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Genetic and phenotypic characterization of commensal and clinical *Candida albicans* isolates reveals heterogeneous distribution of adherence and invasiveness properties

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INTRODUCTION

Considering *Candida albicans* as a primarily clonal model of reproduction, distinct lineages of *C. albicans* could propagate independently, each clonal lineage having possibly variations in biological traits contributing to the pathogenicity of the fungus such as adherence or the yeast-hyphae transition. Indeed, adherence to epithelial cells is considered a virulence trait of *C. albicans* since contributing to the first contact of the fungus with the host's epithelial layers. Moreover the ability of *C. albicans* to switch from the yeast to the hyphal form is an important determinant of the *C. albicans* virulence since favoring invasion of the fungus into the host's tissues.

Using a Multi-Locus Microsatellite Typing (MLMT) approach, we previously addressed the question of the pathogenicity of lineages of *C. albicans* with regard to their origin in three series of *C. albicans* isolates: 82 commensal isolates collected in healthy individuals, 46 invasive bloodstream isolates and 46 of non-bloodstream clinical isolates. In the current study, invasiveness (*i.e.* cytotoxicity effect) and adherence to epithelial cells of a representative set of the 3 series of strains previously characterized at the genotypic level were investigated in the Caco-2 *in vitro* model of human intestinal barrier. Correlations between invasiveness, adherence, genotype and origin were then investigated.

MATERIALS & METHODS

Genotyping

In a previous work (L'Ollivier C. *et al ,2012 , in process of submission),* genotyping of 174 *C. albicans* isolates from three series of different origins (*i.e.* 82 commensal isolates, 46 invasive bloodstream isolates and 46 non-bloodstream clinical isolates) was conducted using eleven microsatellite *loci*. The phylogenetic distribution of these 174 isolates was then analysed using UPGMA algorithms with ape library for R.

Adherence assay

Adherence assays were performed according to a previously described protocol (1). Briefly, Caco-2 cells were inoculated with yeast cells of *C. albicans* SC5314 at about 0.1 MOI. After 30 min of incubation at 37°C, cells were washed three times with PBS to remove non-adherent yeasts, then fixed in 2% glutaraldehyde and stained with calcofluor white. The percentage of adhesion of each strain was determined as the ratio of the number of adherent *C. albicans* cells on the entire surface of the coverslip to the number of *C. albicans* cells inoculated.

Cytotoxicity assay

Caco2 cells were infected with ~5 10^5 *Candida* cells for 24h, at 37°C. Briefly, Caco2 cells were washed with DMEM to remove the non-interacting yeast cells in suspension and stained 10 min with a specific cell death fluorophore (Sytox® orange, invitrogen). For each strain tested, cytotoxicity was expressed as the percentage of variation as compared to the reference strain *C. albicans* SC5314.

Measurement of invasion

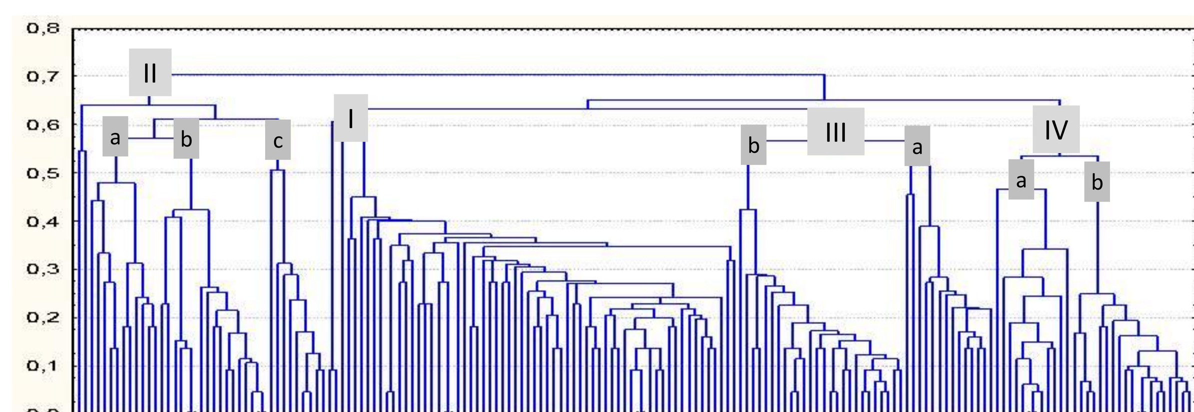
Invasion assays were performed according to a previously described protocol (2). Briefly, Caco2 cells were infected with ~5 10^4 *Candida* cells for 2h, at 37°C, in 24-wells plate culture. The monolayers were rinsed three times to remove fungal cells which were not associated with epithelial cells and fixed in 2% glutaraldehyde. All fungal cells remaining adherent to the surface of the epithelial cells were stained for 1 h with a rabbit anti-*C. albicans* polyclonal antibody (Acris® Antibodies, Germany) and counterstained with secondary anti-rabbit IgG conjugated with Alexa Fluor 488 (Invitrogen). Then, epithelial cells were permeabilized in 0.5% Triton X-100 for 10 min. The adherent and invading parts of fungal cells upon and within epithelial cells (*i.e.* cell-associated) were stained with calcofluor white. The percentage of invading *C. albicans* cells was determined by dividing the number of partially internalized cells by the total number of adherent cells.

Macroscopic and microscopic observations

The *C. albicans* strains were maintained on solid Yeast Extract Peptone Dextrose (YPD) medium at 30°C and routinely grown on liquid YPD at 37°C in a shaking incubator overnight. Hyphal induction was tested in either liquid or solid (plus 2% agar) specific media: YPD plus 10% fetal bovine serum (FBS) (Invitrogen) or Spider medium (microscopic observation with magnification X20).

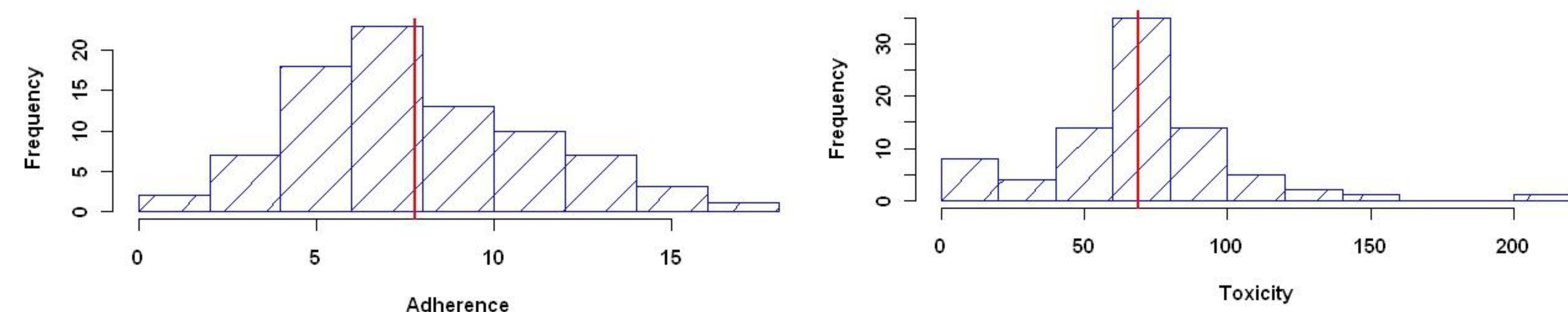
RESULTS

Figure 1. UPGMA phenogram of the 174 *C. albicans* isolates



Using a MLMT approach, **8 phylogenetic groups were identified among 174 *C. albicans* isolates** from 3 different origins.

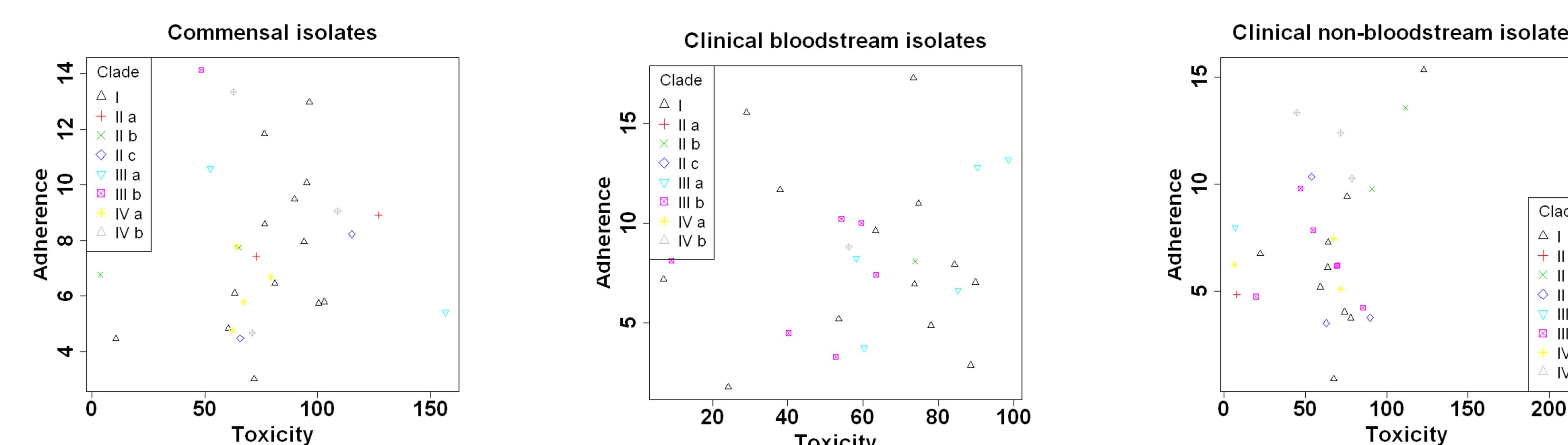
Figure 2. Adherence and cytotoxicity distribution measured in a representative set of this collection (84 strains)



Both adherence and cytotoxicity histograms revealed an heterogeneous distribution of the phenotypic features tested. Interestingly, natural strains displayed very different phenotypic features (*i.e.* 1 to 17% of adherence; 2 to 250% of cytotoxicity) (red bar = median value).

Considering all of theses observations, we investigated the correlation between adherence, cytotoxicity, and origin according to the 8 previously characterized phylogenetic groups (Figure 1).

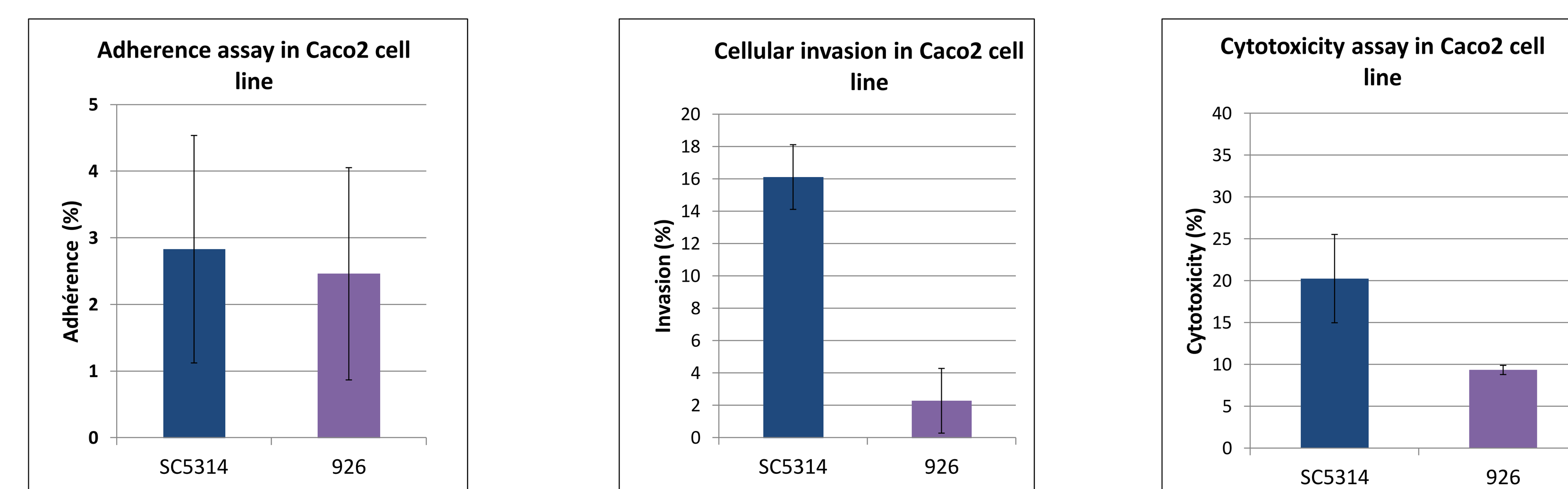
Figure 3. Correlations between adherence, cytotoxicity and genotype with regard to the origin of the strains



To test if the phenotype is related to the phylogenetic groups, a Kruskall-Wallis test was applied to phylogenetic groups and adherence on one side, and phylogenetic groups and cytotoxicity on the other side. None of the tests led to significant results (p-values>0.05), suggesting that **heterogeneity of adherence and cytotoxicity properties are similar whatever the phylogenetic group considered**.

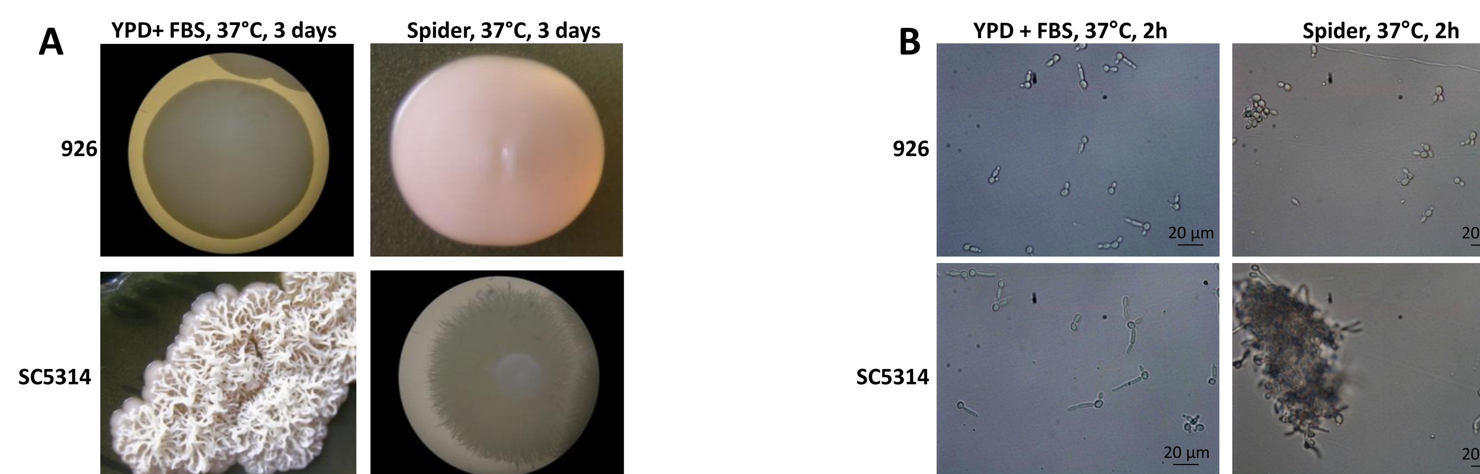
To illustrate this heterogeneity, the clinical strain n°926 (clinical bloodstream isolate) was further characterized *in vitro* (Figure 4).

Figure 4. Comparison of adherence, invasion and cytotoxicity in *C. albicans* n°926 and SC5314 strains



Both **SC5314 and 926 strains** showed **similar adherence** abilities upon the enterocytic Caco2 cell line. Despite this similarity, **the strain 926 was less invasive and cytotoxic** in Caco2 cells than the reference strain. These phenotypic features illustrate the absence of correlation between adherence and invasion/cytotoxic effect in an *in vitro* model of enterocytic cells.

Figure 5. Macroscopic and microscopic observations of 926 strain compared to the reference strain SC5314



Theses observations suggest a normal hyphal growth for *C. albicans* SC5314 whereas **the strain 926 display a defect in filamentation** on solid/liquid specific media. These results suggest that the lower cytotoxicity of the strain 926 is linked to an hyphal growth failing. Moreover, it confirms that the ability to form hyphae is a predominant process in pathogenicity.

CONCLUSIONS

Principal component factor analysis failed to reveal specific correlation between genotypic and phenotypic features for all of the strains studied. Interestingly, some clinical strains presented a decrease in their cytotoxic effect associated with a defect in their filamentation capability.

To illustrate these observations, the clinical strain n°926 (clinical bloodstream isolate) was further characterized *in vitro*. Colony morphology on specific agar media reported a strong defect in its filamentation ability. This was confirmed by microscopic observations in specific liquid media. Despite similar adherence capability to epithelial cells, cytotoxicity of the strain n°926 was significantly lower as compared to the reference strain *C. albicans* SC5314. These data suggest that (i) invasion into epithelial cells is mainly driven by the ability of *C. albicans* to switch from the yeast to the hyphal form and (ii) natural *C. albicans* strains could harbor atypical defect in their filamentation capabilities.

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