New tools for the new bug *Candida auris*

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ABSTRACT *Candida auris* is a recently emerged yeast pathogen of humans causing severe hospital-acquired systemic infections. It is of the utmost importance to understand the genetic and cellular basis of its virulence and pathogenicity. In a recent study, Santana & O'Meara generated forward and reverse genetic tools to manipulate *C. auris*.

Key words: *Candida auris*, genetic manipulation, cellular morphogenesis

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The yeast *Candida auris* was recognized as a novel human pathogen in 2009 and has turned into a major healthcare problem causing systemic infections in patients with pre-existing health conditions [1]. *C. auris* strains can be grouped into four (possibly five) geographically isolated taxonomical groups (clades); strains from different clades differ considerably from each other, genetically and phenotypically [1,2]. Research on *C. auris* in the past decade elucidated aspects of its biology, but we are far away from a comprehensive understanding of its life cycle [2]. A detailed understanding of clinically relevant traits of *C. auris*, such as virulence and antimicrobial resistance, is of particular interest, as this would enable development of new treatments which are needed in clinics to tackle this novel pathogen. Elucidation of the molecular mechanisms underpinning such clinically relevant traits in *C. auris* would require genetic tools that enable the manipulation of its genome. Early efforts have resulted in the establishment of protocols to generate gene deletions and conditional knock-downs via homology-directed repair, and to perform gene editing using CRISPR-Cas9 as an RNA-protein complex [3–5]. Although workable, these approaches have proved laborious and/or technically cumbersome. In this respect, a recent study by Santana & O'Meara [6] has established two novel genetic tools for *C. auris* research: (I) a forward genetic screen assay employing *Agrobacterium*-mediated transformation (AtMT) (Figure 1A), and (II) a DNA-based CRISPR-Cas9 system which is transiently expressed in *C. auris* and facilitates reverse genetic approaches (Figure 1B).

As a proof-of-principle, the AtMT system was used to screen for mutant yeast with an altered colony morphology (Figure 1C) [6]. The genes affected by the insertional mutagenesis of the *Agrobacterium tumefaciens* T-DNA were identified using a whole-genome sequencing approach. These morphogenetic mutants turned out to also affect cellular behaviour indicating that altered colony morphology is a good predictor of changed cellular behaviour. A set of mutants exhibited a cellular aggregation phenotype [6], a trait which previously has also been observed in some clinical *C. auris* isolates and been associated with differences in virulence [7]. A further single mutant displaying constitutive cellular filamentation was also identified [6]; in some fungi, this cellular phenotype is associated with invasive disease. However, in *C. auris*, this has only been described as a response to genotoxic, temperature, and high-salt stress [3,4]. To verify the causative nature of these mutants isolated in the forward genetic screen, Santana & O'Meara then generated full deletions of the affected genes using a DNA-based CRISPR-Cas9 approach inspired by a similar tool developed for *Candida albicans* (Figure 1C) [6,8]. Relying solely on homology-directed, targeted integration for genetic manipulation of *C. auris* results in rather low success rates [3,6]. The frequencies of correct targeting are significantly improved by ~3- to

12-fold when CRISPR-Cas9 is applied. The main strength of the work by Santana & O'Meara is that they explore these genetic tools in *C. auris* strains belonging to the four main clades [6], demonstrating that their strategies are applicable to representatives of the whole species. Intriguingly, in terms of correct targeting efficiency, there seem to be substantial differences between strains from different clades; both with and without the support of CRISPR-Cas9 [6].

The genes of two of the constitutively aggregating mutants from AtMT were identified as orthologs of *Saccharomyces cerevisiae ACE2* and *TAO3* (Figure 1C) [6]. Indeed, the full deletion mutants *ace2*Δ and *tao3*Δ recapitulated the aggregation phenotype of the insertion mutants generated with the AtMT system [6]. Using the CRISPR-Cas9 tool again the wild-type condition was restored, which conclusively demonstrated that these genes are determinants of cellular and colony morphology in *C. auris*. In *S. cerevisiae* Tao3 is a regulator of Ace2 in the RAM (Regulation of *ACE2* morphogenesis) pathway which mediates septum degradation during cell division. Indeed, this function of the RAM pathway is conserved, as mutation of *ACE2* or its upstream regulators, such as *TAO3*, causes a cellular aggregation phenotype in *S. cerevisiae*, *C. albicans*, and *C. auris* [6]. Santana & O'Meara then also demonstrate that in both *ace2*Δ and *tao3*Δ mutants a key enzyme involved in degradation of the septum during cell division, the chitinase Cts1, is downregulated [6]. On a mechanistic level, this would explain the cell aggregation phenotype of *C. auris* that is likely caused by an inability to efficiently degrade the septum thus causing the daughter cells to stick together. Further tests indicated that the *ace2*Δ mutant, in contrast to the *tao3*Δ mutant, did modestly affect virulence. Whereas the *tao3*Δ mutant exhibited increased resistance to some antifungal drugs, while the *ace2*Δ mutant displayed wild-type levels of antifungal drug susceptibility [6].

The mutant isolated from the AtMT screen with a constitutive cellular filamentation and aggregation phenotype affected the ortholog of the *ELM1* gene; again this was confirmed by independently generated deletion mutants and by subsequently restoring the wild-type condition using the CRISPR-Cas9 tool (Figure 1C) [6]. In *S. cerevisiae*, the Elm1 kinase regulates morphogenetic differentiation and cell division; this seems to be conserved in *C. auris* and also in *Candida glabrata* [6]. Intriguingly and in stark contrast to the *ace2*Δ and *tao3*Δ mutants, expression of the Cts1 chitinase is upregulated in the *elm1*Δ mutant [6], indicating that the aggregation phenotype of the latter might have different causes than of the former.

The establishment of *Agrobacterium*-mediated transformation (AtMT) and DNA-based CRISPR-Cas9 in *C. auris* by Santana & O'Meara [6] is a great technical advance. Another forward genetics tool, transposon mutagenesis, has recently been adapted for *C. auris* [9], adding to the growing molecular biology tool box to study this yeast. As a proof-of-principle, Santana & O'Meara screened for and characterized mutants with altered colony and cellular morphology [6]. The screens were by no means exhaustive, and further studies will be needed to follow up on these initial insights. Together with new infection models [10], this will be immensely useful to the budding *C. auris* research community and to the wider medical mycology field, because this is an essential prerequisite for expediting therapeutic development and thus for improving management of life threatening *C. auris* infections in the near future.

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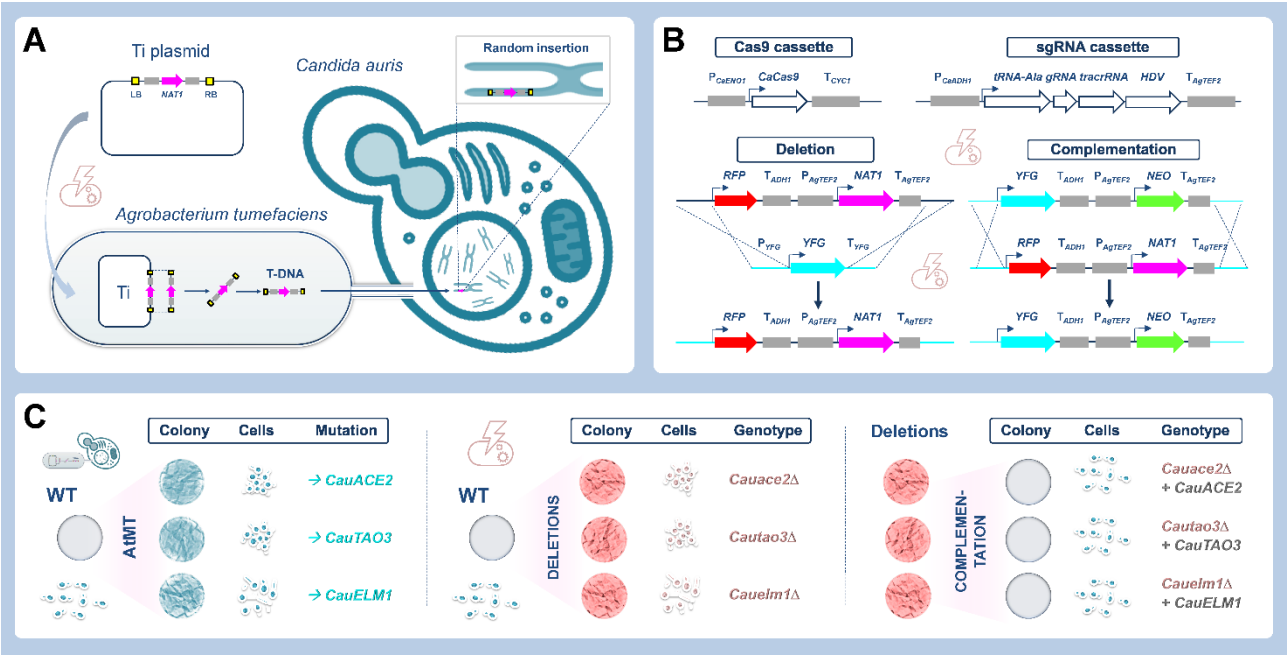

Figure 1. New forward and reverse genetic tools for *C. auris*. **(A) Development of a forward genetic screen assay employing *Agrobacterium*-mediated transformation (AtMT).** The native Ti plasmid from *A. tumefaciens* was modified for transferring nourseothricin resistance (*NAT1* selectable marker) to *C. auris* after co-culture of *A. tumefaciens* and *C. auris*. Note, that in such an approach a single copy of the T-DNA, delimited by both right and left borders (LB and RB), is integrated randomly into the genome of the recipient *C. auris* strain. **(B) CRISPR-Cas9 expression system for targeted transformation of *C. auris*.** The upper panel depicts structures of the Cas9 and sgRNA expression cassettes. *CAS9* is driven by the *C. auris* enolase gene (*ENO1*) promoter and followed by the cytochrome c gene (*CYC1*) terminator. The sgRNA cassette is regulated by the *C. auris ADH1* (alcohol dehydrogenase gene) promoter and the terminator of the *Ashbya gossypii* translational elongation factor 2 gene (*AgTEF2*), and contains *C. auris* tRNA-Ala, 20-bp gRNA sequence, tracrRNA sequence, and hepatitis delta virus (HDV) ribozyme sequence. The lower panel shows the cassette specifically designed for targeted integration in *C. auris* by using homologous recombination. The cassette combines a red fluorescent protein (*RFP*) gene as a marker for correct targeted integration (this is not an essential part of the deletion cassette), *ADH1* terminator, *AgTEF2* promoter, *NAT1* nourseothricin resistance gene, and *AgTEF2* terminator. To achieve correct integration into the genome, this cassette must be flanked by approximately 500 bp 5' and 3' UTR of your favourite *C. auris* gene (*YFG*) to be disrupted. The right lower panel shows the molecular construct developed for functional complementation of deleted genes. The cassette combines *ADH1* terminator, *AgTEF2* promoter, a G418 resistance marker (*NEO*) (novel to *C. auris*), and *AgTEF2* terminator. To drive correct integration into the genome, this cassette must contain at the 5' extremity 500 bp of the 5'UTR plus the complete coding sequence of *YFG* and at the 3' end approximately 500 bp 3' UTR of *YFG* to be reconstituted. **(C) Identification of *C. auris* morphogenesis associated genes.** AtMT first led to the isolation of some morphogenetic mutants. For instance, three of these mutants were found to bear T-DNA integration in the *ACE2*, *TAO3* and *ELM1* loci, respectively. The involvement of these genes in *C. auris* morphogenesis was confirmed by independently disrupting them in *C. auris* strains from different clades, and then reintegrating functional *ACE2*, *TAO3* and *ELM1* genes for complementation.