Staphylococcus aureus adherence to *Candida albicans* hyphae is mediated by the hyphal adhesin Als3p

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The bacterium *Staphylococcus* (*St.*) *aureus* and the opportunistic fungus *Candida albicans* are currently among the leading nosocomial pathogens, often co-infecting critically ill patients, with high morbidity and mortality. Previous investigations have demonstrated preferential adherence of *St. aureus* to *C. albicans* hyphae during mixed biofilm growth. In this study, we aimed to characterize the mechanism behind this observed interaction. *C. albicans* adhesin-deficient mutant strains were screened by microscopy to identify the specific receptor on *C. albicans* hyphae recognized by *St. aureus*. Furthermore, an immunoassay was developed to validate and quantify staphylococcal binding to fungal biofilms. The findings from these experiments implicated the *C. albicans* adhesin agglutinin-like sequence 3 (Als3p) in playing a major role in the adherence process. This association was quantitatively established using atomic force microscopy, in which the adhesion force between single cells of the two species was significantly reduced for a *C. albicans* mutant strain lacking *als3*. Confocal microscopy further confirmed these observations, as *St. aureus* overlaid with a purified recombinant Als3 N-terminal domain fragment (rAls3p) exhibited robust binding. Importantly, a strain of *Saccharomyces cerevisiae* heterologously expressing Als3p was utilized to further confirm this adhesin as a receptor for *St. aureus*. Although the parental strain does not bind bacteria, expression of Als3p on the cell surface conferred upon the yeast the ability to strongly bind *St. aureus*. To elucidate the implications of these *in vitro* findings in a clinically relevant setting, an *ex vivo* murine model of co-infection was designed using murine tongue explants. Fluorescent microscopic images revealed extensive hyphal penetration of the epithelium typical of *C. albicans* mucosal infection. Interestingly, *St. aureus* bacterial cells were only seen within the

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Abbreviations: AFM, atomic force microscopy; CLSM, confocal laser scanning microscopy; ConA–Texas red, concanavalin A conjugated to Texas red; H&E, haematoxylin and eosin; IFA, indirect immunofluorescence; PNA-FISH, peptide nucleic acid probes for fluorescent *in situ* hybridization; SEM, scanning electron microscopy.
A supplementary figure is available with the online version of this paper.

epithelial tissue when associated with the invasive hyphae. This differed from tongues infected with *St. aureus* alone or in conjunction with the *als3* mutant strain of *C. albicans*, where bacterial presence was limited to the outer layers of the oral tissue. Collectively, the findings generated from this study identified a key role for *C. albicans* Als3p in mediating this clinically relevant fungal–bacterial interaction.

INTRODUCTION

Despite their clinical relevance, polymicrobial infections, defined as those infections caused by more than one microbial species, remain largely understudied (Peters *et al.*, 2012). Although they carry serious clinical implications in terms of impact on therapeutic measures, fungal–bacterial mixed infections specifically are poorly understood (Shirtliff *et al.*, 2009). The lack of in-depth investigations into inter-microbial interactions at the molecular level has contributed to our lack of understanding of these complex phenomena.

Candida albicans is the most pathogenic human fungal species, commonly colonizing host mucosal surfaces (Calderone & Clancy, 2012). However, under conditions of immune disruption, *C. albicans* can rapidly transition into a pathogen, causing an array of mucosal and disseminated infections with high mortality (de Repentigny *et al.*, 2004). As a polymorphic species, *C. albicans* is capable of switching morphology between yeast and hyphal forms, a transition crucial to its pathogenesis. While the yeast form is most commonly associated with bloodstream and systemic disease, the hyphae are more adept at adhering to and penetrating host tissue and are therefore responsible for mucosal infections, most commonly oral candidiasis (Sudbery *et al.*, 2004). Similar to *C. albicans*, the bacterial species *Staphylococcus* (*St.*) *aureus* possesses a repertoire of virulence attributes that has contributed to its recent re-emergence as a significant pathogen, particularly in immunocompromised individuals (Tang & Stratton, 2010).

The clinical importance of the intimate association between microbial species in general has remained underappreciated in most diseases; this is particularly significant when involving ubiquitous microbial species with high pathogenic potential such as *C. albicans* and *St. aureus*. In fact, the co-isolation of these diverse organisms from an array of acute and chronic diseases such as burn wounds, ventilator-associated pneumonia and bloodstream infections is well documented (Combes *et al.*, 2002; Klotz *et al.*, 2007a; Mousa, 1997). The majority of these clinical conditions are considered to be biofilm-associated, whereby adherence to and colonization of a surface by the micro-organism is a prerequisite for the development of the infectious process (Archer *et al.*, 2011). Similarly, such infections that are polymicrobial in nature involve the adherence of various microbial species to each other, where some level of interaction or cell–cell communication is expected to occur (Peters *et al.*, 2012). Therefore, elucidating the mechanisms of co-adherence and identification of specific receptors involved in inter-species interactions would aid in the design of novel strategies for impeding such processes.

Given their importance and prevalence as human pathogens and their abilities to co-adhere to surfaces, our initial investigations focused on characterizing the interaction between *St. aureus* and *C. albicans* at the molecular level. Specifically, global proteomic analyses were performed to identify the differential expression of proteins associated with their interaction during *in vitro* biofilm formation. The findings from these studies identified a significant number of proteins to be differentially expressed by these two pathogens, indicating the existence of a complex dynamic interactive process (Peters *et al.*, 2010). Furthermore, a distinct physical association was identified between these organisms, with *St. aureus* adhering to the hyphal filaments of *C. albicans*. However, a defined mechanism behind these interactions had yet to be characterized. To that end, this current study aimed to identify the specific *C. albicans* surface receptor for *St. aureus*.

As microscopic images had revealed a preferential association for *St. aureus* to *C. albicans* hyphae, our efforts focused on Als3p, one of the hyphal-specific adhesins of *C. albicans*. The *ALS3* gene is a member of the *C. albicans* *ALS* (agglutinin-like sequence) gene family, which encodes eight cell surface glycoproteins with an N-terminal domain of adhesive function (Hoyer *et al.*, 2008). The Als proteins diffusely cover the surface of the fungal cell, and therefore are involved in adherence to host and abiotic surfaces. In fact, the role of these adhesins in initiating binding had been confirmed via their heterologous expression in *Saccharomyces* (*Sa.*) *cerevisiae* (Klotz *et al.*, 2007b). Importantly, these surface glycoproteins are also implicated in the adherence of *C. albicans* to bacterial species such as *Streptococcus gordonii* (Holmes *et al.*, 1996; Jenkinson *et al.*, 1990; O'Sullivan *et al.*, 2000). Therefore, based on these previous findings the aim of this current work was to validate the hypothesis that Als3p plays a key role in mediating the adherence of *St. aureus* to *C. albicans* hyphae.

METHODS

Strains and growth conditions. Fungal strains used in this study are listed in Table 1. All media used were purchased from Difco. Strains were stored as frozen glycerol stocks at -80°C and maintained on yeast peptone dextrose (YPD) plates. Cultures were grown in YPD broth overnight at 30°C . Cells were washed with sterile PBS and grown in RPMI 1640 (without phenol red) medium supplemented with glutamine and buffered with HEPES (Invitrogen) or complete yeast nitrogen base (YNB) at 37°C to induce hyphal formation. Meticillin-resistant *St. aureus* strain M2, originally isolated from a patient with osteomyelitis at the University of Texas Medical Branch, was used in all studies (Brady *et al.*, 2006). The strain was stored as a glycerol stock at -80°C and maintained on trypticase soy agar (TSA) containing 5% sheep's blood. Cultures were grown

overnight at 37 °C in trypticase soy broth (TSB), diluted 1:100 in fresh medium and grown to mid-exponential phase prior to use.

Polymicrobial biofilm growth. Microscopic analysis was performed to visualize the interactions between *St. aureus* and *C. albicans* as they co-adhered to a surface during biofilm growth. Polyvinyl chloride (PVC) catheter discs (BenTec Medical) were sterilized by soaking in 100 % ethanol for 10 min, rinsed in sterile water and allowed to dry overnight. Exponential-phase *C. albicans* and *St. aureus* cells were washed in PBS and diluted to OD₅₄₀ 1.0 and OD₆₀₀ 0.12, respectively. Fifty microlitres from each culture was inoculated into the wells of a six-well polystyrene plate containing a catheter disc and 5 ml RPMI medium (1:100 dilution of standardized culture). In some experiments, LabTek II eight-well Permanox chamber slides (Nunc) were used as substrata instead of PVC discs, in which standardized cultures were diluted 1:100 in a volume of 200 µl per well. To assess the effect of serum on the interaction between the two species during polymicrobial biofilm growth, experiments were performed with RPMI containing 25 % bovine serum. Plates or slides were shaken at 120 r.p.m. for 5 min then incubated at 37 °C for 16 h with shaking at 50 r.p.m. Following incubation, media were removed and wells were gently washed with PBS to remove non-adherent cells.

Scanning electron microscopy (SEM) of polymicrobial biofilms. Polymicrobial biofilms were grown on catheter discs as described above, fixed in 4 % paraformaldehyde (PFA) for 1 h, and extensively rinsed in PBS. Discs were immediately processed for SEM by cryofreezing and sputter coating. Biofilms were analysed and imaged using a scanning electron microscope (Quanta 200; FEI) at ×4000 magnification.

Confocal laser scanning microscopy (CLSM) of polymicrobial biofilms. To assess adherence during polymicrobial growth, co-culture biofilms were grown on catheter discs as described above. Prior to fixation with 4 % PFA for 1 h, discs were extensively washed in PBS and stained with a cocktail of fluorescent probes: Calcofluor white (stains chitin in the fungal cell wall), Syto9 (DNA stain) and concanavalin A conjugated to Texas red (ConA-Texas red; stains carbohydrate moieties including microbial matrix).

CLSM to assess *St. aureus* adherence to pre-formed *C. albicans* biofilms. Overnight cultures of *C. albicans* wild-type and adhesin-deficient mutants were adjusted to standardized densities (OD₅₄₀ 0.1) and resuspended in RPMI, and 200 µl was added into chamber slide wells. Chamber slides were incubated at 37 °C overnight to allow for mature biofilm formation. *St. aureus* was grown overnight in TSB as described above. The following day, the stationary-phase *St. aureus* culture was diluted 1:100 in fresh TSB and grown for approximately 3 h until it reached the exponential phase of growth. The *St. aureus* culture was then washed in PBS by centrifugation, homogenized with a PT1200 Polytron homogenizer (Kinematica) and resuspended to OD₆₀₀ 0.12 in PBS. After mature *C. albicans* biofilms had formed on the chamber slide, each well was washed with PBS to remove non-adherent cells. To each well, 100 µl of adjusted *St. aureus* culture was added and incubated for 1 h at 37 °C with gentle shaking. Non-adherent *St. aureus* cells were removed by washing in PBS, and biofilms were then fixed in 4 % PFA after staining with the fluorescent cocktail described above. In all experiments, fluorescence was captured with a Zeiss 510 Meta confocal microscope (Carl Zeiss) using a DAPI/FITC/Texas red filter set.

Adherence ELISA. An ELISA-based assay was developed to confirm inter-species adherence and the involvement of Als3p in the process. The experiments were performed using the Mouse Immunoglobulin Isotyping ELISA kit (BD Pharmingen) according to the manufacturer's protocol, with some modifications. *C. albicans* cells were grown as described above, washed in sterile PBS and diluted to OD₅₄₀

0.1 in fresh RPMI. Aliquots of 100 µl were placed in individual wells of a 96-well high-protein-binding ELISA plate and incubated overnight at 37 °C to allow cells to adhere and coat the surface of the wells. Wells containing no fungal growth were also utilized as controls to account for binding of *St. aureus* to the polystyrene surface. *St. aureus* exponential-phase cells were obtained and standardized as described above. All wells were extensively washed in wash buffer (1 × PBS, 0.05 % Tween 20, 1 % BSA) to remove non-adherent cells. Wells were then blocked with 1 % BSA for 1 h and washed several times in PBS. Exponential-phase *St. aureus* cell suspensions (100 µl) were added to each well and plates were incubated for 1 h at room temperature with gentle agitation. Following incubation, wells were extensively washed with wash buffer to remove non-adherent *St. aureus* and incubated with a 1:1000 dilution of a horseradish peroxidase-conjugated mouse anti-*St. aureus* IgG polyclonal antibody (Pierce Biotechnology). 3,3',5,5'-Tetramethylbenzidine (TMB) reagent was added to each well and plates were incubated for 5 min to allow for colour development indicating *St. aureus* binding to the hyphae-coated wells. A stop solution (1 M H₂SO₄) was added, and absorbance was measured on a Biotek ELx808 plate reader at 550 nm and analysed with the Gen5 software suite.

Crystal violet assay. Biomasses of *C. albicans* parental and adhesin knockout mutant biofilms were assessed via the crystal violet assay to determine whether inter-strain variability in fungal adherence to polystyrene indirectly affects *St. aureus* binding as assessed by ELISA. Fungal biofilms were grown, treated and washed exactly as in the adherence ELISA. In place of the development step, wells were incubated with 200 µl 0.1 % (w/v) crystal violet for 10 min. Following incubation, wells were washed five times in PBS and plates allowed to dry at room temperature for 15 min. Wells were then resolubilized by incubation with 150 µl 95 % ethanol for 15 min. Resolubilized stain was transferred to a new microtitre plate and the absorbance read at 590 nm.

Atomic force microscopy (AFM). AFM was performed to measure force curves between *St. aureus* and selected *C. albicans* strains [1843 (Δ als3) and the parent DAY185] coated on glass slides, as described previously with some modifications (Ovchinnikova *et al.*, 2012). Briefly, adhesion forces were measured in PBS (pH 7) at room temperature with z-scan rates of less than 1 Hz using an optical lever microscope (Nanoscope IV; Digital Instruments) after 0, 5 and 60 s surface delay times on the same, randomly chosen spot on a hypha. To ensure that no bacteria detached from the cantilever during the experiment, 10 control force–distance curves were made with 0 s contact time (immediate retraction of cantilever tip) after each set of measurements. Prior to using a bacterial probe for force measurement, it was used to image hyphae to verify that the probe established single cell contact with the fungal cell. Double contour lines observed in imaging with a bacterial probe indicate double cell contact and probes exhibiting such behaviour were discarded. For each experiment, three bacterial probes were used and median adhesion forces were calculated from at least 80 force–distance curves. Calibration of cantilevers was done using the thermal tuning method (Nanoscope V6.13r1), utilizing spring constants ranging from 0.04 to 0.06 N m^{−1}.

Production of recombinant Als3 N-terminal domain fragment (rAls3p). To determine whether Als3p is required for *St. aureus* adherence to *C. albicans*, a recombinant protein was produced in *Pichia pastoris* and used in some experiments. Detailed information on the production of the protein is provided as supplementary material available online. Following expression, the protein of interest was purified by column chromatography and its identity was confirmed as Als3 by MS. The N-terminal domain of Als1p (amino acids 18–329 of the protein) was produced in *Pichia pastoris* as described previously and used as a control (Coleman *et al.*, 2009).

Table 1. Yeast strains used in this study

Strain	Designation	Genotype	Parent	Reference
<i>C. albicans</i> strains				
SC5314	Wild-type	URA3/URA3 ARG4/ARG4 HIS1/HIS1	—	Gillum <i>et al.</i> (1984)
CAI4	CAI4	ura3/ura3	SC5314	Fonzi & Irwin (1993)
DAY185	Parent	URA3/ura3 ARG4/arg4 HIS1/his1	SC5314	Davis <i>et al.</i> (2000)
1467	$\Delta als1$	ura3/ura3 als1/als1-URA3	CAI4	Zhao <i>et al.</i> (2004)
2757	$\Delta als2$	ura3/ura3 als2/als2-URA3	CAI4	Harriott & Noverr (2010)
1843	$\Delta als3$	ura3/ura3 als3/als3-URA3	CAI4	Zhao <i>et al.</i> (2004)
2034	$\Delta als4$	ura3/ura3 als4/als4-URA3	CAI4	Zhao <i>et al.</i> (2005)
2373	$\Delta als5$	ura3/ura3 als5/als5-URA3	CAI4	Zhao <i>et al.</i> (2007a)
1420	$\Delta als6$	ura3/ura3 als6/als6-URA3	CAI4	Zhao <i>et al.</i> (2007a)
1429	$\Delta als7$	ura3/ura3 als7/als7-URA3	CAI4	Zhao <i>et al.</i> (2007a)
2028	$\Delta als9$	ura3/ura3 als9/als9-URA3	CAI4	Zhao <i>et al.</i> (2007b)
CAH7-1A	$\Delta hwp1$	ura3/ura3 hwp1/hwp1-URA3	CAI4	Staab <i>et al.</i> (1999)
<i>Sa. cerevisiae</i> strains				
UB2155	Wild-type	UB2155 + pBC542 (empty vector)	BY4742	Nobbs <i>et al.</i> (2010)
UB2156	2156	UB2155 + pBC542-ALS3sm	UB2155	Nobbs <i>et al.</i> (2010)
UB2157	2157	UB2155 + pBC542-ALS3lg	UB2155	Nobbs <i>et al.</i> (2010)

Recombinant protein ELISA. To demonstrate that *St. aureus* adherence to hyphae was mediated by Als3p, ELISAs were also performed as described above, except that wells were coated with various concentrations of purified rAls3p, rAls1p or a synthesized control peptide (NH₂-MRFPSIFTAVLFAASSALAAPVNTTTEAEQKILSEE-DLNSAVDHHHHH-OH) diluted in protein storage buffer (Neo-Bioscience) instead of fungal cells. The control peptide included the N-terminal and C-terminal sequences of the recombinant protein but lacked the Als3p sequence. Plates were processed as described above.

Indirect immunofluorescence (IFA) of rAls3p. In addition to ELISA, the generated rALS3p was used for IFA. In these experiments, *St. aureus* cells grown on the surface of Permanox chamber slides (Thermo Scientific) were extensively washed with PBS to remove non-adherent cells and overlaid with 50 µg of purified rAls3p or control peptide. Following incubation for 1 h at room temperature, slides were rinsed three times in PBS, treated with the cell-permeable DNA stain Syto9 (Invitrogen) and fixed in 4% formalin. A primary mouse IgG antibody specific for the c-myc epitope tag on rAls3p (Biolegend) was added and incubated for 4 h at 4 °C. Slides were then washed three times in wash buffer followed by incubation with a Cy3-conjugated secondary goat anti-mouse IgG antibody (Biolegend) for 1 h at room temperature, and then imaged with a Zeiss 510 Meta confocal microscope using differential interference contrast and Cy2/Cy3 line switch filters.

Interactions of *Sa. cerevisiae* with *St. aureus*. To confirm the identification of Als3p as a receptor for *St. aureus* on *C. albicans* hyphae, strains of the yeast *Sa. cerevisiae* heterologously expressing the small (*ALS3sm*) and large (*ALS3lg*) alleles of the protein on their cell surface were utilized (Nobbs *et al.*, 2010). Yeast cells grown for 16 h were washed in PBS, transferred to fresh media and incubated with shaking for 3 h at 30 °C. Aliquots of 1 ml containing 5×10^8 cells of exponential-phase *St. aureus* were added to *Sa. cerevisiae* suspensions and cells were incubated together for 1 h. *Sa. cerevisiae* cells were then washed to remove unattached bacteria, resuspended in one-tenth the original volume and visualized by light microscopy. Numbers of *St. aureus* adhering to *Sa. cerevisiae* were counted, as well as the total number of *St. aureus* per field; these values were divided to calculate percentage attachment. A minimum of 20 fields were counted per strain and the experiment was repeated in triplicate.

An *ex vivo* model of co-infection. To determine whether the interaction between *St. aureus* and *C. albicans* observed on abiotic surfaces can occur on host tissue, an *ex vivo* murine tongue infection model was designed. All animal procedures were approved by the University of Maryland – Baltimore Animal Care and Use Committee. Eight-week-old female CD-1 (ICR) mice purchased from Charles River Laboratories were housed in groups of five and provided *ad libitum* feed and water containing 0.3 mg ampicillin ml⁻¹ (Teknova). Mice were euthanized, and tongues were excised and placed in wells of a 12-well polystyrene plate with 5 ml RPMI. Cell suspensions of *C. albicans* (OD₅₄₀ 1.0) and *St. aureus* (OD₆₀₀ 0.12) strains were standardized as described above, and 50 µl aliquots either alone or in combination were added to the wells with tongues and incubated for 1 h at 37 °C with gentle agitation. Tongues were then washed several times in sterile PBS, transferred to fresh RPMI and incubated overnight at 37 °C with 5% CO₂. Following incubation, tongues were fixed in 4% formalin, embedded in paraffin and sectioned.

Tissue sections were deparaffinized with xylene and stained with haematoxylin and eosin (H&E) for histopathological analysis using light microscopy. Alternatively, sections were also hybridized with species-specific peptide nucleic acid probes for fluorescent *in situ* hybridization (PNA-FISH) according to the manufacturer's protocol (AdvanDx) and examined by confocal microscopy as described above. The *St. aureus*-specific probe was Cy2-labelled and the *C. albicans* probe labelled with Cy3. Experiments were performed in duplicate using five mice per group.

Statistical analysis. Experiments were performed on three separate occasions in triplicate unless noted otherwise. A one-way ANOVA and Dunnett's post-test were used to compare ELISA data. Adhesion forces (>80 measurements per hyphal cell) measured by AFM were not normally distributed according to the Shapiro–Wilk test ($P < 0.05$), and hence results are presented as median and interquartile ranges (Ovchinnikova *et al.*, 2012). The results were compared using a non-parametric Mann–Whitney *U* test. To compare quantitative counts using *Sa. cerevisiae* Als3p-expressing strains, a one-way ANOVA and Bonferroni's post-test were used. For all experiments, a *P* value of <0.05 was considered statistically significant. Statistics were calculated using GraphPad Prism 5.0. Images were processed using Adobe Photoshop CS5.

RESULTS

Electron and confocal microscopy

The physical interaction between *St. aureus* and *C. albicans* was visualized through high-resolution SEM, revealing a three-dimensionally distributed pattern of *St. aureus* hyphal attachment. As can be seen in Fig. 1(a), *St. aureus* cells bordered the basal layer of the hyphae–substratum interface as well as adhered to the upper portion of the hyphal surface. Similarly, confocal fluorescence images (Fig. 1b) not only confirmed the adherence but also identified the involvement of the hyphal Als3p adhesin, as a substantial decrease in the level of adherence was observed for *St. aureus* to the *C. albicans* strain lacking the Als3 protein (Fig. 2c) compared with the parental strain (Fig. 2j). In addition to the *als3* mutant, strains lacking other members of the *ALS* gene family were also examined for their ability to adhere to *St. aureus*. In contrast to the minimal adherence pattern seen with *als3*, *St. aureus* adherence to these other strains was comparable with that seen with the parental strain (Fig. 2a, b, d–i). Images with *C. albicans* alone (Fig. 2k) or *St. aureus* alone (Fig. 2l) are included as controls.

Adherence ELISA

Further semiquantitative analysis for hyphal binding between *St. aureus* and *C. albicans* parental and mutant strains was performed using an immunoassay to validate the qualitative confocal microscopy. Comparisons of the absorbance readings obtained from assays following colour development, the intensity of which reflects the extent of adherence, demonstrated a significant decrease in the level of *St. aureus* adherence to $\Delta als3$ as compared with the parental strain (Fig. 3a); adherence to other *C. albicans* mutant strains was comparable, or in some cases even increased, relative to the parental strain. Binding of *St. aureus* to wells containing no fungal growth (no CA) was significantly less than the parental strain but comparable with staphylococcal adherence to $\Delta als3$. Importantly, biomasses of parental and adhesin-deficient *C. albicans* biofilms were not significantly different (Fig. 3b). Therefore, these results demonstrated

that the lack of appreciable *St. aureus* adherence to the $\Delta als3$ strain is not simply an indirect effect of *C. albicans* adhesion defects on polystyrene.

AFM

In addition to visual microscopy, the adhesion forces between *St. aureus* and the *C. albicans* strains were measured using AFM. Typical force–distance curves of *St. aureus* with *C. albicans* are shown in Fig. 4(a). Adhesion forces between *St. aureus* and hyphae of *C. albicans* $\Delta als3$ increased from 0.1 nN at 0 s contact time (initial contact) to 3.2 nN after 60 s surface delay (Fig. 4b). Irrespective of delay times, these forces were significantly smaller than those between *St. aureus* and the *C. albicans* parental strain, where a 1.3 nN force was generated at initial contact (0 s), increasing to 7.2 nN after 60 s surface delay time (Fig. 4a, b). Adhesion forces between *St. aureus* and the $\Delta als1$ and $\Delta hwp1$ mutants were similar to the *C. albicans* parental strain (data not shown).

ELISA using purified proteins

In addition to using whole *C. albicans* cells, assays were also performed in which various concentrations of the rAls3 protein and its control peptide (lacking the Als3p sequence) were used to coat the wells of ELISA plates. These results demonstrated an rAls3p dose-dependent level of adherence for *St. aureus* to the coated wells ($r^2=0.836$), while staphylococcal binding to the wells coated with the control peptide was nearly absent at all concentrations tested (Fig. 5a). As the structure of Als1p closely resembles that of Als3p, purified rAls1p was also used as a comparative control. Although some adherence was noted with rAls1p, the level was up to fourfold less than that for Als3p, requiring peptide concentrations above $10 \mu\text{g ml}^{-1}$ to support appreciable staphylococcal binding (Fig. 5a).

IFA

An antibody specific for the c-myc epitope on the C terminus of rAls3p was used to image the adherence of this

Fig. 1. Microscopic analysis of the adherence architecture between *St. aureus* and *C. albicans* during polymicrobial biofilm growth. (a) Scanning electron micrograph of catheter discs demonstrating preferential attachment of *St. aureus* to *C. albicans* hyphae (white arrows). Bar, 10 μm . (b) CLSM micrograph of *St. aureus* and *C. albicans* adhering to the surface of chamber slides stained with a cocktail of Calcofluor white (blue), Syto9 and ConA–Texas red (red) stains. *St. aureus* (green) can be seen attached to *C. albicans* hyphae (purple). Bar, 30 μm .

Fig. 2. Comparative analysis of *St. aureus* adherence to *C. albicans* strains as assessed by confocal microscopy. Imaging of *St. aureus* adherence to pre-formed *C. albicans* biofilms demonstrating decreased binding to the $\Delta als3$ strain (c) as compared with the parent strain (DAY185) (j). In contrast, a comparable pattern of adherence to the *C. albicans* parent strain was seen for *St. aureus* with the remaining mutant stains: $\Delta als1$ (a), $\Delta als2$ (b), $\Delta als4$ (d), $\Delta als5$ (e), $\Delta als6$ (f), $\Delta als7$ (g), $\Delta als9$ (h) and $\Delta hwp1$ (i). *C. albicans* parent strain (DAY185) (k) and *St. aureus* (l) grown alone on chamber slides were included as controls. Biofilms were stained with a cocktail of fluorescent dyes: Calcofluor white (blue), Syto9 (green) and ConA–Texas red (red). Bars, 20 μ m.

Fig. 3. ELISA assessment of staphylococcal adherence to *C. albicans* parent and mutant strains. (a) Statistically significant reductions in the levels of bound *St. aureus* ($*P<0.05$) to the strain lacking Als3p ($\Delta als3$) and wells containing no fungal growth (no CA) were demonstrated as compared with the parent; some strains exhibited increases in *St. aureus* binding ($**P<0.05$). (b) Biofilm biomasses, as assessed by the crystal violet assay, were not significantly different between parent and mutant *C. albicans* strains. Groups were compared using a one-way ANOVA and Dunnett's post-test. Error bars, SEM.

Fig. 4. Atomic force measurements of *C. albicans* and *St. aureus* interaction. (a) Typical examples of force–distance curves between *St. aureus* and *C. albicans* strains recorded after 0 s (solid line) and 60 s (dashed line) surface delay time. (b) *C. albicans* mutant lacking Als3p exhibited significant decrease in adhesion force (F_{adh}) with *St. aureus* at all times tested compared with the parent (DAY185). Cantilever tips with *St. aureus* were allowed to interact with a randomly selected hypha and the adhesion forces were measured 10 times; results represent the median of at least 80 individual force–distance curves from three experiments with independently grown cultures (* $P < 0.05$). Groups were compared using a non-parametric Mann–Whitney U test. Bars indicate the median and the error calculated as the interquartile range.

peptide to *St. aureus* cells. The control peptide was also included in these assays. Upon staining with Syto9, all *St. aureus* cells appeared green (Fig. 5b). However, only *St. aureus* cells bound to rAls3p fluoresced red upon incubation with the Als3p-specific antibody and a Cy3-labelled secondary antibody. This was confirmed by images demonstrating that only the addition of rAls3p to the cells resulted in red fluorescence (Fig. 5b), with no red staining observed in the absence of rAls3p.

Heterologous expression of Als3p in *Sa. cerevisiae* mediates interactions with *St. aureus*

To further confirm the *C. albicans* adhesin Als3p as a mediator of coadherence with *St. aureus*, the interaction of a *Sa. cerevisiae* heterologous host strain expressing *C. albicans* Als3p incubated with *St. aureus* was investigated. Micrographs demonstrated that *St. aureus* did not bind parental *Sa. cerevisiae*. However, *St. aureus* bound strongly to cells expressing both the large and the small alleles of Als3p (Fig. 6).

Interactions on host tissue

Tongues excised from euthanized mice were infected *ex vivo* with either species alone or co-infected with both in order to assess the potential implications of this interaction on a host. As expected, examination of PNA-FISH-stained tissue sections demonstrated efficient adherence of *St. aureus* (green) to the outer layers of the tongue epithelium, with no bacteria seen in the subepithelial tissue (Fig. 7a). These images were consistent with those observed with H&E staining, where lack of inflammatory infiltrates confirmed a non-invasive presence of *St. aureus* on the tissue (Fig. 7a). Similar to images from experiments using abiotic surfaces, sections from co-infected tongues showed *St. aureus* adhering to the hyphae (Fig. 7b). Interestingly, however, *St. aureus* also could be seen in the subepithelium in association with the invading hyphae (Fig. 7b, arrows). In contrast, although the hyphae of the $\Delta als3$ strain penetrated the epithelial tissue similarly to the parental strain, minimal *St. aureus* cells were seen attached to the hyphae. Importantly, no staphylococcal presence was detected beneath the outer layers of the tissue (Fig. 7c).

Fig. 5. Evaluation of *St. aureus* adherence to the purified recombinant protein (rAls3p) by ELISA and IFA. (a) *St. aureus* bound to rAls3p (dark-grey bars) in a dose-dependent fashion but not to wells coated with a control peptide lacking the N-terminal Als3p sequence (light-grey bars). Although *St. aureus* bound to rAls1p (white bars), the level of adherence was non-dose-dependent and significantly less than binding to rAls3p (* $P < 0.05$). (b) IFA images of *St. aureus* cells interacting with rAls3p or control peptide stained with the DNA stain Syto9 (green). Following probing with a primary mouse IgG antibody specific for the c-myc epitope tag and a Cy3-conjugated secondary antibody (red), cells demonstrating peptide binding appear yellow due to the merged green–red fluorescence. Bars, 10 μm.

Effects of serum on polymicrobial growth

As it is known that both *C. albicans* and *St. aureus* have receptors to various proteins in serum, we sought to determine whether the observed attachment could be enhanced by cross-bridging independent of adhesin expression (Moreillon *et al.*, 1995; Penn & Klotz, 1994). The use of serum in our polymicrobial experiments showed that both parental and Δ als3 strains supported robust staphylococcal binding during polymicrobial growth. However, in the absence of serum, only the parental strain was capable of binding *St. aureus* (Fig. S1). This noted ability of serum to mediate non-specific co-adherence and/or enhance *St. aureus* growth may explain the discrepancies between our results and those from previous studies, in which, in the presence of serum, *St. aureus* associated equally well when grown with the Δ als3 mutant strain (Harriott & Noverr, 2010).

DISCUSSION

Candida spp. and *Staphylococcus* spp. are among the leading pathogens causing nosocomial and bloodstream infections (Wisplinghoff *et al.*, 2004). Their co-isolation from patients during various disease states is increasingly reported within the context of polymicrobial infections (Baena-Monroy *et al.*, 2005; Cuesta *et al.*, 2010; Klotz *et al.*, 2007a; Valenza *et al.*,

2008). Building on our earlier investigations demonstrating a robust attachment of *St. aureus* cells to *C. albicans* hyphae during polymicrobial biofilm formation, this current study was designed to characterize this inter-species interaction and identify the hyphal receptor for *St. aureus* (Peters *et al.*, 2010). To that end, extensive analyses were performed using several microscopic approaches, immunoassays, protein expression techniques and a tissue infection model.

Images from confocal and electron microscopy clearly demonstrated the close association between these two diverse species (Fig. 1). This association has been reported by Harriott & Noverr (2009), where *St. aureus* was shown to be embedded in the fungal biofilm matrix. Significantly, this close association between *C. albicans* and *St. aureus* in a biofilm conferred resistance to antibiotics upon *St. aureus*. These findings carry significant clinical implications in terms of therapeutic measures, and therefore warranted further investigations to elucidate the mechanism behind this association. Importantly, the identification of a specific adherence receptor would aid in the design of novel strategies for impeding co-adherence and subsequent infection.

The *C. albicans* Als proteins and the hyphal wall protein (Hwp1) are the main cell wall proteins implicated in the adherence of *C. albicans* to host tissue and several bacterial species, namely the oral streptococci (Nobbs *et al.*, 2010;

Fig. 6. Interactions of *St. aureus* with *C. albicans* Als3p adhesin expressed on the surface of surrogate host *Sa. cerevisiae*. (a) *Sa. cerevisiae* cells expressing the candidal adhesin Als3p were incubated for 1 h with staphylococci. Cells were then harvested and visualized by light microscopy. Although *St. aureus* did not bind to the wild-type (WT) *Sa. cerevisiae*, it bound strongly to cells expressing Als3p, both large and small alleles. White arrows show adherent *St. aureus*. (b) Percentage of total bacteria bound to yeast cells, demonstrating a significant increase in the number of *St. aureus* adhering to Als3p-expressing yeast cells as compared with wild-type ($*P < 0.001$). Bars, 10 μ m.

Silverman *et al.*, 2010). The streptococcal surface molecules largely involved in this interaction have been demonstrated to be surface proteins A and B (SspA/SspB) (Holmes *et al.*, 1996). These types of interactions are considered synergistic, as streptococci have been shown not only to enhance *C. albicans* colonization of the oral cavity but also to promote hyphal growth by excreting lactate as a carbon source (Holmes *et al.*, 1996; Jenkinson *et al.*, 1990). In addition to Gram-positive bacteria, the association of *C. albicans* with Gram-negative species has also been reported, most prominently with the pathogen *Pseudomonas* (*Ps.*) *aeruginosa* (Hogan & Kolter, 2002). However, in contrast to the seemingly synergistic interaction with *St. aureus*, that with *Ps. aeruginosa* was described not only to be antagonistic in nature, with the bacteria capable of killing the hyphae, but also to involve quorum-sensing molecules and virulence factors (Cugini *et al.*, 2007; Hogan *et al.*, 2004).

Based on these earlier studies, we tested several *C. albicans* cell wall mutants in an attempt to identify a specific receptor for *St. aureus* on the hyphae. Our initial microscopy-based screen demonstrated that the strain

Fig. 7. *Ex vivo* murine model of co-infection. Mouse tongues were infected *in vitro* with *St. aureus* and in combination with the *C. albicans* parent strain (DAY185) or the *als3* mutant strain. Sectioned tissue was stained with species-specific fluorescent (PNA-FISH) or histopathology (H&E) stains and examined by microscopy. (a) Infection with *St. aureus* (green) was confined to the outer layers of the epithelial surface, with no evidence of tissue invasion as confirmed by a lack of inflammatory infiltrate. (b) *St. aureus* (green) with parent strain (parent + SA) is seen attached to the hyphae (red) and in some instances detected beneath the epithelium associated with penetrating hyphae (white arrow). (c) However, although the *als3* mutant (Δ als3 + SA) hyphae penetrated the tissue, relatively few staphylococci are seen attached to the hyphae, with no bacterial presence noted beneath the epithelial layers. Bars, 10 μ m.

lacking Als3p had a reduced ability to adhere to *St. aureus*, attributing a crucial role to that protein in the adherence process (Fig. 2); these results were further quantified and confirmed by ELISA (Fig. 3). In contrast, the other mutants of Als proteins as well as the strain lacking Hwp1p exhibited adherence levels comparable with that of the parent strain. In some cases, staphylococcal binding was increased to adhesin mutants (Δ als6, Δ als7, Δ als9) as compared with the parental strain (Figs 2f–h and 3). Interestingly, deletion of *als5*, *als6* or *als7* has been linked to increased adhesion to human endothelium and epithelium (Zhao *et al.*, 2007a). Therefore, deletion of these genes may counter-intuitively augment adhesive properties by increasing expression of known adhesins or by exposing other adhesive moieties on the surface of *C. albicans*, resulting in enhanced *St. aureus* adherence. It is important to note, however, that some bacterial cells were seen attached to the hyphae of the *als3* mutant, indicating that additional molecules (including biofilm matrix) may also be involved in the process (Fig. 7c). Alternatively, these observations could also be due to non-specific attachment due to hydrophobic or electrostatic interactions.

Although microscopic images clearly revealed differences in adherence potential between the strains, AFM was utilized to obtain a quantitative measure. AFM is a

powerful tool that has proven useful in determining intraspecies adhesion forces (Dufrêne, 2002; Postollec *et al.*, 2006; Waar *et al.*, 2005). Based on these measurements, deletion of *als3* demonstrated a significant reduction in the staphylococcal–hyphal binding force (Fig. 4). Further confirmation for the involvement of Als3p came from ELISA testing, in which a recombinantly expressed peptide of the N-terminal domain of Als3p containing the receptor-binding function was used for capturing overlaid *St. aureus* cells. These results demonstrated a dose-dependent adherence of *St. aureus* to rAls3p-coated wells (Fig. 5). As Als1p shares significant homology with Als3p, its ability to directly bind *St. aureus* was also tested. Although the *als1* mutant strain did not exhibit reduced capacity to bind to *St. aureus* as demonstrated by microscopy (Fig. 2a) and ELISA measurements (Fig. 3a), when coated on the wells of microtitre plates the purified protein did exhibit some capacity to support staphylococcal binding (Fig. 5a). However, these observations could be due to masking of the contribution of Als1 to *St. aureus* binding by functional Als3 at the fungal surface or conformational differences between the native protein and purified peptide. Collectively, these findings indicated that Als3p is involved in the interaction between *C. albicans* and *St. aureus*. Importantly, this hypothesis was firmly validated using a *Sa. cerevisiae* strain heterologously expressing *C. albicans* Als3p, where *St. aureus* was shown to strongly bind only to the surface of the Als3p-expressing yeast cells (Fig. 6).

Interestingly, although Als3p has been implicated in *C. albicans* adherence to various bacterial species, the mechanism of adherence appears to be vastly different between Gram-negative and Gram-positive bacteria. A study by Brand and colleagues demonstrated that the interaction between *Ps. aeruginosa* and *C. albicans* is independent of Als3p, as strains deficient in the production of the hyphal proteins Hwp1, Als3 and Hyr1 were as susceptible to killing by this bacterial pathogen as the parent strain (Brand *et al.*, 2008). These observations indicate that *Ps. aeruginosa* adherence to hyphae is likely mediated by glycan moieties rather than specific surface proteins.

Perhaps the most significant findings with potential clinical implications come from the experiments examining the ability of *C. albicans* and *St. aureus* to co-adhere to host tissue (Fig. 7). Microscopic images from sectioned mouse tongue tissue paralleled those previously seen *in vitro*, where an extensive network of hyphae from the *C. albicans* parental strain was seen covering the tongue surface with *St. aureus* interspersed throughout the hyphal matrix (Fig. 7b). Importantly, bacterial cells were at times seen beneath the epithelial barrier of the tissue where hyphae had penetrated (Fig. 7b, arrows). In contrast, although the *als3* mutant did not exhibit defects in hyphal formation, minimal bacterial cells were seen adhering to the hyphae (Fig. 7c). These findings are of considerable significance, because although *C. albicans* hyphal filaments are

considered to be highly invasive of mucosal tissue, *St. aureus* infections are strongly correlated with a prerequisite breach of innate biological barriers (Goetz *et al.*, 1999; Scanvic *et al.*, 2001; Troillet *et al.*, 1998). Therefore, it is conceivable that the association of staphylococcal cells with *C. albicans* hyphae, as they penetrate host tissue, may allow *St. aureus* to gain entry into deeper tissues and initiate infection, with dire consequences for the host, particularly in critically ill patients.

In summary, this study identifies the hyphal adhesin Als3p as a receptor for the bacterial pathogen *St. aureus* on *C. albicans* hyphae. The interaction between such diverse and important pathogens holds significant clinical implications, and therefore characterizing their complex interactions is the first step in understanding the nature of their coexistence in the host. The findings generated will not only advance our understanding of the molecular mechanisms underlying the strong interaction between these pathogens, but may also lead to the development of novel strategies to impede microbial co-adherence. Future studies are now focused on identifying the receptors on *St. aureus*.

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