Unequal contribution of *ALS9* alleles to adhesion between *Candida albicans* and human vascular endothelial cells

Xiaomin Zhao, Soon-Hwan Oh and Lois L. Hoyer

Department of Pathobiology, University of Illinois, Urbana, IL 61802, USA

Correspondence

Lois L. Hoyer
lhoyer@uiuc.edu

The *Candida albicans* ALS (agglutinin-like sequence) family includes eight genes (*ALS1* to *ALS7*, and *ALS9*) that share a common general organization, consisting of a relatively conserved 5' domain, a central domain of tandemly repeated sequence units, and a 3' domain of relatively variable length and sequence. To test the hypothesis that the cell-surface glycoproteins encoded by the ALS genes mediate contact between the fungal cell and host surfaces, a set of *C. albicans* mutant strains was systematically constructed, each lacking one of the ALS sequences. Phenotypes of the mutant strains were evaluated, primarily using adhesion assays. *ALS9* is unique within the ALS family due to extensive allelic sequence variation within the 5' domain that may result in functional differences between proteins encoded by *ALS9-1* and *ALS9-2*. Deletion of *ALS9* significantly reduces *C. albicans* adhesion to human vascular endothelial cell monolayers. The mutation was complemented by reintegration of a wild-type copy of *ALS9-2*, but not *ALS9-1*, suggesting allelic functional differences. Complementation of the mutation with a gene fusion between the 5' domain of *ALS9-2* and the tandem repeats and 3' domain of *ALS9-1* also restored wild-type adhesion levels. Analysis of the *als9Δ/als9Δ* mutant phenotype in other assays demonstrated no significant difference from a control strain for adhesion to buccal epithelial cells or laminin-coated plastic plates. The *als9Δ/als9Δ* mutant did not show significant differences from the control for adhesion to or destruction of cells in the reconstituted human epithelium (RHE) disease model, or for cell-wall defects, germ-tube formation or biofilm formation in a catheter model. Analysis of *ALS9* allelic frequency in a collection of geographically diverse clinical isolates showed a distinct preference for *ALS9-2* allelic sequences, within both the 5' and the 3' domain of the *ALS9* coding region. These data suggest greater selective pressure to maintain the *ALS9-2* allele in *C. albicans* isolates and imply its greater relative importance in host–pathogen interactions.

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INTRODUCTION

Shortly after its identification, the first *Candida albicans* agglutinin-like sequence (ALS) gene, *ALS1*, was found to belong to a larger family of eight related genes (Hoyer *et al.*, 1995, 1998; Hoyer, 2001). Because of sequence similarities between Als1p and the *Saccharomyces cerevisiae* cell-surface adhesive glycoprotein α -agglutinin, it was hypothesized that the Als proteins are adhesins (Hoyer *et al.*, 1995). Isolation of *ALS1* and *ALS5* based on their ability to confer adhesive function on the relatively non-adherent *S. cerevisiae* supported this hypothesis (Gaur & Klotz, 1997; Fu *et al.*, 1998). We have taken a systematic approach to investigating Als protein function that involves generating

C. albicans mutant strains, each lacking the coding region for a single ALS gene. Consistent with our initial hypothesis, phenotypic testing of these *C. albicans* mutants has focused on adhesion assays and measured the contributions of Als1p, Als3p (Zhao *et al.*, 2004), Als2p and Als4p (Zhao *et al.*, 2005) to interactions between *C. albicans* and host surfaces.

Completion of the eight ALS gene sequences highlighted common features within the family (Hoyer, 2001). In the centre of each coding region is a domain composed entirely of tandemly repeated copies of a highly conserved 108 bp unit. The number of 108 bp copies present varies considerably between alleles. Flanking this central domain are a 5' domain of approximately 1.3 kb that is 55–90 % conserved across the ALS family and a 3' domain of variable length and sequence. The tandem repeat region

Abbreviations: ALS, agglutinin-like sequence; RHE, reconstituted human epithelium; VB1, variable block 1; VB2, variable block 2.

Fig. 1. Diagram of (a) *ALS9-1*, (b) *ALS9-2* and (c) the *ALS9-2/ALS9-1* fusion. The locations of the ALS domains are shown below *ALS9-1*. The *ALS9-2/ALS9-1* fusion encodes amino acids 1–420 from Als9-2p fused to amino acids 421–1854 from Als9-1p. Capital letters show the approximate location of PCR primers used for allelic genotyping reactions. Primers E and F amplify a 3' domain region (speckled) that contains VB1. Primers G and H amplify a region (basketweave) that contains VB2. Solid shading in each region denotes the extra nucleotides comprised by VB1 and VB2.

and 3' domain encode serine/threonine-rich amino acid sequences that are heavily glycosylated in the mature Als protein (Kapteyn *et al.*, 2000). These sequence features are consistent with a model of Als protein structure that includes a relatively non-glycosylated N-terminal domain that is displayed on the *C. albicans* cell surface by the remainder of the mature protein, which forms an extended stalk, due to its heavy glycosylation. For Als proteins that display adhesive activity in *C. albicans* and/or *S. cerevisiae*, data support the conclusion that most adhesive activity resides within the N-terminal domain (Loza *et al.*, 2004; Zhao *et al.*, 2006). However, recent work in *S. cerevisiae* suggests that the tandem repeat and C-terminal domain of Als5p may have more than a structural role (Rauceo *et al.*, 2006).

The focus of this work is *C. albicans* *ALS9*, which is localized on chromosome 6, immediately downstream of the coding regions for *ALS5* and *ALS1* (Zhao *et al.*, 2003). Analysis of ALS gene expression has shown that *ALS9* is transcribed under a wide variety of *in vitro* growth conditions, and in both human clinical specimens and animal disease models (Cheng *et al.*, 2005; Green *et al.*, 2004, 2005a, b, 2006). Under the *in vitro* conditions assayed by real-time RT-PCR, however, maximal *ALS9* transcript copy number is approximately 10- to 1000-fold less than the maximal copy number observed for other ALS genes such as *ALS1*, *ALS2* or *ALS3* (Green *et al.*, 2005b). Perhaps the most notable feature of *ALS9* is its allelic variability, which is defined based on the sequences of the two *ALS9* alleles from *C. albicans* strain SC5314 (GenBank accession nos for *ALS9-1* and *ALS9-2* are AY269423 and AY269422, respectively). In general, the ALS family is known for its large degree of allelic variability, since each ALS gene exhibits this feature within the central tandem repeat domain (Hoyer, 2001). Certain ALS genes also display allelic sequence variability within the 5' domain (*ALS5*; Hoyer & Hecht, 2001) or 3' domain (*ALS7*; Zhang *et al.*, 2003); however, *ALS9* is the only gene with extensive sequence variability within each coding region domain (Zhao *et al.*, 2003). The 5' domain sequences of *ALS9-1* and *ALS9-2* from strain SC5314 vary by 11% at the

nucleotide level (16% at the amino acid level). Within the 3' domain, allele *ALS9-2* encodes two sequence blocks, called variable block 1 (VB1) and variable block 2 (VB2), which are absent from the 3' domain of allele *ALS9-1* (Zhao *et al.*, 2003). In this work, we test the two *ALS9* alleles in adhesion assays and demonstrate functional differences between them. Here, we associate adhesive function with Als9-2p and demonstrate that the *ALS9-2* allele is more prevalent in *C. albicans* clinical isolates.

METHODS

Construction of a *C. albicans* *als9Δ/als9Δ* strain. *ALS9* coding regions were deleted using the PCR product-directed method of Wilson *et al.* (2000). General methods for creating *alsΔ/alsΔ* strains have been described by Zhao *et al.* (2004). Details specific to the *ALS9* studies are presented here. A diagram of the *ALS9* alleles from strain SC5314 is shown in Fig. 1. An *ALS9* disruption cassette was amplified from the plasmid pDDB57 template (a generous gift from Aaron Mitchell, Columbia University) with primers *ALS9-5DR* and *ALS9-3DR* (Table 1). The disruption cassette was transformed into *C. albicans* strain CAI4 (Table 2). Transformants were selected as described previously (Zhao *et al.*, 2004) and screened by Southern blotting. The Southern blot probe, which hybridizes to nucleotides –473 to –55 upstream of *ALS9*, was PCR-amplified from SC5314 genomic DNA (Table 2) with primers *ALS9upF* and *ALS9upR* (Table 1). Correct transformants were screened by PCR using primer pair *VB2F* and *VB2R* (Table 1) to confirm Southern blot results and determine which *ALS9* allele was deleted. Strain 1976, from which the *ALS9-2* allele was deleted, was grown on medium containing 5-fluoroorotic acid (5-FOA; Boeke *et al.*, 1984) to select a *Uri[–]* strain (Wilson *et al.*, 2000). Strain 1978 (Table 2) was identified by Southern blotting and used for deletion of the *ALS9-2* allele with the PCR-generated disruption cassette, as described above. Southern blotting indicated that both *ALS9* alleles were deleted in strain 2028. The deletion region extended from 15 bp upstream of the *ALS9* coding region to 29 bp downstream of the *ALS9* stop codon. A *Uri[–]* version of strain 2028, named 2030 (Table 2), was used for subsequent transformation steps.

Real-time RT-PCR analysis was used to assess transcriptional levels for each *ALS9* allele in the strains constructed for this study. Methods for the assay and data analysis have been described previously (Green *et al.*, 2005b). Primers *QRT9-1F* and *QRT9-1R* detected *ALS9-1* transcript specifically, while primers *QRT9-2F* and *QRT9-2R* were

Table 1. Oligonucleotide primers used in this study

Primer name	Forward/ reverse	Label in Fig. 1*	Primer sequence (5'–3')
ALS9-5DR	F		CTGTTGACATTGATAAAAAAATATATAAAAGGCATCTATTTCCATGCA- GAGGGACGTGTGTTTTCCAGTCACGACGTT
ALS9-3DR	R		AACTACAAAAAGAAAACCGAAAAAGACAAAATATAATAACATCACGAAG- AAATAATAAAAGTGTGGAATTGTGAGCGGATA
ALS9upF	F		TTTTTCTTTTCCTTGTTTTCC
ALS9upR	R		TTGAAATTCCTGGTGATTAAG
VB2F	F	G	CATCATTTGTGTCTACAACCTGCTG
VB2R	R	H	GAACCCCTTGTCTGAATATGG
QRT9-1F	F		CATTTTACTCATCAGAAGCACAGTCG
QRT9-1R	R		AGGTATTACTCGAATCAATTTCTAAACTCG
QRT9-2F	F		AATATTTCACTCGTCAGAACCACATTAT
QRT9-2R	R		AATCACTGGAATCAAAGTCTGAGCTAT
ALS9dnF2	F		ACCTTCTATTTAGAGATAGTGA
ALS9dnR	R		CTCTCAAATGACGCGACTCATG
ALS9FullF	F		CCCCCTAGGGGAAAAAATGAGAGAAAAAAGGAA
ALS9R	R		CCCCTCGAGTCACTATCTCTAAATAGAAGG
ALS9URCF	F		CCCACTAGTATAGGAATTGATTTGGATGG
ALS9dnRCR2	R		CCCAAGCTTCTCTCAAATGACGCGACTCATG
ALS9F2	F		CCCCCTAGGGGAAAAAATGAGAGAAAAAAGGAA
1499-PinAIR	R		CCCCTCGAGTTTACCGTGGGATTAGTATAAGTAGTGG
2096-PinAIFm	F		CCCACCGGTTCTGTAGATACTGTTATTGTGCGAAC
ALS9-XhoIR	R		CCCCTCGAGTTAAATAAACAAAAATAATATTGTGACCA
ALS9-1F	F	A	CAACTCATTAACTTGGGCCAATG
ALS9-1R	R	B	GAAGTTCGACAATAACAGTATCTACA
ALS9-2F	F	C	TGACTCATTGACATGGACTAGAT
ALS9-2R	R	D	GAATTTGCACAATAACAGTGTCTATG
VB1F	F	E	CGCTATGTCAACACCACAATCTCC
VB1R	R	F	TGGAACGTAGGAACAAACATGACC

*The location for some primers within the *ALS9* coding region is shown in Fig. 1, in which letter labels are used instead of primer names.

Table 2. *C. albicans* strains used in this study

Strain	Parent	Genotype	Doubling time (h)	Source/reference
SC5314		Wild-type clinical isolate		Gillum <i>et al.</i> (1984)
CAI4	SC5314	<i>iro1-ura3Δ</i> : : <i>limm</i> ⁴³⁴ / <i>iro1-ura3Δ</i> : : <i>limm</i> ⁴³⁴		Fonzi & Irwin (1993)
CAI12	CAI4	<i>iro1-ura3Δ</i> : : <i>limm</i> ⁴³⁴ / <i>iro1-ura3Δ</i> : : <i>limm</i> ⁴³⁴ / <i>IRO1 URA3</i>	1.59 ± 0.04	Porta <i>et al.</i> (1999)
1976	CAI4	<i>iro1-ura3Δ</i> : : <i>limm</i> ⁴³⁴ / <i>iro1-ura3Δ</i> : : <i>limm</i> ⁴³⁴ <i>ALS9-1/</i> <i>als9-2Δ-URA3</i>		Zhao <i>et al.</i> (2003)
1978	1976	<i>iro1-ura3Δ</i> : : <i>limm</i> ⁴³⁴ / <i>iro1-ura3Δ</i> : : <i>limm</i> ⁴³⁴ <i>ALS9-1/</i> <i>als9-2Δ-ura3</i>		This study
2028	1978	<i>iro1-ura3Δ</i> : : <i>limm</i> ⁴³⁴ / <i>iro1-ura3Δ</i> : : <i>limm</i> ⁴³⁴ <i>als9-1Δ/</i> <i>als9-2Δ-URA3</i>	1.55 ± 0.04	This study
2030	2028	<i>iro1-ura3Δ</i> : : <i>limm</i> ⁴³⁴ / <i>iro1-ura3Δ</i> : : <i>limm</i> ⁴³⁴ <i>als9-1Δ/</i> <i>als9-2Δ-ura3</i>		This study
2740	2030	<i>iro1-ura3Δ</i> : : <i>limm</i> ⁴³⁴ / <i>iro1-ura3Δ</i> : : <i>limm</i> ⁴³⁴ <i>als9-1Δ/</i> <i>als9-2</i> : : <i>ALS9-1-URA3</i>	1.60 ± 0.04	This study
2064	2030	<i>iro1-ura3Δ</i> : : <i>limm</i> ⁴³⁴ / <i>iro1-ura3Δ</i> : : <i>limm</i> ⁴³⁴ <i>als9-1Δ/</i> <i>als9-2</i> : : <i>ALS9-2-URA3</i>	1.61 ± 0.04	This study
2767	2030	<i>iro1-ura3Δ</i> : : <i>limm</i> ⁴³⁴ / <i>iro1-ura3Δ</i> : : <i>limm</i> ⁴³⁴ <i>als9-1Δ/</i> <i>als9-2</i> : : <i>ALS9-1-ALS9-2</i> : : <i>URA3</i>	1.58 ± 0.04	This study

specific for *ALS9-2* (Table 1). In strain CAI12, which is wild-type for *ALS9*, transcription of *ALS9-1* was 1.1 ± 0.2 -fold of *ALS9-2* transcript levels, suggesting that the alleles were transcribed equally. In strain 2028, transcription of *ALS9-1* was below the detection limit of this assay, while transcription of *ALS9-2* was 0.005 ± 0.003 -fold relative to strain CAI12. These results are consistent with the deletion of both *ALS9* alleles in strain 2028.

Reintegration of wild-type *ALS9* alleles into the *als9 Δ /als9 Δ* strain. Because the large sequence differences between *ALS9-1* and *ALS9-2* might reflect functional differences in the encoded proteins, each *ALS9* allele was reintegrated separately into the *als9 Δ /als9 Δ* strain. To construct the *ALS9-2* reintegration cassette, primers *ALS9dnF2* and *ALS9dnR* (Table 1) were used to amplify 710 bp of sequence downstream of *ALS9*. This fragment was digested with *Sst*II/*Ngo*MIV and cloned into plasmid pUL (Zhao *et al.*, 2004) to yield plasmid 1320. Full-length *ALS9-2* with 602 bp of upstream sequence was amplified from SC5314 with primers *ALS9FullF* and *ALS9R* (Table 1) and cloned into pCRBlunt (Invitrogen) to yield plasmid 1499. Primers *ALS9URCF* and *ALS9dnRCR2* (Table 1) were used to amplify the *URA3* gene and *ALS9* downstream sequence cassette from plasmid 1320. This product was digested with *Spe*I/*Hind*III and cloned into plasmid 1499 downstream of *ALS9-2* to yield plasmid 1541. The *ALS9-1* reintegration cassette was produced by amplifying full-length *ALS9-1* with 475 bp of upstream sequence (primers *ALS9F2* and *ALS9R*; Table 1) from strain 1976, which has an *ALS9-2* deletion. The PCR product was digested with *Avr*II/*Xho*I and cloned into plasmid 1320 (Zhao *et al.*, 2004) to yield plasmid 2729. Digestion with *Avr*II/*Ngo*MIV released the *ALS9-1* cassette from plasmid 2729; *Hind*III/*Xho*I released the *ALS9-2* cassette from plasmid 1541. These fragments were transformed individually into strain 2030 to yield strain 2740 (*ALS9-1* reintegrated) and strain 2064 (*ALS9-2* reintegrated). Correct transformants were confirmed by Southern blotting. DNA sequencing of both the transformation cassette and PCR-amplified sequences from the correct transformants verified the integrity of the *ALS9* alleles in these strains. PCR and DNA sequencing were used to confirm integration of *ALS9-1* under control of its own promoter in strain 2740 and integration of *ALS9-2* under control of its own promoter in strain 2064. Real-time RT-PCR (Green *et al.*, 2005b) was used to demonstrate that transcription levels from the reintegrated genes were similar to those in the CAI12 control strain. Transcription of *ALS9-1* in strain 2740 was 1.8 ± 0.2 -fold of CAI12 levels, while transcription of *ALS9-2* in strain 2740 was 0.001 ± 0.001 -fold compared to that of CAI12. Transcription of *ALS9-1* in strain 2064 was 0.005 ± 0.004 -fold of CAI12 levels, while transcription of *ALS9-2* was 1.1 ± 0.3 -fold relative to CAI12.

Construction of a *C. albicans* strain that produces a cell-surface Als9-2p/Als9-1p fusion protein. Approximately 500 bp of *ALS9-2* upstream sequence and the 5'-most 1266 bp of the *ALS9-2* coding region were amplified from plasmid 1499 using primers *ALS9F2* and *1499-PinAIR* (Table 1). Primer *1499-PinAIR* includes a silent mutation (T to C at nt 1260 of the *ALS9-2* coding region) that creates a *PinAI* site. The PCR product was digested with *Avr*II/*Xho*I and cloned into plasmid 2729 to replace the *ALS9-1* sequence. The resulting construct was called plasmid 2760. The tandem repeat and 3' domains of *ALS9-1* were amplified from plasmid 2096 using primers *2096-PinAIFm* and *ALS9-XhoIR* (Table 1). The same silent mutation described above was included in the *2096-PinAIFm* primer to create a *PinAI* site in the PCR product. The PCR product was digested with *PinAI*/*Xho*I and cloned into plasmid 2760 to yield the *ALS9-2/ALS9-1* fusion plasmid 2765. This construct encodes a chimeric Als9 protein that includes amino acids 1–420 of Als9-2p followed by amino acids 421–1854 of Als9-1p. The fusion construct was verified by DNA sequencing. Digestion with *Avr*II/*Ngo*MIV released the fragment that included the fused genes and the *URA3* selectable marker, flanked by *ALS9* upstream and downstream

sequences to direct its integration into strain 2030 (Table 2). Southern blotting and DNA sequencing identified the correct transformant (strain 2767; Table 2). PCR demonstrated that the fusion construct was integrated under control of the *ALS9-2* promoter. The real-time RT-PCR primers for *ALS9-1* were used to measure transcription of the fused allele relative to *ALS9-1* transcription in strain CAI12. Transcript levels for the fused allele were 1.8 ± 0.5 -fold of CAI12 levels, indicating similar transcriptional activity between the strains.

Germ-tube formation assay. The germ-tube assay format was changed from the one used in our previous work in order to require fewer input cells and to include multiple time points. Strains for testing were streaked from -80°C glycerol stocks to YPD plates (1 % yeast extract, 2 % peptone, 2 % glucose with 2 % agar for plates), incubated for 24 h at 37°C and stored at 4°C for no more than 1 week. A single *C. albicans* colony was inoculated into 10 ml YPD and incubated for 16 h at 37°C with 200 r.p.m. shaking. Cells were washed in Dulbecco's PBS without calcium or magnesium (DPBS; Cambrex catalogue no. 17-512Q) and counted, and 5×10^5 cells were added to 3 ml of either RPMI 1640 with L-glutamine (Invitrogen catalogue no. 11875-085) or Lee medium (Lee *et al.*, 1975) pre-warmed to 37°C in a 25 ml Erlenmeyer flask. The culture flasks were incubated at 37°C with 200 r.p.m. shaking for 20, 40 or 60 min. The entire volume of the flask was collected by vacuum filtration over a $1\text{ }\mu\text{m}$ pore-size polycarbonate filter (Fisher Scientific). Filters were washed with 5 ml DPBS to remove residual medium. Filters were removed from the vacuum filtration unit, inverted onto glass slides and dried. Following removal of the filter from the slide, slides were heat-fixed, stained with crystal violet for 2 min, washed with tap water and dried. *C. albicans* yeast cells with a germ tube longer than one diameter of the mother cell were considered positive. The percentage of cells forming germ tubes was calculated by visually counting all cells present on five randomly selected fields on the slide. Approximately 200 cells were counted from each slide. Duplicate assays for each condition were conducted on two different days. Statistical significance was assessed using PROC MIXED in SAS (SAS Institute) with $P \leq 0.05$ considered significant.

Testing for cell-wall defects. *C. albicans* strains were tested for defects in the *C. albicans* cell wall by growing them on YPD agar plates containing one of the following compounds: NaCl (1, 1.5 M), SDS (0.02, 0.1 %), Calcofluor White (Sigma; 20, 50 $\mu\text{g ml}^{-1}$), Congo red (Sigma; 100, 250 $\mu\text{g ml}^{-1}$) or Hygromycin B (Invitrogen; 75, 200 $\mu\text{g ml}^{-1}$). *C. albicans* strains were grown for 16 h at 37°C and 200 r.p.m. shaking, as described above. Cells were harvested, washed in DPBS, counted and resuspended at 1×10^8 cells ml^{-1} . Tenfold serial dilutions were prepared and 5 μl of each spotted onto the YPD agar plates with the appropriate test substance included. Plates were incubated for 24 h and growth of the test strains was compared to growth of a CAI12 positive control.

Other phenotypic assays. Key strains used in this analysis were evaluated to ensure that their growth rate was not statistically different from that of the CAI12 control strain (Table 2), and that the degree of cellular aggregation observed did not increase compared to that of CAI12. Zhao *et al.* (2004) have described methods for these analyses. Formation and analysis of biofilm on a silicone elastomer catheter substrate followed the method of Kuhn *et al.* (2002). Methods for measuring adhesion to vascular endothelial cells, adhesion to buccal epithelial cells and relative pathogenicity in the reconstituted human epithelium (RHE) tissue model have been published (Zhao *et al.*, 2004). Adhesion and cellular aggregation assays used *C. albicans* yeast forms inoculated from a YPD stock plate prepared as described above. A single colony was resuspended in 1 ml YPD; 10 μl of the suspension was inoculated into a 10 ml culture, which was incubated for 16 h at 37°C and 200 r.p.m. shaking. Cells

Fig. 2. Adhesion assay data. Histograms showing the adherence of *C. albicans* control (CAI12), *als9Δ/als9Δ* (2028), *als9Δ/als9Δ* :: *ALS9-1* (2740), *als9Δ/als9Δ* :: *ALS9-2* (2064) and *als9Δ/als9Δ* :: *ALS9-1/ALS9-2* fusion (2767) strains to (a) human umbilical vein endothelial cell (HUVEC) monolayers, (b) buccal epithelial cells (BEC) and (c) laminin-coated plastic plates. Adhesion assays used *C. albicans* yeast forms that were grown for 16 h in YPD at 37 °C as described in Methods. Asterisks indicate significant differences from wild-type adhesion ($P \leq 0.05$).

were washed in DPBS prior to the assay. Methods for real-time RT-PCR analysis of ALS gene expression have been published (Green *et al.*, 2005b).

Adhesion to laminin was evaluated in a six-well plate format using modifications of the fibronectin adhesion assay described by Zhao *et al.* (2004). Briefly, laminin (Sigma catalogue no. L-2020) was diluted to $10 \mu\text{g ml}^{-1}$ in DPBS and 1 ml added to each tissue-culture well. Plates were incubated overnight at 37 °C. *C. albicans* yeast forms were cultured in YPD as described above, washed in DPBS and resuspended at a density of 500 cells ml^{-1} . Laminin solution was removed immediately before the assay and each well rinsed twice with 2 ml DPBS. One millilitre of the yeast suspension was added to each coated well and plates were incubated at 37 °C for 30 min. Washing and counting of adherent colonies, calculation of percentage adherence, experimental replication and statistical analysis were the same as described for the fibronectin adhesion assay (Zhao *et al.*, 2004).

Collection of clinical *C. albicans* isolates and PCR genotyping reactions. The collection of 196 isolates used in this study was obtained from three previously analysed populations (Pujol *et al.*, 1997, 2002; Blignaut *et al.*, 2002). The collection included 100 bloodstream isolates, 81 oral isolates, 10 vaginal isolates and five from other body sites. PCR genotyping was used to determine which *ALS9* domains were present in each strain. Primers ALS9-1F and ALS9-1R were used to detect the *ALS9-1* 5' domain, while primers ALS9-2F and ALS9-2R were used to detect the *ALS9-2* 5' domain (Table 1, Fig. 1). The size of VB1 was determined using primers VB1F and VB1R (Table 1, Fig. 1). A smaller product is characteristic of the *ALS9-1* prototype allele as defined in strain SC5314 (GenBank accession no. AY269423), while a larger product is associated with the *ALS9-2* allele (GenBank accession no. AY269422; Zhao *et al.*, 2003). The presence of both PCR product sizes suggests that both allelic sequences are present, but does not associate a specific VB1 sequence with a particular 5' domain. Similarly, the size of VB2 was assessed using primers VB2F and VB2R with a smaller product amplified from the *ALS9-1* allele and a larger product from the *ALS9-2* allele (Fig. 1). Amplification of both product sizes was observed for heterozygous strains. PCR products were separated on agarose gels using a SC5314 standard to judge relative fragment sizes and assign allelic designations.

RESULTS

Effect of *ALS9* deletion on *C. albicans* adhesion

Our main goal in phenotypic analysis of the *als9Δ/als9Δ* mutant (strain 2028) was to assess its adhesion to cell types relevant to *C. albicans* host–pathogen interactions. These assays used *C. albicans* yeast forms that were grown at

37 °C in YPD medium for 16 h, as described in Methods. *ALS9* is transcribed in *C. albicans* cells grown in this manner (Green *et al.*, 2005b), suggesting that the growth condition is appropriate for assessing Als9p function. The *als9Δ/als9Δ* strain was significantly less adherent to human vascular endothelial cell monolayers than the control strain CAI12 ($P=0.05$; Fig. 2a). Although reintegration of a wild-type *ALS9-1* allele did not complement the mutant phenotype ($P=0.02$), transformation of *ALS9-2* into the mutant strain restored wild-type adhesion levels (Fig. 2a). These results suggest functional differences between the proteins encoded by the *ALS9* alleles. One possible explanation for this result is that the reintegrated *ALS9-1* allele had defects and did not produce a viable protein. Since antibodies specific for Als9p are not available, other verification steps were pursued. DNA sequencing of *ALS9-1* that was PCR-amplified from the reintegrant strain verified that its sequence was identical to that derived from the parental strain. Further, the adhesion phenotype of strain 1976 was tested. Strain 1976 is hemizygous (*ALS9-1/als9-2*) and was derived during construction of the *als9Δ/als9Δ* mutant (Table 1). Compared to the percentage adherence for the CAI12 control (15.8 ± 1.6), the percentage adherence for the hemizygous strain 1976 (9.7 ± 1.6) was similar to those of the mutant strain 2028 (9.1 ± 1.6) and the *ALS9-1* reintegrant strain 2740 (9.9 ± 1.6). The percentage adherence for strains 1976, 2028 and 2740 was significantly lower than that of the CAI12 control ($P \leq 0.006$). These results are consistent with the conclusion that there is an unequal contribution of these *ALS9* alleles to adhesion between *C. albicans* and human vascular endothelial cells.

A fusion construct between the N-terminal domain of Als9-2p (amino acids 1–420) and the tandem repeats and C-terminal domain of Als9-1p (amino acids 421–1854) was constructed in *C. albicans als9Δ/als9Δ* strain 2028 (Fig. 1). The resulting strain, called 2767, had wild-type adhesion levels, suggesting that the fusion protein restored the missing Als9p function. This result suggests that adhesive function for Als9-2p largely resides within the N-terminal domain.

The *als9Δ/als9Δ* mutant was also tested for adhesion to buccal epithelial cells (Fig. 2b) and laminin-coated plastic plates (Fig. 2c). Testing for adhesion to laminin was

Fig. 3. Light micrographs of buccal RHE inoculated with either (a) the *C. albicans* control (CAI12) or (b) the *als9Δ/als9Δ* mutant strain (2028) and grown for 8 h at 37 °C. The similar appearance of RHE following incubation with either strain suggested that Als9p does not contribute to epithelial damage in this experimental model.

prompted by a report that *S. cerevisiae* becomes adherent to laminin when overexpressing *ALS9* (Sheppard *et al.*, 2004). In each assay, deletion of *ALS9* did not have a significant effect on the phenotype of the *C. albicans* strain, suggesting that although Als9-2p may play a measurable role in adhesion to endothelial cells, the Als9 proteins are not important for buccal cell or laminin adhesion.

Other phenotypic analyses of the *C. albicans als9Δ/als9Δ* strain

The *C. albicans als9Δ/als9Δ* strain 2028 was tested in other assays to assess its phenotype. Inoculation of the buccal RHE model with the *als9Δ/als9Δ* mutant and a control strain showed no difference between the strains with respect to epithelial destruction, adherence or hypha formation (Fig. 3). Comparison between the *als9Δ/als9Δ* strain and the CAI12 control strain showed no differences in growth on YPD agar that contained cell-wall-stressing agents, including NaCl, SDS, Calcofluor White, Congo red or Hygromycin B. The lack of an effect of these agents on growth of the *als9Δ/als9Δ* mutant suggests that cell-wall structure and glycosylation are not affected by *ALS9* deletion (Elorza *et al.*, 1983; Kopecka & Gabriel, 1992; Dean, 1995; Machi *et al.*, 2004). The *als9Δ/als9Δ* mutant strain also did not exhibit any significant differences in germ-tube formation over the course of 1 h when grown in RPMI 1640 or Lee medium (Table 3). Finally, although growth of the *als9Δ/als9Δ* strain in a model of biofilm

formation on a silicone elastomer catheter surface (Kuhn *et al.*, 2002) resulted in decreased biomass compared to the CAI12 control strain (1.21 ± 0.16 versus 1.4 ± 0.16 mg, respectively), this difference was not significant statistically ($P=0.06$). Compensatory changes in expression of other ALS genes in the *als9Δ/als9Δ* strain were ruled out using real-time RT-PCR analysis of ALS gene expression (Green *et al.*, 2005b).

Genotyping of clinical isolates

Endothelial cell adhesion assays showed a clear difference in function between Als9-1p and Als9-2p (Fig. 2). In a previous study, PCR genotyping of the *ALS9* alleles in 30 *C. albicans* clinical isolates showed that, in each strain, at least one allele had *ALS9-2* sequences in the 5' domain (Zhao *et al.*, 2003). This analysis was expanded to 196 *C. albicans* clinical isolates from various body sites (Table 4). PCR primers were used that could distinguish between the *ALS9-1* and *ALS9-2* sequences within the 5' domain and also within the VB1 and VB2 regions of the 3' domain (Table 1, Fig. 1). Within the 5' domain, approximately half of the isolates were positive for *ALS9-1*, while all isolates had at least one *ALS9-2* allele (Table 4). Therefore, among the *C. albicans* isolates tested, approximately half were heterozygous (*ALS9-1/ALS9-2*), while the others appeared to be *ALS9-2* homozygotes, although it is formally possible for these strains to encode other *ALS9* alleles. Within VB1 of the 3' domain, *ALS9-2* sequences were common, while

Table 3. Percentage of *C. albicans* cells forming germ tubes over time in different growth media

Duplicate assays were conducted on two different days. Means $\pm$ SEM are reported. There were no significant differences between the CAI12 control strain and any of the other strains in percentage germ-tube formation at each time point ($P>0.05$ for each comparison).

Growth medium	Time (min)	Strain				
		CAI12	2028	2064	2740	2767
RPMI 1640	20	8.7 ± 0.5	8.1 ± 0.5	8.3 ± 0.5	9.1 ± 0.5	9.0 ± 0.5
	40	51.3 ± 1.1	51.1 ± 1.1	50.9 ± 1.1	49.7 ± 1.1	50.3 ± 1.1
	60	90.6 ± 0.4	90.1 ± 0.4	90.2 ± 0.4	90.0 ± 0.4	89.5 ± 0.4
Lee	20	7.4 ± 0.3	6.3 ± 0.3	6.9 ± 0.3	6.6 ± 0.3	6.6 ± 0.3
	40	27.7 ± 1.3	26.4 ± 1.3	27.5 ± 1.3	27.5 ± 1.3	26.0 ± 1.3
	60	40.6 ± 2.0	40.9 ± 2.0	39.8 ± 2.0	40.8 ± 2.0	41.0 ± 2.0

Table 4. Allelic frequencies for *ALS9* domains in a geographically diverse collection of clinical isolates

Pos., positive; Neg, negative.

Source	5' domain				3' domain							
	<i>ALS9-1</i>		<i>ALS9-2</i>		VB1				VB2			
	Pos.	Neg.	Pos.	Neg.	Both	<i>ALS9-1</i>	<i>ALS9-2</i>	Neither	Both	<i>ALS9-1</i>	<i>ALS9-2</i>	Neither
Bloodstream	51	49	100	0	50	2	49	0	36	1	64	1
Oral	45	36	81	0	46	2	31	1	13	3	63	0
Vaginal	4	6	10	0	5	0	4	1	1	1	8	0
Other	4	1	5	0	3	1	1	0	1	0	4	0
Total	104	92	196	0	104	5	85	2	51	5	139	1

ALS9-1 sequences were rather rare (Table 4). Nearly half of the *C. albicans* isolates examined had both *ALS9-1* and *ALS9-2* sequences within VB1. Within the VB2 region, *ALS9-2* sequences were far more common than *ALS9-1* sequences, and nearly one-quarter of the strains examined had both *ALS9-1* and *ALS9-2* sequence types (Table 4). Within both VB1 and VB2, a low number of strains had sequences that did not conform to either the *ALS9-1* or *ALS9-2* types that were defined from strain SC5314. This result suggests even greater sequence variability within the *ALS9* 3' domain.

DISCUSSION

Phenotypic assays of the *C. albicans als9Δ/als9Δ* mutant strain showed that the most notable effect of deleting *ALS9* was a significant decrease in adhesion to human vascular endothelial cell monolayers. Reintegration of the *ALS9-2* allele complemented the mutation, while the *ALS9-1* allele did not. Fusion of the Als9-2p N-terminal domain (amino acids 1–420) to the tandem repeats and C-terminal domain of Als9-1p (amino acids 421–1854) produced a protein that was also capable of complementing the mutant phenotype. This observation is consistent with the conclusion that adhesive function resides within the Als9p N-terminal domain. In previous work, fusion of the Als3p N-terminal domain (amino acids 1–430) to the C-terminal 323 amino acids of *S. cerevisiae* α -agglutinin was sufficient to increase adhesion of a *C. albicans als3Δ/als3Δ* strain to buccal epithelial cells (Zhao *et al.*, 2006). Although most adhesive activity is believed to be associated with the N-terminal domain, experiments that involve overexpression of ALS genes in *S. cerevisiae* suggest that some adhesive activity may be associated with other domains in Als proteins (Loza *et al.*, 2004; Rauceo *et al.*, 2006).

Assay of *C. albicans alsΔ/alsΔ* mutant strains and overexpression of ALS genes in *S. cerevisiae* are the two main methods that have been utilized to evaluate Als protein function (Loza *et al.*, 2004; Rauceo *et al.*, 2006; Zhao *et al.*, 2004, 2005). In some cases, results from the two methods are in agreement; however, more often, the two methods provide different views of Als protein function. Advantages

of the *S. cerevisiae* system include working in a tractable model system using relatively simple genetic manipulations, and the ability to address hypotheses regarding adhesive function in a low-adhesion background. These advantages are gained at the expense of differences in genetic code (Santos & Tuite, 1995) and glycosylation patterns (Herrero *et al.*, 2002), the tendency to draw functional conclusions from testing a single ALS allele, the need to utilize artificial gene-expression levels and patterns, the potential for protein overproduction to create cell-wall artefacts including mislocalized products, the inability to address *C. albicans*-specific Als protein function and/or interaction of Als proteins with others, and the limited ability to address non-adhesive phenotypes. While these disadvantages of *S. cerevisiae*-based studies are not present when working with the native *C. albicans*, *C. albicans* is inherently sticky, which complicates adhesion assays. Working in *C. albicans* also requires an understanding of ALS gene-expression patterns to select assay conditions, and phenotypic changes may be masked by compensatory gene-expression changes (Zhao *et al.*, 2005). Differences in results between the *C. albicans*- (Fig. 2) and *S. cerevisiae*-based (Sheppard *et al.*, 2004) approaches to studying Als9p function are obvious. When overexpressed in *S. cerevisiae*, *ALS9* did not confer adhesion to vascular endothelial cell monolayers (Sheppard *et al.*, 2004). Because the allele tested was not specified, it is possible that *ALS9-1* was used, which would account for the difference in results compared to our *C. albicans* analysis (Fig. 2). Overexpression of *ALS9* in *S. cerevisiae*, which presumably coats the cell surface in heterologous protein, resulted in adhesion to laminin (Sheppard *et al.*, 2004). Although statistically significant, this percentage adhesion was very low (~3%) suggesting, at best, a minimal contribution to laminin adhesion from the Als9 protein.

A key issue in extrapolating *S. cerevisiae*-based Als protein functional data to the *C. albicans* system is how well the *S. cerevisiae* model mimics native *C. albicans*. High-level overexpression of ALS genes in *S. cerevisiae* would best mimic *C. albicans* growth stages at which a specific ALS gene is highly expressed. While some ALS genes are known to undergo large changes in expression level from nearly

undetectable to very high (*ALS1* and *ALS3*, for example; Zhao *et al.*, 2004), others appear to be transcribed only at very low levels (*ALS6* and *ALS7*; Green *et al.*, 2004, 2005a, b, 2006). *ALS9* ranks in the middle of this ALS gene-expression hierarchy: clearly transcribed under all of the conditions tested, but to date, observed at expression levels that are well below those of the most highly expressed ALS genes (Green *et al.*, 2004, 2005a, b, 2006). For each of the Als proteins, the protein abundance and localization on the cell surface are almost unknown. Many variations in Als protein localization can be envisioned, from large areas or small focal patches of high protein concentration, to diffuse arrangements of a sparse number of proteins across the cell surface. Knowing the Als protein localization on the *C. albicans* cell surface would provide a better understanding of how well the *S. cerevisiae* model mimics the native system.

In many of the phenotypic assays conducted, the *als9Δ/als9Δ* strain failed to show a difference from the wild-type control strain. One potential explanation for these results is compensatory expression of other *C. albicans* ALS genes to confer a wild-type phenotype. This possibility is suggested by work that shows a relationship between expression of *ALS2* and *ALS4* in which a reduction in the transcriptional level of one gene results in increased transcription of the other (Zhao *et al.*, 2005). Compensatory ALS gene expression in response to *ALS9* deletion has been ruled out by real-time RT-PCR analysis (Green *et al.*, 2005b). Compensatory expression of non-ALS genes is possible, but if applicable to the *als9Δ/als9Δ* mutant, would have to involve genes that function in all processes tested except endothelial cell adhesion. These limitations decrease the likelihood that compensatory expression is a factor in functional analyses of the *als9Δ/als9Δ* strain.

Within the ALS family, *ALS9* is the most extreme example of allelic variability that has been described. While all eight ALS genes have variability in the number of tandem-repeat copies within the central coding domain, sequence variability in the non-tandem-repeat domains is less documented. Examples include *ALS5*, which has sequence variability within the 5' domain that surpasses the mean sequence variability present in a representative list of *C. albicans* genes (Hoyer & Hecht, 2001), and *ALS7*, which has a complex repeated structure within the 3' domain that lends considerable allelic variability to this locus (Zhang *et al.*, 2003). For *ALS3*, the presence of 12 instead of nine tandem-repeat copies in the central domain results in a higher percentage adherence of *C. albicans* to both endothelial and epithelial cell monolayers (Oh *et al.*, 2005). For *ALS9*, allele-specific function is associated with the large sequence differences (11 % at the nucleotide level; 16 % at the amino acid level) within the *ALS9* 5' domain. These differences may affect key residues within the Als9p N-terminal domain that are involved in binding to endothelial cells. Structural analysis of the Als N-terminal domain will answer such questions and could exploit the sequence and functional differences described here to better understand Als9p interaction with host cells.

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