Using Genomics to Shape the Definition of the Agglutinin-Like Sequence (ALS) Family in the Saccharomycetales

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The Candida albicans agglutinin-like sequence (ALS) family is studied because of its contribution to cell adhesion, fungal colonization, and polymicrobial biofilm formation. The goal of this work was to derive an accurate census and sequence for ALS genes in pathogenic yeasts and other closely related species, while probing the boundaries of the ALS family within the Order Saccharomycetales. Bioinformatic methods were combined with laboratory experimentation to characterize 47 novel ALS loci from 8 fungal species. AlphaFold predictions suggested the presence of a conserved N-terminal adhesive domain (NT-Als) structure in all Als proteins reported to date, as well as in S. cerevisiae alpha-agglutinin (Sag1). Lodderomyces elongisporus, Meyerozyma guilliermondii, and Scheffersomyces stipitis were notable because each species had genes with C. albicans ALS features, as well as at least one that encoded a Sag1-like protein. Detection of recombination events between the ALS family and gene families encoding other cell-surface proteins such as Iff/Hyr and Flo suggest widespread domain swapping with the potential to create cell-surface diversity among yeast species. Results from the analysis also revealed subtelomeric ALS genes, ALS pseudogenes, and the potential for yeast species to secrete their own soluble adhesion inhibitors. Information presented here supports the inclusion of SAG1 in the ALS family and yields many experimental hypotheses to pursue to further reveal the nature of the ALS family.

Keywords: ALS genes, adhesion, comparative genomics, protein structure, fungi, repeated sequences

INTRODUCTION

Most knowledge about agglutinin-like sequence (Als) protein structure and function comes from the study of Candida albicans (reviewed in Hoyer and Cota, 2016). C. albicans Als proteins are adhesins that promote interactions between fungi and host cells, establishing colonization that provides the opportunity to cause disease. Als proteins also function in C. albicans adhesion to
abiotic surfaces such as indwelling medical devices, and between fungi and other microbes such as bacteria, seeding polymicrobial biofilm development (Peters et al., 2012).

Using Als proteins feature known at the time, Hoyer and Cota (2016) suggested the NT/T/TR/CT model for proteins in the Als family with each protein possessing an NT-Als adhesive domain (NT; Lin et al., 2014), followed by a Thr-rich region (T), tandemly repeated sequences (TR), and a C-terminal (CT) domain rich in Ser and Thr. Other key features include a secretory signal peptide and GPI anchor addition sequence that direct the mature protein to β-1,6-glucan crosslinkage in the fungal cell wall (Lu et al., 1994). Display of the NT-Als adhesive domain on the cell surface is key for its ability to interact with other cells and materials (Lin et al., 2014).

The crystallographic structure of the NT-Als adhesive domain revealed protein folding driven by formation of 4 disulfide bonds (8 Cys); an invariant Lys is located at the end of the NT-Als binding pocket (Salgado et al., 2011). The positively charged Lys establishes a salt bridge with the C-terminal carboxylic acid of an incoming peptide ligand. Als binding affinity is at the micromolar level; avidity of Als protein interactions is increased by a peptide sequence with high β-aggregation potential (called the amyloid-forming region; AFR; Lipke et al., 2018).

The first ALS gene was named for the similarity of its predicted protein to Saccharomyces cerevisiae alpha-agglutinin (Sag1; Hoyer et al., 1995). At that time, considerably less DNA sequence information was available in public databases. Sag1 is an adhesin that promotes cell-cell contact between haploid yeasts during the mating process (Wojciechowicz et al., 1993). Sag1 binds the carboxyl-terminal peptide of a-agglutinin with nanomolar affinity (Cappellaro et al., 1994; Zhao et al., 2001). In contrast to Als proteins, the sequence of the N-terminal region of Sag1 has 6 Cys and an Arg residue at the position of the invariant Lys. The structure of Sag1 was not solved experimentally although molecular modeling and biochemical analyses contributed considerable insight into protein function and potential structure (Chen et al., 1995; Lipke et al., 1995; Grigorescu et al., 2000). Despite inherent differences between Als proteins and Sag1, various authors recognized the common features between these proteins and proposed that Sag1 is part of the Als family (Hoyer and Cota, 2016; Lipke, 2018).

The rich foundation of experimental evidence from C. albicans was used to define ALS genes and their encoded proteins in other fungal species including Candida dubliniensis (Jackson et al., 2009), Candida parapsilosis (Oh et al., 2019), Candida orthopsilosis (Lombardi et al., 2019), Candida metapsilosis (Oh et al., 2019) and Candida tropicalis (Oh et al., 2021). The burgeoning number of publicly available draft genome sequences provides the opportunity to locate other ALS sequences using a simple BLAST search. However, lack of proper gene assembly is a frequent stumbling block due to the tendency for each species to have multiple ALS loci and for ALS genes to encode long stretches of highly conserved, tandemly repeated sequences. Computer assembly of short-read sequences often breaks down in ALS coding regions because the length of repeated sequences is similar to the length of sequence reads (Lombardi et al., 2019). Emergence of long-read DNA sequencing technology provided datasets with more-accurate ALS assemblies, however, PCR amplification and Sanger sequencing are still frequently required to complete ALS loci (Oh et al., 2019; Oh et al., 2021).

The initial focus of this work was to derive an accurate census and sequence for the ALS genes in the human pathogenic Saccharomycetales species. Closely related species, including some with biotechnological importance, were also studied because their genome sequences are incorporated into available analysis tools (Maguire et al., 2013). Information presented here expands the list of known ALS loci, adding 47 carefully validated genes from 8 fungal species. Four long-read-based genome assemblies were generated. Predicted proteins were evaluated in the context of the model C. albicans Als and S. cerevisiae Sag1 characteristics. The availability of new, accurate structural prediction tools (Jumper et al., 2021) provided the opportunity to assess similarities within this group of proteins. Recent release of many new genome sequences for budding yeasts in the subphylum Saccharomycotina (Shen et al., 2018) allowed new insight into whether C. albicans Als proteins and S. cerevisiae Sag1 arose from a common ancestor. The resulting information redefines the ALS family, its diversity, and taxonomic boundaries. Information presented here also provides the basis for new hypotheses regarding Als protein function.

MATERIALS AND METHODS

Fungal Strain and Public Genome Sequence Resources

To ensure use of authenticated materials, Lodderomyces elongisporus strain NRRL YB-4239 and Spasothpora passalidarum strain NRRL Y-27907 were obtained from the Agricultural Research Service Culture Collection (Peoria, IL; https://nrl.ncaur.usda.gov). Meyerozyma guilliermondii strain ATCC 6260 and Scheffersomyces stipitis strain CBS 6054 (ATCC 58785) were purchased from the American Type Culture Collection (https://www.atcc.org). Table 1 lists genome sequences used for this study.

Genome Sequencing and Assembly

New genome assemblies were constructed for L. elongisporus NRRL YB-4239 (ASM1362098v1), M. guilliermondii ATCC 6260 (ASM6942125v1), S. stipitis CBS 6054 (ASM694211v1), and S. passalidarum NRRL Y-27907 (ASM362096v1) using a combination of Illumina MiSeq and Oxford Nanopore MinION data. Details of the method and genome assembly are in Supplementary Files S1–S4, respectively. Similar methods were described previously (Oh et al., 2019; Oh et al., 2021) and were reproduced here for the reader’s convenience.

Fungal cells were stored at -80°C and streaked to YPD agar plates (per liter: 10 g yeast extract, 20 g Bacto peptone, 20 g dextrose, 20 g Bacto agar). A single colony was inoculated into YPD liquid medium and the culture grown 16 h at 30°C and 200 r/min shaking. Genomic DNA was isolated according to
molar concentration. The pooled libraries were made using the Hyper Library construction kit (Roche) and pooled in equimolar concentration. The pool was sequenced library with the LSK-108 kit (Oxford Nanopore) with the United States). The sheared DNA was converted to a shotgun MiniSpin plus microcentrifuge (Eppendorf, Hauppauge, NY, United States). The sheared DNA was converted to a shotgun MiniSpin plus microcentrifuge (Eppendorf, Hauppauge, NY, United States). The sheared DNA was converted to a shotgun MiniSpin plus microcentrifuge (Eppendorf, Hauppauge, NY, United States).

Individuals barcoded shotgun libraries were made using the Hyper Library construction kit (Roche) and pooled in equimolar concentration. The pooled libraries were quantitated by qPCR and sequenced on one MiSeq flowcell for 48 h using a MinION MK1 sequencer. Basecalling was done with software MinKNOW version 1.7.7 and demultiplexing was performed with the Albacore software version 1.2.4.

Detailed assembly steps are outlined in Supplemental Files S1–S4 for the four genomes. In brief, trimming was performed using a simple Perl script to remove 60 nt from the ends of the reads, retaining reads longer than or equal to 1000 nt for the final assembly. Canu v1.5 (Koren et al., 2017) was used for assembly with the following parameters: “canu -p asm -d useGrid = false -nanopore-raw genomeSize=GENOME_SIZE <TRIMMED_FASTQ> -s index”, using the relevant quality-trimmed reads from Porechop and the following estimated genome sizes as input: S. stipitis = 15.4 Mb, L. elongisporus = 15.5 Mb, M. guillermondii = 10.6 Mb, S. passalidarum = 13.2 Mb.

Trimmed Oxford Nanopore reads were then aligned against their associated assembly using bwa v0.7.5 (Li, 2018) by first indexing the assembly using “bwa index <ASSEMBLY>” then aligning reads using the parameters “bwa mem -x ont2d <ASSEMBLY_INDEX> <TRIMMED_READS>”. The alignment was then used to polish the assembly using nanopolish v0.7.1 (Senol Cali et al., 2018). Quality-trimmed MiSeq data were used to further polish the assembly using Pilon v1.22 for error correction (Walker et al., 2014). Assembly names and GenBank accession numbers for the genome sequences for L. elongisporus NRRL YB-4239, M. guillermondii ATCC 6260, S. passalidarum NRRL Y-27907, and S. stipitis CBS 6054 are located in Table 1.

**Table 1** | Principal genome sequences used for ALS gene detection.

| Species | Strain | Assembly Name | GenBank Assembly Accession | Reference |
|---------|--------|---------------|---------------------------|-----------|
| *Candida albicans* | SCS314 | ASM18296v3 | GCA_000182965.3 | (Jones et al., 2004) |
| *Candida dubliniensis* | CD36 | ASM2694v1 | GCA_000026945.1 | (Jackson et al., 2009) |
| *Candida parapsilosis* | CDC317 | ASM18276v2 | GCF_00182765.2 | (Butler et al., 2009) |
| *Candida orthopsilosis* | Co 90-125 | ASM31587v1 | GCA_000315875.1 | (Riccombeni et al., 2012) |
| *Candida metapsilosis* | ATCC 96143 | ULC_Meta_2.0 | GCA_009804065.2 | (Butler et al., 2009) |
| *Candida tropicalis* | MYA-3404 | ASM335v3 | GCA_000033563.3 | (Butler et al., 2009) |
| *Meyerozyma guilliermondii* | ATCC 6260 | ASM14942v1 | GCA_000149425.1 | (Butler et al., 2009) |
| *Clavispora lusitaniae* | ATCC 42720 | ASM385v1 | GCA_000038535.1 | (Butler et al., 2009) |
| *Scheffersomyces stipitis* | CBS 6054 | ASM14942v1 | GCA_000038535.1 | (Butler et al., 2009) |
| *Lodderomyces elongisporus* | NRRL YB-4239 | ASM14968v1 | GCA_000149685.1 | (Butler et al., 2009) |
| *Yamadazyma tenuis* | ATCC 10573 | Candida tenuis v1.0 | GCA_000223465.1 | (Wohlbach et al., 2011) |
| *Spathaspora passalidarum* | NRRL Y-27907 | S passalidarum v2.0 | GCA_000223485.1 | (Wohlbach et al., 2011) |
| *Pichia kudriavzevi* | ATCC 6258 | ASM30544v1 | GCA_000305445.1 | (Douglass et al., 2018) |
| *Candida glabrata* | CBS138 | ASM254v2 | GCA_00002545.2 | (Douglass et al., 2018) |
| *Saccharomyces cerevisiae* | BG2 | ASM14211v1 | GCA_000014211v1 | (Butler et al., 2009) |
| *S. passalidarum* | CBS 6054 | ASM14942v1 | GCA_000149425.1 | (Butler et al., 2009) |
| *Candida auris* | MYA-3404 | ASM14942v1 | GCA_000149425.1 | (Butler et al., 2009) |
| *Saccharomyces cerevisiae* | S288C | RN4 | GCA_000142545.2 | (Goffeau et al., 1996) |
| *S. kudriavzevi* | B8441 | Cand_auris_B8441_V2 | GCA_002759435.2 | (Lockhart et al., 2017) |

Sherman et al. (1986). Briefly, cells were spheroplasted with zymolyase and lysed with sodium dodecyl sulfate. The lysate was extracted with phenol, DNA precipitated with isopropanol, and the final preparation treated with Proteinase K. DNA shearing was minimized by use of wide-bore pipet tips and gentle mixing. Agarose gel electrophoresis was used to verify the presence of high-molecular-weight DNA prior to subsequent library preparation.

Libraries were constructed and sequenced at the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. Individually barcoded shotgun libraries were made using the Hyper Library construction kit (Roche) and pooled in equimolar concentration. The pooled libraries were quantitated by qPCR and sequenced on one MiSeq flowcell for 251 cycles from each end of the fragment using a MiSeq 500-cycle sequencing kit (version 2). PhiX DNA was used as a spike-in control for MiSeq sequencing runs. FASTQ files were generated and demultiplexed with bcl2fastq Conversion Software (Illumina, version 2.17.1.14). MiSeq reads were quality trimmed using Trimmomatic v0.36 (Bolger et al., 2014) to remove adaptors from the 3’ end of the reads prior to assembly, retaining all reads longer than or equal to 50 nt in length.

Libraries for Oxford Nanopore long-read sequencing used 1 μg of genomic DNA that was sheared in a gTube (Covaris, Woburn, MA, United States) for 1 min at 6,000 r/min in a MiniSpin plus microcentrifuge (Eppendorf, Hauppauge, NY, United States). The sheared DNA was converted to a shotgun library with the LS-108 kit (Oxford Nanopore) with the Expansion barcoding kit (EXP-NBD103). The libraries were pooled in equimolar concentration and the pool was sequenced on two SpotON MK I (R9.5) flowcells for 48 h using a MinION MK1 1B sequencer. Basecalling was done with software MinKNOW version 1.7.7 and demultiplexing was performed with the Albacore software version 1.2.4. Sixty bp were trimmed from each end of the reads to remove barcodes using a custom script.
Identification of ALS Genes and Predicted Als Protein Features

Methods for identifying ALS genes and deducing predicted protein features were nearly identical to those reported in previous publications (Oh et al., 2019; Oh et al., 2021). Details were reproduced here for the reader's convenience. BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify potential ALS genes and Als proteins in the genome sequences (Table 1). BLAST searches were also conducted using the Candida Genome Database (www.candidagenome.org; Skrzypek et al., 2017). Query sequences included all C. albicans ALS genes as reported by Oh et al. (2019). As new ALS/Als sequences were identified, they were also used as BLAST queries until search reports failed to reveal new sequences.

ALS gene sequences were verified or corrected by Sanger sequencing of PCR-amplified products. Primers were designed using the Primer Quest Tool (https://www.idtdna.com) and primers purchased from Integrated DNA Technologies (Corvallis, OR). PCR used Q5 High Fidelity DNA Polymerase (New England Biolabs) according to the manufacturer's instructions. Extension time was adjusted to promote amplification of the desired product size. PCR products were purified using the MultiScreen HTS 96-well Filtration System (Millipore), then Sanger sequenced at the Roy J. Carver Biotechnology Center (University of Illinois at Urbana-Champaign). A list of primer sequences is located in Supplementary Table S1.

Structural Predictions

Protein structural predictions were derived using AlphaFold (Jumper et al., 2021), accessed using AlphaFold Colab (https://colab.research.google.com/github/deepmind/alphafold/blob/main/notebooks/AlphaFold.ipynb). ColabFold (Mirdita et al., 2021; https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb) was used for more-rapid generation of structural prototypes. ColabFold utilized MMSeqs2 (UniRef+Environmental), as well as a custom MSA file constructed using Hhblits Toolkit server (Zimmerman et al., 2018; Gabler et al., 2020).

Clustal Omega (Madeira et al., 2019) was used to align amino acid sequences from Supplementary File S5 with those from 4LE8, the Protein Data Bank (https://www.rcsb.org) entry for the crystallographic structure of C. albicans NT-Als3 (chain A, 299 amino acids). This molecule did not include the secretory signal peptide at the N-terminal end nor the amyloid-forming region at the C-terminal end. Sequences in Supplementary File S5 were trimmed according to the Clustal Omega alignment and the trimmed sequences were used for AlphaFold analysis. Sequences for other fungal cell-surface proteins were included as controls in the structural analysis: C. albicans Hyr1 (NCBI Reference Sequence: XP_722183.2, amino acids 21 to 309) and S. cerevisiae Flo1 (NCBI Reference Sequence: NP_009424.1; amino acids 25 to 273). Structural visualization and alignments used the PyMOL Molecular Graphics System, Version 2.5.2 (Schrödinger, LLC).

RESULTS

Detection and Assembly of ALS Sequences in Eight Fungal Species

Examination of new and existing genome sequence data (Table 1) provided the information needed to locate, assemble and/or validate the sequence for 47 ALS loci (5 from Lodderomyces elongisporus NRRL YB-4239; 3 from Candida auris B8441; 3 from Scheffersomyces stipitis CBS 6054; 4 from Meyerozyma guilliermondii ATCC 6260; 29 from Spathaspora passalidarum NRRL Y-27907; and 1 each from Clavispora lusitaniae ATCC
42720, Yamadazyma tenuis ATCC 10573, and Debaryomyces hansenii CBS 767). These sequences are presented here in the context of previously described ALS loci including 8 from Candida albicans SC5314 (Hoyer et al., 2008), 7 from Candida dubliniensis CD36 (Jackson et al., 2009), 13 from Candida tropicalis MYA-3404 (Oh et al., 2019), 3 from Candida orthopsilosis Co 90-125 (Lombardi et al., 2019), and 4 from Candida metapsilosis ATCC 96143 (Oh et al., 2019). Supplementary Table S2 details all gene names, GenBank accession numbers, and predicted protein characteristics. Gene names were taken from locus tags associated with the annotated reference genome for each species. For genes that were not annotated in the reference genome, "0.5" was added to the name, indicating that the ORF was located between two previously annotated genes. Figure 1 displays the phylogenetic relationship between the species described here.

**L. elongisporus**

*L. elongisporus* is an infrequent human pathogen and, as accurately portrayed in Figure 1, closely related to C. parapsilosis (Lockhart et al., 2008). Two *L. elongisporus* genome sequences were available through the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov) database; both were for strain NRRL YB-4239 (Table 1). Assembly ASM14968v1 was comprised of 145 contigs arranged into 28 scaffolds (i.e. composed of contigs and gaps that are indicated by insertion of “NNN”) while ASM1362098v1 had 53 contigs. ALS gene names were derived from the original annotation of the reference genome (ASM14968v1). Although a *L. elongisporus* karyotype was not located in the literature, the number of contigs/scaffolds was far higher than an expected number of chromosomes, indicating that the genome assemblies required refinement.

BLAST analysis using ALS query sequences consistently revealed 6 *L. elongisporus* loci (Supplementary File S6). LeALS734 was located in the middle of a > 3 Mb supercontig in each genome assembly. LeALS2536, LeALS2716 and LeALS2721 were located in the center of another supercontig in ASM14968v1 with the latter two genes located near each other and transcribed in the same direction (Figure 2). The remaining genes (LELG_04272, LeALS5708) were each on their own supercontig.

Proteins predicted from five loci (LeALS734, LeALS2536, LeALS2716, LeALS2721, and LeALS5708) had features expected for an Als protein including a secretory signal peptide, NT-Als-rich sequences, and GPI anchor addition site (Supplementary Table S2). Although the protein translated from the sixth locus (LELG_04272) had tandemly repeated sequences like those in Als proteins, the ORF did not predict an NT-Als domain (Supplementary File S6). BLAST of sequences N-terminal to the LEALS_04272 tandem repeats best matched M. guilliermondii PGUG_02520, a 660-amino acid protein of unknown function. The hybrid LEALS_04272 was likely the result of recombination between an ALS open reading frame (ORF) and another gene. Such recombination was observed previously between ALS and IFF/HYR genes in *C. metapsilosis* CmALS2265 which encoded an Als N-terminal domain on the stalk of an Iff/Hyr protein (Oh et al., 2019). Because LELEG_04272 lacked an NT-Als adhesive domain, it was not given an Als family name.
Similar to *C. metapsilosis*, recombination between ALS and IFF/HYR genes was evident in *L. elongisporus* (Supplementary File S6). *LeALS734* and *LeALS2716* each had the 5’ end of an ALS gene and the 3’ end of an IFF/HYR gene. Consensus tandem repeat sequences for each locus closely matched that for *CmALS2265* (Oh et al., 2019). The remaining *LeALS* genes did not include a central domain of tandemly repeated sequences.

The predicted amino acid sequence of the NT-Als domain was highly conserved between *LeAls2536*, *LeAls2721*, and *LeAls5708* (70-80% identity; Supplementary File S6). *LeAls734* and *LeAls2716* were approximately 40% identical to each other and to the other *L. elongisporus* Als proteins. Since the crystallographic structure of *C. albicans* NT-Als3 is known (Lin et al., 2014), the CaAls3 sequence was included for comparison to newly characterized proteins. The *LeAls* proteins were 33-45% identical to the CaAls3 NT-Als sequence.

The incomplete *L. elongisporus* genome assemblies provided multiple opportunities for repairing ORFs. *LELG_04272* lacked a GPI anchor addition site in its original annotation in ASM14968v1 (Supplementary File S6). Primer design, PCR amplification, and Sanger DNA sequencing indicated that the downstream *LELG_04271* was actually the 3’ end of the *LELG_04272* coding region. The full sequence was deposited into GenBank (accession number OK172378). The region encoding *LeALS2716* and *LeALS2721* was also assembled poorly in the available genome sequences (Figure 2). Insertion of many NNN (designating unknown sequence) in this region of assembly ASM14968v1 made it difficult to locate the gene boundaries. The size of the verified *LeALS2716* was larger than predicted in assembly ASM14968v1, but smaller than suggested in assembly ASM1362098v1. The difference was possibly attributable to variation in tandem repeat copy number between alleles in the diploid *L. elongisporus*. A similar scenario was encountered for completing the sequence of *LeALS734*: several previously designated ORFs were combined into the final *LeALS734*, yet the sequence deposited into GenBank was shorter than anticipated from the genome assemblies that guided PCR primer design (details not shown).

**M. guilliermondii**

In addition to its role as a human pathogen, *M. guilliermondii* has applications in bioprocessing (e.g. production of riboflavin and xylitol) and bioremediation (reviewed in Papon et al., 2013). Eight *M. guilliermondii* genome assemblies were reported in the NCBI database. Two sequences were available for strain ATCC 6260, which was the focus of this work. ASM14942v1 consisted...
of 71 contigs arranged on 9 scaffolds. ASM694215v1 had 9 contigs.

Two of the 4 Als loci in strain ATCC 6260 (MgALS2302, MgALS3259) were on the same chromosome, approximately 1.7 Mb away from each other at opposite telomeres, transcribed in opposite directions (Supplementary File S7). The 3' end of the 9681-bp MgALS2302 was located approximately 10 kb from one telomere while the 3' end of the 6963-bp MgAls3259 was about 7 kb from the opposite telomere. The other two MgAls loci (MgALS673 and MgALS3330) were each on other large contigs, likely representing M. guilliermondii chromosomes.

The coding region for MgALS3259 predicted two potential start sites, one 60 bp upstream from the other. The second predicted Met start site provided a clear secretory signal peptide MgALS2302 assembly included the length of upstream start site instead. Another difference between GenBank deposits (9681 bp vs. 3840 bp). The new gene name protein (CauAls4112) had a central domain of tandemly repeated sequences from previously characterized Candida genes (Supplementary File S7). The most-common repeat unit size was 35 amino acids with a range of 34-36 amino acids. The predicted MgAls2302 and MgAls3259 NT-Als protein sequences were the most similar to each other. They shared 57% identity while other NT-Als comparisons were only about 30% identical.

C. auris
Availability of genome sequences for over 100 isolates of C. auris in the NCBI database reflected its emergence as an important pathogen (Du et al., 2020). Strain B8441 (18 contigs arranged into 15 scaffolds; assembly Cand_auris_B8441_V2) was used for this work (Table 1). The three Als loci in strain B8441 were on different scaffolds with CauAls2582 in a subtelomeric location (Supplementary File S8). Only one predicted C. auris Als protein (CauAls4112) had a central domain of tandemly repeated (34-39 amino acids) sequences. C. auris NT-Als sequences were 38-50% identical to each other and 25-33% identical to C. albicans NT-Als3. BLAST searching of other C. auris genomes showed that many were missing the CauAls2582 locus. These observations matched the report of Muñoz et al. (2021) that described loss of subtelomeric adhesins such as CauAls2582 in clade II C. auris isolates.

S. stiptitis
S. stiptitis is studied because of its ability to ferment xylose, and thus its potential to contribute to biofuel production from hemicellulose (Jeffries et al., 2007). Because S. stiptitis is within the CUG clade of the Family Debaryomycetaeae, the genome of this beetle-associated commensal yeast is often included in comparisons that feature human-pathogenic species (Wohlbach et al., 2011; Maguire et al., 2013). The NCBI database included three S. stiptitis genome sequences: two for strain CBS 6054 (ASM20916v1 = 9 contigs arranged on 8 scaffolds; ASM694211v1 = 30 contigs) and one for strain NRRL Y-7124. Efforts here focused on strain CBS 6054 (Table 1).

Strain CBS 6054 had three Als loci (Supplementary Table S2). Two (SsALS2386 and SsALS2786) were on chromosome 2, approximately 700 kb apart and transcribed in opposite directions (Supplementary File S9). The third (SsALS4579) was on chromosome 4. NT domains of the predicted chromosome 2 proteins were 58% identical. However, each was only 27-29% identical to SsAls4579. Both SsAls2386 and SsAls2786 had a central domain of tandemly repeated sequences that resembled those from previously studied Candida species.

BLAST searches and text searches of the NCBI database revealed other repeat-containing loci that were annotated as Als genes in assembly ASM20916v1. For example, PICST_4391 (called “ALS1”) was a fragment of SsAls2786 described above. PICST_4539 (called “ALS1.2”) was a fragment of SsAls2386 and PICST_31759 (called “ALS6”) corresponded to SsAls4579. PICST_31095, despite being named “ALS2”, belonged to the IFF/HYR family. Another, called “ALS4” (PICST_30411), was comprised only of repeated sequences. It was flanked by sequences that contained many stop codons with a low likelihood of encoding a larger ORF. In assembly ASM20916v1, PICST_30411 was adjacent to an IFF/HYR gene in a subteromeric region on chromosome 2.

S. passalidarum
The S. passalidarum genome was also sequenced to study xylose metabolism in fungal species (Wohlbach et al., 2011). Because initial BLAST searching revealed an unusually high number of Als loci in S. passalidarum, additional analysis was pursued. Two genome assemblies for strain NRRL Y-27907 were located in the NCBI database (Table 1). One was created by the DOE Joint Genome Institute (Spaspassalidarum v2.0; 26 contigs arranged onto 8 scaffolds) and the other was part of the current work (ASM1362069v1; 10 contigs).

BLAST searching revealed 29 physical loci with sequence similarity to Als genes (Supplementary Table S2). Seven of the 8 contigs from assembly Spaspassalidarum v2.0 had at least 1 Als locus, and as many as 7 Als loci (Table 2). Four locations featured Als genes adjacent to each other: SpAls64434 and SpAls64435 on scaffold 1, SpAls50348 and SpAls50349 on scaffold 3, SpAls15305 and SpAls15305.5 on scaffold 4, and SpAls68952 and SpAls68952.5 on scaffold 8. Two Als genes were subtelomeric: SpAls131476 on scaffold 1 and SpAls137089 on scaffold 3.

Examination of the encoded protein sequences showed that many did not have repeated sequences in the center (Supplementary File S10). When present, repeated sequences were unusually long (Supplementary Table S2), highly variable in the length of the repeat unit, and did not necessarily have tandemly arrayed repeat copies. A consensus of the 80-amino-acid and 89-amino-acid versions of the S. passalidarum repeated sequence resembled the T (Thr-rich) domain of C. albicans Als proteins that is located between the end of NT-Als and the start.
of the tandem repeat region (reviewed in Hoyer and Cota, 2016). Some manuscripts depict this region separately from “NT-Als” for which the crystal structure is known (e.g. Oh et al., 2019). Other manuscripts, like this one, include the T domain in the “NT-Als” region for each protein.

*S. passalidarum* had several partial ALS genes (Supplementary File S10). In previous analyses, partial ALS genes indicated poor genome assembly that could be corrected by PCR amplification and Sanger sequencing (Oh et al., 2021). Here, however, PCR amplification and Sanger sequencing validated the accuracy of the partial ALS genes including the contiguous loci SpALS50348 and SpALS50349 that each only encoded an NT-Als domain with a secretory signal peptide. A clear stop codon between SpALS68952.5 and SpALS68952 created pseudogenes from a perhaps previously long, functional gene. Some predicted proteins lacked features such as a secretory signal peptide that would direct the protein toward cell-surface localization. Signal-peptide-encoding sequences were noted upstream of SpALS66147 and SpALS134590.5 but were separated from the ORF by at least one clear stop codon. Other predicted proteins (SpAls49824, SpAls61022.5, SpAls140483, and SpAls140900; Supplementary File S10) showed only a weak signal for GPI anchor addition, suggesting that the mature proteins may not be localized to the cell surface. Among the predicted proteins, to have both sequential signal peptide and strong GPI anchor addition signal, many were short, raising the question of how far the adhesive NT-Als domain could be projected away from the *S. passalidarum* cell surface to contact binding partners.

Comparison between the NT-Als domains for the predicted *S. passalidarum* proteins showed considerable sequence divergence from *C. albicans* NT-Als3 and from each other (Supplementary File S10). Many comparisons were in the range of 22-45% identity although greater sequence conservation was observed for some pairs (60-80% range). Contiguous loci (37-62% identity) were not necessarily more conserved than other comparisons. The most-conserved pair was SpAls55077 and SpAls134590 (89%) potentially indicating a gene duplication.

### Y. tenuis

Interest in the *Y. tenuis* genome also arose from its xylose fermentation capabilities (Wohlbach et al., 2011). A single complete ALS locus was detected in the only *Y. tenuis* genome assembly available in the NCBI database (strain ATCC 10573; Candida tenuis v1.0; Table 1). The predicted YtAls93631 protein was > 3000 amino acids and included a central domain of tandemly repeated sequences (32-45 amino acids; Supplementary File S11). The most common repeat unit was 35 amino acids. The *Y. tenuis* genome sequence was quite fragmented, consisting of 74 contigs arranged onto 25 scaffolds. A second potential ALS locus was detected at the end of a scaffold sequence (CANTEScaffold_00010; 12.1 kb total length) suggesting that other *Y. tenuis* ALS loci may be revealed as the genome assembly matures.

### C. lusitaniae

*C. lusitaniae* is a less-frequent cause of human candidiasis (reviewed in François et al., 2001). Of the species considered here, *C. lusitaniae* was the most closely related to *C. auris* (Figure 1). More than 30 *C. lusitaniae* genome assemblies were available in the NCBI database. Observations here focused on strain ATCC 42720 (ASM383v1) which was comprised of 88 contigs arranged onto 9 scaffolds. One long ALS gene (> 8 kb) was detected in this sequence (Supplementary Table S2). BLAST of other available genomes also suggested a single ALS locus, although incomplete genome assemblies with many contigs may obscure the presence of other loci. Two different types of repeated DNA sequences were found in the center of CIALS3274 (Supplementary File S11). BLAST
searching of the non-redundant nucleotide database suggested that the repeats predicting a 33 amino acid sequence were unique. The other repeated sequences were primarily 67 amino acids in length, although some repeat units expanded to 102 amino acids. Sequences within these longer repeats were reminiscent of motifs found in the Als consensus repeat from C. parapsilosis/Orthopsilosis/C. metapsilosis, as well as the 80-amino-acid consensus repeat from S. passalidarum Als proteins (Supplementary File S10).

D. hansenii
Rather than being studied as a pathogen, D. hansenii is discussed commonly in the context of food production (summarized in Gori et al., 2011). D. hansenii growth requires surface adhesion (Mortensen et al., 2005), a property that might involve Als proteins. The NCBI database displayed 12 D. hansenii genome sequences, representing 11 different strains. This work focused on CBS 767 (ASM644v2), which was designated as the reference genome and had 15 contigs arranged on 8 scaffolds. A single ALS locus was detected, located on the chromosome G contig (Supplementary Table S2). The predicted protein, DhAls2178, did not contain tandemly repeated sequences (Supplementary File S11).

C. glabrata
C. glabrata is well-recognized as a human pathogen, particularly for its ability to rapidly evolve resistance to antifungal drugs (Vale-Silva and Sanglard, 2015). Eighteen C. glabrata genomes were present in the NCBI database. BLAST of the strain CBS 138 sequence using Als and Als queries revealed CAGL0G04125g, which was annotated to indicate similarity to S. cerevisiae Sag1 (Supplementary File S11). Like Sag1, the C. glabrata predicted protein did not encode a central domain of tandemly repeated sequences.

NT-Als Structural Predictions
The C. albicans NT-Als3 structure was solved crystallographically (Lin et al., 2014; Protein Data Bank accession number 4LE8), providing experimental information that can be used to produce structural predictions for other NT-Als proteins. A crystallographic structure has not been reported for S. cerevisiae Sag1. The recent development of the highly accurate protein structural prediction program AlphaFold (Jumper et al., 2021) and widespread access to its use (e.g. Mirdita et al., 2021) provided the opportunity to assess predicted structural relatedness between the diverse set of NT-Als sequences and their similarity to Sag1.

Figure 3 shows the crystallographic structure of C. albicans NT-Als3 from 4LE8 (Figure 3A) and the AlphaFold-predicted structure for the same amino acid sequence (Figure 3B). The root-mean-square-deviation (RMSD) value for alignment of the two structures was 0.53, indicating the ability of AlphaFold to reproduce the crystallographic structure of NT-Als3 from its amino acid sequence. AlphaFold was then used to predict the structures of other NT-Als proteins, particularly those with limited sequence identity to C. albicans NT-Als3 (Figures 3C–G). Strikingly, a similar structure was predicted for each protein, including Sag1. Analysis of each NT-Als sequence in Supplementary File S5 produced the same general protein shape (data not shown) suggesting that all, including Sag1, were unified as a family. To demonstrate diversity in structural predictions, the amino acid sequences for the N-terminal domain of the cell-surface proteins C. albicans Hyl1 (amino acids 21 to 309) and S. cerevisiae Flo1 (amino acids 25 to 273) were entered into AlphaFold. The resulting structural predictions were different from the Als family and from each other (Figures 3H, I).

Adhesive Activity Predictions From NT-Als Protein Features
In order to provide biologically meaningful adhesive activity on the fungal cell surface, Als proteins need a secretory signal peptide and GPI anchor addition site to achieve an effective localization (Lu et al., 1994). The crystallographic structure of C. albicans NT-Als3 (Lin et al., 2014) demonstrated the involvement of 8 Cys residues in folding of the mature protein and the function of an invariant Lys at the end of the binding cavity that sinks the negative charge of incoming peptide ligands. Other work demonstrated the importance of the amyloid-forming region (AFR) for increasing the avidity of aggregative interactions (Ho et al., 2019). Supplementary Table S2 evaluated each of these features for the previously characterized and newly introduced Als proteins. The data were summarized in Figure 4 from which various hypotheses emerged.

The cluster of dots in the top center of Figure 4 contained proteins with 8 Cys and an invariant Lys. Each protein represented in the top center cluster of dots also had an AFR with high predicted β-aggregation potential and a location immediately C-terminal to the NT-Als domain as in C. albicans Als proteins (Salgado et al., 2011; Lipke et al., 2018). The top center cluster of dots included 7 of the 8 C. albicans Als proteins, 6 of 7 from C. dubliniensis, and 9 of 13 from C. tropicalis suggesting robust potential for functional adhesive interactions by these species. Also present in the top center cluster of dots were 2 of the S. stipitis Als proteins, 2 from S. passalidarum, and 1 each from C. parapsilosis, C. metapsilosis, L. elongisporus, and Y. tenuis.

Viewing potential Als function according to these criteria provided predictions about the adhesive activity in other species. For example, C. parapsilosis, C. orthopsilosis, and C. metapsilosis Als proteins almost universally had a weaker or absent AFR leading to the hypothesis that these species are functionally less adherent than C. albicans, C. dubliniensis, and C. tropicalis. Data in Figure 4 suggested a similar hypothesis for S. passalidarum: despite its overabundance of ALS-like loci, S. passalidarum encoded only two proteins (SpAls138016 and SpAls156463; Supplementary File S10) that possessed the functional features present in NT-Als3 and therefore may not be a very adherent species. However, nine SpAls proteins had AFR sequences 20-30 amino acids C-terminal to the location where they are found in C. albicans Als3 (Supplementary Table S2). Experimentation is required to evaluate whether sequences with high β-aggregation potential in this alternative location contribute to S. passalidarum aggregative interactions. Using the criteria in Figure 4, C. lusitaniae was an outlier. Its sole Als protein,
ClAls3274, had only 4 Cys residues and no AFR. Despite these key differences, the predicted structure for the NT-ClAls3274 domain was similar to those from other species (Figure 3D). The contribution of ClAls3274 remains to be tested, as does the overall adhesion profile for the species.

Proteins with features that more closely resemble Sag1 than C. albicans Als3 segregated to the left column of the diagram. Substitution of Arg (R printed in the dot) in place of the invariant Lys was a common theme. Three of the four M. guilliermondii Als proteins were in the left column. Perhaps these proteins have more-specific, higher-affinity adhesive interactions that more-closely resemble those of Sag1, rather than NT-Als3. The same prediction could be made for two of the C. auris proteins, as well as the sole protein from D. hansenii.

Information presented in Figure 4 and Supplementary Table S2 showed that some species had a complement of Als proteins that possessed features of both S. cerevisiae Sag1 and C. albicans Als3. For example, S. stipitis SsAls4579 had Sag1-like features (lower left, Figure 4) while SsAls2386 and SsAls2786 looked more like C. albicans Als3 (top center, Figure 4). The line diagrams in Supplementary File S9 reinforced this idea, illustrating the extensive regions of tandemly repeated sequences in the latter two proteins (C. albicans Als-like) and lack of a repeated domain in SsAls4579 (Sag1-like). M. guilliermondii was a similar mixing bowl of Als proteins: MgAls673 was the most Sag1-like of the group. Each of the other MgAls proteins had a long stalk of tandemly repeated units with NT domains that perhaps were more like Sag1 than Als3 (Supplementary File S7). L. elongisporus NT-Als proteins also appeared in more than one column in Figure 4 with one (LeAls734) sharing greater similarity with Sag1. The recombinant nature of some of the LeAls proteins presented a more-complex picture than for S. stipitis or M. guilliermondii (Supplementary File S6).
Drawing a Tree to Visualize NT-Als Relatedness

In addition to comparing NT-Als proteins based on the features described above, more-global comparisons were made. Clustal Omega alignment of the NT-Als sequences (Supplementary File S5) showed identity values ranging from approximately 20% to 90% (data not shown). A maximum likelihood tree was drawn to visualize the results (Figure 5).

The topology of the tree reflected the overall diversity in NT-Als sequence identity. For example, NT-Als sequences from the closely related species *C. parapsilosis/C. orthopsilosis/C. metapsilosis* appeared on short branches in tight groups. In contrast, many branches showing the relationship between the highly diverse *S. passalidarum* NT-Als sequences were quite long, most notably for SpAls153035.5, which was the least related to other proteins in that species.

Als sequences from the same species tended to cluster together which made the overall tree organization similar to the species tree in Figure 1. The close relationship between *C. albicans* and *C. dubliniensis* (Jackson et al., 2009), as well as that between *C. parapsilosis/C. orthopsilosis/C. metapsilosis* (Tavanti et al., 2005), were reflected by the proteins from these groups being localized to distinct regions of the tree. *C. tropicalis* sequences were similarly clustered as were most of the *S. passalidarum* sequences. *C. auris* and *C. lusitaniae* sequences were grouped closely, similar to their position on the species tree (Figure 1). Within the groupings of closely related species, orthologs were obvious (e.g. CaAls6/CdAls86290, CaAls7/CdAls86150, CpaAls660/CoAls800 and CoAls4210/CmAls4210-1). Examples of paralogs, likely the result of gene duplication, included CpaAls4770/CpaAls4780/CpaAls4800, CtrAls3791/CtrAls2293/CtrAls3786, and SpAls64434/SpAls64435.

Similar to predictions made from the Figure 4 diagram, NT-Als sequences from some species were spread across the tree. The four *M. guilliermondii* Als sequences were found in three different locations: MgAls673 was grouped more closely to...
sequences like ScSag1; MgAls2302 and MgAls3259 were clustered together near proteins from C. auris; and MgAls3330 was on its own branch elsewhere on the tree. Low bootstrap support for the latter location suggested that MgAls3330 could easily be located differently on the tree. The three sequences from S. stipitis were also dispersed with one near ScSag1 and the two others closer to the C. albicans/C. dubliniensis proteins. The five L. elongisporus Als proteins were split into two groups: the first including LeAls2536/LeAls5708/LeAls2721 (likely paralogs) and the second including LeAls734/LeAls2716 (recombinants between Als and Iff/Hyr). The Sag1-like features for SsAls4579 and MgAls673 were reflected on the tree (Figure 5), while LeAls734 and LeAls2716 (both Als recombinants with Iff/Hyr) clustered together in a different location.

The tree in Figure 5 was drawn from a portion of each protein sequence. Another way to evaluate phylogenetic relationships is
via conservation of physical location (synteny) as depicted using the Candida Gene Order Browser (CGOB; Maguire et al., 2013). CGOB includes all species in Figure 5 except C. glabrata (CAGL0G04125g). A screenshot from one region of the CGOB was included in Figure 2C. It showed genes from several different fungal species that were orthologs of Ca. albicans ALS6 and ALS7. Orthologs reported by CGOB are shown in Table 3. Yellow hexagons with alphabetical labels were used to display the CGOB relationships on the tree in Figure 5.

CGOB ortholog designations were most reliable in closely related species such as C. albicans and C. dubliniensis; each C. albicans locus had a C. dubliniensis ortholog except CaALS3 and CaALS5 which are missing from the C. dubliniensis genome (Jackson et al., 2009). These ortholog groups were frequently augmented with genes from other species such as Group A which included SsALS2386. Groups E and F were the largest, suggesting ortholog relationships between genes in 7 and 6 species, respectively.

CGOB also proposed ortholog groups that did not include C. albicans or C. dubliniensis sequences. One example was Group J that included the SAG1-like MgALS673 and SsALS4579, as well as DhAls2178, the lone ALS sequence from the primarily haploid D. hansenii. Examination of the predicted protein sequence showed lack of a tandem repeat domain as well as other Sag1-like features (Supplementary File S11 and Figure 4).

Some CGOB ortholog designations were perhaps problematic. For example, PICST_31095 (Group D) belonged to the IFF/HYR family instead of ALS and ScFLO1 (Group G) encoded a flocculin with a mannos-binding N-terminal domain that was structurally diverse from the Als family (Figure 3). Perhaps the most intriguing ortholog designation was Group K which included ScSAG1 and CdALS65010. CdALS65010 was nearly 100% identical to CdALS64800, which was included in ortholog Group B. These loci are near each other on C. dubliniensis chromosome 6 and the appear to be paralogs (i.e. recently duplicated; Jackson et al., 2009). As species become more divergent, assessing ortholog designations becomes more challenging. However, the suggestion of orthology between ScSAG1 and a C. dubliniensis ALS gene supported the general idea that the genes were part of the same family and arose from a common ancestor.

Probing the Taxonomic Boundaries of the ALS Family

The rapidly increasing number of genome sequences present in public databases provided an opportunity to further explore the breadth and potential origin of the ALS family. The NCBI database can be queried using taxonomic identification (taxid) numbers to detect potential ALS loci more broadly across the phylogenetic tree. Table 4 lists the taxids for designations of the major Families within the Order Saccharomycetales. Inputs from NCBI (lifemap-ncbi-univ-lyon1.fr), Shen et al. (2018), and Li et al. (2021) were included although the taxonomic designations were not identical among the sources. The portion of the

Table 3: Orthologous ALS loci reported on Candida Gene Order Browser (CGOB).

| Reference Gene | Reported Ortholog | Group* | Reference Gene | Reported Ortholog | Group* |
|----------------|-------------------|--------|----------------|-------------------|--------|
| CaALS1         | CdALS64210        | A      | CaALS7         | CdALS6150        | F      |
|                | SsALS2386"        |        |                | CtrALS2228       |        |
|                |                   |        |                | CdALS64000       |        |
|                |                   |        |                | CoALS4220        |        |
|                |                   |        |                | LeALS2721        |        |
| CaALS2         | CdALS64800        | B      | CaALS9         | CdALS64220       | G      |
|                | CtrALS3786        |        |                | LELG_04272"      |        |
|                | SpALS69592        |        |                | ScFLO1"          |        |
| CaALS3         | YtALS93631        | C      |                |                   |        |
|                | SpALS134590       |        |                |                   |        |
| CaALS4         | CdALS64610        | D      | CtrALS2229     |                   |        |
|                | CauALS2582        |        | CoALS4780      |                   |        |
|                | CIALS3274         |        |                |                   |        |
|                | PICST_31095"      |        |                |                   |        |
|                | MgALS3259         |        |                |                   |        |
| CaALS6         | CdALS68290        | E      | DhALS2178"     |                   |        |
|                | CtrALS2229        |        | SsALS4579      |                   |        |
|                | CprALS4790        |        | MgALS673       |                   |        |
|                | CmALS4220"        |        |                |                   |        |
|                | LeALS2716         |        |                |                   |        |
|                | SpALS141433       |        |                |                   |        |

*Group designations were noted within yellow hexagons in Figure 5.

1For clarity, gene names from Supplementary Table S2 were used here instead of the ORF designations on CGOB. SsALS2386 was called PICST_4539 on CGOB; CmALS4220 was called CMET_1838 on CGOB, and CmALS6800 was called CMET_3970.

2PICST_31095 belonged to the IFF/HYR family instead of the ALS family. LELG_04272 lacked an NT-Als domain but had tandem repeats and C-terminal sequence features similar to ALS genes. ScFLO1 belongs to a family of floculins (Goossens et al., 2011). Although floculins also have a central domain of repeated sequences, the structure of the mannos-binding N-terminal domain is clearly different from NT-Als (Figure 3).

3Although not ALS genes, CaFGR23 (orf19.1616), Cdb2280, and Ctr2409 were also in this ortholog pillar.
TABLE 4 | ALS gene representation within the Order Saccharomycotales*.

| Family | Genus/species | # ALS Loci |
|--------|---------------|------------|
| Debaryomycetaceae (taxid:766764); included in CUG-Ser1 (Figure 6) | *Candida albicans* (taxid:5476) | 8 |
| | *Candida dubliniensis* (taxid:43273) | 7 |
| | *Candida tropicalis* (taxid:5482) | 13 |
| | *Candida parapsilosis* (taxid:5480) | 5 |
| | *Candida orthopsilosis* (taxid:273371) | 3 |
| | *Candida metapsilosis* (taxid:273372) | 4 |
| | *Lodderomyces elongisporus* (taxid:36914) | 5 |
| | *Debaryomyces Hansenii* (taxid:4959) | 1 |
| | *Meyeromyza guillermondii* (taxid:4929) | 4 |
| | *Schéffersomyces stipitis* (taxid:4924) | 3 |
| | *Spathaspora passalidarum* (taxid:340170) | 29 |
| | *Yamadazyma tenuis* (taxid:2315449) | 1 |
| | *Clavispora lusitaniae* (taxid:36911) | 1 |
| | *Candida auris* (taxid:498019) | 3 |
| Metschnikowiaceae (taxid:27319); included in CUG-Ser1 (Figure 6) | *Saccharomyces cerevisiae* (taxid:4932) | 1 |
| | *Candida glabrata* (taxid:5478) | 1 |
| | *Pichia kudriavzevii* (taxid:4909) | 0 |
| Pichiaceae (taxid:1156497) | *Saccharomyces cerevisiae* (taxid:241407) | 37 |
| | *Yamadazyma tenuis* (taxid:2315449) | 1 |
| | *Clavispora lusitaniae* (taxid:36911) | 1 |
| | *Candida auris* (taxid:498019) | 3 |
| | *Spathaspora passalidarum* (taxid:340170) | 29 |
| | *Yamadazyma tenuis* (taxid:2315449) | 1 |
| | *Clavispora lusitaniae* (taxid:36911) | 1 |
| | *Candida auris* (taxid:498019) | 3 |
| | *Spathaspora passalidarum* (taxid:340170) | 29 |
| | *Yamadazyma tenuis* (taxid:2315449) | 1 |
| | *Clavispora lusitaniae* (taxid:36911) | 1 |
| | *Candida auris* (taxid:498019) | 3 |

*Family designations were from lifeman-ncbi-univ-lyon1.fr, as well as the work of Shen et al. (2018) and Li et al. (2021). Taxid numbers were from the NCBI Taxonomy Database (https://www.ncbi.nlm.nih.gov/taxonomy). Numbers of ALS loci (right column; top half of table) were taken from the genome sequences examined in this study (Table 1). Numbers of species with an available genome sequence in the NCBI Database (Accessed September 2021) are presented (left column; bottom half of table). Numbers of species with potential ALS loci are presented (right column; bottom half of table).

Table 4 indicates the position of some common ancestor (red dot) for the ALS family that would include both *S. cerevisiae* SAG1 (in the *Saccharomycetales*) and genes more closely resembling *C. albicans* ALS loci (in CUG-Ser1). BLAST searching using taxids did not reveal ALS loci in available genome sequences from the Lipomycetaceae, Trigonopsidaceae, Dipodascaceae, Trichomonascaceae, and the Sporopachydermia clade (Table 4). CUG-Ser1 was comprised of the Debaryomycetaceae, Metschnikowiaceae, and Cephaloascaceae (Shen et al., 2018). BLAST searching using taxids suggested that there were numerous additional ALS genes among the 88 Debaryomycetaceae species genomes in NCBI (accessed September 2021). Among the 62 Metschnikowia species, ALS sequences were found primarily in species closely related to *C. auris* and *C. lusitaniae* including *Candida haemulonii*, *Candida dublubulaeulonii*, and *Candida pseudohaemulonii*, all of which have been observed as pathogens (Jurardo-Martín et al., 2020). *C. intermedia*, noted for its ability to ferment xylene, as well as a rare cause of catheter-related fungemia (Ruan et al., 2010; Geijer et al., 2020) also had ALS sequences. No ALS sequences were detected in the Cephaloascaceae, although only 2 genomes were available (Table 4).

*Pichia kudriavzevii* (formerly *Candida kruzi*; Douglass et al., 2018) was included in Table 4 and in Figure 1 since the species is among those associated with clinical disease (Gomez-Gaviria and Mora-Montes, 2020). However, ALS genes were not detected in *Pichia kudriavzevii*, nor in the genomes of 60 other species from the Family Pichiaceae that were available in the NCBI database (Table 4). No ALS sequences were detected in the species included in the CUG-Ala clade (Figure 6). The lone BLAST hit in the Saccharomycopsisidaceae (Table 4; overlap with CUG-Ser2 in Figure 6) was based more on the presence of Ser/Thr-rich repeated sequences than on an NT-Als adhesive domain. Additional work would be required to validate the sequence and determine if an NT-Als domain was present.

Potential ALS genes in the Phaffomycetaceae (Table 4) were located in two species each in the genera Wickerhamomyces and Cyberlindnera; each locus encoded a potential NT-Als domain. Many additional ALS sequences were found among the 104 Saccharomycetaceae genomes available in NCBI. This family included *S. cerevisiae*, for which SAG1 was the only BLAST hit.
For other species such as Torulospora delbrueckii, a species of interest for wine making and baking (Fernandes et al., 2021), more than one ALS sequence was detected in the genome. One (TDEL_0E04030) predicted a protein that more closely resembled S. cerevisiae Sag1, while another (TDEL_0G04810) encoded an NT-Als domain (validated using AlphaFold; data not shown) attached to a complex C-terminal Ser/Thr-rich sequence. This predicted C-terminal domain was notable because it included 5 copies of the 45-amino acid tandem repeat found in S. cerevisiae Flo1 (Soares, 2010), suggesting recombination between the ALS and FLO families. A predicted protein in Lachancea thermotolerans (KLTH0C00440p) suggested an even-more-convincing recombinant that had an NT-Als domain attached to >30 copies of the Flo1 tandem repeat. These sequences require verification to ensure the recombinants were part of the genome, rather than an artifact of computational genome assembly. ALS sequences were also detected in Kazachstania species extending the representation of ALS sequences throughout the Saccharomycetaceae as defined by Shen et al. (2018). Many of these sequences appeared partial, suggesting the need for PCR amplification and repair to reveal the true nature of their encoded proteins. No ALS sequences were detected among the 21 Saccharomycodaceae species queried.

The taxonomic category Saccharomycetales incerta sedis had four ALS sequences, all in the species Diutina rugosa (formerly Candida rugosa), which is noted as an infrequent human pathogen (Ming et al., 2019). Evaluation of the best hit (DIURU_002000) showed a predicted secretory signal peptide and GPI anchor addition site. Within the NT-Als region, DIURU_002000 was 37% identical to C. albicans NT-Als3, had 8 Cys in the expected locations, Arg instead of the invariant Lys, and a strong predicted AFR. AlphaFold produced the same general structure for the DIURU_002000 NT-Als protein as for those shown in Figure 3 (data not shown).

Collectively, these observations pinpointed the location of the ALS family among currently available genome sequences and suggested potential taxonomic boundaries that will continue to be tested as more genome sequences are added to this region of the phylogenetic tree.

**DISCUSSION**

Availability of genome resources for pathogenic yeasts, as well as other closely related species, provided a comparative view of the ALS family across a broader expanse of the phylogenetic tree than previously investigated. Despite advances in long-read DNA sequence technologies, many ALS loci still required PCR amplification and Sanger sequencing to derive an accurate gene count and sequence. The availability of the unprecedented...
protein structural prediction accuracy of AlphaFold (Jumper et al., 2021) provided the opportunity to demonstrate conservation of NT-Als domain shape for the proteins listed in Supplementary Table S2. Recent addition of more than 200 new budding yeast genome sequences into the NCBI database (Shen et al., 2018) further bridged the gap between S. cerevisiae and C. albicans and provided a clearer picture of the ALS family breadth. In total, the information leads to the conclusions that S. cerevisiae Sag1 is an Als protein and the Als family is far more diverse than previously described.

Presence of the NT-Als domain is universal among the proteins in Supplementary Table S2, and clearly is a defining characteristic for inclusion in the Als family. The AFR is recognized as part of the NT-Als domain (Lin et al., 2014); this sequence was absent, weaker, or perhaps in another location in many of the newly characterized Als proteins described here. Immediately following the AFR in C. albicans Als proteins is a short Thr-rich region that some publications refer to as the T domain (reviewed in Hoyer and Cota, 2016). The T domain in C. albicans Als proteins ends at the start of the tandem repeat region. Designating a T domain in many of the proteins described here would require arbitrary judgement calls so the region was not included in any of the line diagrams (Supplementary Files S6-S11). NT-Als domains were drawn to include all amino acids up to the start of the repeated sequences or Ser/Thr-rich regions.

In some proteins (e.g. SpAls55077, SpAls134590, CIAls3274), sequences similar to the C. albicans T domain are expanded into repeated sequences of variable length in the center of the protein. While all C. albicans Als proteins have a region of highly conserved, 36-amino-acid tandemly repeated sequences, this part of the Als protein is quite variable among those from other species. Variations include tandem copies of novel lengths and sequences, repeated sequences that are not necessarily tandem copies, and complete lack of repeated sequences. The latter presentation is characteristic of S. cerevisiae Sag1 and other proteins more closely related to ScSag1 on the Figure 5 tree. Sequences most C-terminal in the Als family are also diverse, many featuring an enriched Ser/Thr composition. Ser/Thr richness promotes O-glycosylation that can form an extended structure on which the NT-Als domain is displayed on the cell surface (Jentoft, 1990). Some of the Als proteins have other amino acids (e.g. Glu, Gly, Pro) that are also enriched in the C-terminal portion of the protein or in novel, short repeated sequences.

Inclusion in the Als family is focused on the presence of an NT-Als domain that has the same general shape as the proteins described here (Figure 3). This Als family definition also recognizes instances of recombination between ALS genes and others encoding cell-surface proteins. A previous report (Oh et al., 2019) described CmALS2265, the product of recombination between ALS genes and the IFF/HYR family (Bailey et al., 1996; Bates et al., 2007; Chaffin, 2008; Boisramé et al., 2011; Riccombeni et al., 2012). In the present study, additional recombinants between ALS and IFF/HYR genes were noted in L. eponigsporus (LeALS734 and LeALS2716). The tantalizing possibility of ALS/FLO recombinants in T. delbrueckii and L. thermotolerans suggests even-greater interfamily recombination as a mechanism for generating diversity on the fungal cell surface. Recombination, as the mechanism to generate novel adhesins, has been discussed previously (Verstrepen et al., 2004; Verstrepen et al., 2005) although these descriptions involved recombination between genes in the same family. In contrast, interfamly recombination places the functional NT domain from one family onto the glycosylated stalk from another. Predicted structures for the NT domains of Als, Iff/Hyr, and Flo proteins were included in Figure 3 to highlight their obvious differences. Potential for hybrid function expands if the glycosylated stalk is more than just a pedestal for displaying the NT domain. These observations provide ample experimental hypotheses to pursue to better understand cell-surface diversity among the yeast species.

Another novel observation from the current work is the subtelomeric localization of ALS genes in multiple species including M. guilliermondii (MgALS2302, MgALS3259), C. auris (CauALS2582), and S. passalidarum (SpALS134176, SpALS137089). C. albicans subtelomeric regions are described as hotspots for genomic rearrangement, although adhesin-encoding genes are not located there (Dunn and Anderson, 2019). Subtelomeric location of adhesin genes is well-documented in other species such as C. glabrata (Xu et al., 2020). However, analysis of long-read-based genome sequences from three C. glabrata isolates suggested that changes in subtelomeric adhesin-encoding genes is due to break-induced replication, a relatively rare event that affects only the gene closest to the telomere (Xu et al., 2021). Muñoz et al. (2021) noted C. auris subtelomeric regions rich in genes encoding Iff/Hyr proteins, candidate cell wall proteins unique to that species, and CauAls2582. Analysis of genome sequences from 12 C. auris isolates representing diverse clades showed that Clade II isolates lose large subtelomeric regions and the cell wall genes associated with them (Muñoz et al., 2021). The consequences of ALS gene location in the subtelomeric region remain to be further explored in species such as M. guilliermondii and S. passalidarum.

By including Sag1 in the Als family, the definition of Als function also becomes more diverse. Sag1 binds a very specific ligand (the C-terminal peptide of α-agglutinin; Cappellaro et al., 1994) with high affinity. In comparison, C. albicans Als proteins can bind many ligands with lower affinity and rely on AFR-mediated protein aggregation to increase avidity (reviewed in Hoyer and Cota, 2016; Ho et al., 2019). Similar to those in C. albicans, several of the more-recently described Als proteins in other species function in adhesion to host cells or protein-coated surfaces (Bertini et al., 2016; Neale et al., 2018; Zoppo et al., 2018; Lombardi et al., 2019; Zoppo et al., 2020a; Zoppo et al., 2020b). However, considerable effort is still required to understand the specifics of these interactions. Information in Figures 3, 5 can be used to select proteins from along the newly defined Als family spectrum to better understand how structure contributes to functional variation. Experiments involving purified proteins and biophysical techniques could be pursued, or perhaps individual genes or chimeric constructs could be expressed in model systems such as the recently described ALS-negative C. var.
orthopsilosis strain (Zoppo et al., 2019). Experimentally demonstrated structural solutions could also be pursued, and in place of the difficult-to-crystallize ScSag1, proteins close to ScSag1 on the Figure 5 tree may serve as informative surrogates (e.g. Group J and CAGLOG04125G, which may be mating glycoproteins from the haploid species M. guilliermondii, D. hansenii, and S. stipitis).

The ALS alleles listed in Supplementary Table S2 are quite long; many are at least 5 kb and one is over 15 kb. In C. albicans, where multiple strains and ALS alleles have been examined, these long ORFs are maintained without interruption. One notable example is C. albicans ALS7 that experiences frequent expansions and contractions of multiple different repeated sequences and is transcribed at an extremely low level that may not produce sufficient protein for biologically meaningful adhesive interactions (Zhang et al., 2003; Green et al., 2005). In contrast, the current study revealed the first example of ALS pseudogenes. They are found in S. passalidarum that has amplified the ALS family to 29 loci, well beyond the census in other characterized species (Supplementary File S10). In addition to the ALS pseudogenes, many of the other S. passalidarum ALS loci encode proteins that are predicted to mislocalize compared to the C. albicans model: lack of a secretory signal peptide suggests protein that accumulates inside the cell rather than on the surface. The presence of loci that encode proteins with secretory potential, but lacking the ability to crosslink into the cell wall (i.e. no GPI anchor addition site), suggests the potential for S. passalidarum to export its own adhesion inhibitors. For example, a secreted, high-affinity binding protein could inhibit S. passalidarum adhesion to host cells, or perhaps release individual cells from host interactions or from polymicrobial structures such as biofilms. Wohlbach et al. (2011) pointed out that the wood-boring-beetle-associated S. passalidarum is also a commensal, similar to the typical relationship between humans and many of the fungi described here. However, only two of the SpAls proteins include all NT-Als features (8 Cys, invariant Lys, AFR) that are important for function of C. albicans Als3 (Supplementary Table S2: Supplementary File S10; Figure 4). The long branches associated with the S. passalidarum ALS sequences in Figure 5 could result from sequence substitutions due to lack of selective pressure or could indicate functional diversification that serves S. passalidarum in its ecological niche. The role of the Als proteins in S. passalidarum is another topic with many hypotheses for future exploration.

Additional hypotheses to test involve allelic variability and gene expression. Information reported here is limited to one allele from each species. Previous work in C. albicans revealed tremendous allelic variation, often involving copy numbers of repeated sequences, but sometimes affecting the NT-Als coding region (reviewed in Hoyer et al., 2008). Considerable variation is frequently observed between alleles within the same strain of a diploid species (Zhao et al., 2003; Zhao et al., 2007). It is reasonable to hypothesize that allelic variation exists among the genes and species described here. It is also reasonable to expect examples of differential gene expression with growth stage and morphology, gene expression variations between strains of the same species, and gene expression patterns that differ between in vitro and in vivo growth conditions (reviewed in Hoyer et al., 2008; Coleman et al., 2012; Galán-Ladero et al., 2019; Lombardi et al., 2019). There are many exciting avenues for investigation as the nature of the ALS family continues to be discovered.

**DATA AVAILABILITY STATEMENT**

The genome assembly for Lodderomyces elongisporus strain NRRL YB-4239, generated using Illumina MiSeq and Oxford Nanopore MinION data, is available in GenBank under BioProject accession number PRJNA432415, BioSample accession number SAMP08448784, and Genome Assembly accession number GCA_013620985.1 (ASM1362098v1). The genome assembly for Meyerozyma guilliermondii strain ATCC 6260, generated using Illumina MiSeq and Oxford Nanopore MinION data, is available in GenBank under BioProject accession number PRJNA432394, BioSample accession number SAMN08448312, and Genome Assembly accession number GCA_006942155.1 (ASM694215v1). The genome assembly for Scheffersomyces stipitis strain CBS 6054, generated using Illumina MiSeq and Oxford Nanopore MinION data, is available in GenBank under BioProject accession number PRJNA432364, BioSample accession number SAMN08446935, and Genome Assembly accession number GCA_006942115.1 (ASM694211v1). The genome assembly for Spathaspora passalidarum strain NRRL Y-27907, generated using Illumina MiSeq and Oxford Nanopore MinION data, is available in GenBank under BioProject accession number PRJNA432249, BioSample accession number SAMN08439027, and Genome Assembly accession number GCA_013620965.1 (ASM1362096v1).

**AUTHOR CONTRIBUTIONS**

LH conceptualized the study, acquired funding, and administered the project. KS, VV, CF, and AH conducted formal analysis. S-HO, AI, RR-B, CF, AH, and LH performed the investigation. CF, AH, and LH developed the study methodology. S-HO, KS, CF, AH, and LH wrote the original draft. All authors contributed to the article and approved the submitted version.

**FUNDING**

This work was funded by R15 DE026401 from the National Institute of Dental and Craniofacial Research, National Institutes of Health.

**ACKNOWLEDGMENTS**

This article is dedicated to the memory of Professor W. LaJean Chaffin, an outstanding scientist, mentor, and friend. Professor...
Chaffin shared her enthusiasm for medical mycology with many young researchers and helped them become established investigators. AI and RR-B were part of an Undergraduate Project in Fungal Genomics, which is a collaboration between the University of Illinois at Urbana-Champaign and Millikin University. Other students who contributed to this effort included Kaia Ball, Madeline Batek, Anton Bershanskiy, Stephen DeMartini, Erica Forbes, Zeidy Garcia, Jillian Jones, Jessie Kirk, Mariah McNamer, Deniz Namik, Quinn Nguyen, and Brooke Smith. The authors thank Drs. Travis Wilcoxen and Laura Zimmerman, Millikin University Department of Biology, for coordinating student participation and supervising research credit for students in the program.

**SUPPLEMENTAL MATERIAL**

The Supplemental Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.794529/full#supplementary-material
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