Development of a High Throughput Cytotoxicity Assay of Caco-2 Cells using a *Candida albicans* Homozygous Deletion Library

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INTRODUCTION

The opportunistic yeast *Candida albicans* gains access to the bloodstream mainly through translocation across the intestinal barrier. Using an *in vitro* model of interaction with the enterocytic-like cell line Caco-2, it has been shown that *C. albicans* is capable of adhering to, invading and damaging intestinal cells, probably through a combination of phenotypic properties including adherence, yeast to hyphae transition and secretion of lytic enzymes (1). However, the sequence of events together with the genes involved in these processes remain to be clarified.

The aim of our study was to screen a *C. albicans* isogenic collection of homozygous knock-out strains for genes involved in the infectivity of enterocytes using a Caco-2 *in vitro* model.

A high throughput screening tool adapted from our Caco-2 model was used for testing the infectivity of 662 *C. albicans* homozygous deletion strains (equivalent to ~ 11% of the genome) (2). Infectivity was measured as the cytotoxic effect of individual mutants upon Caco-2 cell monolayers. For each of the mutants tested, cytotoxicity was expressed as the percentage of variation as compared to the *C. albicans* SC5314 reference strain.

MATERIALS & METHODS

***Candida albicans* homozygous deletion library**

The library includes 662 *C. albicans* homozygous deletion strains. Two collections of independent mutants were used to screen and validate genes involved in cytotoxicity. These collections were supplied by S. Noble (2).

Cytotoxicity assay & screening method

Caco-2 cells were infected with ~1x10^5 *Candida* cells for 24h, at 37° C , in 96-wells plate culture. Briefly, Caco-2 cells were washed with DMEM to remove the non-interacting yeast cells in suspension and then stained 10 min with a specific cell death fluorophore (Sytox® orange, Invitrogen).

Fluorescent intensity was measured 9 times per well, in quadruplicate for the first screening step. The validation step was performed three times independently using at least 4 technical replicates per experiment.

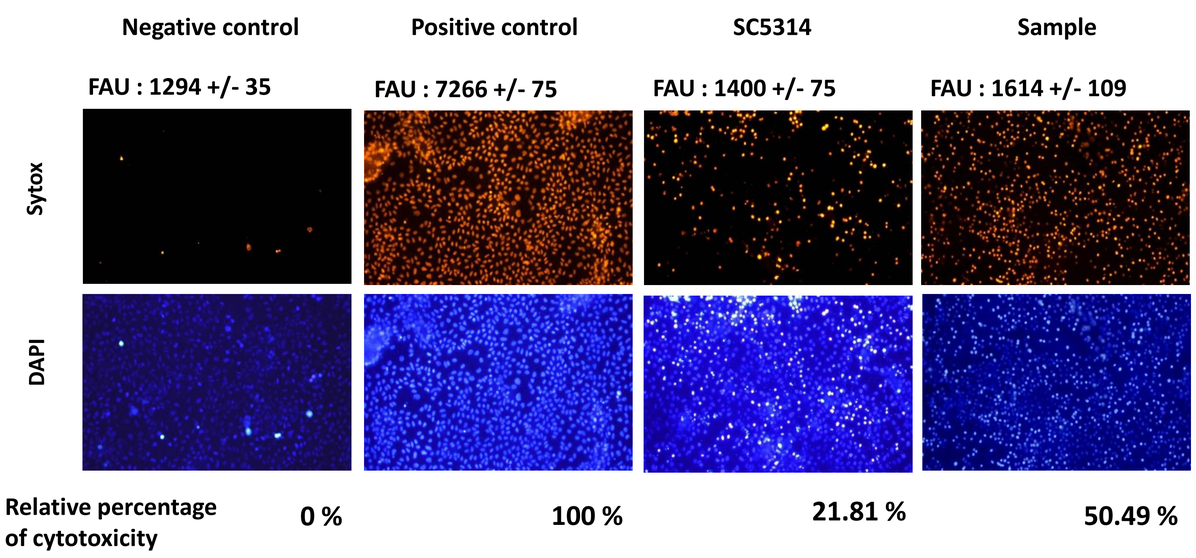
For each strain tested, cytotoxicity was expressed as the percentage of variation relative to the reference strain *C. albicans* SC 5314.

Statistics and data analysis

For data analysis, the median of the replicates for each of the 662 strains was used. Cytotoxicity values of each experiment were centered around 0 and scaled to have a standard deviation value of 1. Mutants with either increased or decreased virulence were chosen as the 5% population on each side of the Gaussian distribution. The phenotypes were then validated using a second independent set of the same mutants. Cytotoxicity variations were calculated as compared to the reference strain *C. albicans* SC5314. A Wilcoxon test was used to test the differential cytotoxicity conferred by every mutant as compared to the SC5314 strain.

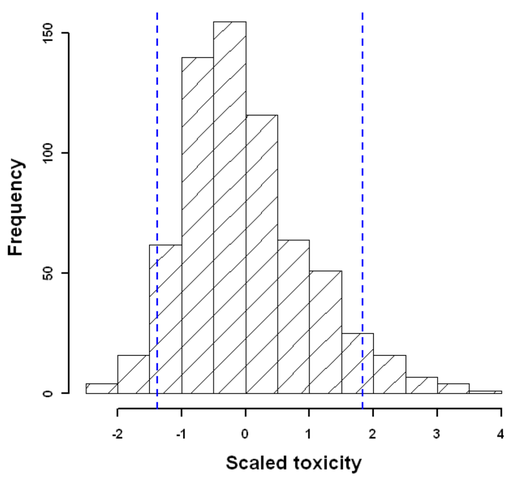
RESULTS

Figure 1. Development of a high throughput screening tool for cytotoxicity analysis in Caco-2 cell line.



Using the fluorescent Sytox® dead cell stain, we can visualize Caco-2 cell death by means of fluorescence microscopy followed by quantification on a microplate reader. **Figure 1 shows the correlation between the Fluorescent Arbitrary Units (FAU) measured and the number of dead cells**. Thus, we can determine the relative percentage of cytotoxicity according to the positive and negative controls. In this example, the *Candida albicans* SC5314 reference strain displays 21.81% of cytotoxicity after 24 hours of infection with 1x10^5 *Candida* cells.

Figure 2. First step of the selection process: screening of the entire collection (662 mutants).

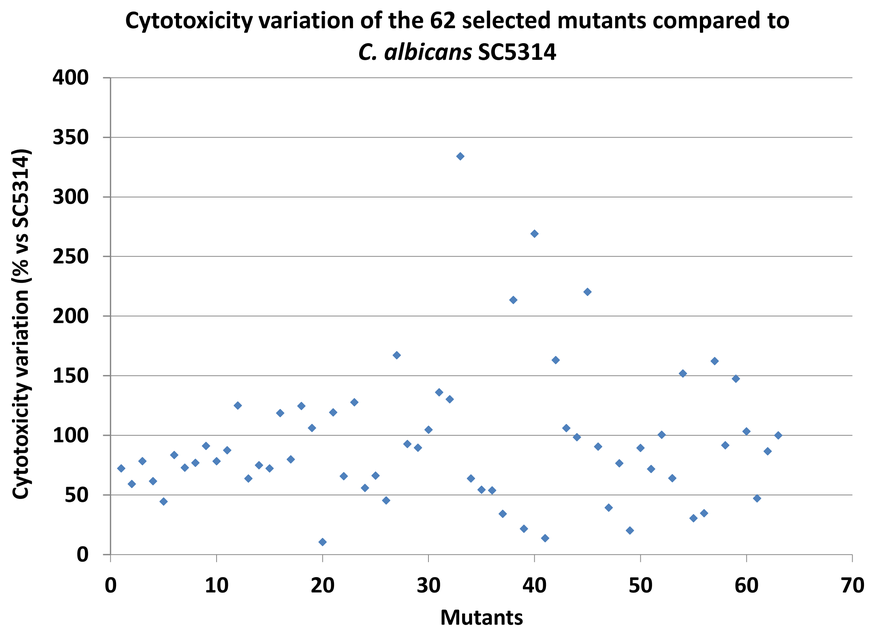


Cytotoxicity distribution histogram (with mean 0 and standard deviation 1). The vertical dashed lines correspond to the 5% and 95% quantiles.

We identified **62 mutants out of 662 conferring marked increase or decrease in Caco2-cell cytotoxicity**.

These results were the basis of our screening process. In a second step, a second collection of independent mutants was used to validate the formerly selected mutants.

Figure 3. Validation of the 62 selected mutants with a second independent K-O collection.



These results consolidate the previous screening, however many strains (21) displayed a small change in cytotoxicity towards the reference (100%). This underlines the importance of performing validation steps with at least an additional independent set of mutants. A total of 41 strains was selected based on a p-value cutoff of <0.05.

Despite the relevance of the statistical analysis, we selected mutants whose phenotype varied by at least about two-fold compared to the reference strain. This allows to take into account the biological relevance of these results. Using the overall criteria, 18 mutants displayed altered virulence, including **14 mutants with reduced virulence and 4 with increased virulence**.

A gene ontology term analysis of the 18 ORFs (analysis was conducted in Feb. 2012 using www.candidagenome.org) showed functional enrichment of genes involved in filamentous growth (p-value of 0.0067, false discovery rate of 0%) among the 662 ORFs tested.

SUMMARY and CONCLUSIONS

This high throughput assay allowed us to identify 62 *C. albicans* mutants conferring Caco-2 cytotoxicity out of 662 strains. Using a second independent knock-out collection, these phenotypes were validated in 41 strains (p-value <0.05) out of 62. Among these, 30 had attenuated virulence whereas 11 had increased virulence, as compared to the SC5314 strain. In this selection, 18 mutants displayed a virulence variation of at least 2-fold as compared to the reference strain, mainly involving genes in filamentous growth. These observations suggest that *C. albicans* hyphal development is an important determinant of Caco2-cell cytotoxicity.

Our study demonstrated the potential of our screen to identify genes involved in *C. albicans* ability to cause enterocyte cell damage. Known genes and yet uncharacterized ORFs have been identified for which deletion increases or decreases *C. albicans* virulence *in vitro*. Further studies will be conducted to confirm the involvement of these genes or ORFs in *in vivo* models of disseminated candidiasis originating from the gastrointestinal tract.

Acknowledgement: This work has been supported by Agence Nationale de la Recherche (ANR -08-MIEN-033-01)

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Poster number : **P212**