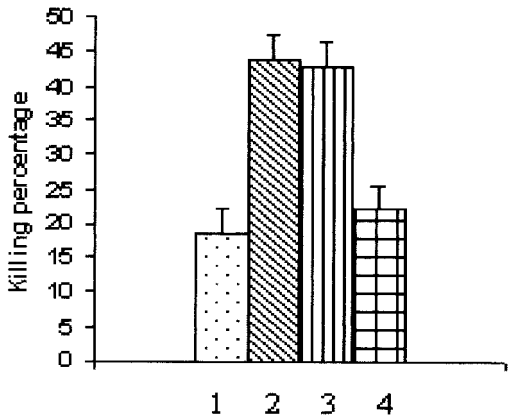

FIG. 2. Killing of *C albicans* strains CAF2-1 (parental, bar 1), SSK21 (bar 2), SSK24 (bar 3), and SSK23 (bar 4) by human neutrophils. See Table 1 for the genotypes of each strain. SSK21 and SSK24 versus CAF2-1, $P < 0.01$; SSK21 and SSK24 versus SSK23, $P < 0.01$; CAF2-1 = SSK23. Data represent averages for three experiments.

Superoxide production in infected neutrophils. We followed methods previously described for human monocytes and neutrophils (27, 40). In 96-well microtiter dishes, *C. albicans* yeast cells (10^6 per 50 μl) were mixed with neutrophils (10^5 per 50 μl), each prepared in Hanks' balanced salt solution. In other wells, neutrophils or *C. albicans* yeast cells were incubated alone. All cell suspensions were incubated for 1 to 2 h at 37°C. Absorbancy units in cocultures of yeasts and neutrophils were corrected for neutrophil- or *C. albicans*-only controls. O_2^- was assayed spectrophotometrically by the cytochrome *c* reduction method as described by Roilides et al. (40). The cell mixtures were incubated at 37°C with 50 μM cytochrome *c* for 1 h. Subsequently, absorbance was measured at 550 nm, and superoxide anion was then calculated with the extinction coefficient for reduced cytochrome *c*.

Fungicidal assays with HNP-1. All fungicidal assays were performed in 96-well microtiter plates. For assays with HNP-1, a final concentration of either 4.15 or 8.3 μM was used with yeast cells (10^5 cells in a total volume of 100 μl of phosphate-buffered saline). All cell suspensions were incubated for 90 min in triplicate at 37°C with gentle shaking. Following incubation, 40 μl was removed from each well, diluted in water, and plated on YPD agar for determinations of viability. The candidacidal activity of HNP-1 for each strain was determined by counting colonies of the organism following incubation on YPD agar for 24 h at 30°C. Results are expressed as killing of each strain with the formula [(CFU without HNP-1) − (CFU with HNP-1)]/(CFU without HNP-1) × 100.

Statistical analysis. One-way analysis of variance and Dunnett's modification for multiple comparisons were used. Differences were considered significant when P was < 0.05.

RESULTS

Killing of *C. albicans* strains by human PMNs. These experiments were predicated by our observation that the *ssk1* mutant of *C. albicans* is more sensitive than wild-type cells and a gene-reconstituted strain in vitro to hydrogen peroxide, menadione, *t*-butyl hydroperoxide, and KNO_2 (11). Therefore, killing of the *ssk1* mutant (SSK21), a gene-reconstituted strain (SSK23), the SSK21 strain reconstituted with *URA3* at its locus (SSK24) (Fig. 1), and wild-type cells (CAF2-1) by human PMNs was determined (Fig. 2). We found that that the killing of SSK21 and SSK24 was approximately 43.9 and 42.9%, respectively, compared to the reduced killing of CAF2-1 and SSK23, 18.8 and 22.1%, respectively ($P < 0.01$ for SSK21 and SSK24 versus CAF2-1 and SSK21 and SSK24 versus SSK23;

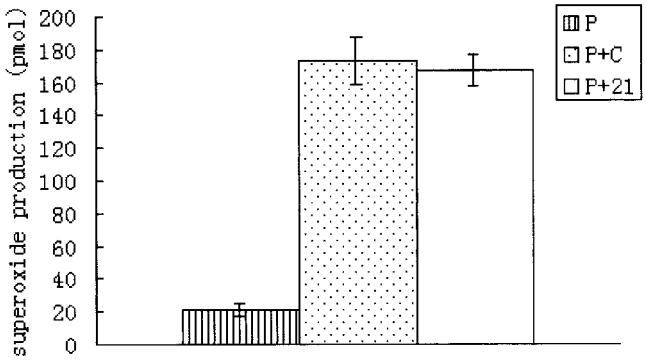

FIG. 3. Production of O_2^- by neutrophils infected with CAF2-1 or SSK21. P = neutrophils only; P+C = neutrophils infected with CAF2-1; P+21 = neutrophils infected with SSK21.

CAF2-1 = SSK23). The data presented in Fig. 2 represent an average of three experiments with standard deviations indicated.

The killing by PMNs was not due to differences in the phagocytosis of each strain since approximately 30% of CAF2-1, SSK24, and SSK21 cells were phagocytosed (data not shown). While significantly greater killing was observed in neutrophils infected with the *ssk1* mutant (strain SSK21), total superoxide production by neutrophils was similar when infected with either CAF2-1 or SSK21 (Fig. 3).

MAP kinase signaling in infected neutrophils. Phosphorylation of the MAP kinases p44/42 and p38 was evaluated in PMNs infected with either CAF2-1 or SSK21 in order to determine if the increased killing of SSK21 was associated with signaling events. Unphosphorylated p44/42 and p38 (a loading control) were measured from each sample with a polyclonal antibody to each protein (Fig. 4, lower set of Western blots).

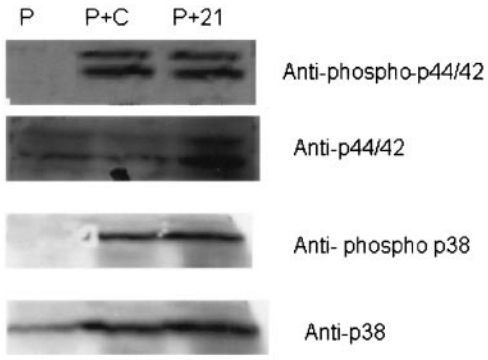

FIG. 4. MAP kinase activation of PMNs cultured with strains of *C albicans*. Human neutrophils (P) were cocultured with yeast cells of strain CAF2-1 (P+C) or SSK21 (P+21) for 5 min; proteins were extracted, separated by SDS-PAGE, and transferred for Western blotting with polyclonal antibodies to the unphosphorylated MAP kinases p38 and p44/42 (lower Western blot profiles). Then, the blots were stripped and reacted with monoclonal antibodies (anti-phospho-p44/42 or anti-phospho-p38) to each MAP kinase (upper Western blot profiles). Both proteins were phosphorylated in neutrophils infected with either strain of *C. albicans* (P+C and P+21) but not in uninfected neutrophils. Phosphorylated proteins also were not detected in extracts of *C. albicans* cells incubated without neutrophils (not shown). These experiments were done three times with similar results.

TABLE 2. Microarray analysis of inflammatory gene response from PMNs infected with CAF2-1 or SSK21

Protein family	Gene	Increase (-fold)[a]	
		CAF2-1 + PMNs/PMNs	SSK21 + PMNs/PMNs
Cell surface receptors			
	L-selectin	1.2	2.4
	E-selectin	1.8	3.4
Signal transduction			
	CDC25A	0	3.2
Chemokines, cytokines, and receptors			
	MIP-2a	1.8	2.9
	CX3CR1	1.6	3.9
	IL-8	1.3	2.8
	4-1BB	1.9	2.8
	IL-10	2.5	2.4

[a] Data from a single RNA extraction are presented, but the analysis of a second RNA extraction gave similar results.

Then, the blots were stripped and reacted with polyclonal antibodies to each phosphorylated protein (Fig. 4, upper set of Western blots). Our results indicated that these proteins were not phosphorylated in uninfected PMNs (Fig. 4). When PMNs were infected, phosphorylation of p44/42 and p38 MAP kinases was observed, although there were no apparent differences in signal intensity in PMNs infected with either strain (Fig. 4, upper set of Western blots, compare P+C and P+21). Similar studies were done to measure phosphorylation of the JNK MAP kinase, but we could not detect a signal in any of the cell mixtures (data not shown). As a control, we performed Western blots with *C. albicans* cells incubated alone with the antibodies described above, but no phosphorylation signal was detected with proteins of each strain (data not shown).

Inflammatory gene expression profiles in infected neutrophils. The expression of approximately 385 inflammatory response genes was compared in PMNs infected with either CAF2-1 (wild type) or SSK21 (*ssk1* mutant) cells relative to PMNs incubated without the *Candida* strains. Five categories of genes were examined, including those that encoded cell surface factors, transcription factors, response genes, signal transduction, and chemokines and chemokine receptors. The increase in expression of genes for each cell suspension was calculated as [(CAF2-1 + PMN)/PMN] or [(SSK21 + PMN)/PMN]. Data were normalized to *ACT1* for each cell suspension, including PMNs incubated without *Candida*. The changes that occurred in expression were consistent for both array analyses, but of the total genes measured, only seven were upregulated in the SSK21 mutant compared to wild-type-infected PMNs (Table 2).

Expression of the cell surface receptors E-selectin and L-selectin, the signal transduction protein CDC25A, the chemokines MIP-2a and CX3CR1, and the cytokines IL-8 and 4-1BB increased in PMNs infected with strain SSK21 compared to PMNs infected with CAF2-1 cells, while an increase in the cytokine IL-10 was observed in PMNs infected with both strains. Microarray data for the top three genes that were upregulated in neutrophils infected with SSK21 were verified by reverse transcription-PCR (Fig. 5). We observed increases in mRNA amounts in neutrophils infected with strain SSK21 of

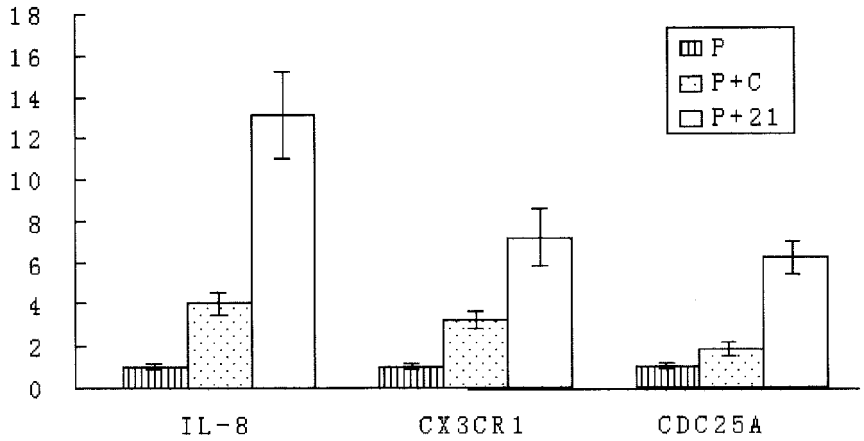

FIG. 5. Reverse transcription-PCR analysis of IL-8, CX3CR1, and CDC25A transcription. Transcription was performed with the Superarray reverse transcription-PCR kit. Expression was normalized by establishing the luminosity of β-actin as a standard for each sample. Data are expressed relative to uninfected neutrophils (relative value of 1.0). P = neutrophils only; P+C = neutrophils infected with CAF2-1; P+21 = neutrophils infected with SSK21.

approximately 3.5-fold (IL-8), 2.5-fold (CX3CR1), and 3-fold (CDC25A) compared to neutrophils infected with CAF2-1.

Killing of *C. albicans* strains by HNP-1. The increased killing of strain SSK21 by human PMNs could be due in part to nonoxidative mechanisms. To test this, we compared the killing of the same strains used in the neutrophil experiments by HNP-1 (Table 3). The *ssk1* mutant (SSK21) was killed to a significantly greater extent than CAF2-1 or SSK23 at a concentration of 8.3 μM of HNP-1 (defensin-1). Even at a concentration of 4.15 μM, the mutant was killed more than CAF2-1 (Table 3). The killing of strain SSK23 was similar to CAF2-1 at each concentration of HNP-1. The sensitivity of the mutant to HNP-1 does not indicate a general change in uptake of compounds by the SSK23, since this strain has the same sensitivity as wild-type cells to a number of antifungal drugs and other inhibitors (11).

DISCUSSION

We show that *SSK1* of *C. albicans*, which encodes a putative two-component signal transduction response regulator protein, is important to the survival of *C. albicans* in human PMNs. Most likely, this protein regulates adaptation of the organism to oxidative stress through the *HOG1* (hypersomotic glycerol) MAP kinase signal transduction pathway, as in previous studies we showed that Ssk1p phosphorylates Hog1p during oxidant stress (11). The role of Ssk1p in the adaptation to oxidant stress has also been demonstrated by microarray analysis, since an upregulation of genes associated with oxidant adaptation in the mutant occurred (11). Furthermore, the mutant is less adherent to human esophageal tissue in vitro, and, interestingly, transcriptional profiling indicates that genes encoding cell wall functions (*ALS1*, *CHK1*, *MNN4*, and *FLO1*) are altered compared to CAF2 (11). Als1p, a cell surface glycoprotein that provides an adherence function for *C. albicans* (26), is downregulated in the mutant; that observation may explain the decreased levels of adherence by this strain (4, 32). The apparent multiple functions (adherence and oxidant and nonoxidant adaptation) are not unexpected given the cross-talk that occurs among signal transduction systems (30). For example, Ssk1p negatively regulates the histidine kinase Chk1p, which may not be a protein of the *HOG1* MAP kinase pathway (31).

TABLE 3. Effect of HNP-1 on killing of *C. albicans*[a]

Strain	% Killing at HNP-1 concn:	
	4.15 μM	8.3 μM
CAF2-1	4.3 ± 1.4	13.1 ± 2.0
SSK21	8.7 ± 2.3	31.2 ± 4.1
SSK23	6.3 ± 1.6	14.5 ± 2.8

[a] SSK21 > CAF2-1, $P < 0.01$; SSK21 > SSK23, $P < 0.01$; SSK23 = CAF2-1.

Importantly, the current studies also demonstrate that the phenotype of the *ssk1* mutant (increased killing by human PMNs) is due to the *ssk1* gene deletion and not a consequence of a *URA3* positional effect, since strain SSK24, an *ssk1* mutant but with *URA3* integrated at its own locus, is phenotypically similar to strain SSK21 which is an *ssk1* mutant with the *URA3* gene located in the *SSK1* locus (6). Phenotypic changes associated with *URA3* positional effects have been described in *C. albicans* following construction of deletion mutants with the Urablaster procedure (12).

We attempted to determine the mechanism(s) by which the *ssk1* mutant is killed more significantly by human PMNs than wild-type cells. Along with the sensitivity of the mutant to H_2O_2, menadione, and defensin HNP-1, transcriptional profiling of PMNs infected with the *ssk1* mutant indicated an upregulation of several inflammatory response genes compared to PMNs infected with wild-type cells. Of the genes that were upregulated, IL-8 (or its receptor) has been shown to play an important role in protection against some forms of candidiasis (2, 17, 18, 42, 43). IL-8 also plays an important role in neutrophil phagocytosis of *Aspergillus fumigatus* in vitro and protection against paracoccidioidomycosis (36, 39, 52). Similarly, the L- and E-selectin genes have also been shown to be important in immunity to systemic candidiasis (34). Expression data of other investigations did not always correlate with our PMN

array analysis. For example, both IL-17 and IL-2 are thought to be required for protection against systemic candidiasis, but the expression of these gene products was unchanged in our studies (23, 38, 45). IL-10 has been shown to inhibit phagocytosis and hyphal killing of human neutrophils, while in our study IL-10 levels were similar in wild-type- and mutant-infected PMNs (40).

While the killing of *C. albicans* SSK21 compared to CAF2-1 is associated with several PMN inflammatory gene products, phosphorylation of PMN MAP kinase proteins and superoxide production by neutrophils was similar for both the wild-type and mutant strains. Data by other groups indicate that p44/42 but not p38 MAP kinase activity is required for phagocytosis and is associated with PMN killing of *C. albicans* (56). Interestingly, a mouse macrophage cell line that was ineffective in killing *C. albicans* did not process signal transduction events via the p38 MAP kinase (24). Perhaps the lack of killing in macrophages in comparison to PMNs which are more candidacidal is in part due to differences in signaling among the two phagocytes. In our experiments, we detected phosphorylated p44/42 and p38 but failed to detect a signal with the JNK/MAP kinase. Thus, it is possible the JNK pathway plays a minor role in the candidacidal activity of PMNs. In conclusion, our data establish a role for the Ssk1p response regulator protein in the survival of *C. albicans* confronted with human PMNs.

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