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Functional regions of *Candida albicans* hyphal cell wall protein Als3 that determine interaction with the oral bacterium *Streptococcus gordonii*

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The opportunistic pathogen *Candida albicans* colonizes the oral cavity and gastrointestinal tract. Adherence to host cells, extracellular matrix and salivary glycoproteins that coat oral surfaces, including prostheses, is an important prerequisite for colonization. In addition, interactions of *C. albicans* with commensal oral streptococci are suggested to promote retention and persistence of fungal cells in mixed-species communities. The hyphal filament specific cell wall protein Als3, a member of the Als protein family, is a major determinant in *C. albicans* adherence. Here, we utilized site-specific in-frame deletions within Als3 expressed on the surface of heterologous *Saccharomyces cerevisiae* to determine regions involved in interactions of Als3 with *Streptococcus gordonii*. N-terminal region amino acid residue deletions Δ166–225, Δ218–285, Δ270–305 and Δ277–286 were each effective in inhibiting binding of *Strep. gordonii* to Als3. In addition, these deletions differentially affected biofilm formation, hydrophobicity, and adherence to silicone and human tissue proteins. Deletion of the central repeat domain (Δ434–830) did not significantly affect interaction of Als3 with *Strep. gordonii* SspB protein, but affected other adherence properties and biofilm formation. Deletion of the amyloid-forming region (Δ325–331) did not affect interaction of Als3 with *Strep. gordonii* SspB adhesin, suggesting this interaction was amyloid-independent. These findings highlighted the essential function of the N-terminal domain of Als3 in mediating the interaction of *C. albicans* with *S. gordonii*, and suggested that amyloid formation is not essential for the inter-kingdom interaction.

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INTRODUCTION

The pleiomorphic fungus *Candida albicans* commonly exists as a commensal of the skin, and of mucosal tissues of the oral cavity, urogenital and gastrointestinal tracts. Pathogenesis of *C. albicans* is facilitated by environmental changes, including alterations to the resident microbiota, modified physiology (e.g. reduced saliva flow sometimes resulting from medical intervention) and impaired immune defence. *Candida* species are the fourth most common micro-organisms recovered from nosocomial bloodstream infections in the USA, and infections often require long and costly hospital stays (Zaoutis *et al.*, 2005; Moran *et al.*, 2009).

Abbreviations: AFR, amyloid-forming region; CRD, central repeat domain; ECM, extracellular matrix; HA, haemagglutinin; Ig, immunoglobulin; PBC, peptide-binding cavity; RFU, relative fluorescence unit.

Two supplementary tables and three supplementary figures are available with the online Supplementary Material.

In the oral cavity, colonization by *C. albicans* occurs as a result of direct contact with host tissues and with the salivary pellicle that coats most surfaces (Cannon & Chaffin, 1999). Infections by *C. albicans* can be enhanced by the formation of biofilms on denture materials (Minagi *et al.*, 1985) as well as in catheters (Hawser & Douglas, 1994), and on plastics (Klotz *et al.*, 1985). The hyphal form of *C. albicans* is essential for biofilm development, and hyphae express a range of specific cell wall proteins (e.g. Als3 and Hwp1) that enable adhesion and biofilm formation (Nobile *et al.*, 2006, 2008). In addition, *C. albicans* is rarely detected in isolation and co-exists with a diversity of micro-organisms (Shirtliff *et al.*, 2009). Interactions of *C. albicans* with Gram-positive bacteria include *Staphylococcus aureus* (Harriott & Noverr, 2009), *Streptococcus* spp. (Jenkinson *et al.*, 1990; Xu *et al.*, 2014a, b) and *Actinomyces* spp. (Grimaudo *et al.*, 1996). Gram-negative bacteria also interact with *C. albicans*, and include *Pseudomonas aeruginosa* (Hogan & Kolter, 2002), *Acinetobacter* spp. (Gaddy *et al.*, 2009) and *Fusobacterium* spp. (Grimaudo & Nesbitt, 1997). These associations may be deleterious to

C. albicans, such as interactions with *P. aeruginosa* (Hogan & Kolter, 2002; Holcombe *et al.*, 2010), or beneficial for both organisms involved (Carlson & Johnson, 1985; Bamford *et al.*, 2009; Harriott & Noverr, 2009; Xu *et al.*, 2014a). The relationship of *C. albicans* with streptococcal species is potentially synergistic (O'Sullivan *et al.*, 2000; Bamford *et al.*, 2009; Diaz *et al.*, 2012; Xu *et al.*, 2014b) as lactate production by streptococci can act as a carbon and energy source for the yeast (Jenkinson *et al.*, 1990; Xu *et al.*, 2014a), whilst *C. albicans* reduces oxygen tension to levels preferred by streptococci (O'Sullivan *et al.*, 2000; Jenkinson & Douglas, 2002). Dual-species biofilms of *C. albicans* and *Streptococcus gordonii* have greater biomass compared with those produced by individual species (Bamford *et al.*, 2009; Dutton *et al.*, 2014; Xu *et al.*, 2014a).

As a primary colonizer of the oral cavity, the bacterium *Strep. gordonii* attaches to mucosal or hard surfaces within the mouth (Nyvad & Kilian, 1990; Frandsen *et al.*, 1991). Adherence of *C. albicans* to *Strep. gordonii* potentially provides an additional means for colonization of the oral cavity by *C. albicans*. Previous studies have identified cell surface *Strep. gordonii* polypeptides SspA and SspB as receptors for *C. albicans* hyphae (Holmes *et al.*, 1998; Silverman *et al.*, 2010). We have also identified several *C. albicans* surface proteins, including Eap1, Hwp1 and Als3, that bind *Strep. gordonii* cells when they are expressed on the surface of surrogate host *Saccharomyces cerevisiae* (Nobbs *et al.*, 2010). A direct role for Als3 in interactions of *C. albicans* hyphae with *Strep. gordonii* was suggested by observing that an *als3*Δ/*als3*Δ (null) mutant was unable to bind *Strep. gordonii* cells or interact with *Strep. gordonii* SspB polypeptide expressed on the surface of surrogate host strain *Lactococcus lactis* (Silverman *et al.*, 2010).

Als3 is a hypha-specific cell wall protein and a member of the Als protein family in *C. albicans* (Hoyer *et al.*, 1998; Green *et al.*, 2005). Als3 is able to bind extracellular matrix (ECM) proteins, epithelial and endothelial cells (Fu *et al.*, 1998; Sheppard *et al.*, 2004; Zhao *et al.*, 2004; Liu & Filler, 2011), induce endocytosis via adherence to E- or N-cadherins (Phan *et al.*, 2013), and mediate trafficking to the brain (Fu *et al.*, 2013). The ability of *C. albicans* to form biofilms on a variety of substrata including the salivary pellicle, polystyrene and silicone elastomer is also influenced by expression of Als3 (Nobile *et al.*, 2006, 2008; Nobbs *et al.*, 2010). The primary structure of Als3 (Fig. 1a) contains an N-terminal domain with signal peptide, an immunoglobulin (Ig)-like region (~300 aa), a conserved Thr-rich region, a central Thr-rich repeat domain (~470 aa residues), a Ser/Thr-rich C-terminal domain and a C-terminal glycosylphosphatidylinositol modification (Gaur & Klotz, 1997; Sheppard *et al.*, 2004; Phan *et al.*, 2007). The central repeat domain (CRD) consists of imperfect

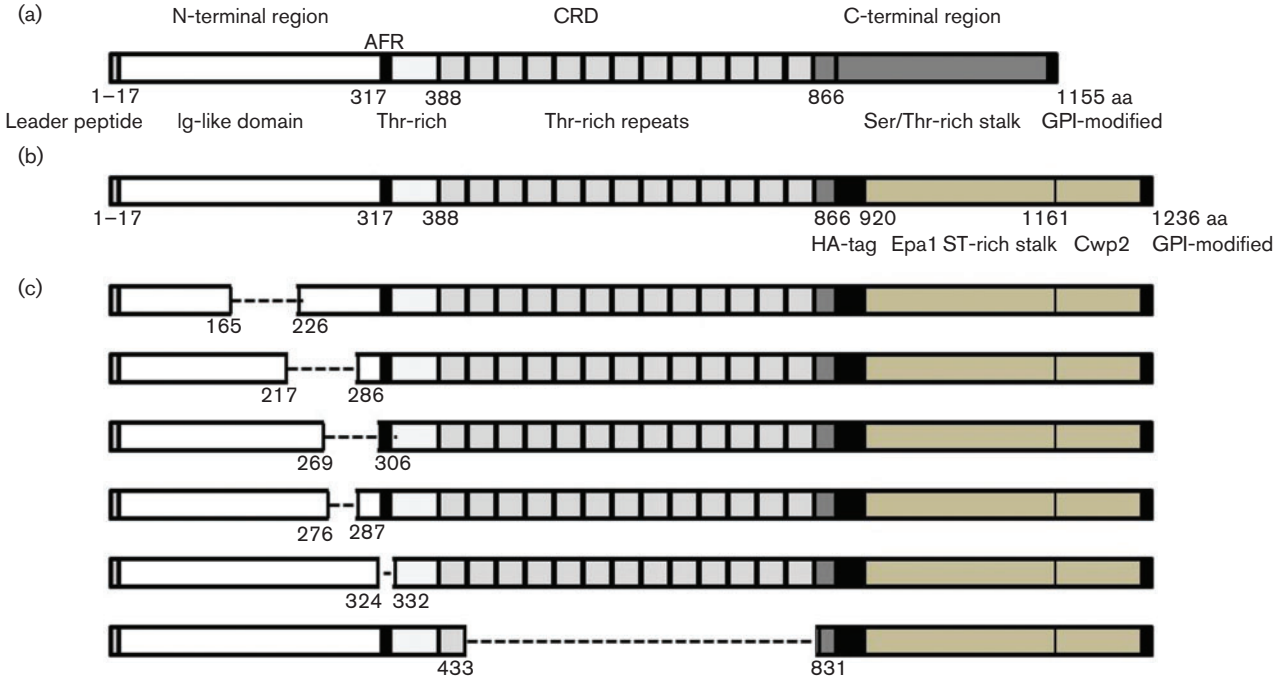

Fig. 1. Diagrammatic representation of the Als3 polypeptide showing (a) overall structure and domains based upon primary sequence, (b) corresponding protein expressed on the surface of *Sacch. cerevisiae* with insertion of a HA-tag and replacement of the C-terminal region with an Epa1 Ser/Thr-rich stalk from *Candida glabrata* and (c) proteins expressed by *als3* plasmid deletion mutants and expressed on the surface of *Sacch. cerevisiae*. Numbers indicate amino acid residues. Amino acid residues 325–331 corresponded to the predicted β-amyloid sequence IVIVATT (AFR). GPI, glycosylphosphatidylinositol.

Thr-rich tandem repeat regions, each of 36 aa residues (Frank *et al.*, 2010). The number of repeat units varies in Als3 proteins from different *C. albicans* strains and the two *ALS3* alleles in *C. albicans* SC5314 encode proteins with different numbers of repeats (Oh *et al.*, 2005). The C-terminal Thr/Ser-rich region of Als3 is heavily glycosylated and the glycosylphosphatidylinositol modification localizes the protein to within the hyphal cell wall (Liu & Filler, 2011).

The main adhesive region of Als3 has been mapped to the N-terminal domain (Zhao *et al.*, 2006) and antibodies to N-terminal Als3 peptides were able to block adhesion of *C. albicans* to buccal epithelial cells. The N-terminal domain is predicted to form two Ig folds (Phan *et al.*, 2007) with a peptide-binding cavity (PBC) for interaction with potential substrates, such as the flexible C-termini of polypeptides (Salgado *et al.*, 2011; Lin *et al.*, 2014). At the boundary of the Ig-like domain and conserved Thr-rich region is an amyloid-forming region (AFR) (Fig. 1a) that is implicated in cell–cell aggregation and adhesin clustering (Garcia *et al.*, 2011; Beaussart *et al.*, 2012), but may not be involved directly in *C. albicans* adherence to buccal epithelial cells (Lin *et al.*, 2014). The CRD is believed to enhance the presentation of the adhesive N-terminal region away from the cell surface (Rauceo *et al.*, 2006; Frank *et al.*, 2010). In this study, we aimed to identify regions of Als3 that were necessary for binding *Strep. gordonii* and for interacting with the *Strep. gordonii* SspB cell wall adhesin. Specific amino acid sequences were deleted from within Als3 and the resulting polypeptides were surface-expressed on *Sacch. cerevisiae*. Our results provided evidence that regions within the N-terminal domain of Als3 were responsible for interaction of Als3 with *Strep. gordonii* cells and with *Strep. gordonii* SspB cell wall protein. However, the major AFR of Als3 appeared not to be necessary for the interaction of *C. albicans* Als3 protein with *Strep. gordonii* SspB protein – a primary mechanism for this inter-microbial association.

METHODS

Microbial strains and culture conditions. The bacterial and yeast strains used in this study are listed in Table S1 (available in the online Supplementary Material). Streptococcal cells were grown in brain heart infusion (BHI) medium (Lab M) supplemented with 5 mg yeast extract ml^{-1} statically in a candle jar for 16 h at 37 °C. *Escherichia coli* was grown in Luria–Bertani medium (Becton Dickinson) at 37 °C with vigorous shaking (200 r.p.m.). Ampicillin (100 μg ml^{-1}) was included when necessary. *L. lactis* was grown in M17 medium (Difco) supplemented with 0.5 mg glucose ml^{-1} statically in a candle jar for 16 h at 30 °C. Erythromycin (5 μg ml^{-1}) was added as appropriate. *Sacch. cerevisiae* strains were grown with shaking (200 r.p.m.) at 30 °C in complete synthetic medium (CSM) without uracil (ForMedium) supplemented with 0.67 % Yeast Nitrogen Base (Difco) and 2 % glucose (CSM-Glc). Uridine (25 μg ml^{-1}) was added to the medium for growth of the *Sacch. cerevisiae* BY4742 parent strain (Brachmann *et al.*, 1998). *C. albicans* was grown in YPD medium with shaking (200 r.p.m.) at 37 °C (Nobbs *et al.*, 2010) and induced to form hyphae by transfer to YPT-Glc medium (Dutton *et al.*, 2014).

Heterologous expression of Als3. The *ALS3* gene (larger allele) (GenBank accession number AY223552) minus the C-terminal stalk region (Fig. 1a) was amplified by PCR from *C. albicans* SC5314 chromosomal DNA with primers ALS3attB1 and ALS3attB2 (Gillum *et al.*, 1984; Nobbs *et al.*, 2010). The product was mixed with Gateway BP enzymes (Invitrogen), inserted into donor vector pDONR207 and transformed into *E. coli* OmniMAX 2-T1. Using Gateway LR enzymes, the *C. albicans* gene fragment was recombined with the destination vector pBC542 (Zupancic *et al.*, 2008) to generate an expression vector and electroporated into *Sacch. cerevisiae* BY4742. To generate recombinant Als3 polypeptides with defined regions deleted, the plasmid pBC542-$ALS3_{lg}$ (Nobbs *et al.*, 2010) was extracted from *E. coli* using a QIAquick Spin Miniprep Purification kit and used as template for inverse PCR with an Expand Long Template PCR System (Roche) and oligonucleotide primers as listed in Table S2. PCR products were purified using a QIAquick PCR Purification kit (Qiagen). Oligonucleotides were designed with *Bgl*II restriction sites at their termini to enable the coding sequence for Als3 to be religated with required deletions. Resulting plasmids were transformed into *E. coli* OmniMAX 2-T1 and deletions were confirmed by sequencing (Fig. S1). Plasmids with the correct sequences were linearized and transformed into *Sacch. cerevisiae* BY4742 by electroporation, with pBC542 and pBC542-*ALS3* as controls. PCR and sequencing were again utilized to confirm successful cloning and authenticity.

Surface expression of Als3. *Sacch. cerevisiae* cells were pelleted by centrifugation at 5000 ***g*** for 5 min and washed in PBS. The OD_{600} of the cell suspension was adjusted to 1.0 ($\sim 1 \times 10^7$ cells ml^{-1}) and the cells were fixed with 4 % paraformaldehyde for 30 min at 22 °C (room temperature). After paraformaldehyde was removed, cells were blocked with heat-inactivated rabbit serum for 30 min at 22 °C. The cells were washed with PBS and then incubated for 1 h at 22 °C with mouse anti-heamagglutinin (HA) antibody (Zymed) at 10 μg ml^{-1}. FITC-conjugated rabbit anti-mouse antibodies (Dako) diluted 1 : 100 were then added and the cells incubated for 1 h at 22 °C. Unbound antibodies were removed by washing with PBS. HA-tagged protein expression was visualized by fluorescence microscopy, and quantified by transferring portions into black 96-well microtitre plates (Greiner) and measuring relative fluorescence units (RFU) with a Molecular Devices SpectraMax M2 microtitre plate reader. The values were then adjusted to account for different levels of total protein solubilized from whole cells with 1 M NaOH and measured by the Lowry method, as described previously (Nobbs *et al.*, 2010).

Interactions of *Sacch. cerevisiae* with *Strep. gordonii* or *L. lactis* expressing SspB. *Sacch. cerevisiae* cells were harvested by centrifugation at 5000 ***g*** for 5 min and washed twice in CSM-Glc. Cell suspensions (2 ml) were adjusted to OD_{600} 0.5, transferred to glass tubes and incubated with vigorous shaking (200 r.p.m.) for 3 h at 30 °C. *Strep. gordonii* or *L. lactis* cells were collected by centrifugation of cultures at 5000 ***g*** for 10 min, washed with CSM-Glc and fluorescently labelled by incubating cells with 1.5 mM FITC in 0.05 M Na_2CO_3 containing 0.1 M NaCl for 1 h at 22 °C. Labelled cells were collected by centrifugation, washed with CSM-Glc and adjusted to OD_{600} 0.5 ($\sim 5 \times 10^8$ cells ml^{-1}). Labelled bacteria (1 ml) were added to *Sacch. cerevisiae* cells and the suspensions incubated with gentle shaking (50 r.p.m.) for 1 h at 30 °C. Cells were harvested by brief centrifugation at 5000 ***g*** for 2 min, concentrated 10-fold, and imaged by transmitted light and fluorescence microscopy. Bacterial attachment to yeast cells was semi-quantified using imaging software (Cell^D; Olympus) and expressed as the mean number of fluorescent pixels bound per *Sacch. cerevisiae* cell (minimum cell count 200).

Biofilm formation on salivary pellicle. Collection of saliva from human subjects was approved by the National Research Ethics Committee South Central Oxford C. (08/H0606/87 + 5). Pooled human saliva from at least six adult subjects who provided written

informed consent was treated for 10 min on ice with 2.5 mM DTT. Mucins and bacteria were sedimented by centrifugation at 10 000 ***g*** for 10 min, and clarified saliva supernatant was sterilized by passage through a 0.2 μm filter and then diluted to 10 % with sterile distilled H_2O. Aliquots (1 ml) were transferred to wells of a 24-well plastic plate, 13 mm diameter sterile glass coverslips were added and plates were incubated for 16 h at 4 °C. Excess saliva was removed by washing with PBS. *Sacch. cerevisiae* cell suspensions in CSM-Glc at OD_{600} 1.0 were added (0.4 ml) to wells containing saliva-coated coverslips and incubated for 4 h at 30 °C with gentle rotation (50 r.p.m.). Cell suspensions were aspirated, coverslips were washed gently with PBS (1 ml), fresh CSM-Glc was added (1 ml) and then plates were incubated for 20 h at 30 °C. Coverslips were then washed carefully with PBS and removed from the wells to air-dry before staining with 0.5 % crystal violet for 2 min. Excess crystal violet was removed with distilled H_2O and the biofilms were imaged by transmitted light microscopy. Alternatively, coverslips were transferred to new plate wells to release the crystal violet with 10 % acetic acid for A_{595} measurements, proportional to total biomass (Jakubovics *et al.*, 2005; Nobbs *et al.*, 2010).

Hydrophobicity assay. *Sacch. cerevisiae* cells were prepared as described above, and cell suspensions (0.1 ml) added directly to wells of a 96-well microtitre plate and incubated for 4 h at 30 °C with gentle agitation (50 r.p.m.). Wells were washed thoroughly with TBS (10 mM Tris/HCl, 0.15 M NaCl, pH 8) and cells were fixed by addition of 25 % formaldehyde for 30 min at 22 °C. Biomass measurements of adherence to polystyrene, as a determinant of hydrophobicity, were then performed with crystal violet assay as described above.

Biofilm formation on silicone. Silicone elastomer squares (1 cm^2) were added to wells of a 24-well plate containing heat-inactivated horse serum (1 ml) and incubated for 16 h at 4 °C. The silicone squares were then washed with PBS, transferred to new wells and incubated with *Sacch. cerevisiae* cell suspension (OD_{600} 1.0) in CSM-Glc for 4 h at 30 °C with gentle agitation (50 r.p.m.). Cell suspensions were carefully aspirated, and silicone squares were washed twice with PBS, air-dried and biomass measurements recorded using crystal violet as above.

Host protein adherence and biofilm assays. Human fibrinogen (Calbiochem), fibronectin (Roche) or type IV collagen (Sigma) were dissolved in coating buffer (20 mM Na_2CO_3, 20 mM $NaHCO_3$, pH 9.3), added to wells of a 96-well plate (10 μg ml^{-1}) and incubated for 16 h at 4 °C. Wells were washed and blocked with 1 % gelatin in

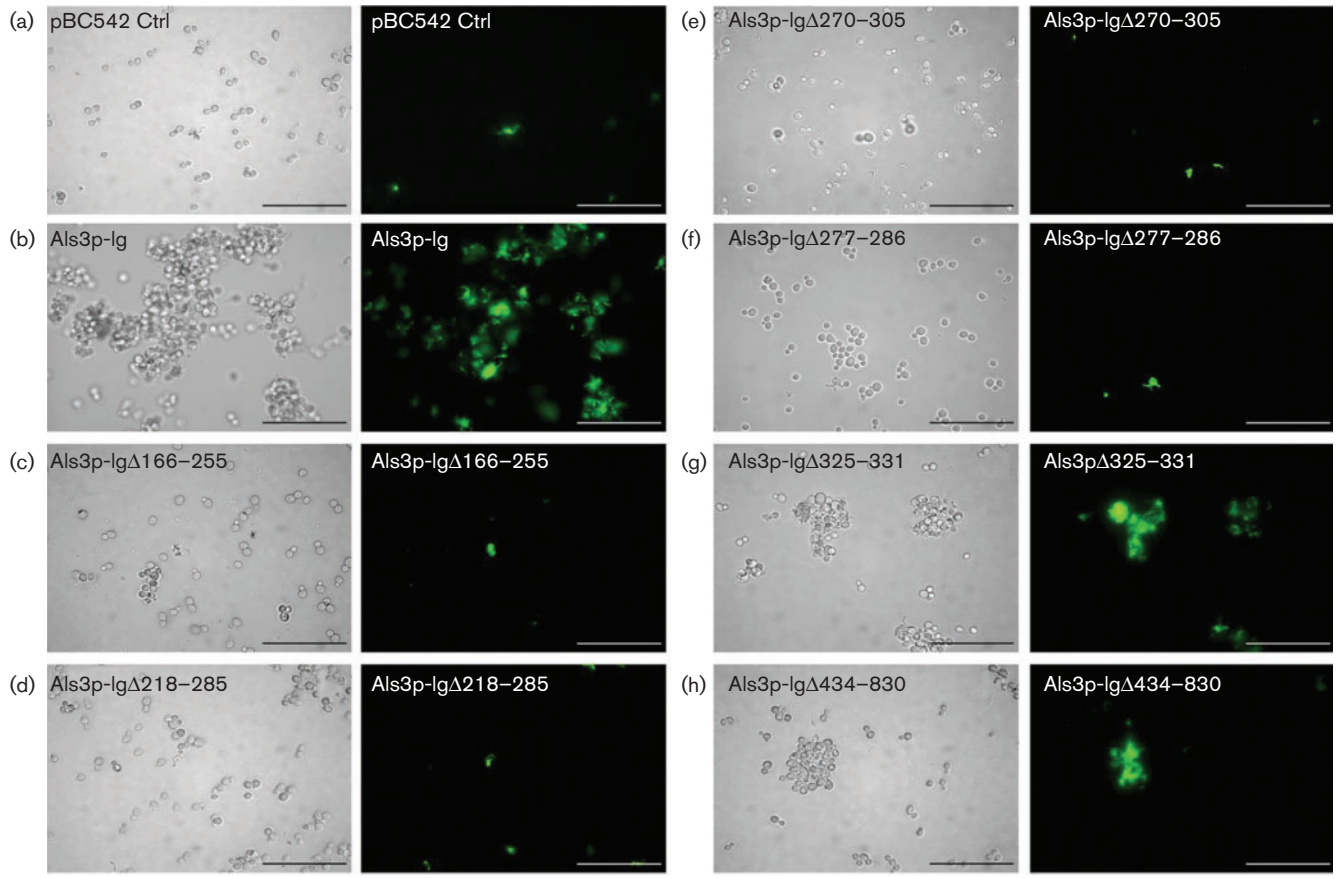

Fig. 2. Interactions of *Sacch. cerevisiae* expressing Als3 or Als3Δ polypeptides with *Strep. gordonii* DL1. *Sacch. cerevisiae* cells were incubated for 3 h at 30 °C and then with FITC-labelled *Strep. gordonii* for 1 h. Interactions were visualized by transmitted light microscopy and by fluorescence microscopy. (a–h) Light and corresponding fluorescence images of *Sacch. cerevisiae* BY4742 strains carrying pBC542 [vector-only control (Ctrl)] (a), pBC542-Als3 (b), pBC542-Als3Δ166–225 (c), pBC542-Als3Δ218–285 (d), pBC542-Als3Δ270–305 (e), pBC542-Als3Δ277–286 (f), pBC542-Als3Δ325–331 (g) and pBC542-Als3Δ434–830 (h). Bar, 50 μm.

TBSC buffer (10 mM Tris/HCl, pH 7.6, 150 mM NaCl, 5 mM $CaCl_2$) before incubation with *Sacch. cerevisiae* cell suspensions as described above and measurement of biomass. Values were corrected for biomass values obtained for *Sacch. cerevisiae* cells binding to gelatin alone.

Statistics. For adhesion and biofilm assays, statistical analyses were performed with Student's *t*-test using the Bonferroni correction for multiple comparisons.

RESULTS

Surface expression of Als3 in heterologous host *Sacch. cerevisiae* BY4742

Evidence suggests that the hyphal filament-specific Als3 protein plays a major role in the binding of *Strep. gordonii* by *C. albicans* (Silverman *et al.*, 2010). To address the question of which regions of the Als3 protein were necessary for this interaction, deletions were generated in recombinant Als3 and expressed from plasmid pBC542 (Nobbs *et al.*, 2010) on the surface of *Sacch. cerevisiae* BY4742 (Fig. 1b). This strain of *Sacch. cerevisiae* does not bind *Strep. gordonii* (Nobbs *et al.*, 2010; Silverman *et al.*, 2010).

Specific amino acid residue sequences were selected for deletion based upon a number of criteria. Deletion of aa 218–285 and 277–286 from within the Als1 protein of *C. albicans* resulted in reduced adherence of expressing *Sacch. cerevisiae* strains to endothelial cells (Loza *et al.*, 2004). Corresponding regions of Als3 have 85 and 90 % identity, respectively, and were therefore targeted in this study (Fig. 1c). Deletions of aa 166–225 and 270–305 were also generated (Fig. 1c) within the Ig-like N-terminal region, as detailed in Fig. S2. We also produced a deletion (aa 325–331) across the AFR sequence IVIVATT (Otoo *et al.*, 2008) that has been proposed to induce adhesin clustering (Beaussart *et al.*, 2012). Lastly, most of the CRD (aa 434–830) was deleted (Figs 1c and S3) to determine the role of this domain in directing adhesive interactions. The Als3 protein deletions were confirmed by sequencing (Fig. S1). Surface expression levels of proteins on *Sacch. cerevisiae* were measured by immunofluorescence assay using antibodies to the C-terminal HA-tag (Fig. 1) as described previously (Nobbs *et al.*, 2010). There were small but statistically not significant differences in expression levels per microgram total protein for all the *Sacch. cerevisiae* strains generated (data not shown).

Effects of Als3 deletions on interactions with *Strep. gordonii*

The deletions within Als3 were utilized to investigate which regions were necessary for binding *Strep. gordonii*. *Sacch. cerevisiae* cells expressing Als3 or deletion constructs were incubated with FITC-labelled *Strep. gordonii* cells. Coaggregation was visualized by transmitted light and fluorescence microscopy, and was quantified by calculating the mean numbers of fluorescent pixels (RFU) per *Sacch. cerevisiae* cell (Nobbs *et al.*, 2010). *Sacch. cerevisiae* $Als3^+$ cells showed the strongest ability to coaggregate with *Strep. gordonii* when compared with the vector-only control (Fig. 2a, b). All of the deletions from within the N terminus of Als3 (aa 166–225, 218–285, 270–305 and 277–286) affected binding to *Strep. gordonii* (Fig. 2c–f) and binding levels were reduced by >90 % compared with intact Als3 (Fig. 3). However, deletion of the AFR (aa 325–331) resulted in ~50 % reduction in binding of *Strep. gordonii* (Figs 2g and 3). The Als3 construct with deleted CRD (aa 434–830) also showed ~50 % reduction in ability to bind FITC-labelled *Strep. gordonii* cells compared with the *Sacch. cerevisiae* $Als3^+$ strain (Figs 2h and 3). These results suggested that the N-terminal region of Als3 was mainly responsible for binding *Strep. gordonii*, but that the CRD may also have played a role, either in direct binding or stabilizing or folding of the N-terminal adhesive region.

Effects of Als3 deletions on interactions with SspB

As the *Strep. gordonii* SspB protein specifically has been shown to interact with Als3 (Silverman *et al.*, 2010), we then tested the ability of *Sacch. cerevisiae* strains to interact with *L. lactis* expressing SspB. *L. lactis* control strain containing empty vector did not interact with *Sacch. cerevisiae* expressing Als3 (data not shown), whilst there was extensive interaction between *Sacch. cerevisiae* $Als3^+$

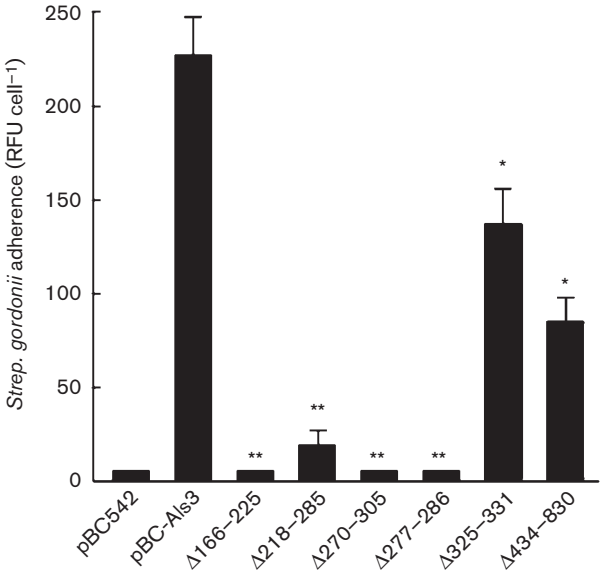

Fig. 3. Binding levels of *Strep. gordonii* DL1 to *Sacch. cerevisiae* expressing Als3 or Als3Δ polypeptides. *Sacch. cerevisiae* cells were incubated for 3 h at 30 °C and then with FITC-labelled *Strep. gordonii* for 1 h. *Strep. gordonii* adherence is expressed as mean ± SD ($n=3$) RFU per *Sacch. cerevisiae* cell, as described in Methods. Statistical significance relative to pBC-Als3: *$P<0.05$, **$P<0.005$.

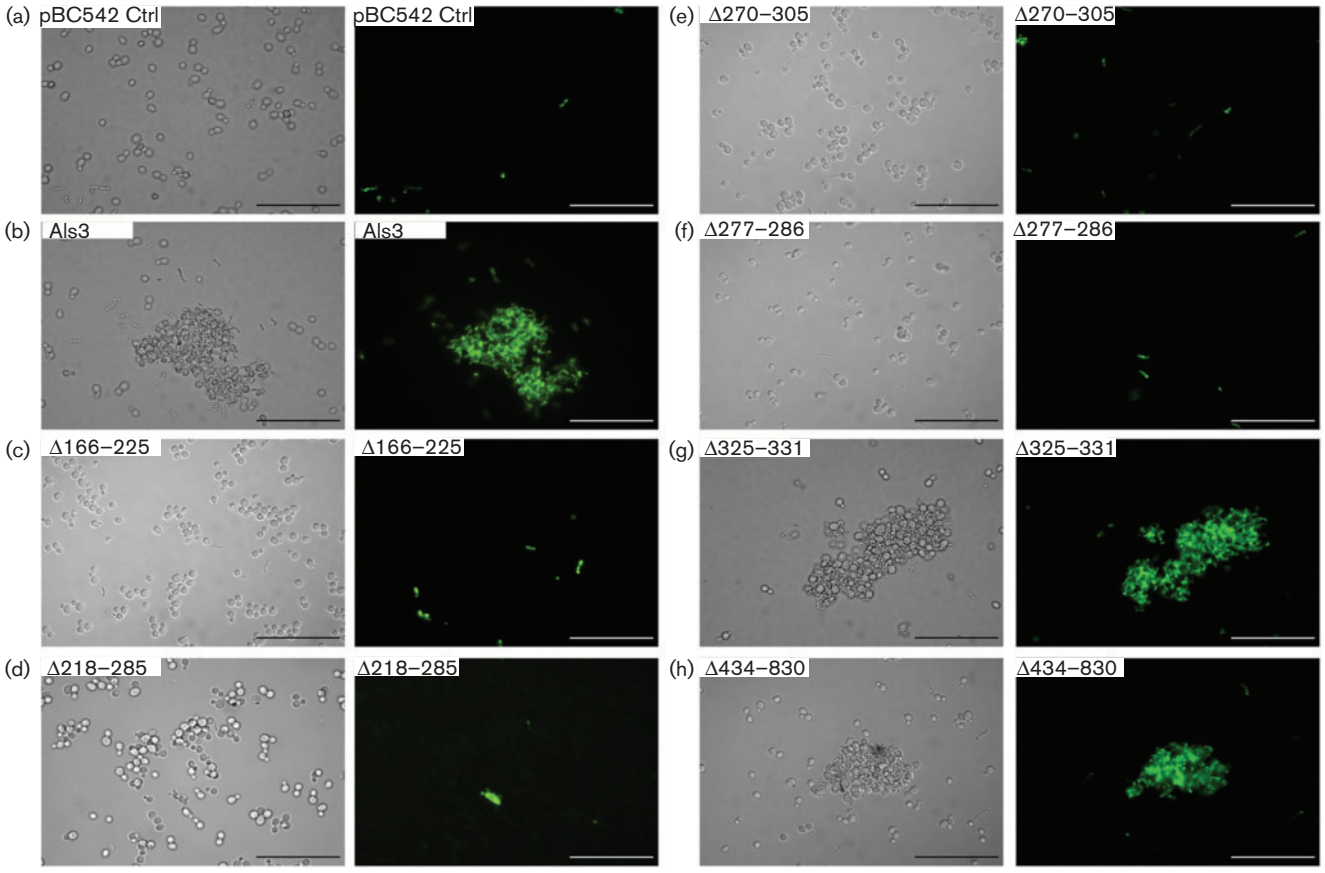

Fig. 4. Interactions of *Sacch. cerevisiae* expressing Als3 or Als3Δ polypeptides with surrogate host *L. lactis* cells expressing SspB polypeptide adhesin from *Strep. gordonii*. *Sacch. cerevisiae* cells were incubated for 3 h at 30 °C and then with FITC-labelled *L. lactis* for 1 h. Interactions were visualized by transmitted light microscopy and by fluorescence microscopy. (a–h) Light and corresponding fluorescence images of *Sacch. cerevisiae* BY4742 strains carrying pBC542 [vector-only control (Ctrl)] (a), pBC542-Als3 (b), pBC542-Als3Δ166–225 (c), pBC542-Als3Δ218–285 (d), pBC542-Als3Δ270–305 (e), pBC542-Als3Δ277–286 (f), pBC542-Als3Δ325–331 (g) and pBC542-Als3Δ434–830 (h). Bar, 50 μm.

and *L. lactis* SspB$^+$ (Fig. 4). The patterns of SspB binding by the *Sacch. cerevisiae* strains expressing the various Als3 deletions (Fig. 5) closely mirrored those of binding to *Strep. gordonii* (Fig. 3). All N-terminal region deletions, except deletion of the AFR, ablated binding to *L. lactis* SspB$^+$ cells (Figs 4a–f and 5). Deletions of the AFR or CRD had no effects on binding of SspB (Figs 4g, h and 5). These results confirmed the notion that SspB is a major determinant of *Strep. gordonii* binding to *C. albicans* hyphae expressing Als3. However, the AFR and CRD, whilst involved at least in part in the Als3–*Strep. gordonii* interaction, did not appear to play a direct role in the Als3–SspB interaction.

Biofilm formation by *Sacch. cerevisiae* strains expressing Als3 deletion constructs

Expression of Als3 by *Sacch. cerevisiae* confers upon *Sacch. cerevisiae* the ability to form biofilms (Nobbs *et al.*, 2010). To determine the effects of Als3 deletions on adherence and biofilm formation, saliva-coated glass coverslips were incubated with *Sacch. cerevisiae* strains for 24 h, washed and stained with crystal violet, and examined by microscopy or assayed for biomass (see Methods). Vector-only *Sacch. cerevisiae* was unable to form a biofilm (Fig. 6a), whilst *Sacch. cerevisiae* Als3$^+$ cells formed a dense biofilm (Fig. 6a, b). Deletions of aa 166–225, 218–285, 277–286 and 434–830 all led to significantly reduced biofilm biomass values (60–85 % reduced) (Fig. 6b), whilst deletion of aa 270–305 had less effect (40 % reduction), and deletion of the AFR (aa 325–331) resulted in reduced, but not statistically significant, biomass accumulation (Fig. 6b).

Effects of Als3 deletions on adherence to polystyrene and silicone elastomer

A role for Als3 in adherence and biofilm formation by *C. albicans* on man-made materials, including polystyrene and silicone elastomer, has also been demonstrated (Nobile *et al.*, 2008; Nobbs *et al.*, 2010). To test the effects of Als3

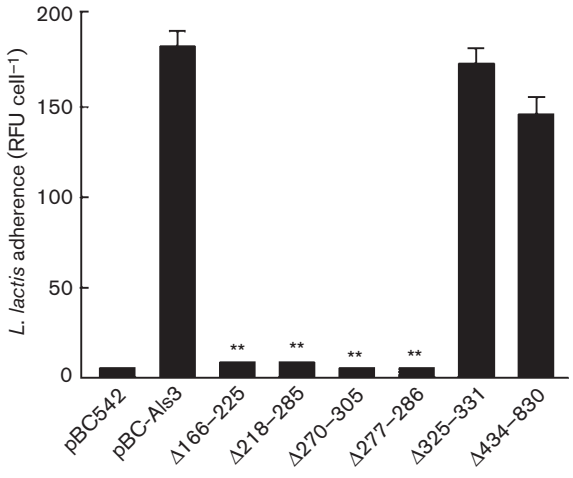

Fig. 5. Binding levels of *L. lactis* expressing *Strep. gordonii* SspB protein to *Sacch. cerevisiae* expressing Als3 or Als3Δ polypeptides. *Sacch. cerevisiae* cells were incubated for 3 h at 30 °C and then with FITC-labelled *L. lactis* for 1 h. *L. lactis* adherence is expressed as mean ± SD (n=3) RFU per *Sacch. cerevisiae* cell, as described in Methods. Statistical significance relative to pBC-Als3: **P<0.005.

deletions on adherence to polystyrene (an indirect measurement of surface hydrophobicity), *Sacch. cerevisiae* cells were incubated with microtitre plate wells for 4 h at 30 °C and adherence levels determined by crystal violet stain assay. *Sacch. cerevisiae* Als3$^+$ cells adhered strongly to polystyrene when compared with vector-only controls (Fig. 7a). Deletion of Als3 aa 166–225 or CRD (aa 434–830) partly reduced adherence biomass (Fig. 7a), whilst the aa 277–286 deletion had no effect. Intriguingly, deletions of aa 218–285, 270–305 and 325–331 (AFR) resulted in significantly higher levels of attachment to polystyrene (Fig. 7a).

Silicone elastomer is a material commonly used for indwelling medical devices. Adherence by Als3-expressing *Sacch. cerevisiae* cells compared with vector-only control was measured after 4 h at 30 °C as described above. All of the Als3 deletions, except deletion of the AFR (aa 325–331), resulted in significant reductions in ability to adhere to serum-coated silicone (Fig. 7b).

Adherence to ECM proteins

Als3 interacts with fibronectin and a range of other matrix proteins (Liu & Filler, 2011). Significant reductions in

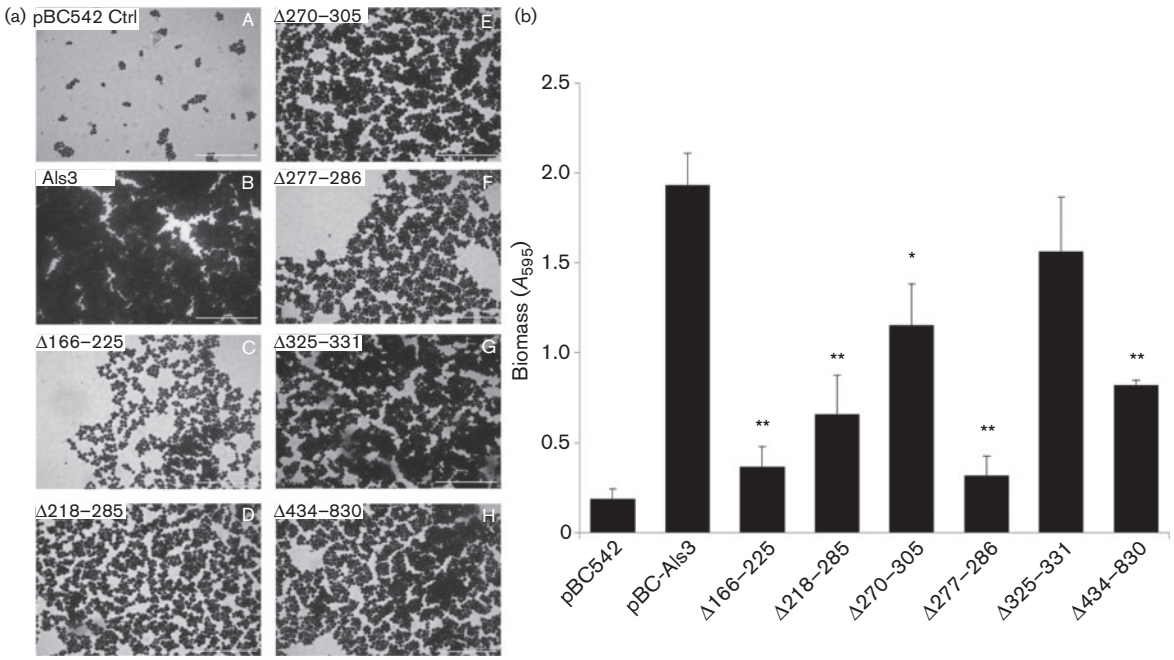

Fig. 6. Biofilm formation by *Sacch. cerevisiae* cells expressing Als3 or Als3Δ polypeptides. *Sacch. cerevisiae* cells were incubated with saliva-coated coverslips for 24 h at 30 °C. After washing and air-drying, cells were stained with crystal violet and imaged by light microscopy. (a) Crystal violet stained biofilms formed by *Sacch. cerevisiae* BY4742 strains carrying pBC542 [vector-only control (Ctrl)] (A), pBC542-Als3 (B), pBC542-Als3Δ166–225 (C), pBC542-Als3Δ218–285 (D), pBC542-Als3Δ270–305 (E), pBC542-Als3Δ277–286 (F), pBC542-Als3Δ325–331 (G) and pBC542-Als3Δ434–830 (H). Bar, 50 μm. (b) Corresponding biomass values for biofilms measured by crystal violet assay (see Methods). Values shown represent mean ± SD of three experiments performed in triplicate. Significant differences in biomass from the *Sacch. cerevisiae* Als3-expressing strain: * P<0.05, **P<0.005.

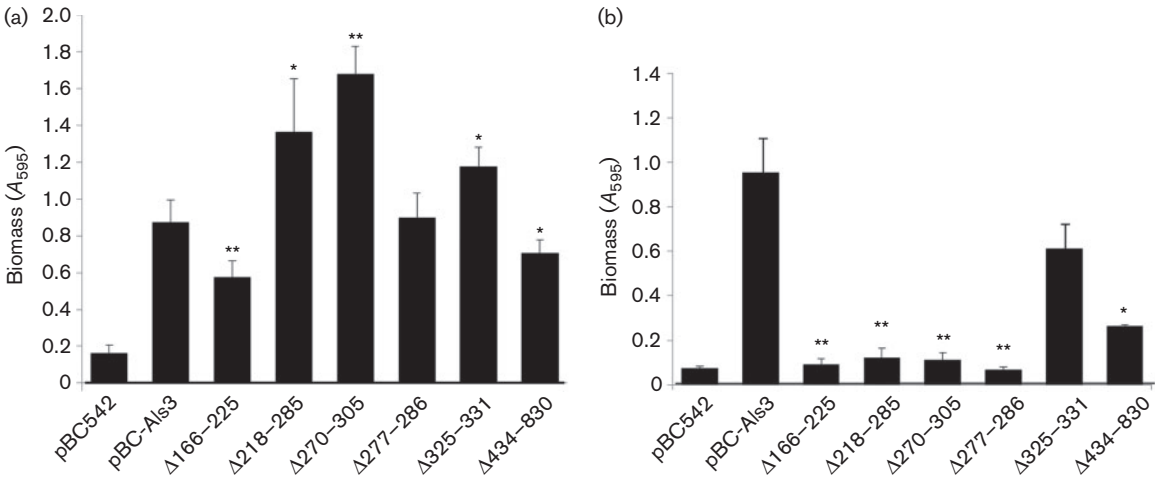

Fig. 7. Early stage biofilm formation on (a) polystyrene or (b) serum-coated silicone elastomer by *Sacch. cerevisiae* cells expressing Als3 or Als3Δ polypeptides, or carrying empty vector (pBC542). (a) *Sacch. cerevisiae* cells were incubated in polystyrene microtitre plate wells for 4 h at 30 °C. Non-adherent cells were removed and adhered biomass measured by crystal violet staining. (b) *Sacch. cerevisiae* cells were incubated with serum-coated silicone elastomer squares (1 cm^2) for 4 h at 30 °C. Non-adherent cells were removed and total biomass was measured by crystal violet assay. Values shown represent mean ± SD of three experiments performed in triplicate. Significant differences in biomass from the *Sacch. cerevisiae* pBC-Als3$^+$-expressing strain: *$P<0.05$, **$P<0.005$.

adherence and biofilm formation (biomass) on collagen type IV, fibrinogen and fibronectin were observed for all *Sacch. cerevisiae* strains expressing Als3 deletion polypeptides as compared with *Sacch. cerevisiae* Als3$^+$ (Fig. 8). In these experiments, deletion of the AFR (aa 325–331) significantly reduced the ability to form biofilms on all three matrix proteins, but the reduction was not as great as for other Als3 deletions tested.

DISCUSSION

Als3 is a key hyphal adhesin in *C. albicans*, and has been shown to mediate a wide range of interactive properties with host tissue proteins, mammalian cells and bacteria (Xu *et al.*, 2014a; Liu & Filler, 2011; Peters *et al.*, 2012). It is widely believed that the N-terminal region determines adhesive and invasive properties of the Als proteins. For example, the recombinant N-terminal region of Als3 is sufficient to mediate endocytosis by epithelial and endothelial cells (Phan *et al.*, 2007). Cadherins can activate this pathway and in this respect are thought to function as host cell receptors for Als3 (Phan *et al.*, 2005). Molecular modelling of the interactions of Als3 with E- or N-cadherin suggested that the Ig domains of Als3 interacted with the Ig domains of the cadherins. As the proposed interaction mimics E-cadherin molecules binding to each other, it has been suggested that Als3 functions as a molecular mimic of mammalian E-cadherin (Phan *et al.*, 2007).

As a result of the deletion analyses, it seemed evident that the N-terminal domain was of paramount importance in the interactions of *C. albicans* Als3 with *Strep. gordonii* and with the *Strep. gordonii* adhesin SspB. By contrast, deletion of the AFR had less effect on binding *Strep. gordonii* and no effect on binding SspB. This supports previous observations that the *β*-amyloid-disrupting compound Congo red had little effect upon Als3 interactions with *Strep. gordonii* (Nobbs *et al.*, 2010). It was noted that SspB also carried putative *β*-amyloid sequences (AFRs) as predicted by the TANGO algorithm (Silverman *et al.*, 2010). However, the data here imply a higher specificity of interaction between SspB and Als3 than aggregate formation driven by hydrophobicity and side-chains of AFRs. Deletion of the CRD (aa 434–830) had no statistically significant effect on the binding interactions of *C. albicans* with *L. lactis* SspB$^+$, but did significantly reduce association with *Strep. gordonii* cells. Thus, whilst the CRD does not seem to play a role in the Als3–SspB interaction, it may facilitate interactions with alternative streptococcal adhesins. Support for a second interactive mechanism comes from the observation that *Strep. gordonii* Δ*sspAB* is impaired but not ablated in binding surrogate host *Sacch. cerevisiae* expressing Als3 (Silverman *et al.*, 2010).

Recent work describing the crystal structure of the N-terminal region of Als3 (Lin *et al.*, 2014) has suggested the presence of a PBC that accommodates the C-terminal amino acid residues of polypeptides, thus mediating protein–protein interactions. It is proposed that differences in Als3 specificity for various substrates might be due to variations in C-terminal peptide sequences that can be accommodated in the PBC. In this model, proteolysis mediated by *C. albicans* secreted aspartyl proteases such as

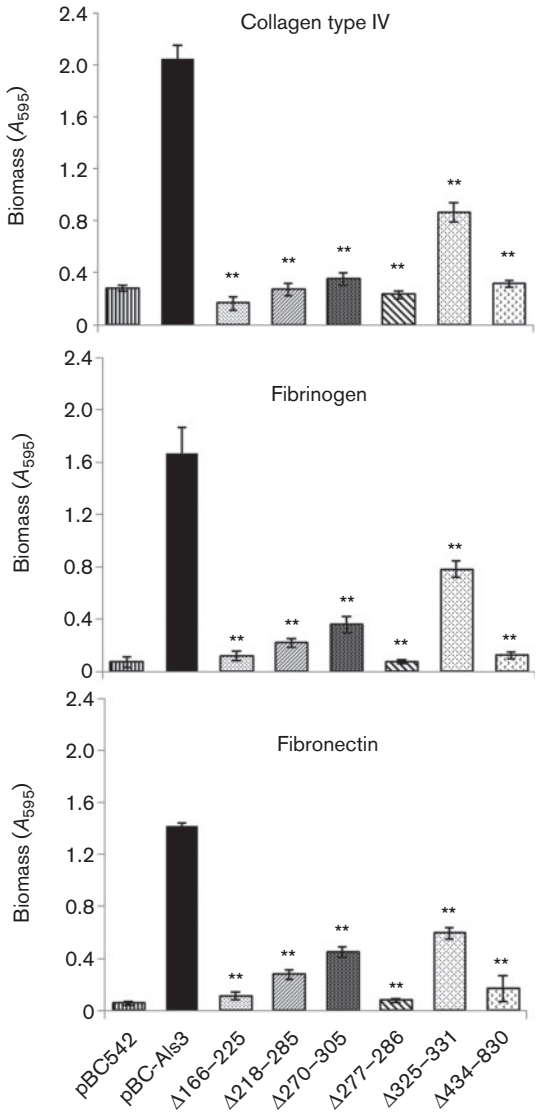

Fig. 8. Early-stage biofilm formation on ECM proteins (collagen type IV, fibrinogen or plasma fibronectin) by *Sacch. cerevisiae* cells expressing Als3 polypeptides. *Sacch. cerevisiae* cells were incubated in microwells coated with 0.5 μg ECM protein for 4 h at 30 °C. Non-adherent cells were removed and total biomass values were estimated by crystal violet staining. Values shown represent mean ± SD of three experiments performed in triplicate. Significant differences in biomass from the *Sacch. cerevisiae* pBC-Als3$^+$-expressing strain: $^{**}P<0.005$.

Sap9 would generate exposed C-termini of proteins on other *C. albicans* cells or on host tissues (Salgado *et al.*, 2011; Dutton *et al.*, 2014). However, in surrogate expression experiments, the specificity of Als3 (expressed on *Sacch. cerevisiae*) for *Strep. gordonii* SspB, as opposed to *Strep. gordonii* adhesin Hsa, was demonstrated clearly (Bamford *et al.*, 2009). These observations suggest that the specificity of Als3 for SspB resides more than just with the PBC. Moreover, the SspB adhesin is covalently anchored to bacterial cell wall peptidoglycan via the C terminus, so there would theoretically be no C-terminal peptide ligand available. It seems more likely that interaction of SspB with Als3 occurs independently of the PBC mechanism proposed for interaction of Als3 with human epithelial cells (Lin *et al.*, 2014). There is also the possibility that force-induced unfolding of the N-terminal domain, resulting from mechanical contact with cells or surfaces, exposes cryptic sequences involved in this protein–protein interaction (Alsteens *et al.*, 2010; Beaussart *et al.*, 2012; Dutton *et al.*, 2014).

Deletions within the Als3 N-terminal region, and of the CRD, had the most significant inhibitory effects on the ability of Als3 to interact with the salivary pellicle or ECM proteins. This was also seen for attachment to serum-coated silicone, probably indicating Als3 targeting of serum ECM proteins such as fibronectin in this interaction. These data correlate with studies on Als1, for which elimination of some or all of the tandem repeats was shown to considerably reduce adhesive function (Loza *et al.*, 2004). This suggests that the CRD may provide a secondary role in adhesion, either in presentation of the Als adhesive regions away from the cell surface or in folding of the N-terminal region. Interestingly, only deletion of the CRD, or of aa 166–225, impaired Als3 binding to polystyrene, whilst other N-terminal deletions (aa 218–285 or 270–305) actually promoted the interaction. This finding is in keeping with the evidence that the CRD (of Als5) has exposed hydrophobic surfaces that support binding to polystyrene substrata (Frank *et al.*, 2010). Our results also suggest that CRD disruption in Als3 may concomitantly modulate presentation of the N-terminal domain structure to result in increased exposure of hydrophobic residues, thereby compensating for the loss of CRD.

The N-terminal region of Als3 comprises two IgG-type Ig domains designated N1 and N2 (Salgado *et al.*, 2011). An invariant lysine residue (K_{59}) within N1 (Fig. S3) is proposed to interact with the C-terminal carboxylate of peptide ligands. The PBC is formed and covered by a loop from domain N1, providing broad specificity for peptide binding. Amino acid residues in the mature protein between aa 166–172 and 294–298 have side-chains that are in close proximity to ligand within the PBC (Lin *et al.*, 2014). All N-terminal deletions in Als3 spanning 166–305 residues resulted in major inhibitory effects upon *Strep. gordonii* binding, biofilm formation and adherence to human tissue proteins. These deletions would be expected to affect the overall conformation of the N-terminal region and probably disrupt formation of the PBC (Fig. S3).

The AFR in Als5 is important for efficient binding of *Sacch. cerevisiae* cells expressing Als5 to polystyrene (Garcia *et al.*, 2011). Point mutation of the AFR sequence IVIVATT to INIVATT suppressed amyloidogenic potential and significantly impaired attachment to polystyrene. We observed in the present work that removal of the β-amyloid-like motif

IVIVATT did not affect Als3 interactions with polystyrene, so Als3 may have additional hydrophobic residues that compensate for this deletion. As the AFR deletion also did not affect Als3 binding to SspB, it would appear to suggest that amyloid formation does not drive these adhesive interactions. We conclude that formation of amyloid-like structures may serve in some way to stabilize interactions of Als3 N-terminal functional domains with such substrata. Intriguingly, where deletion of AFR did impair Als3 function was in binding to ECM proteins, although these effects were weaker than those observed for disruption of the N-terminal region or CRD. There is precedent for interactions of ECM proteins such as fibrinogen or collagen with amyloids in neuropathies or cognitive conditions such as Alzheimer's (Misumi *et al.*, 2012; Muradashvili *et al.*, 2014).

CONCLUSIONS

Our findings highlight the essential function of the N-terminal domain of Als3 in mediating binding of *Strep. gordonii* and of some human tissue proteins. Heterologous expression has allowed us to focus on Als3 properties in the absence of other determinants present on the *C. albicans* hyphal filaments that might influence interactions with *Strep. gordonii*. Further mutagenesis studies of Als3 in *C. albicans* would reveal the *Strep. gordonii* binding activity of Als3 in the context of other *C. albicans* cell wall components. The AFR plays a role in these reactions and is known to act along with the CRD in the force-induced clustering of surface adhesins to form nanodomains on the *C. albicans* cell surface (Alsteens *et al.*, 2010; Chan & Lipke, 2014). Our data support the notion that the AFR could stabilize and strengthen adhesive interactions. We acknowledge that the deletion strategy that we utilized is a less precise means of determining regions of function than is, for example, site-directed mutagenesis and that deletions lead to overall conformational changes in proteins. However, our findings can now be developed to determine more accurately the residues and structure required for interactions of Als3 with SspB, and other bacterial receptors. The deletion strategy showed that the AFR and CRD both play a role in the interaction of Als3 with *Strep. gordonii* cells, but the AFR and CRD seem to be unnecessary for direct interaction of Als3 with SspB protein. Further understanding of these interactions will assist in the development of new drug or clinical strategies to manage *C. albicans* carriage and infection in association with bacteria in biofilms.

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