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Note

A standardized toolkit for genetic engineering of CTG clade yeasts

Tatiana A. Defosse[a,b], Vincent Courdavault[b], Alix T. Coste[c], Marc Clastre[b], Thomas Dugé de Bernonville[b], Charlotte Godon[a], Patrick Vandeputte[a,d], Arnaud Lanoue[b], Antoine Touzé[e], Tomas Linder[f], Samir Droby[g], Carlos A. Rosa[h], Dominique Sanglard[c], Christophe d'Enfert[i], Jean-Philippe Bouchara[a,d], Nathalie Giglioli-Guivarc'h[b], Nicolas Papon[a,*]

[a] Groupe d'Etude des Interactions Hôte-Pathogène (EA 3142), GEIHP, UNIV. Angers, Université Bretagne-Loire, Angers, France
[b] Université François-Rabelais de Tours, Biomolécules et Biotechnologies Végétales, EA 2106, Tours, France
[c] Institute of Microbiology, University Hospital Center, University of Lausanne, Lausanne, Switzerland
[d] Laboratoire de Parasitologie - Mycologie, Centre Hospitalier Universitaire d'Angers, Angers, France
[e] Université François-Rabelais de Tours, Biologie des Infections à Polyomavirus, Molecular virology and immunology, ISP 1282, INRA, Tours, France
[f] Swedish University of Agricultural Sciences, Department of Molecular Sciences, Uppsala, Sweden.
[g] Department of Postharvest Science, Agricultural Research Organization, the Volcani Center, Bet Dagan, Israel
[h] Departamento de Microbiologia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil
[i] Unité Biologie et Pathogénicité Fongiques, Institut Pasteur, INRA, Paris, France

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ABSTRACT

We have developed a series of synthetic constructs suitable to genetically manipulate a broad range of yeast species belonging to the fungal CTG clade. This molecular toolbox notably allows heterologous gene expression, single or dual fluorescence labeling and construction of luciferase-expressing strains for bioluminescence imaging.

The fungal CTG clade includes a broad series of well-known yeasts of clinical importance and/or high biotechnological potential. Prominent species posing risks for human health include for instance *Candida albicans, Candida parapsilosis, Candida dubliniensis, Candida auris, Clavispora lusitaniae, Diutina rugosa,* and *Lodderomyces elongisporus*. These species causing cutaneous, mucosal and/or systemic infections termed candidiasis are actively studied in order to elucidate molecular events underlying pathogenicity, host-pathogen interaction and antifungal resistance (Turner and Butler, 2014). Besides, the CTG clade encompasses several species of particular interest in various areas of biotechnology such as *Scheffersomyces stipitis*, *Debaryomyces hansenii, Candida maltosa, Millerozyma farinosa, Spathaspora passalidarum* (Hittinger et al., 2015). These fungi are considered to be convenient producers of industrial enzymes, single-cell protein, biofuel, vitamins, sweeteners, lipids as well as other metabolites of medicinal and nutritional values. Interestingly, some of these yeasts are able to grow on a variety of carbon sources including phenols, fatty acids, *n*-alkanes, pentoses and several other carbohydrates conferring serious advantages for implementation of low-cost production processes. In addition, some species from the CTG clade (*e.g. Candida tropicalis*) have an important potential in bioremediation, particularly through their ability to produce biosurfactants that promote the cellular assimilation of many hydrocarbon types (petroleum, motor oils). Finally, some of these microorganisms (*e.g. Meyerozyma guilliermondii, Metschnikowia fructicola*) are also used as antagonist yeasts for post-harvest biological control of spoilage fungi during storage of plant-derived products (Papon et al., 2014).

The recent whole-genome sequencing of more than twenty species from the CTG clade clearly reflects the increasing interest of the scientific community in these yeasts, both for their clinical importance and their biotechnological potential (Hittinger et al., 2015). These massive genomics resources thus represent a major step to support genetic approaches in these species, particularly functional genomics and metabolic engineering. However, it is well-known that one of the major drawbacks with that perspective remains the occurrence in the fungal CTG clade of an Alternative Yeast Codon Usage (AYCU) (Mühlhausen and Kollmar, 2014). This precludes the direct use of most of the molecular tools dedicated to bacteria or *Saccharomyces cerevisiae* for genetics in the CTG clade. As a consequence, increasing effort has been made over the last fifteen years to adapt some drug-resistant markers,

* Corresponding author.
E-mail address: nicolas.papon@univ-angers.fr (N. Papon).

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Table 1
List of fungal CTG clade species experimented in this study and some of their characteristics.

Species[a]	Clinical relevance [b]	Biotechnological potential[c]	Genome sequence	CMI (μg/ml)[d]		
				Nourseothricin	Hygromycin B	Mycophenolic acid
Candida albicans SC 5314	+ + + +	−	Available	> 300 (R)	> 500 (R)	< 50 (S)
Candida parapsilosis ATCC 22019	+ +	+ +	Available	< 150 (S)	500 (I)	< 50 (S)
Candida tropicalis MYA3404	+ +	+ +	Available	< 150 (S)	< 300 (S)	< 50 (S)
Candida auris VPCI 479/P/13	+ +	−	Available	> 300 (R)	> 500 (R)	< 50 (S)
Candida dubliniensis CD36	+	−	Available	< 150 (S)	< 300 (S)	< 50 (S)
Clavispora lusitaniae CBS 6936	+	−	Available	< 150 (S)	< 300 (S)	< 50 (S)
Lodderomyces elongisporus CBS 2605	+	−	Available	< 150 (S)	< 300 (S)	100 (I)
Meyerozyma guilliermondii ATCC 6260	+	+ + + +	Available	< 150 (S)	500 (I)	> 100 (R)
Debaryomyces hansenii CBS 767	+	+ + + +	Available	< 150 (S)	< 300 (S)	< 50 (S)
Diutina rugosa UFMG-CM-Y349	+	+ +	−	> 300 (R)	< 300 (S)	100 (I)
Scheffersomyces stipitis CBS 6054	−	+ + + +	Available	< 150 (S)	< 300 (S)	< 50 (S)
Spathaspora passalidarum CBS 10155	−	+ + + +	Available	< 150 (S)	< 300 (S)	< 50 (S)
Millerozyma farinosa CBS 7064	−	+ +	Available	300 (I)	< 300 (S)	100 (I)
Metschnikowia fructicola CBS 8853	−	+ +	Available	< 150 (S)	< 300 (S)	< 50 (S)
Candida maltosa UFMG-CM-Y350	−	+ +	Available	< 150 (S)	< 300 (S)	< 50 (S)

[a] Yeast strains were routinely cultivated in liquid YPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 30 °C under shacking (150 rpm) except for *D. hansenii* (25 °C).
[b] + + + +, high clinical relevance; + +, moderate clinical relevance; +, low clinical relevance; −, no clinical relevance.
[c] + + + +, high biotechnological potential; + +, moderate biotechnological potential; −, no biotechnological potential.
[d] Concentrations of drugs that inhibit the growth of each yeast species were determined according to the conditions described previously (Defosse et al., 2016; Basso et al., 2010). S, susceptible; I, intermediate; R, resistant.

fluorescent protein variants, and luciferase genes to allow their expression in species related to the CTG clade. Until now, this approach was carried out in a case-by-case basis and only for a restricted number of yeast species (Papon et al., 2012). With this in mind, and because standardization of molecular cloning greatly facilitates advanced DNA engineering, parts sharing, and collaborative efforts, we were here particularly interested in developing a global approach for providing a series of synthetic constructs to genetically engineer a broad range of interesting CTG clade species.

The list of species used to test the versatile molecular toolbox is provided in Table 1. These species were selected for (i) their known affiliation to the fungal CTG clade, (ii) their importance in human health and/or their biotechnological potential and (iii) apart from *D. rugosa*, the availability of a sequenced genome (Hittinger et al., 2015). The architecture of the standardized pAYCU series of plasmids in the toolkit is illustrated in Fig. 1. The basic structure consists of two main functional modules: a drug resistance cassette and a reporter gene expression cassette. First, it is possible to choose in the synthetic construct among three different selectable makers including *SAT1* (resistance to nourseothricin), *HPH*$^{\#}$ (resistance to hygromycin B), and Mg*IMH3.2* (resistance to mycophenolic acid) (Defosse et al., 2016). This appears essential because our preliminary tests of susceptibility revealed a markedly variable profile among our set of species toward the three drugs commonly used in CTG yeast genetics (Table 1). As reporter genes, the toolkit offers a range of codon-adapted fluorescent protein variants including the yeast enhanced green, yellow, cyan, and red fluorescent proteins (*yeGFP, yeYFP, yeCFP*, and *yemCherry*, respectively) but also the *gLUC59* corresponding to the codon-optimized version of the surface-exposed *Gaussia princeps* luciferase and the *Streptococcus thermophilus lacZ* (St*lacZ*) gene encoding a *β*-galactosidase (Courdavault et al., 2011; Uhl and Johnson, 2001; Enjalbert et al., 2009). Importantly, all the DNA parts are interchangeable thanks to the presence of commonly used and rare-cutter restriction sites (Fig. 1). The features of particular interest provided by the backbone plasmid include: (i), the whole drug resistance cassette exchange following a single step of digestion/ligation with *Not*I; (ii), the multiple and compatible endonuclease restriction sites located on both sides of fluorescent protein variants for facilitating N- and C-terminus fusion strategies; and (iii), if required, exchange of driving promoters in the drug resistance or the expression cassettes.

Interestingly, during the testing phase of this standardized toolkit, we found that some specific adaptation (*i.e.* some DNA parts exchanges) in the pAYCU212 plasmid that we initially developed for *M. guilliermondii* were necessary to extend its applicability in all listed species (Tables 1 and 2). First, we demonstrated that both *M. guilliermondii* phosphoglycerate kinase (P_{MgPGK1}) and Actin 1 (P_{MgACT1}) gene promoters are also functional in *C. lusitaniae* (Fig. 2A–B), *C. parapsilosis*, *S. stipitis*, *M. farinosa*, and *M. fructicola* since these species were successfully transformed with the pAYCU212 to nourseothricin resistance and expressed the ye*YFP* reporter gene. By contrast, the P_{MgPGK1} was not functional in the remaining species of the list (Tables 1 and 2) because no transformants were obtained when drug resistance genes (*SAT1, HPH*$^{\#}$, and Mg*IMH3.2*) were driven by this promoter. By a single exchange of the P_{MgPGK1} with the *C. dubliniensis* translation elongation factor 1 gene promoter (P_{CdTEF1}) in the pAYCU212 (yielding pAYCU228, Table 2), the synthetic construct was found fully functional in *C. dubliniensis* but also in *C. albicans*, *C. tropicalis*, *C. maltosa*, and *D. rugosa*. In addition, specific promoter exchanges in the drug resistance cassette were necessary to recover full functionality of the synthetic construct in *S. passalidarum* (Fig. 2G–H) and *D. hansenii* (pAYCU229 and pAYCU244, Table 2). Finally, exchanges of both the promoters driving the resistance and the reporter genes were necessary to recover a full functionality of the synthetic construct in *L. elongisporus* (pAYCU254, Table 2) (Fig. 2C–D). Thus, with the exception of *L. elongisporus* (for which a particular genomic organization is known) (Butler et al., 2009), these results suggest the broad potential of the P_{MgACT1} for controlling gene expression in yeast species displaying the AYCU. These results also demonstrate that few DNA part exchanges are finally required for pAYCU plasmid functionality in a large range of species belonging to the CTG clade. On the basis of these preliminary observations, and as a proof of concept, a series of plasmids harboring different DNA part combinations was generated (compiled in Table 2). The functionality of each of these synthetic constructs was tested and validated by the genetic transformation of species indicated in the last column of Table 2. A panel of representative pictures of experiments demonstrating the expression of the reporter genes in several yeast species is provided in Fig. 2. For instance, the pAYCU268 vector (including the nourseothricin resistance marker for the selection of transformants and the *yeGFP* as reporter gene) was shown to be functional not only in *C. tropicalis* (Fig. 2E-F) but also in *C. albicans, C. dubliniensis, C. maltosa, D. rugosa* as indicated in Table 2. The pAYCU211 plasmid (harboring the hygromycin B resistance marker for the selection of transformants and the