OTKA NN100347

Final Report

Understanding colonisation and the transition to pathogenic dissemnination by *Candida* species: towards early diagnostic and therapeutic approches. (ERA-NET PathoGenoMics)

Candida fajok kolonizációjának és patogén disszeminációjának vizsgálata, a korai diagnosztika és terápiás lehetőségek kifejlesztésének érdekében.
(ERA-NET PathoGenoMics)

Attila Gacser PhD
University of Szeged
Department of Microbiology

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Summary

Candida albicans (Ca) and Candida parapsilosis (Cp) are the two main yeast species that are both commensal of humans and responsible for devastating disseminated infections. The aim of the CandiCol (ERA-NET-PathoGenoMics, OTKA NN100374) project was to identify the attributes that are shared by or distinguish these three species and contribute to the transition from commensalism to dissemination. To this aim, we established or further developed ex vivo and in vivo models to study host colonisation and systemic infections by Ca, or Cp. Macrophages play key roles in killing pathogenic *Candida* species. Having established C. albicans-macrophage interaction models (killing and phagocytosis assays, as well as gene reporter systems), this model has been extended to other *Candida* species. We established a model of human peripheral blood mononuclear cell (PBMCs) model to monitor the induced immune response of the host. This model is currently used to evaluate the striking differences in the host response upon *C. parapsilosis* and *C. albicans* infection. To further explore the virulence of the *C. parapsilosis*, we established a killing assay and examined the pathogen elimination and host cell-damaging capacity using primary human PBMC-derived macrophages. Using these models several fungal transcriptional factors have been identified using RNA-Seq experiments, that were overexpressed during host-pathogen interactions. Based on these data, we generated a knock-out collection *in C. parapsilosis*, the selected target genes encode transcription factors, kinases and genes that are involved in cell wall biosynthesis. Majority of our results were already published (see detailed publication list attached to the final report), below we report about the unpublished data. Importantly, the preparation of two manuscripts describing the below discussed data is also in progress. Estimated publishing time is mid 2015.

The detailed Report about the unpublished results:

Knock-out library preparation

The preparation of knock out library including the genes mentioned above is now in progress. We have recently adapted a gene knock out strategy from the work of Susanne Noble et al. (PMID: 15701792) that enables us to generate homozygous deletion mutants more efficiently and faster than other gene deletion strategies such as the flipper cassette method (PMID: 17853941). Fusion PCR method was applied to generate gene specific deletion constructions to disrupt genes from the genome of *C. parapsilosis* CLIB leu-/his- auxotrophic strain.

Methods

First of all we generated the gene specific flanking PCR products for the upstream and downstream regions of each of these genes.

For amplifying the upstream flanking region, upstream sequence specific ‘primer 1’ and ‘primer 3’ were used and for the downstream flanking region downstream sequence specific ‘primer 4’ (as forward primer) and ‘primer 6’ (as reverse primer) were used (Figure 1/A). To amplify the selection marker sequences the primer ‘universal 2’ and ‘primer 5’ were used, the latter

carrying the gene specific barcode sequence (Figure 1/A). We used *C. dubliniensis HIS1* marker from plasmid vector pSN52, and *C. maltosa LEU2* from plasmid vector pSN40. Gene specific primers 'primer 1' and 'primer 6' were used for joining the separate regions (Figure 1/B). In each case, the fusion sequences are provided by the 'primer 3' and 'universal primer 2' in order to join the upstream flanking region with the selection marker. For joining the selection marker with the downstream flanking sequence, the fusion sequences are carried by the 'primer 5' and 'primer 4'. In order to replace both of the targeted gene's allels by homologous recombination (Figure 1/C), the double auxothropic strain of the *C. parapsilosis* clinical isolate CLIB 214 was transformed with the fusion PCR products using chemical transformation, including polyethylene glycol treatment. After the PEG treatment, the cells were kept at 30°C overnight. Heat shock was applied at 44°C, and the cells were then washed with YPD liquid medium prior to plating on either histidine or leucine containing selective medium. To confirm the total deletion of each of the genes we used colony PCR. In order to check the right integration of the deletion construction, both sides of the integration place were checked using gene surrounding region specific primers ('5'Check' and '3'Check') and marker specific primers (Figure 1/D). For LEU2 integration we used 'LEU Check1' and 'LEU Check2' primers, for HIS1 integration 'HIS Check 1' and 'HIS Check 2' primers. All of the transformants were barcoded using a 20bp tag in order to be able to identify the mutants during later in vivo infections. The right transformants of each of the targeted genes were then selected for further examination.

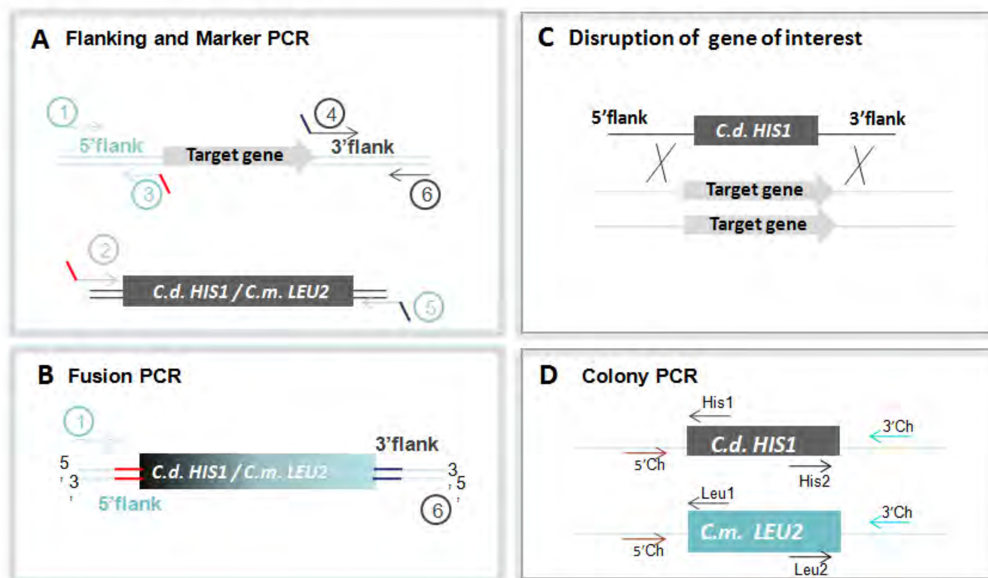


Figure 1.: Representing the generation of the deletion constructions for the disruption of a gene of interest from the genome of *Candida parapsilosis.* (A) Amplification of the gene specific upstream (primer 1 and primer 3) and downstream (primer 4 and primer 6) surrounding regions, and the selection marker sequences (primer 2 and primer 5). *C. dubliniensis HIS1* marker was amplified from plasmid vector pSN52, and *C. maltosa LEU2* from plasmid vector pSN40. (B) Generation of the deletion construction with fusion PCR (primer 1 and primer 6). (C) Process of gene disruption using the deletion construction. Gene deletion is based on homologous recombination. (D) Confirmation of transformants with colony PCR by checking both sites of the integration place.

Results

So far we have generated null mutants for 29 genes and heterozygous mutants for further 23 genes with two independent deletion mutants (Table 1.).

Table 1.: List of the available heterozygous (23) and homozygous (29) mutants involved in the *C. parapsilosis* knock – out library.

Homozygous mutants		Heterozygous mutants	
CPAR2_202040	CPAR2_400270	CPAR2_802400	CPAR2_602950
CPAR2_502720	CPAR2_602840	CPAR2_406400	CPAR2_204320
CPAR2_200040	CPAR2_209520	CPAR2_100610	CPAR2_805310
CPAR2_200390	CPAR2_602430	CPAR2_THR1	CPAR2_105250
CPAR2_108410	CPAR2_304080	CPAR2_205060	CPAR2_703840
CPAR2_303700	CPAR2_704370	CPAR2_209240	CPAR2_602720
CPAR2_401150	CPAR2_704330	CPAR2_205220	CPAR2_107020
CPAR2_300080	CPAR2_204840	CPAR2_407830	CPAR2_207310
CPAR2_800040	CPAR2_100470	CPAR2_503290	
CPAR2_302400	CPAR2_303240	CPAR2_804640	
CPAR2_503760	CPAR2_500180	CPAR2_206440	
CPAR2_104420	CPAR2_501400	CPAR2_108840	
CPAR2_100540	CPAR2_500360	CPAR2_806950	
CPAR2_602820	CPAR2_107240	CPAR2_601590	
CPAR2_602370		CPAR2_DED81	

Knock – out Library - Characterization

Response to antifungal drugs and stressors

Response to the presence of the commonly used antifungal agent, fluconazole has been examined in case of the null mutant strains. All of the available strains were further characterized for a variety of stress-related phenotypes, including oxidative (H_2O_2), osmotic (SDS) or cell wall (Congo red, calcofluor white, caffeine) stressors. Response to cell wall stressors was confirmed by both survival in solution and on solid media.

Methods

Microdilution test with fluconazole

Candida strains were grown overnight at 30°C in YPD solution with shaking (180rpm). Cells were washed 3 x with PBS and diluted to the concentration of $3x10^3$ cells /ml. 64 µg/ml of fluconazole was used as stock solution. Two fold dilutions were prepared in ten step of the stock. 100µl cell suspension was then mixed with a 100µl of fluconazole solved and diluted in RPMI-MOPS. Measurements were made at OD_{600} after 24 hours and 48 hours of co-incubation at 30°C.

Stress induced growth response

Following the overnight culturing and PBS washing steps, cells were standardized to $5x10^4$ yeast cell/ml. 95µl of yeast cells were then mixed with 5 µl of stressor and incubated at 30°C for 16 hours. For the experiments, cell wall stressor calcofluor white (2mg/ml stock) , congo red (2mg/ml stock) and caffeine (1M) were used, along with the osmotic stressor SDS (2% stock), oxidative stressor H_2O_2 (200mM stock) and also Hygromycin B (0,312mg/ml stock) in six serial two-fold dilutions. End point measurement was made at OD_{600} after 16 hours of co-incubation at 30°C w/o shaking.

Results

Among the strains tested, 10 showed increased resistance to fluconazole ($MIC_{90} \geq$ 4µg/ml) in comparison with the wild type ($MIC_{90} \geq$ 2µg/ml) both after 24 hours and 48 hours of incubation (Table 2). During the stress related phenotypical characterization (representative image shown on Figure 2), various responses were detected in case of several deletion null mutant strains comparing to the CLIB 214 wild type strain. During the characterization of the 29 strains 2 strains were susceptible, 1 was resistant to the presence of hydrogen-peroxide. Further 6 showed different survival growth in the presence of cell wall stressors and 2 were susceptible to SDS. Additionally 2 strains were found to be sensitive to Hygromycin B in comparison with the wild type strain (Table 3).

Table 2.: Fluconazole susceptibility - MIC_{50}

Strain	24h (µg/ml Caspofungin)	48h (µg/ml Caspofungin)	Strain	24h (µg/ml Caspofungin)	48h (µg/ml Caspofungin)
CLIB 214 wt	2	2	Δ/Δ 202040	2	2
Δ/Δ 303700	8	8	Δ/Δ 502720	2	2
Δ/Δ 300080	8	8	Δ/Δ 200040	2	2
Δ/Δ 602840	4	8	Δ/Δ 602370	2	2
Δ/Δ 302400	4	4	Δ/Δ 304080	2	2
Δ/Δ 602820	4	4	Δ/Δ 108410	2	2
Δ/Δ 400270	4	4	Δ/Δ 401150	2	2
Δ/Δ 204840	4	4	Δ/Δ 503760	2	2
Δ/Δ 704370	4	4	Δ/Δ 104420	2	2
Δ/Δ 209520	4	4	Δ/Δ 100540	2	2
Δ/Δ 602430	4	4			
Δ/Δ 100470	2	2			
Δ/Δ 200390	2	2			

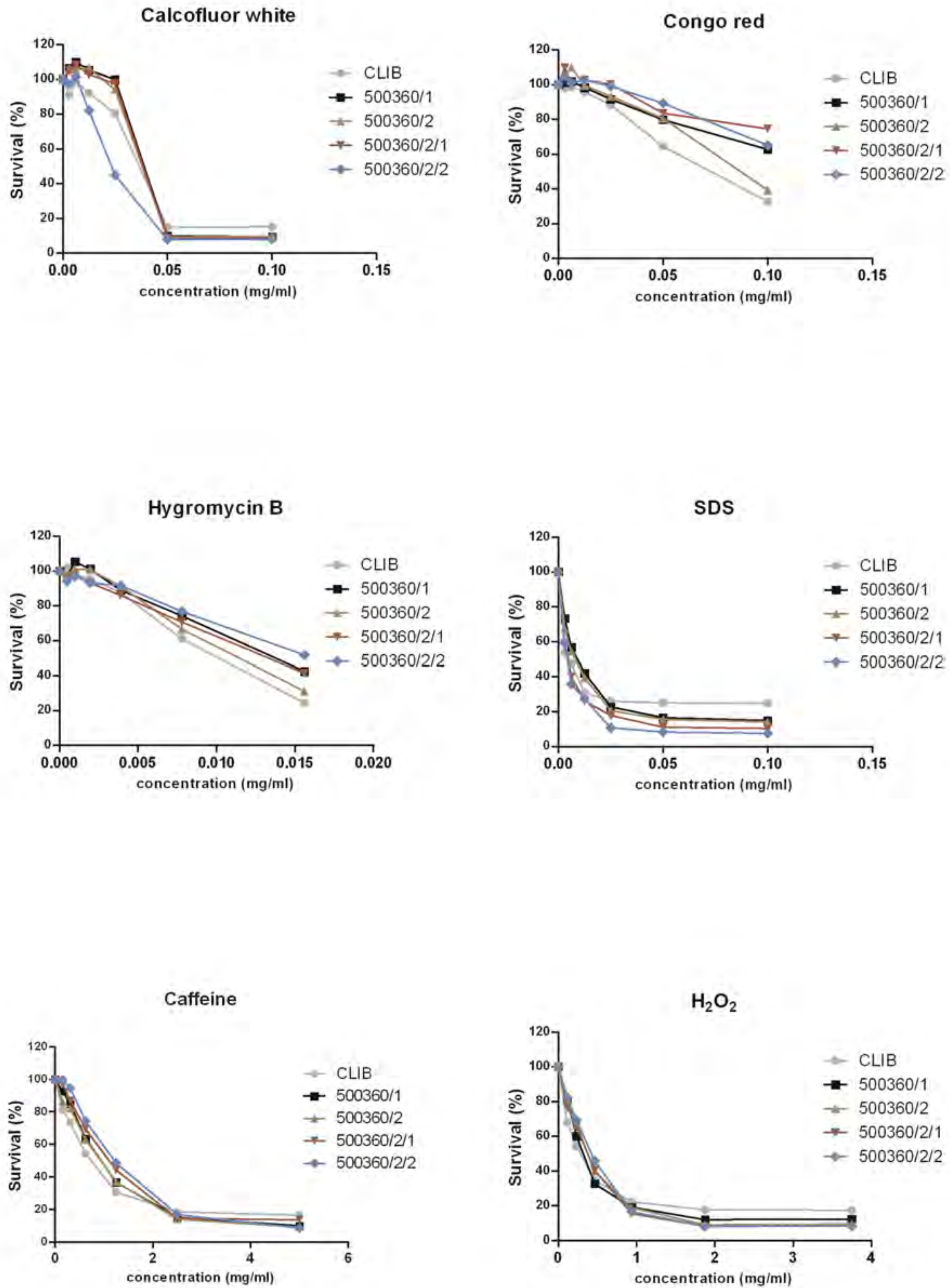


Figure 2.: Representative image of the stress induced growth response (percentage of survival) of the CPAR2_500360 deletion strains. Heterozygous mutant strains: 500360/1, 500360/2; homozygous mutant strains: 500360/2/1, 500360/2/2.

Table 3: Various stress induced growth responses. CW – calcofluor white; CR – congo red, Hyg B- Hygromycin B.

	Stressors					
Mutant	**CW**	**CR**	**Hyg B**	**Caffeine**	**SDS**	**H_2O_2**
Δ/Δ 200390	R	R	S	R	-	R
Δ/Δ 303700	-	-	-	-	S	S
Δ/Δ 602840	-	-	-	-	-	S
Δ/Δ 503760	-	S	-	-	-	-
Δ/Δ 200040	R	-	-	S	-	-
Δ/Δ 401150	-	-	-	S	-	-
Δ/Δ 602430	S	S	S	-	-	-
Δ/Δ 501400	S	S	-	-	S	-

General phenotyping

All of the available null mutant strains were tested in different conditions such as growth abilities on different temperatures (20, 30, and 37°C) and on different medias (YPD, YNB+ glucose, YCB + BSA, YNB + FBS + glucose, a representative image is shown on Figure 3). Furthermore we examined their growth on solid media with different pH-s as well (pH4, pH5, pH6, pH7 and pH8, shown on Figure 2. Growth kinetics of each of the null mutants has also been examined (represented on Figure 4).

Methods

Prior to experiment, strains were grown overnight in YPD solution at 30°C with shaking (180 rpm). Cells were washed three times with PBS and standardized to $2x10^6$, $2x10^5$, $2x10^4$, $2x10^3$ cell / ml. Yeast cells were then plated onto differently supplemented solid media and incubated at 30°C for 2 days. The following medias were used: YPD, YCB supplemented with 10% BSA; YNB supplemented with 10% FBS and 1% glucose, and YNB supplemented with 1% glucose.

To examine growth kinetics, $5x10^5$cells / ml cells were inoculated in YPD solution and optical density was measured at 600nm for 24 hours, with shaking (180 rpm) applied before each measurement.

Results

During the examination, growth deficiencies and morphology changes were detected among the mutant strains tested. A total of 7 gene's null mutants showed decreased growth under the applied conditions, and 5 gene's homozygous strains switched colony morphology comparing to the wild type. Differences in biofilm formation of certain null mutants were also detected in comparison with the wild type strain. Altogether 8 strains showed decreased biofilm forming ability, in case of further two strains increased biofilm formation was detected (Table 4). All of

these changes can influence the pathomechanism of the fungi that we confirmed by using both in vitro and in vivo infection models.

During the analyses we found null mutants that show differences in appearance such as increased pseudohyphae forming (Δ/Δ 200390, Figure 5), regressed growth on different temperatures (Δ/Δ 303700, Figure 6) and alkali-phobic phenotype (Δ/Δ 100540, Figure 7). Therefore the deletion mutants of CPAR2 200390, CPAR2 303700 and CPAR2100540 were chosen for further characterization using both *in vitro* and *in vivo* infection models.

Table 4.: General phenotyping of deletion mutants

Growth deficiencies	Changed morphology	Decreased biofilm formation	Increased biofilm formation
Δ/Δ 100540	Δ/Δ 100540	Δ/Δ 204840	Δ/Δ 303700
Δ/Δ 200390	Δ/Δ 200390	Δ/Δ 300080	Δ/Δ 602840
Δ/Δ 303700	Δ/Δ 303700	Δ/Δ 302400	
Δ/Δ 602840	Δ/Δ 500180	Δ/Δ 502720	
Δ/Δ 602820	Δ/Δ 501400	Δ/Δ 503760	
Δ/Δ 202040		Δ/Δ 304080	
Δ/Δ 501400		Δ/Δ 400270	
		Δ/Δ 401150	

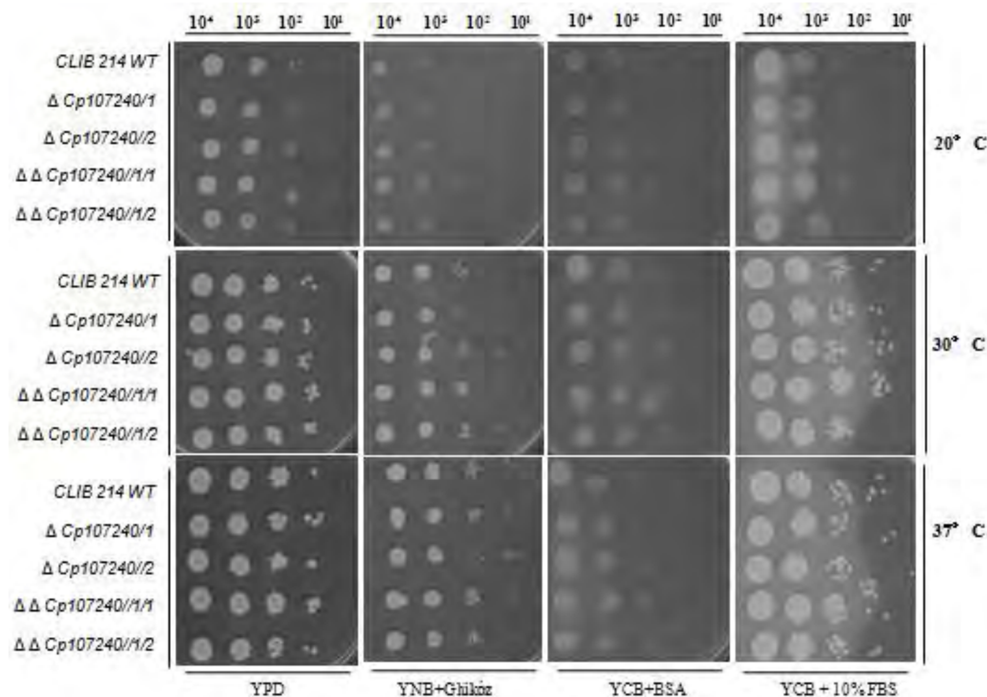


Figure 3: A representative image of phenotypical characterization of CPAR2_107240 deletion mutants. Heterozygous mutants: 107240/1, 107240/2; homozygous mutants: 107240/1/1, 107240/1/2. Strains were incubated for 48 hours under the shown conditions.

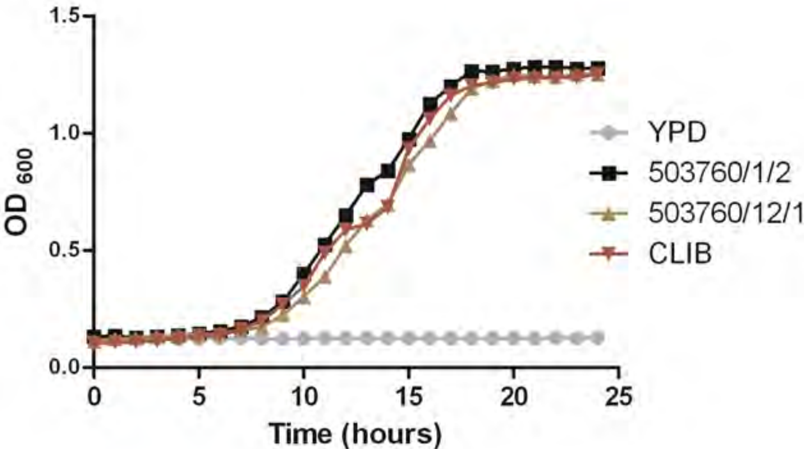


Figure 4: Growth kinetics of the CPAR2 503760 homozygous deletion strains.

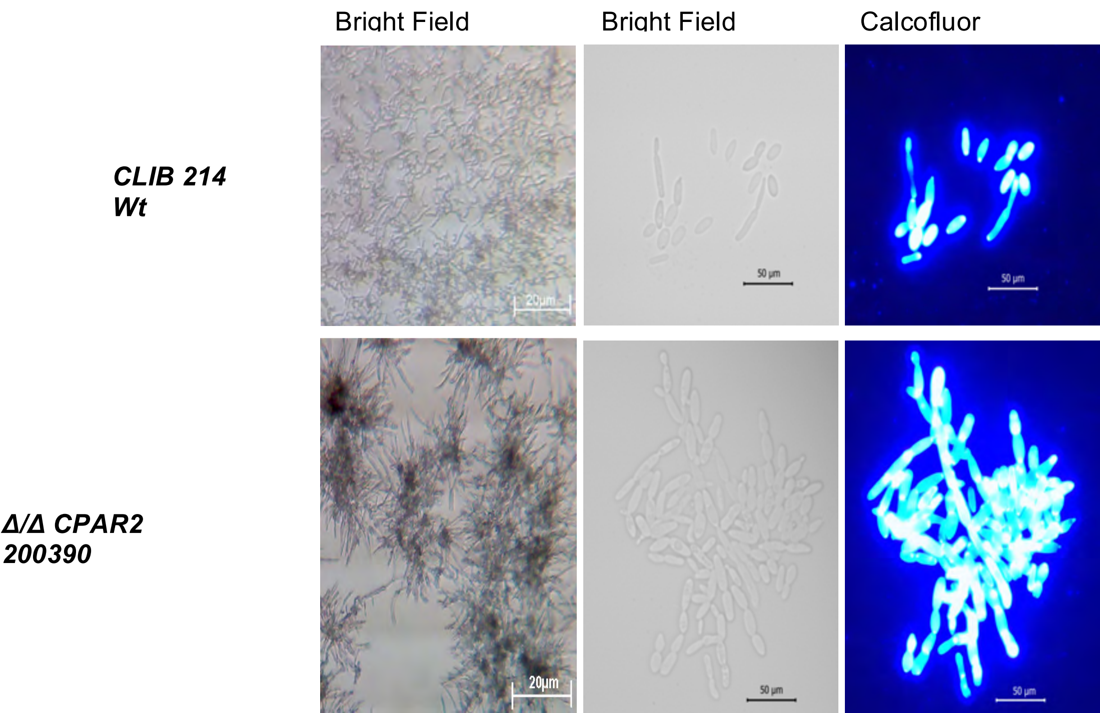


Figure 5.: Bright field and fluorescent microscopic images of the CPAR2 200390 null mutant strain. Cells were inoculated in FBS supplemented DMEM solution. Images were taken after 24 hours of incubation.

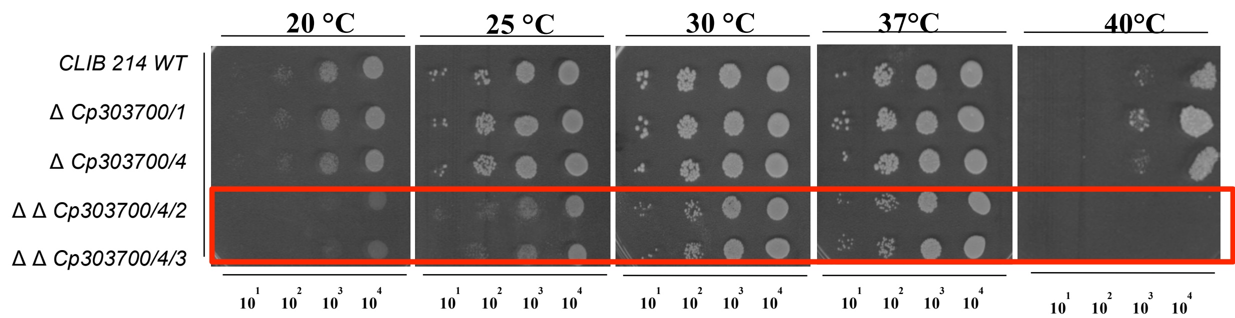


Figure 6.: Images represent the growth deficiency of CPAR2 303700 mutant strains at different temperatures. Heterozygous mutants: 303700/1, 303700/4; homozygous mutants: 303700/4/2, 303700/4/3.

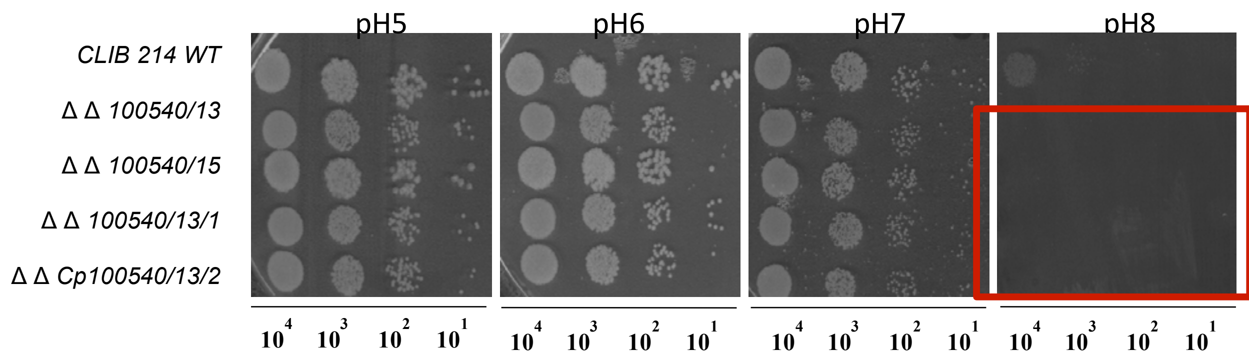


Figure 7.: Images represent the alkali-phobic phenotype of CPAR2 100540 mutant strains. Heterozygous mutants: 100540/13, 100540/15; homozygous mutants: 100540/13/1, 100540/13/2.

Characterization of selected mutants of interest

Results

The null mutant strains of **CPAR2 303700** showed increased sensitivity to the change of temperature and also to the presence of SDS and H_2O_2. The knock out strains of this gene further showed increase in doubling time in comparison with the wild type (Figure 8/A). We observed a significant temperature induced increase of pseudohyphae formation (Figure 8/B) and also increased biofilm formation (Figure 8/C).

There was no difference in killing of yeast cells by murine macrophages, but there was a decrease in the phagocytosis of the knock out strains relative to the reference strain (Figure 8 /D). Following in vivo infection of a murine model with the null mutants significant clearance occurred on both liver and spleen, in contrast a significant increase of fungal burden occurred in the kidneys in comparison with the original strain (Figure 9).

As described above, the null mutant strains of the **CPAR2 100540** showed sensitivity to pH8 and slightly decreased growth on minimal media when comparing to the reference strain.

Morphology change was also observable. Following in vitro infections, these homozygous deletion strains were less killed by murine macrophages (Figure 10/A), however, more macrophages were found phagocytosing than in the presence of the wild type strain (Figure 10/B). Following infection of a murine in vivo model, significant clearance occurred on both liver and spleen between day 3 and day 7 (Figure 11).

As shown above, the null mutant strains of the **CPAR2 200390** showed hyperfilamentous phenotype, along with morphology change and increased doubling time in comparison with the wild type. Also significantly increased resistance was detected to the cell wall stressor congo red, calcofluor white and also caffeine.

The homozygous deletion mutants showed decreased biofilm formation (Figure 12/A). Furthermore, in contrast with the reference strain, these mutants were less killed by murine macrophages in vitro (Figure 12/B). Similarly to CPAR2 303700 significant clearance was detected on both liver and spleen 3 days after the infection (Figure 13).

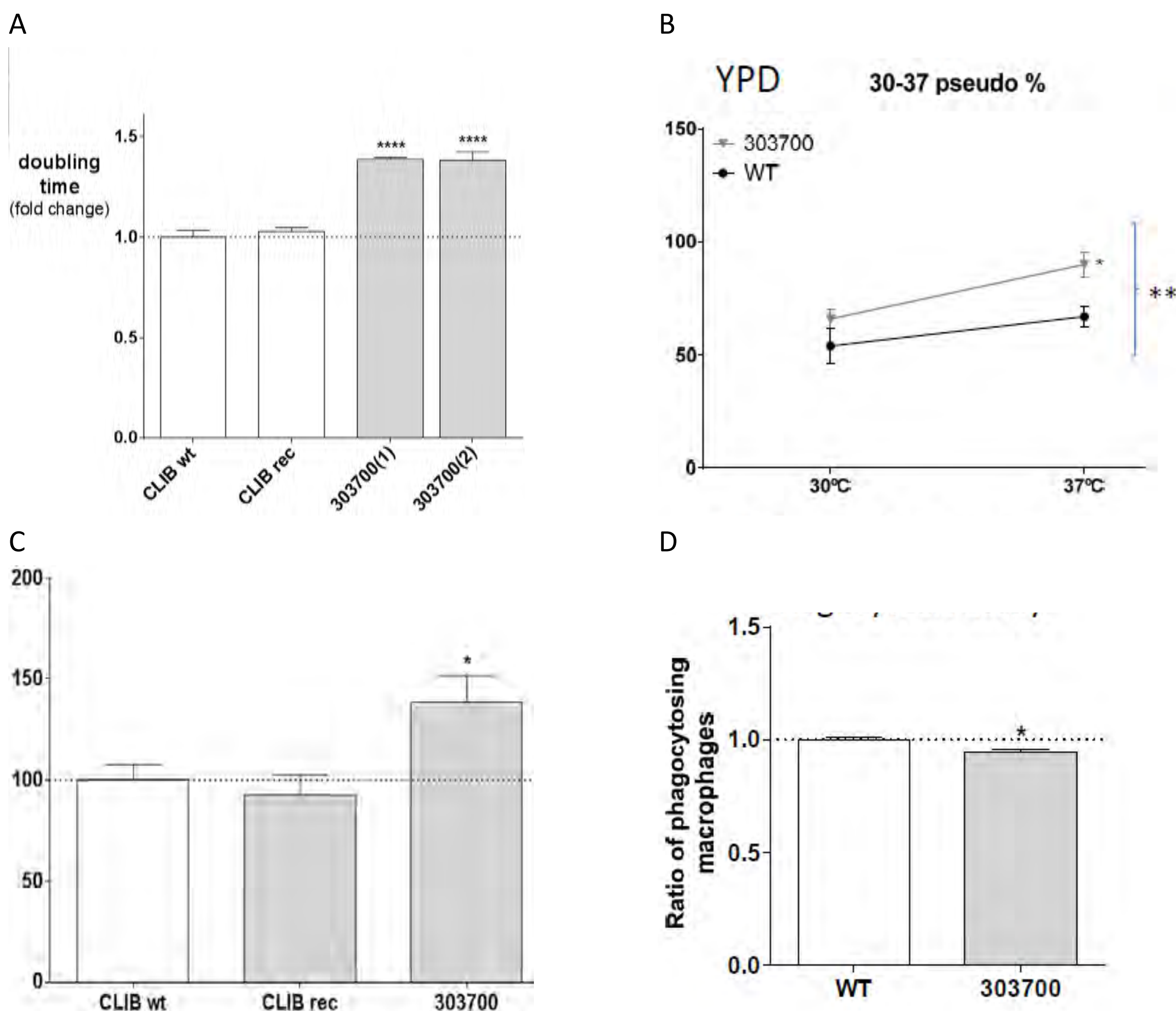


Figure 8.: *In vitro* characterization of CPAR2 303700

A - Doubling time; B – Temperature induced pseudohypha formation; C – Biofilm formation; D - Phagocytosis

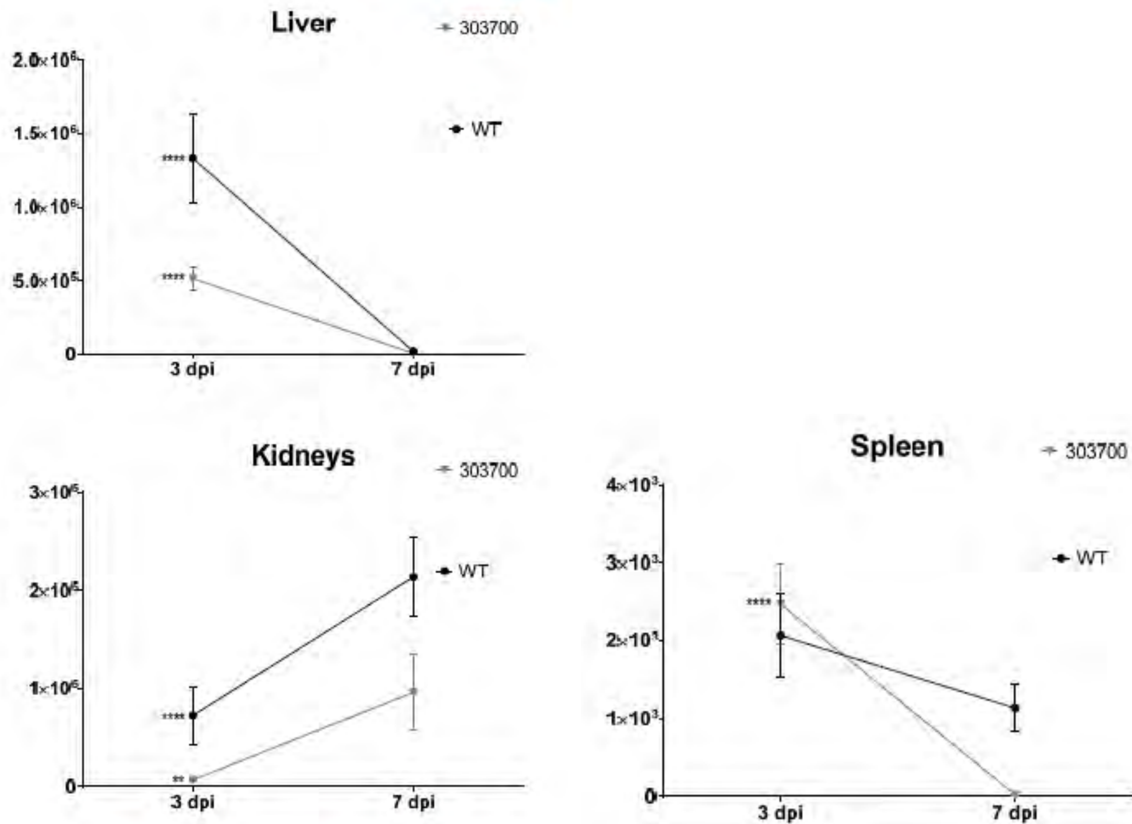
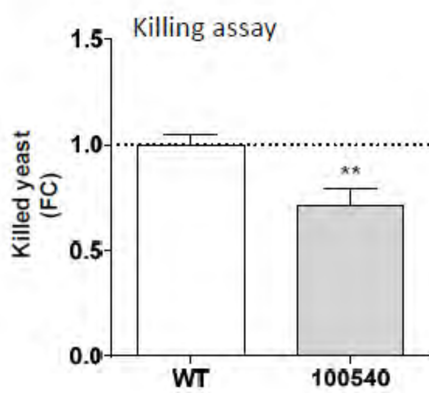


Figure 9.: *In vivo* characterization of CPAR2 303700

A – Killing assay



B – Phagocytosis assay

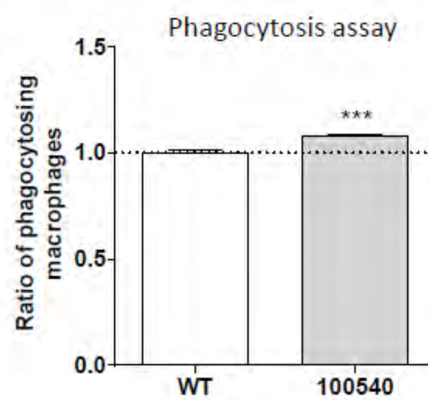


Figure 10.: *In vitro* characterization of CPAR2 100540 null mutants.

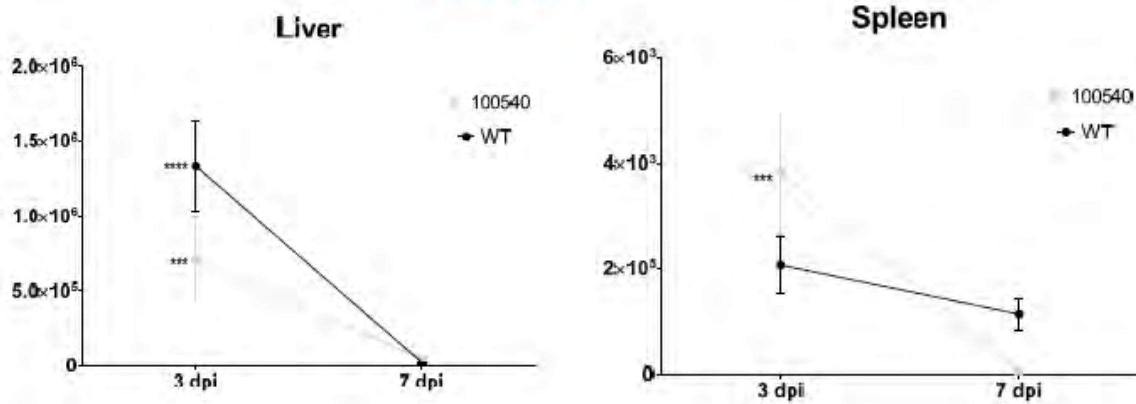
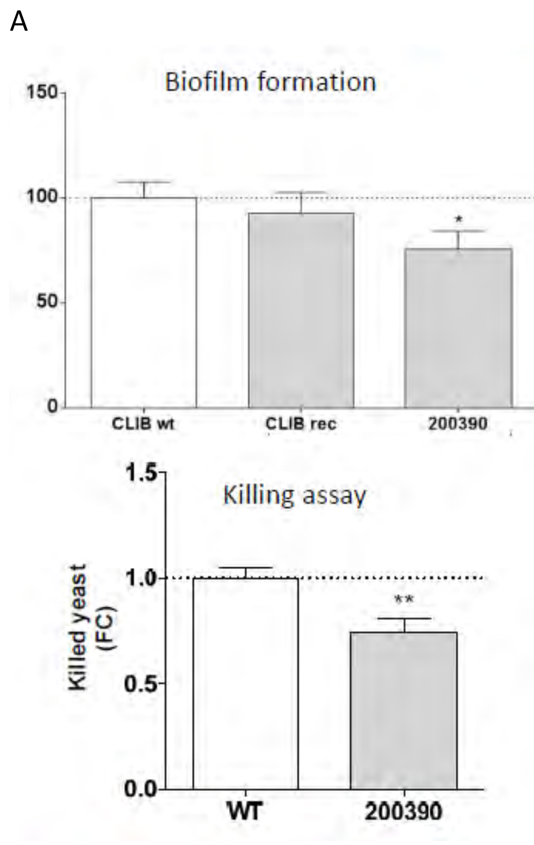


Figure 11.: *In vivo* characterization of CPAR2 100540 null mutants.

A



B

Figure 12.: *In vitro* characterization of CPAR2 200390 null mutants. A – Biofilm formation; B – Killing assay

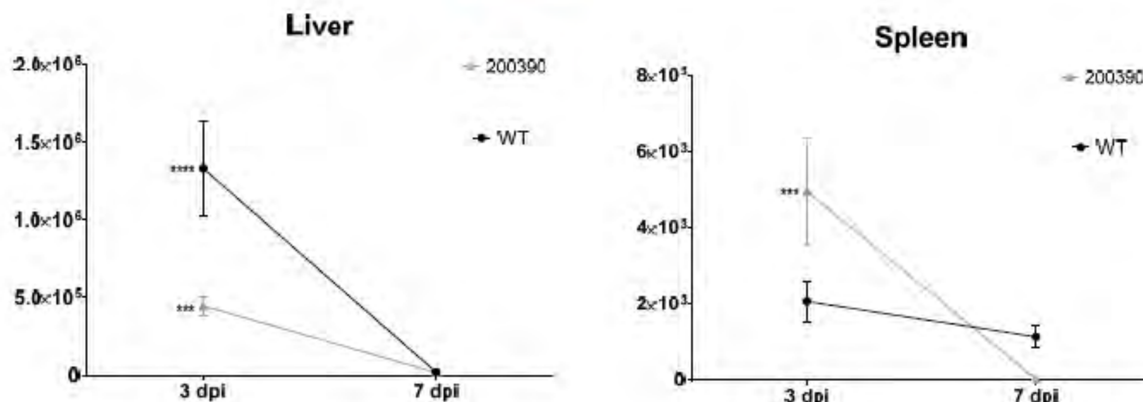


Figure 13.: *In vivo* characterization of CPAR2 200390 null mutants.

Over-expression library

Another approach for studying gene function is to generate strains that over-express the genes of interest under the control of a strong promoter. Recently over-expression strategies have been applied to investigate *Candida albicans* gene function, interaction with the host and virulence attributes (PMID: 23049891; PMID: 223283878). This high-throughput system (Figure 14) was adopted and optimized in *C. parapsilosis* to create an overexpression library. Instead of the traditional method -with restriction endonucleases and ligase enzymes- this technology utilizes two enzyme mixes to clone the gene of interest into different vectors. The system mimics the integration and the excision of the lambda phage into/from *E. coli* genome at a specific recombination site (*att*). Two enzyme mixes catalyse the recombination of specific *att* sites of different sequences.

Methods

The targeted gene's ORF is amplified with forward and reverse primers carrying *attB1* and *attB2* sites at their 5' ends respectively. Each reverse primer is further expanded with a unique 20 nucleotide long barcode that can be used later for identification. The BP clonase® enzyme mix catalyses the recombination between the *attB1*/*attB2* sites of the PCR product and the *attP1*/*attP2* sequences of the pDONR vector. As a result the pENTRY vector is generated carrying the gene of interest flanked by *attL1*/*attL2* sites. The newly formed recognition sites are formed by the combination of *attB1*/*attP1* and *attB2*/*attP2* sequences.

For the LR clonase® reaction the pENTRY and pDESTINATION vectors are required. The destination vector provides the high modularity of the Gateway® system as it carries all the essential components for an efficient overexpression. It contains the promoter, selection marker and the target site of the recombination. Addition of a unique tag to the native protein is also possible. All of the components can be changed / replaced by conventional cloning techniques. After the LR reaction the newly formed pEXPRESSION vector contains the ORF

under the regulation of the desired promoter, the correct selection marker and the target site of the integration.

Results

In our previous work several fungal transcriptional factors have been identified using RNA-Seq data that were overexpressed during host-pathogen interactions. Based on the RNA-Seq data mentioned above we used the Gateway™ technology to generate *C. parapsilosis* strains that over-express our genes of interest. For this, *C. parapsilosis* CLIB 214 leu- strain was used. Using the *caSAT1* flipper system we have integrated the *RP10* locus of *C. albicans* SC5314 to the *RP10* locus of *C. parapsilosis* CLIB 214 leu- strain (Figure 15). With this integration we were able to adopt the *TDH3p-CLP10* over-expression system established in *C. albicans*. A *TDH3p-CLP10-GFP* construct was used to test whether this system is able to express the genes of interest in *C. parapsilosis*. For entry vectors the pDONR 207 was used, while for destination vectors the *TDH3p-CLP10* containing vectors (PMID: 223283878) were applied. All of the transformants were barcoded using a 20bp tag.

In order to make the Gateway system available for a wide range of *C. parapsilosis* isolates the *TDH3p-CLP10* containing destination vector was redesigned. The recognition site of *Stu*I restriction endonuclease was integrated into the *C. paraspsilosis* RP10 (*CpRP10*) amplicons using PCR with two sets of primer pairs. In order to avoid the endless generation of acceptor strains for each isolate, we exchanged the *C. albicans* RP10 with the newly generated C. *parapsilosis* RP10 sequence in the destination vector.

For the same reason auxotrophy based selection was also changed to a dominant selection method. Therefore a new destination vector carrying a dominant selection marker (CaSAT1 gene, NAT resistance) was constructed. The constitutive promoter, TDH3 and the terminator region of the plasmid were kept intact. Thus two types of destination vectors are now available including different types of selection: pDESTINATION *TDH3-CpRP10-NAT* and pDESTINATION *TDH3-CpRP10-LEU2.*

To amplify *C. parapsilosis* ORFs the state of the art Phusion High-Fidelity DNA polymerase (Thermo Scientific) was used. Amplicons were cloned into the pDONR221-Kanamycin vectors and transformed into the *E. coli* 2T1 strain. After the isolation and checking of the correct entry vectors they were sent for sequencing. Only vectors containing ORFs without a missense mutation were used for LR cloning with the destination vectors. Expression vectors were then transformed to *E. coli* 2T1, selected according to restriction map and digested overnight. *C. parapsilosis* transformation was carried out by either chemical transformation or electroporation. Transformants were tested by both colony PCR and Southern-blot. Overexpression is usually checked by qRT-PCR.

So far the ORF of 86 genes has been amplified. The first set of targeted genes was selected according to the previously mentioned RNA-Seq data, gained from the infection of the THP-1 human monocyte cell line with *C. parapsilosis.* Furthermore, additional genes involved

in the primary list are hypothetical genes responsible for cell-wall biosynthesis, biofilm formation, morphological changes, antibiotic resistance or maintenance of lipid metabolism. So far, 67 pENTRY are ready for characterizing, out of these 40 carries sequences without missence mutations. 19 pEXPRESSION vectors were created with either NAT or LEU2 selection marker, and 11 were already used for transformation. There are 6 mutants checked with colony PCR. Southern-blot and real-time PCR are now in progress.

The construction of a doxycyclin inducible, sophisticated Tet-on/Tet-off system is now in progress, along with GFP and RFP tagged destination vectors.

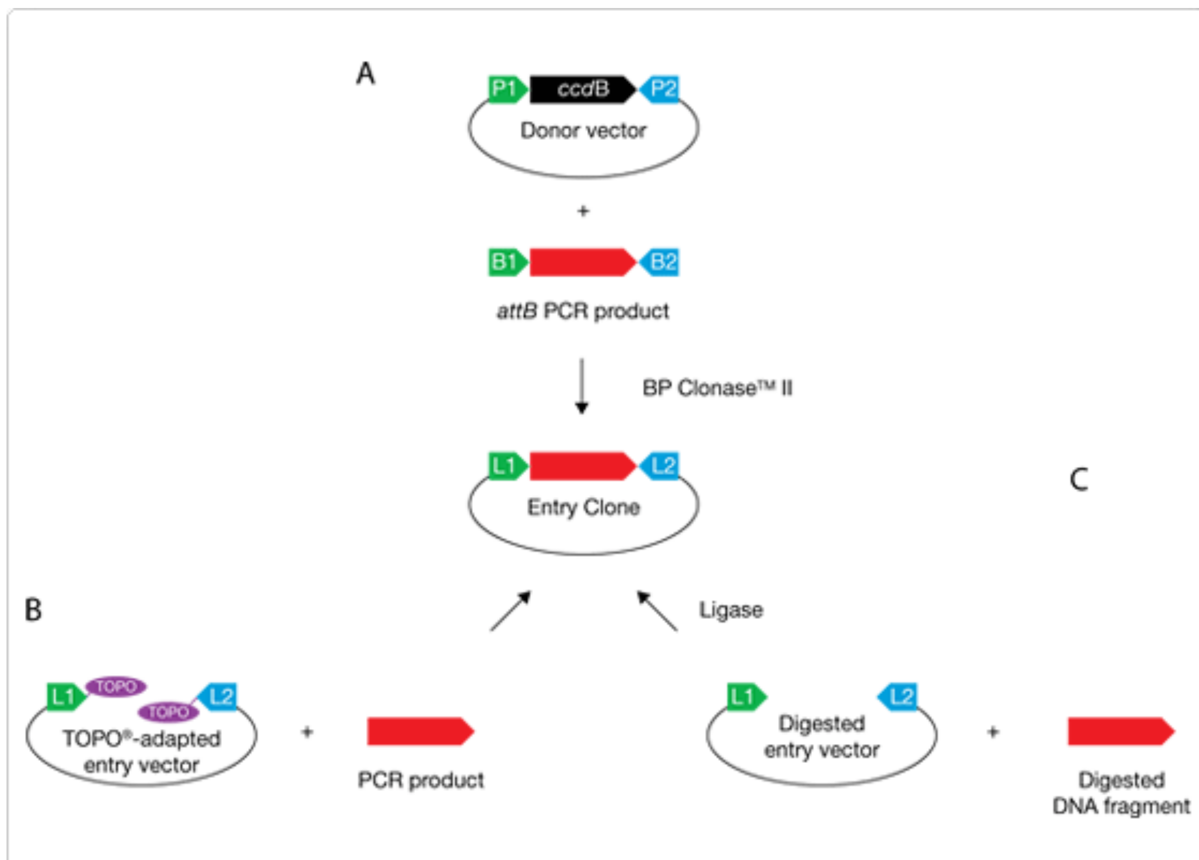


Figure 14.: The Gateway technology. Source: http://www.lifetechnologies.com