1 *CpALS4770* and *CpALS4780* contribution to the virulence of *Candida*
2 *parapsilosis*
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Abstract

The ability of yeast to adhere to biotic and abiotic surfaces represents an essential trait during the early stages of infection. Agglutinin-like sequence (Als) cell-wall proteins play a key role in adhesion of *Candida* species. *Candida parapsilosis* genome encompasses 5 *ALS* members, of which only the role of *CPAR2_404800* has been elucidated. The present project was aimed at investigating the contribution of *C. parapsilosis* Als proteins by generating edited strains lacking functional Als proteins. *CPAR2_404770* and *CPAR2_404780*, further indicated as *CpALS4770* and *CpALS4780*, were selected for the generation of single and double edited strains using an episomal CRISPR/Cas9 technology. Phenotypic characterization of mutant strains revealed that editing of both genes had no impact on the *in vitro* growth of *C. parapsilosis* or on morphogenesis. Notably, *CpALS4770*-edited strain showed a reduction of biofilm formation and adhesive properties to human buccal cells (HBECs). Conversely, single *CpALS4780*-edited strain did not show any difference compared to the wild-type strain in all the assays performed, while the double *CpALS4770-CpALS4780* mutant revealed an increased ability to produce biofilm, a hyper-adhesive phenotype to HBECs, and a marked tendency to form cellular aggregates. Murine vaginal infection experiments indicated a significant reduction in CFUs recovered from BALC/c mice infected with single and double edited strains, compared to those infected with the wild-type strain. These finding clearly indicate that CpAls4770 plays a role in adhesion to biotic and abiotic surfaces, while both *CpALS4770* and *CpALS4780* genes are required for *C. parapsilosis* ability to colonize and persist in the vaginal mucosa.

1. INTRODUCTION

Candida parapsilosis is commonly associated with systemic fungal infections among immunocompromised individuals, especially premature infants. Adhesion to host surfaces is a key factor in pathogenesis, but this process has not been extensively studied in *non albicans Candida* species. It is well known that *C. albicans* adhesion is mediated by a number of cell wall proteins and among them, the agglutinin-like sequence (Als) proteins are one of the best studied (Cota and Hoyer, 2015). Cross-hybridization analysis comparing *C. albicans ALS* sequences and genomic DNA with other *Candida* species suggested that similar genes are found in closely related fungi, including *C. parapsilosis* where 5 putative *ALS* genes were identified (Hoyer et al., 2001; Butler et al., 2009). Among these, the function of *CPAR2_404800* (*CpALS4800*) has been investigated through targeted gene disruption (Bertini et al., 2016) and the encoded adhesin has been demonstrated to play a role in *C. parapsilosis* virulence and pathogenicity (Bertini et al., 2016; Neale et al., 2018). Although a complete description of their structure and nomenclature has recently been proposed (Oh et al., 2019), functions of the remaining Als proteins in *C. parapsilosis* still need to be established.

The development of the CRISPR/Cas9 system as gene-editing technology has revolutionized gene function studies in *Candida* research, allowing to rapidly generate knock-outs and knock-ins in specific genetic *loci* and to simultaneously inactivate multiple members of gene families (Enkler et al., 2016; Grahl et al., 2017; Norton et al., 2017; Lombardi et al., 2019a; Zhu et al., 2019). The first evidence of the application of CRISPR/Cas9 system for gene editing in *C. albicans* was developed in 2015 by Vyas *et al.*(Vyas et al., 2015). This system was further improved in 2017, with the development of an episomal CRISPR/Cas9 system for *C. parapsilosis* gene editing, with a self-processing gRNA composed by the Hammerhead (HH) and hepatitis delta virus (HDV) ribozymes (Gao and Zhao, 2014) under the regulation of a RNA polymerase II promoter (Lombardi et al., 2017). Plasmid cure following the editing event allowed to minimize Cas9 expression, and therefore, the likelihood of off-target events. The same group recently implemented this system by replacing the HDV ribozyme with a tRNA sequence and used this approach for the efficient gene editing in the *C. parapsilosis* species complex (Lombardi et al., 2019a; Morio et al., 2019).

In the present work, the role of two uncharacterized *C. parapsilosis ALS* genes, *CPAR2_404770* and *CPAR2_404780*, further referred as to *CpALS4770* and *CpALS4780*,

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was investigated through the creation of single and double edited strains using the ribozymes-based CRISPR/Cas9 system (Lombardi et al., 2017) described above. The panel of mutant isolates was characterized for phenotypic traits such as growth rate, ability to produce pseudohyphae, biofilm formation both on plastic surfaces and glass beads, and adhesion on human epithelial buccal cells. Real-time RT PCR assay was used to evaluate the transcriptional levels of *C. parapsilosis ALS* expression following *CpALS4770* and *CpALS4780* inactivation. Finally, the pathogenic potential of the mutant collection was assessed in a murine model of vaginal candidiasis.

2. MATERIALS AND METHODS

2.1 *Candida parapsilosis* strain used in this study

A highly adhesive clinical isolate of *C. parapsilosis*, CP50 (Bertini et al., 2013), was used for the creation of single and multiple edited strains. Yeast cells were grown in YPD medium at 30°C. Selection of edited clones was performed on YPD agar plates supplemented with 100 μg/ml Nourseothricin (Nou, Werner BioAgents, Jena, Germany) (YPD-Nou). Sabouraud Dextrose medium (1% mycological peptone, 2% dextrose, Sigma Aldrich, Saint Louis, USA) (SAB) was used for morphology tests and biofilm formation experiments.

2.2 Computational design of guide RNA

For each target gene, gRNA selection was performed according to a bioinformatics analysis. We designed guide sequences corresponding to each annotated gene in the genome of *C. parapsilosis*, using updated guide selection and scoring methods (Doench et al., 2016). We listed all 20 nucleotides (nt) followed by NGG (or CCN plus 20 nt) in the genome. We kept only the guides that were hitting the coding regions of genes. To find putative off-targets, we took the 14 nt closest to the protospacer-adjacent-motif (PAM) sequence, constructed the four possible sequences of the PAM, and mapped these with Bowtie (http://bowtie-bio.sourceforge.net/index.shtml), allowing up to 3 mismatches. For each original guide, we calculated the on-target score using rule set 2 for sgRNA on-target activity script (described in Doench et al.(Doench et al., 2016)) as well as all the possible off-target scores for all the places in the genome that the 14-nt NGG hit with ≤3 mismatches (cutting frequency determination [CFD] scoring) (Doench et al., 2016). Guides were defined as having no off-targets if the off-target scores at other locations were lower

than 0.2. According to these criteria, 5' AAGCACAAAGGCAATAAACG 3' (- strand, Cas9 cutting site far 31 nt from the beginning of the gene) and 5' GGAGGCTCCAATACCAACCC 3' (+ strand, Cas9 cutting site 554 nt from the beginning of the gene) were selected for *CpALS4770 and CpALS4780* gene targeting, respectively.

2.3 Construction of pSAT1-Ribo plasmid for *CpALS4770 and CpALS4780* gene editing

The creation of *C. parapsilosis* edited strains relied on the use of the episomal CRISPR/Cas9 system described in *C. parapsilosis* (Lombardi et al., 2017). Plasmids used in this study, pUC57_HH_HDV_sgADE2B and pSAT1, were kindly provided by Geraldine Butler, University College Dublin, Dublin. Plasmid pUC57_HH_HDV_sgADE2B was linearized using primers GA7_pUC57F/GA7_pUC57R (Supplementary Table 1). The selected gRNA sequences for *CpALS4770* and *CpALS4780* gene editing were generated through primer extension using primers TOP-sgRNA-Cp4770/BOTTOM-sgRNA-Cp4770 and TOP-sgRNA-CPAR2_4780/BOTTOM-sgRNA-CPAR2_4780 (Supplementary Table 1) respectively, bearing 20 bp overlapping sequences at the 3' ends. The resulting 100 bp DNA fragment and the linearized pUC57_HH_HVD_sgADE2B plasmid were assembled together using Gibson Assembly® Master Mix (New England Biolabs, Massachusetts, USA), according to manufacturer's instructions. The resulting plasmids, harbouring the gRNA sequences specific for *CpALS4770* and *CpALS4780* gene targeting, were respectively named pUC57_RIBO_CpALS4770 and pUC57_RIBO_CpALS4780. The ribozyme cassette encompassing the GAPDH promoter and terminator, the specific gRNA sequence, and the HH-HDV ribozymes, was PCR amplified using primers GA_pSAT1_F/GA_pSAT1_R (Supplementary Table 1). The amplified fragment was introduced by Gibson Assembly into pSAT1 plasmid, previously linearized with NruI (New England Biolabs), thus generating the final constructs, pRIBO-sgCpALS4770 and pRIBO-sgCpALS4780, respectively.

2.4 Repair template synthesis

In order to introduce specific mutation in *CpALS4770* and *CpALS4780* gene sequences, two different repair templates, further referred as RT-4770 and RT-4780, were generated through primer extension. Primers RT_2xSTOP_CPAR2_4770 F/RT_2xSTOP CPAR2_4770R and RT_2xSTOP_CPAR2_4780F/RT_2xSTOP CPAR2_4780R

(Supplementary Table 1) were used for *CpALS4770* and *CpALS4780* gene editing, respectively. Each repair template generated had 40 bp homology regions arms at both ends and a 12 bp insertion in the middle encompassing two stop codons (TAA, TAG) and the KpnI restriction site.

2.5 *C. parapsilosis* transformation and screening of recombinant clones

Plasmids pRIBO-sgCpALS4770 and pRIBO-sgCpALS4780, harbouring the gRNA specific for *CpALS4770* and *CpALS4780,* respectively*,* were transformed in *C. parapsilosis* parental strain CP50 through electroporation. *C. parapsilosis* strain was grown overnight (ON) at 30°C in 5 ml of YPD broth. The following day cells were washed and suspended in 2 ml TE pH 7.5 supplemented with 10 mM Lithium Acetate and 60 μl of DTT 1M. After 1h incubation at 30°C with shaking, *Candida* cells were washed twice with ice-cold water and once with sorbitol 1M. The electro-competent pellet was then resuspended in 400 μl of sorbitol 1M and kept on ice. 5 μg of pRibo plasmid and 5 μg of the corresponding RT were mixed with 40 μl of competent *Candida* cells. Electroporation was performed using 0.2 cm cuvettes (Cell Projects Ltd, Harrietsham, United Kingdom) and Bio-Rad Gene Pulser (Bio-Rad, Hercules, USA), set at 1.8 kV, 25 μFD and 200 Ω. Following electroporation, cells were let recover for 3h at 30°C under static condition and then plated onto YPD-Nou agar plates.

CpALS4770 and *CpALS4780* transformed clones were screened by allele-specific PCR using primers 4770_EF/4770mut_R and 4780mut_F/4780R (Supplementary Table 1), respectively, recognizing the edited sequences. Genomic DNA (gDNA) from *C. parapsilosis* colonies that resulted positive by colony PCR screening was extracted. Q5 High-Fidelity DNA polymerase (New England Biolabs) was used to perform the PCR reaction using primers 4770EF/4770R for *CpALS4770* and 4780EF/4780R for *CpALS4780* (Supplementary Table1). Purified DNA fragments were then digested with KpnI (New England Biolabls) and sequenced (LIGHTRUN, GATC-Biotech, Konstanz, Germany).

2.6 Growth ability and pseudohyphal formation

The growth ability of *C. parapsilosis* edited strains was compared to their respective parental wild-type strains in YPD liquid media at 37°C. Briefly, a single colony for each strain was inoculated in 10 ml of YPD broth and incubated ON at 30°C with shaking. Following 18 h incubation, the optical density was measured at 600nm (OD_{600}) with a

1   spectrophotometer (Biophotometer, Eppendorf, Hamburg, Germany) and suspensions were

2   diluted in 100 ml YPD in order to reach an OD_{600} of 0.1. *C. parapsilosis* strains were

3   incubated at 37°C with shaking (220 rpm) and optical density was measured each hour in

4   disposable cuvettes (10 mm path length) (Kartell Labware, Noviglio, Italy).

5   The panel of *C. parapsilosis* mutant strains obtained in this study and the wild-type strain

6   was analyzed for their ability to undergo morphogenesis under inducing condition (YPD

7   broth supplemented with 10% FBS) according to a previously standardized protocol

8   (Bertini et al., 2016).

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10 **2.7 Biofilm formation assay on plastic surfaces**

11 Biofilm formation by *C. parapsilosis* strains was evaluated as previously described

12 (Tavanti et al., 2007). Briefly, strains were grown in SAB broth at 30°C, and the

13 concentration of each suspension, microscopically determined, was adjusted to 1.5×10^7

14 cell/ml. Twenty microliters of yeast suspension were added to 180 µl of SAB broth

15 supplemented with 8% glucose in a 96-well (round bottom) polystyrene microtiter plate

16 (Corning, New York, USA). Following a 24h incubation, biofilm biomass was evaluated

17 by spectrophotometric measurement, determining optical density of both biofilm matrix

18 and sessile cells embedded in the matrix. To this end, 100 µl PBS were added to each well

19 and optical density at 405 nm (OD_{405}) was measured using an automated plate reader

20 (Microplate Reader 550, Bio-Rad). Mean background values (negative control wells) were

21 subtracted from the mean value obtained for biofilm formed by each strain. Biofilm

22 biomass was expressed as biofilm biomass index (ratio between the OD_{405} detected for the

23 edited strain and the OD_{405} detected for CP50).

24 The cellular metabolic activity was evaluated by the XTT/menadione assay (Ramage et al.,

25 2001). Briefly, a 0.5 g/l XTT (Sigma-Aldrich) solution in PBS was mixed with a 1 µM

26 menadione (Sigma-Aldrich) solution in acetone. An aliquot of 100 µl of XTT/menadione

27 solution was added to each well and incubated in the dark at 37°C. Following a 2 h

28 incubation, 80 µl of the supernatant were then transferred into a new 96-well plate to

29 measure colorimetric development at 490 nm (OD_{490}). Background optical density of

30 negative control wells was subtracted from the mean value obtained from each strain and

31 metabolic activity was expressed as biofilm metabolic index (ratio between the OD_{490}

32 detected for the edited strain and the OD_{490} detected for CP50).

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2.8   Biofilm formation on glass beads and scanning electron microscopy (SEM) imaging

For biofilm formation on glass beads, overnight yeast suspension was diluted in SAB broth supplemented with 8% glucose (final concentration $10^5 \times$CFU/ml) and incubated in the presence of porous sintered glass beads (ROBU®, Germany) at 37°C for 24 h. The ratio between beads and diluted yeast suspension was 1 bead:1 mL. The presence of attached cells to the glass beads was evaluated by CFUs counting after the sonication of the beads. First, each bead was washed three times with 1ml sterile sodium-phosphate buffer (PBS) to remove unbound yeasts and placed in a 2ml-eppendorf tube containing 1ml of fresh PBS. Then, samples were vortexed for 30 seconds and sonicated in a BactoSonic ultrasound bath (BANDELIN electronic GmbH & Co. KG, Germany) for 1 minute, followed by an additional 30 seconds vortexing. Ten-fold serial dilutions of the sonication fluid were plated onto Sabouraud agar plates and the plates were incubated for 24 h at 30 °C for colony counting. For SEM imaging, yeast biofilms were grown on porous glass beads as described above. Afterwards, all beads were washed in ddH$_2$O to remove unbound fungal cells and fixed for 24h at 4°C (4% paraformaldehyde, 2.5 % glutaraldehyde in 0.05 M HEPES). Subsequently, the samples were dehydrated in 30, 50, 70, 90, 95, 100% ethanol, critical point dried, mounted on aluminium stubs, sputter coated with a 20 nm layer of gold palladium and examined in the SEM (ZEISS 1530 Gemini, Carl Zeiss Microscopy GbmH, Germany) operating using the in-lens electron detector. For picture processing images have been cropped, adjusted for optimal brightness and contrast (applied to the whole image) using Photoshop Lightroom (Adobe Systems, San Jose, Ca, USA).

2.9 Adhesion to human buccal epithelial cells (HBECs)

Adhesive properties of the mutant and wild-type strains were evaluated according to previously standardize method (Bertini et al., 2013; Bertini et al., 2016; Zoppo et al., 2018). Yeast cells were grown in YPD medium ON at 30°C, collected by centrifugation, washed and resuspended in PBS (pH 7.4) at a density of 1×10^8 cells/ml. Similarly, HBECs were washed in PBS (pH 7.4) then adjusted to a density of 1.0×10^5 cells/ml. HBECs and yeast cells were co-incubated at a ratio of 1:1000 for 45 min at 37°C with gentle shaking. Buccal cells were then collected by filtration through polycarbonate filters (pore diameter of 12 μm, Millipore), gram stained and examined by light microscopy at 1000× magnification. The number of yeast adherent to 100 HBECs was counted. At least three independent experiments were performed; in each experiment, co-incubation of yeast

cells and HBECs was performed in triplicate The donor of exfoliated buccal cells signed an informed consent in accordance with the Declaration of Helsinki. Local ethical Committee (Committee on Bioethics of the University of Pisa) approval was received for this set of experiments (Ref. no. 6/2019, Prot. No. 0012910/2019).

2.10 Gene expression analysis by real-time RT-PCR

Transcriptional levels of *C. parapsilosis ALS* genes were evaluated by real-time reverse transcription PCR (RT-PCR) from total RNA. Each strain was inoculated in 10 ml of YPD at 30°C ON and 500 μl of this suspension were inoculated in 20 ml of YPD for 24 h at 30°C. Cells were recovered by centrifugation at 4500 rpm for 5 min and washed twice with 10 ml of PBS and total RNA was extracted from 5×10^7 cells using Nucleospin RNA (Machery Nagel, Düren, Germany), according to manufacturer's instructions. Spectrophotometric analysis, using UVette® (10 mm path length, Eppendorf, Milan, Italy), was used to determine the quantity of extracted RNA and to check for the presence of protein contamination. cDNA was synthetized, with random primers, using 0,7-1 μg of total RNA as template, through the Reverse Transcription System kit (Promega, Madison, USA), following manufacturer's instructions. Gene expression levels were analyzed by real-time PCR, using primers CPAG_05054NF/CPAG_05054NR for *CpALS4790*, CPAG_05056F/CPAG_05056R for *CpALS4800*, CPAG_05314F/CPAG_05314R for *CpALS660*, CPAG_00368F/CPAG_00368R for *CpALS4770*, CPAG_00369F /CPAG_00369R for *CpALS4780* and lastly ACT1_RT/ACT2_RT for actin, used as reference housekeeping gene (Table 2.2). SsoAdvancedTM universal SYBR Green supermix (Bio- Rad, Hercules, USA) was used to perform the Real-time PCR in 96 well plates on CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, USA), following manufacturer's instruction. Each primer pair produced a single amplicon with a uniform melting curve. The transcription level of detected genes was calculated using the formula of $2^{-\Delta\Delta Ct}$ (Bertini et al., 2016; Zoppo et al., 2018; Lombardi et al., 2019b).

2.11 Murine model of vaginal candidiasis

The pathogenicity of *C. parapsilosis* parental and edited strains was tested in a murine model of vaginal candidiasis. *In vivo* experiments were conducted following the ethical protocol approved by the committees on the Ethics of Animal Experiments of the "Istituto Superiore di Sanità", Rome, Italy (Permit number: DM 227/2016-B dated 22/12/2016).

Briefly, groups of 5 BALB/c mice were intravaginally inoculated with a 1×10^6 yeast/20 μl. Fungal vaginal burden was monitored over an infection period of 3 weeks. 100 μl of undiluted and serially diluted vaginal fluids, taken at designated times (0, 1, 2, 5, 7, 14 and 21 days), were seeded, in triplicate, onto chloramphenicol (50 mg/L) supplemented Sabouraud dextrose agar plates. Cultures were incubated at 30°C for 48-72 h.

2.12 Statistical analysis

Statistical analysis of mean adhesion was performed using GraphPad Prism software (version 6.05 for Windows, La Jolla, CA USA). One-way ANOVA followed by Bonferroni's post-hoc test was used to evaluate the differences in biofilm formation, adhesion ability, gene expression levels and CFU values obtained in murine infection experiments. A P value <0.05 was considered statistically significant.

3. RESULTS

3.1 Screening and identification of *C. parapsilosis CpALS4770* and *CpALS4780* edited clones

An episodic CRISPR/Cas9 strategy was used in order to inactivate *CpALS4770* and *CpALS4780* genes using the *C. parapsilosis* highly adhesive clinical isolate CP50 as parental strain. Screening was performed by allele specific PCR using primers 4780mut_F/4770R recognizing the correct editing of the *CpALS4780* gRNA according to the repair template used yielding a PCR product of 428 bp (Supplementary Fig. 1A).

The genotype of *C. parapsilosis* clones that resulted positive by colony PCR screening was confirmed by restriction analysis. As shown in Fig. 1A, the PCR product obtained with primers 4780EF/4780R (1400 bp) was digested with KpnI generating two fragments of 993 bp and 407 bp. This result confirms the correct editing of the *CpALS4780* gene, attesting the presence of a KpnI site and therefore, the use of the repair template RT-4780 for the repair of the DBS induced by Cas9 (Fig. 1A). Among all the clones resulted positive to the restriction analysis one clone was selected for sequencing of the gRNA region. Sequencing results obtained with primers 4780EF/4780R confirmed the expected genotype, with the insertion of two stop codons and a KpnI restriction site in the coding sequence of *CpALS4780* mutant strain (Fig. 1B). The edited strain was named CP50-ed4780.

CP50 and the single edited strain CP50-ed4780, were used as parental strains for the creation of *CpALS4770* single and double edited strains, respectively. Edited clones were screened by allele specific PCR using primers 4770_EF/4770mut_R. A DNA band of the expected molecular weight (372 bp) was obtained for all genomic backgrounds used (Supplementary Fig. 1B). Amplification with primers 404770_EF/404770R and subsequent digestion of the PCR product (914 bp) with KpnI generated DNA bands with the expected molecular weight (363 bp and 551 bp) (Fig. 1C). These results attest the correct integration of the repair template RT-4770 in *CpALS4770*. Sequencing of the gRNA region obtained with primers 4770EFF/4770R (Fig. 1D) confirmed the expected genotype. As a result, the edited strains obtain from CP50 and CP50-ed4770 parental strains were named CP50-ed4770 and CP50-ed4770/4780, respectively.

3.2 Effect of *CpALS4770* and *CpALS4780* gene inactivation on growth ability and *in vitro* morphological properties of *C. parapsilosis*

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The impact of the inactivation of genes encoding for cell wall anchored proteins on *C. parapsilosis* growth ability was evaluated in YPD medium at 37°C for 24 hours. The graph shown in Fig. 2A shows the results obtained for parental strain CP50, CP50-ed4770, CP50-ed4780, CP50-ed4770/4780. Growth curves obtained from the individual strains indicate no difference between the wild-type and edited strains of *C. parapsilosis*.

Fungal cell morphology of CP50 strain and related mutant strains (CP50-ed4770, CP50-ed4780, CP50-ed4770/4780) grown in SAB or YPD media at 37°C was observed with bright field light microscopy. As shown in Fig. 2B, which reproduces representative micrographs of each strain grown in SAB, CP50 and CP50-ed4780 presented a typical *C. parapsilosis* round morphology. Interestingly, CP50-ed4770/4780 and, to a lesser extent CP50-ed4770, produced elongated cells which tended to form cellular aggregates that were particularly evident in the case of the double mutant strain. When grown in YPD medium, only CP50-ed4770/4780, but not CP50-ed4770, showed slightly elongated cells and a mild tendency to form cellular aggregates (Figure 2C).

Unlike *C. albicans*, *C. parapsilosis* is unable to form true hyphae, but under inducing conditions, such as incubation at 37°C and the presence of serum, cells produce elongated forms with constrictions at the cell-cell junctions, also known as pseudohyphae. As illustrated in Fig. 2C, after 24h at 37°C in YPD broth supplemented with 10% FBS, all the edited *C. parapsilosis* strains were equally able to form pseudohyphae, demonstrating that inactivation of *CpALS4770* and *CpALS4780* or both genes contextually did not affect morphogenesis. When grown in YPD medium, the double CP50-ed4770/4780 mutant strain, and to a lesser extent Cp50-ed4770, produced elongated cells, which tended to form cellular aggregates, even in the absence of FBS (Fig. 2C).

3.3 Impact of *CpALS4770* and *CpALS4780* editing on *C. parapsilosis* ability to form biofilm on abiotic surfaces.

Biofilm formation assays were set up to evaluate the ability of all the edited strains obtained in this study to produce biofilm on abiotic surfaces, such as plastic surfaces and glass beads. Biofilm production on plastic surfaces was performed in 96-well microtiter plates in the presence of SAB supplemented with 8% glucose. Following 24h incubation at 37°C, biofilm formation was evaluated assessing both biofilm cellular density and cellular metabolic activity. As illustrated in Fig. 3A, while CP50-ed4780 produced the same extent of biofilm as the parental strain, CP50-ed4770 showed a marked reduction in its ability to form biofilm as compared to its parental strain. Surprisingly, the double edited strain

CP50-ed4770/4780 displayed a higher capacity to produce biofilm as compared to the wild-type strain. Metabolic activity results (Fig. 3B) confirmed the trend observed for biomass data.

Biofilm production by the panel of parental and mutant strains was also evaluated on glass beads. *C. parapsilosis* wild type and edited strains were grown in SAB broth supplemented with 8% glucose and incubated at 37°C for 24 h in the presence of glass beads. Fig. 4 shows the biofilm produced by *C. parapsilosis* parental and edited strains on glass beads using SEM microscopy. As shown in Figure 4, *C. parapsilosis* single mutant strains, CP50-ed4770 (Fig.4C-D) and CP50-ed4780 (Fig.4E-F), and double mutant strain CP50-ed4770/4780 (Fig.4G-H), adhered and produced biofilm on glass beads to the same extent as the wild-type strain (A-B). No significant differences were observed in CFU number of wild type and mutant strains dislodged from biofilm under these experimental conditions (data not shown).

3.5 Inactivation of *CpALS4770* and *CpALS4780* differently affects the adhesion properties of *C. parapsilosis* on human buccal epithelial cells (HEBCs)

An *in vitro* adhesion assay to HBECs was used to evaluate the adhesive properties of the mutant and parental strains, in order to better understand the role played by *CpALS4770* and *CpALS4780* in *C. parapsilosis* adhesion to host surfaces.

As illustrated in Fig. 5, *CpALS4770* gene editing caused a reduced ability of *C. parapsilosis* mutant strain to adhere to HBECs cells as compared to the parental strains (3,9-fold reduction). Conversely, *CpALS4780* gene editing did not affect the adhesion of *C. parapsilosis* to HBEC. Interestingly, the simultaneous inactivation of *CpALS4770* and *CpALS4780* resulted in an enhanced ability of *C. parapsilosis* to adhere on HBECs. As shown in Fig. 5, the double mutant strain CP50-ed4770/4780 showed a hyper-adhesive phenotype, with a 2,6-fold increase in the adhesion index as compared to the corresponding parental strain (P<0.0001, one-way ANOVA followed by Bonferroni's *post-hoc* test). Such a hyper-adhesive phenotype observed for the mutant strains also correlated with a marked tendency to form cellular aggregates, as also visible from the micrographs shown in Fig. 5, below the histograms.

3.6 *ALS* gene expression profiles in *CpALS4470* and *CpALS4780* gene edited strains

To evaluate how the lack of functional CpAls4770p and CpAls4780p impacts on the expression profile of *CpALS* genes, quantitative gene expression analysis was performed

on *C. parapsilosis* wild type and mutant strains. *C. parapsilosis* strains were grown in YPD medium and in the same *in vitro* conditions used for preparation of fungal suspensions in HBEC adhesion assays. As shown in Fig. 6, in the experimental conditions tested, reduced levels of *CpALS4780* and *CpALS4800* mRNA were observed in CP50-ed4770. In contrast, CP50-ed4780 mutant strain did not show differences in the expression level of any *CpALS* gene. Notably, an overexpression of *CpALS4780* was detected in the CP50 double mutant strain. Even with marked trends detected, the results obtained did not reach the level of statistical significance, due to the high experimental variability observed.

3.11 *In-vivo* model of vaginal infection

The pathogenic potential of the *C. parapsilosis* parental and edited strains was assessed in an *in-vivo* model of murine vaginal candidiasis. Following vaginal challenge, BALB/c mice were monitored for fungal vaginal burden over an infection period of 3 weeks. As shown in Fig. 7, the number of CFUs recovered from mice infected with CP50-ed4770, CP50-ed4780 and CP50-ed4770/4780 was significantly lower with respect to those recovered from wild type (CP50). Differently from CP50-ed4780 and CP50- ed4770/4780 where this trend was maintained throughout the entire course of the vaginal monitoring, reduction of CP50-ed4770 recovered from infected mice was statistically significant only in the early stages of the infection, while significance was lost at late time-points. These findings suggest that the lack of CpAls4770p or CpAls4780p or their simultaneous inactivation results in a significant reduced ability to colonize and persist on the vaginal mucosa.

4. DISCUSSION

In this study we described the inactivation of two functionally uncharacterized *ALS* genes of *C. parapsilosis, CpALS4770* and *CpALS4780.* The episomal CRISPR/Cas9 system (Lombardi et al., 2017) and a repair template carrying two stop codons were used for the successful creation of single edited strains (CP50-ed4770, CP50-ed4780) in *C. parapsilosis* CP50 strain, a clinical isolate previously characterized as highly adhesive to HBECs (Bertini et al., 2013) and a double edited strain (CP50-ed4770/4780) generated in CP50-ed4780 genetic background.

Microscopical analysis of edited and parental strains indicated a tendency for CP50-ed4770 and CP50- ed4770/4780 strains to form elongated cells and to produce cellular

aggregates when grown in Sabouraud broth. This phenotype was conserved in YPD medium for CP50-ed4770/4780, but not for CP50-ed4770. This cellular behaviour, which was more pronounced in the double mutant strain, was also observed by Zhao and colleagues in *C. albicans ALS5*, *ALS6* or *ALS7* knock-out strains (Zhao et al., 2007). A possible explanation could reside in the structural rearrangements of the fungal cell-wall proteins that occur in response to the lack of functional CpAls4770 and CpAls4780 proteins resulting in the exposure of other adhesive moieties on the *C. parapsilosis* surfaces.

Phenotypic analysis of wild type and edited strains confirmed that the deletion of *CpALS4770* and *CpALS4780* did not affect the growth rate of the yeast in YPD medium. Since in *C. albicans ALS* genes are associated with hyphal formation (Hoyer et al., 1998; Argimón et al., 2007), a morphogenesis assay was used to evaluate the effect of the inactivation of *CpALS4770*, *CpALS4780* or both in the ability of *C. parapsilosis* strains to produce pseudohyphae. All mutant strains were able to undergo morphogenesis under inducing conditions, indicating that neither *CpALS4770* nor *CpALS4780* play a major role in this process. This finding is in agreement with results previously observed for *C. parapsilosis* deletion of *CpALS4800* and for *C. orthopsilosis CoALS4210* inactivation (Zoppo et al., 2018). A biofilm formation assay (Ramage et al., 2001; Tavanti et al., 2007) was used to investigate a possible role for *CpALS4770* and *CpALS4780* in *C. parapsilosis* biofilm formation process, as previously reported for *ALS3* in *C. albicans* (Nobile et al., 2006). Both CP50 and CP50-ed4780 strains were able to produce biofilm with the same extent while CP50-ed4770 had a statistically significant reduction in both biofilm biomass and metabolic activity. Notably, the double *CpALS4770*/*CpALS4780* mutant displayed a marked increase in biofilm production as compared to wild type strain. These results indicate that, while *CpALS4770* seems to be important in biofilm formation, *CpALS4780* does not appear to be directly implicated in this process. The peculiar phenotype of CP50-ed4770/4780 could be explained by the up-regulation of other members of the *ALS* gene family resulting in a more adherent strain. Alternatively, the lack of CpAls4770p and CpAls4780p may also have caused an alteration of the cell-wall structure organization that exposes other adhesive moieties of the *C. parapsilosis* surfaces, resulting in an increased aggregative phenotype. As supported by SEM analysis, a minimum extent of biofilm formation could be detected on glass beads for all *C. parapsilosis* parental and edited strains; however, no difference was observed between the wild type and edited strains biofilm production. The involvement of *C. albicans* Als proteins on the adhesion to glass

surfaces has been already described by Aoki and colleagues (Aoki et al., 2012) through heterologous expression experiments performed in *Saccharomyces cerevisiae*. In this study, each *ALS* gene was found to contribute to the adhesion on glass surfaces. Therefore, the effect of the inactivation of *CpALS4770* and *CpALS4780* on glass beads biofilm could have been masked by the adhesion ability of the remaining *ALS* genes of *C. parapsilosis*.

The role of *CpALS4770* and *CpALS4780* in the adhesion of *C. parapsilosis* to biotic surfaces was assessed in an *in vitro* model, of co-incubation of yeast cells in the presence of human buccal epithelial cells (HBECs) (Bertini et al., 2013; Bertini et al., 2016; Zoppo et al., 2018). While *CpALS4770* seemed not to be involved in the adhesion of *C. parapsilosis* to epithelial human surfaces, a reduction in the adhesion ability of CP50-ed4770 mutant strains if compared with their relative parental strain was observed. Similarly to what observed in biofilm production assays, the contextual inactivation of *CpALS4770* and *CpALS4780* resulted in significantly increased adhesion to HBECs. Such peculiar phenotype was already described by Zhao and co-workers (Zhao et al., 2007) when adhesion of *C. albicans* mutant strains of *ALS5*, *ALS6* or *ALS7* was tested on human vascular endothelial cell monolayers and buccal epithelial cells. As previously mentioned for biofilm formation, this finding could be linked to a re-organization of the cell-wall structure or to an up-regulation of other *ALS* genes in the double mutant.

To verify the last hypothesis, the transcription levels of *C. parapsilosis ALS* genes were evaluated through real-time RT-PCR in wild type and mutant strains. Even though the alterations in expression levels were conserved in all three independent experiments, no difference reached statistical significance, due to experimental variability. However, CP50-ed4770 showed a reduction in *CpALS4790* and *CpALS4800* mRNA levels, which could at least partially explain the reduced adhesion ability observed with the HBECs assay. Both *CpALS4800* (Bertini et al., 2016) and *CpALS4790* (unpublished results) are in fact required for the adhesion of *C. parapsilosis* to buccal epithelium. Moreover, the double mutant strain, CP50-ed4770/4780, displayed a 27-fold overexpression of *CpALS4780,* suggesting an attempt of *C. parapsilosis* to overcome the presence of truncated CpAls4780 protein. In *C. albicans* it has been shown that *ALS1* overexpression leads to extensive flocculation and an aggregative phenotype (Huang, 2012). In this case, the only overexpressed gene in the double mutant strain is the edited *CpALS4780,* which produces a non-functional adhesin. Since this truncated protein encompasses only the first 185 amino acids, we can exclude the possibility that the amyloid-forming region (AFR) is responsible for the aggregative phenotype observed in the double mutant strain (Garcia et al., 2011; Oh et al., 2019). A

proteomic analysis of the cell wall structure of single and double edited strains coupled with a measurement of hydrophobic forces involved in cell adhesion by atomic force microscopy (AFM) may help to elucidate the observed phenotype (El-Kirat-Chatel et al., 2015).

Finally, data obtained from the murine model of vaginal candidiasis clearly indicated that the single or contextual deletion of these genes causes a significant reduction in the ability of *C. parapsilosis* to persist in the vaginal mucosa, compared with the wild type strain. To conclude, these findings confirm that both *CpALS4770* and *CpALS4780* contribute to the pathogenesis of murine vaginal candidiasis.

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Supplementary Table 1: Primer sequences used in this study.

Primer	Sequence 5' →3'	Usage
Generation of pRIBO-sgCpALS4770 and pRIBO-sgCpALS4780		
GA7_pUC57F (Lombardi et al., 2017)	TTTAGAGCTAGAAATAGCAAGTTAA AATAAGGCTA GTCC	Linearization of pUC57_HH_HDV_sgA DE2B
GA7_pUC57R (Lombardi et al., 2017)	GAATTTGGAAAAGAAAGAAAGAAA GAAGGAAGG AAGG	Linearization of pUC57_HH_HDV_sgA DE2B
TOP sgRNA Cp4770	TTCTTTCTTTTCCAAATTCAGTGCTT CTGATGAGTCCGTGAGGACGAAAC GAGTAAGCTCGTC	Generation of the *CpALS4770* sgRNA
BOTTOM sgRNA Cp4770	GCTATTTCTAGCTCTAAAACCGTTT ATTGCCTTTGTGCTTGACGAGCTTA CTCGTTTCGT	Generation of the *CpALS4770* sgRNA
TOP sgRNA CPAR2_4780	TTCTTTCTTTTCCAAATTCAGCCTCC CTGATGAGTCCGTGAGGACGAAAC GAGTAAGCTCGTC	Generation of the *CpALS4780* sgRNA
BOTTOM sgRNA CPAR2_4780	GCTATTTCTAGCTCTAAAACGGGTT GGTATTGGAGCCTCCGACGAGCTTA CTCGTTTCGT	Generation of the *CpALS4780* sgRNA
GA_pSAT1_F (Lombardi et al., 2017)	CAGTGAATTGGAGATCGGTACTTCG GTACTTTGGTGTAACTGG	Amplification of the ribozyme cassette
GA_pSAT1_R (Lombardi et al., 2017)	GTGTACCGGTATCTCGACGCATTCG CTTTATTTATAAACTCATATACGAA AAATATATAAAC	Amplification of the ribozyme cassette
Repair template synthesis		
RT_2xSTOP_CPAR2_4 770 F *	CCACAAATCCAATGATTAAACAATT ATCATTTGCC**TAATAG**==GGTACC==TT	*CpALS4770* repair template synthesis
RT_2xSTOP_CPAR2_4 770 R *	TGCTTGAACGAAGGTTGTAAGCACA AAGGCAATAAA==GGTACC==**CTATTA**	*CpALS4770* repair template synthesis
RT_2xSTOP_CPAR2_4 780 F *	ACCACTGCAAACTTCCAAGGAGGCT CCAATACCAAC**TAATAG**==GGTACC==	*CpALS4780* repair template synthesis
RT_2xSTOP_CPAR2_4 780 R *	ACACGATTGTTGAAGACAATGTTAT TAGTTGATGTG==GTACC==**CTATTA**GT	*CpALS4780* repair template synthesis
Screening and sequencing of edited clones		
4770_EF	CACCTCCTTTACGGCATTGT	*CpALS4770* sequencing
4770R	TGCTGTATACGATATCTTCG	*CpALS4771* sequencing

4770mut_R	ACAAAGGCGGTACCTTACTA	*CpALS4770* allele specific PCR
4780mut_F	CAATACCAACTAATAGGGTACC	*CpALS4780* allele specific PCR
4780R	CTCCCCATTTTATTGATTGTGAG	*CpALS4780* sequencing
4780EF	CCAACTCGTGGATTGCTTCT	*CpALS4780* sequencing
Real-time PCR		
CPAG_00368F	TGTCCTCGACAACTCCAGCTT	*CpALS4770* real-time amplification
CPAG_00368R	GGTTCTAAAATGGGTGGAATGG	*CpALS4770* real-time amplification
CPAG_00369F	AACGTCCAACAGGTCAAGTG	*CpALS4780* real-time amplification
CPAG_00369R	CTCCCCATTTTATTGATTGTGAG	*CpALS4780* real-time amplification
CPAG_05054NF	TCGAGTTCCTAATGGTGCAG	*CpALS4790* real-time amplification
CPAG_05054NR	CCTTCTTCACCCCAGTTTTG	*CpALS4790* real-time amplification
CPAG_05314F	GGGATCAGCAAATTCTGTCGA	*CpALS660* real-time amplification
CPAG_05314R	CCAGCGGTAAAACATTGGGA	*CpALS660* real-time amplification
CPAG_05056F	AAAGTCACCACCACCGAGGTT	*CpALS4800* real-time amplification
CPAG_05056R	CGGCGCAGATGTGCTAATG	*CpALS4800* real-time amplification
ACT1_RT	AGTGTGACTTGGATGTCAGAAAGGAATTGT	Actin real-time amplification
ACT2_ RT	ACAGAGTATTTTCTTTCTGGTGGAGCA	Actin real-time amplification

*: Stop codons are in red and bold; KpnI restriction site is highlighted in yellow.

FIGURE LEGENDS

Supplementary Fig. 1: Screening of *CpALS4770* and *CpALS4780* edited clones. **A)** Screening of *C. parapsilosis CpALS4780* recombinant clones obtained using CP50 as parental strain. Correct editing of the *CpALS4780* was evaluated by allele specific PCR using primers 4780mut_F/4780R. The upper panel shows a schematic representation of wild type and edited *CpALS4780* gene, primer position and expected molecular weight (428 bp) of the PCR product. The lower panel shows the PCR result. 1-14: recombinant clones; 15: CP50 gDNA; NC: negative control (no gDNA); M: 1Kb Gene Ruler 1kb DNA ladder (Thermo Scientific). **B)** Screening of *C. parapsilosis CpALS4770* recombinant clones obtained using CP50 and CP50-ed4780 as parental strains. Correct editing of the *CpALS4770* was evaluated by allele specific PCR using primers 4770_EF/4770mut_R. The upper panel shows a schematic representation of wild type and edited *CpALS4770* gene, primer position and expected molecular weight (372 bp). The lower panel shows the PCR result. 1-5: recombinant clones obtained using CP50 as parental strain; 6-10: recombinant clones obtained using CP50-ed4780 as parental strain ; 11: CP50 gDNA; NC: negative control (no gDNA); M: 1Kb Gene RulerTM 1kb DNA ladder (Thermo Scientific).

Fig. 1: KpnI restriction analysis and sequencing of *CpALS4780* and *CpALS4770* transformant clones. **A)** *CpALS4780* gRNA region was amplified in the parental strain (CP50) and transformant clones with primers 4780EF/4780R yielding a PCR product of 1400 bp. As shown in the electrophoresis gel, digestion with KpnI generates two fragments of 993 bp and 407 bp, respectively, only if the DSB caused by Cas9 is repaired using RT-4780. M: 1Kb Gene Ruler 1kb DNA ladder (Thermo Scientific); 1: KpnI digestion products of *CpALS4780* gRNA region obtained from putative edited clone; 2: KpnI digestion product of *CpALS4780* gRNA region obtain from CP50 parental strain. **B)** Sequencing results of *CpALS4780 locus* confirmed the presence of two stop codons (TAA and TAG) and the KpnI site (GTTACC) allowing the identification of the CP50-ed4780 edited strain. **C)** *CpALS4770* gRNA region was amplified with primers 4770EF/4770R yielding a PCR product of 914 bp. KpnI digestion led to the generation of two fragments of 551 bp and 363 bp only if the DSB caused by Cas9 is repaired using RT-4770. M: 1Kb Gene Ruler 1kb DNA ladder (Thermo Scientific); 1: KpnI digestion product of *CpALS4770* gRNA region obtained from ATCC 22019; 2, 3, 4: KpnI digestion products of *CpALS4780* gRNA region obtained from putative edited *CpALS4770* and *CpALS4770/ CpALS4780* clones, respectively. **D)** Sequencing

results of *CpALS4770 locus* confirmed the presence of two stop codons (TAA and TAG) and the KpnI site (GTTACC) allowing the identification of CP50-ed4770 and CP50ed4770/4780 strains.

Fig. 2: Growth abilities and morphognesis. A) Growth ability of parental and edited strain. Growth curves of CP50, CP50-ed4770, CP50-ed4780, CP50-ed4770/4780 strains in YPD medium at 37°C. **B)** Optical microscopy imaging of CP50 and edited strains. Pictures were taken *from C. parapsilosis* wild type and edited clones grown for 24 h in Sabouraud broth at 37°C. Scale bars denote 10 μm. **C)** Production of pseudohyphae by the panel of edited strains. *C. albicans* SC5314 was included as positive control for filamentation (true hyphae). Morphogenesis was induced in YPD broth in presence of 10% FBS. Following 24 h of incubation at 37°C, 10 μl of each culture was directly observed with an optical microscope at 400 × magnification. Scale bars denote 10 μm.

Figure 3: **Evaluation of biofilm formation in the panel of reference and edited *C. parapsilosis* strains obtained in his study. A)** Total biofilm biomass determined by measuring optical density at 405 nm, following an incubation in Sabouraud+8% glucose for 24 h at 37°C; **B)** Cellular metabolic activity was measured with the XTT/menadione assay, with colorimetric development measured at 490 nm on biofilm formed as described for biomass evaluation. Error bars represent standard error of means of 2 independent experiments, each performed in at least eight replicates. One-way ANOVA followed by Bonferroni's post-hoc test was used to evaluate differences biofilm formation (*******: P<0.001; ******: P<0.01).

Fig. 4: SEM microscopy performed on biofilm produced on glass beads.
Each panel shows *C. parapsilosis* parental and edited strains adherent to glass beads surfaces. Left column: (overview images) biofilm grown between sintered glass particles of the glass bead. Scale bars denote 20 μm. Right column: (close-up images) *C. parapsilosis* cells with varying amounts of network-like extracellular matrix. Scale bars denotes 2 μm. CP50 (A, B); CP50-ed4770 (B, C); CP50-ed4780 (E, F); EP50-4770/4780 (G, H).

Fig. 5: Evaluation of the adhesive properties of reference and edited *C. parapsilosis* strains to HBECs. Adhesive properties of the *C. parapsilosis* edited strains obtained in this study are compared to their corresponding parental strain. Bars represent adhesion index mean ± standard error of mean. At least three biological replicates were used; *******: P<0.001; ******: P<0.01. Micrographs below bars show representative Gram-stained *C. parapsilosis* yeast cells from each strain adhered to a buccal cell observed at a magnification of 1000 ×. Scale bars denote 10 μm.
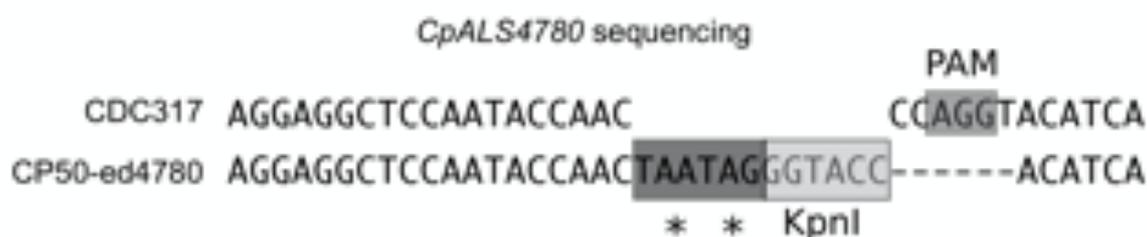
Fig. 6: Transcriptional levels of *CpALS* genes in *C. parapsilosis* CP50, CP50-ed4770, CP50-ed4780 and CP50-ed4770/4780 strains (A-E). Relative expression of each gene in edited strains is normalized on actin and expressed as relative to wild-type values. Error bars represent standard error of three independent experiments, each performed in triplicate. *****: P<0.05

Fig.7: Time course of murine vaginal candidiasis. Five BALB/c mice for each strain (CP50, CP50-ed4770, CP50-ed4780, CP50- ed4770/4780) were intra-vaginally infected with $10^6 \times$ cells/20 μl. Bars represent mean values ± standard error of the mean of *C. parapsilosis* CFUs recovered from vaginal fluids at different time points. *P<0.05; **P< 0.01, ***P< 0.001.
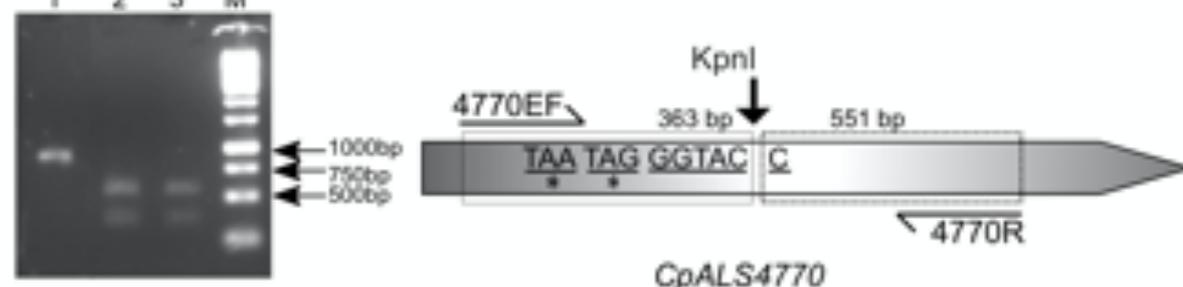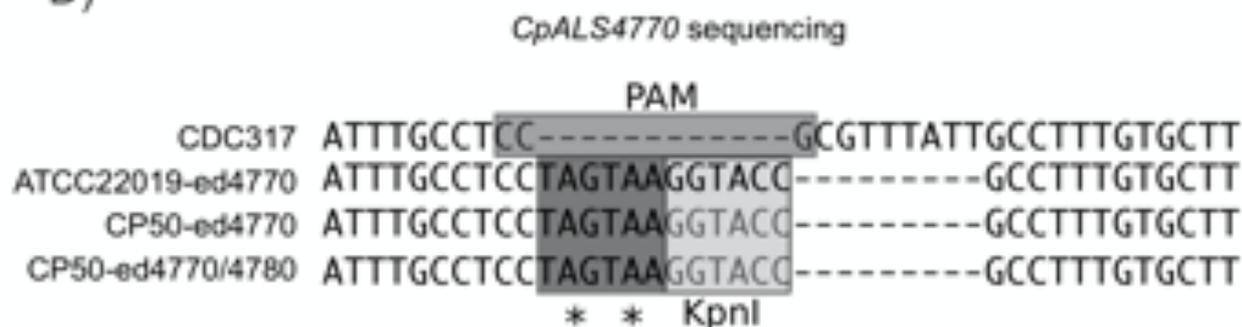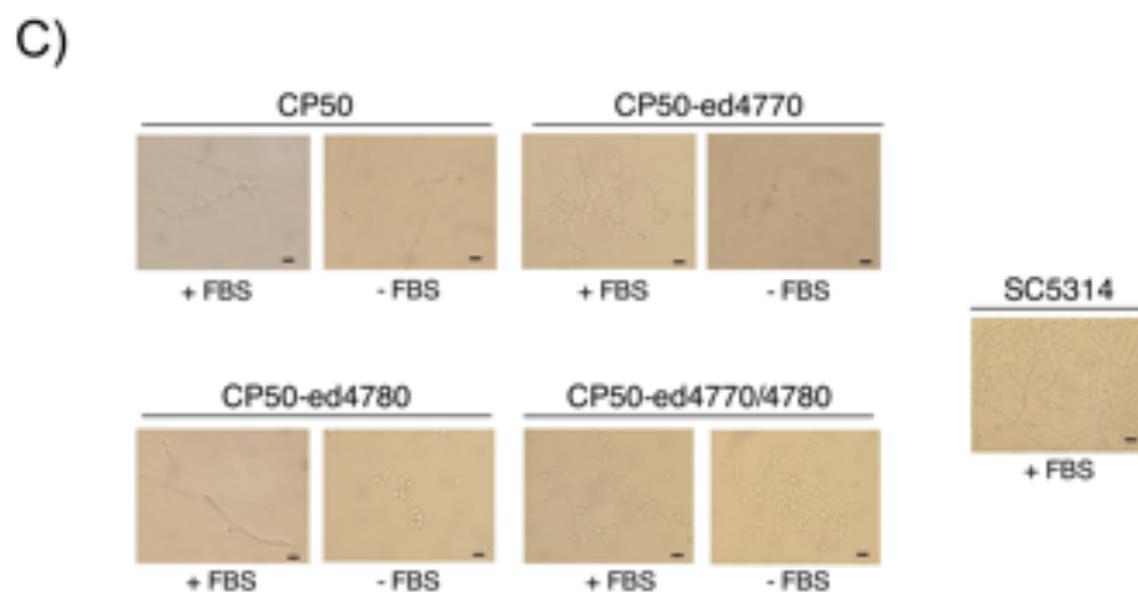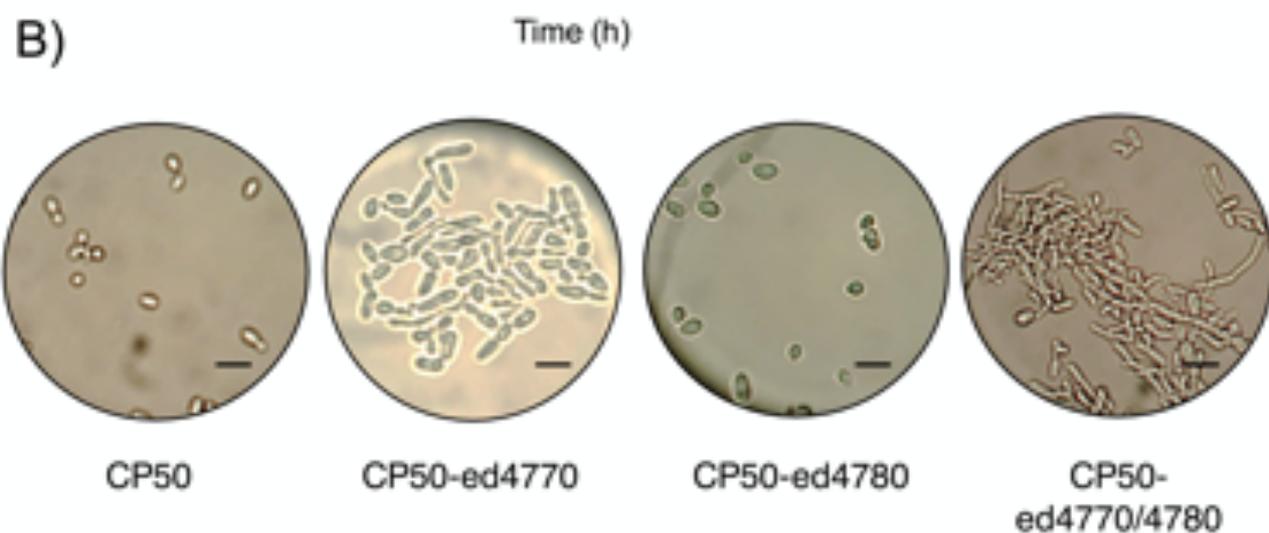
A)

M   1   2

4780EF
993bp   KpnI   407bp

TAA TAG GGTAC   C

*   *

CpALS4780

B)

CpALS4780 sequencing

PAM

CDC317   AGGAGGCTCCAATACCAAC   CCAGGTACATCA

CP50-ed4780   AGGAGGCTCCAATACCAACTAATAGGGTACC-------ACATCA

                                 *   *   KpnI

C)

1   2   3   M

1000bp
750bp
500bp

4770EF
363 bp   KpnI   551 bp

TAA TAG GGTAC   C

*   *

CpALS4770

D)

CpALS4770 sequencing

PAM

CDC317   ATTTGCCTCC-------------GCGTTTATTGCCTTTGTGCTT

ATCC22019-ed4770   ATTTGCCTCCTAGTAAGGTACC---------GCCTTTGTGCTT

CP50-ed4770   ATTTGCCTCCTAGTAAGGTACC---------GCCTTTGTGCTT

CP50-ed4770/4780   ATTTGCCTCCTAGTAAGGTACC---------GCCTTTGTGCTT

                                 *   *   KpnI

A)

B)

CP50   CP50-ed4770   CP50-ed4780   CP50-ed4770/4780

C)

A)

Biomass

B)

XTT assay

A)

4780mut_F

CpALS4780 *CpALS4780* wild type allele

4780R

mut4780_F

428 bp

TAATAGGGTACC
Kpnl site

CpALS4780 *CpALS4780* edited allele

4780R

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 NC

500 bp
250 bp

B)

4770_EF

CpALS4770 *CpALS4770* wild type allele

4770mut_R

4770_EF

372 bp

TAATAGGGTACC
Kpnl site

CpALS4770 *CpALS4770* edited allele

4770mut_R

CP50   CP50-ed4780

1 2 3 4 5 6 7 8 9 10 11 NC M

500 bp
250 bp