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Review

Combinatorial optimization of CRISPR/Cas9 expression enables precision genome engineering in the methylotrophic yeast *Pichia pastoris*

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ABSTRACT

The methylotrophic yeast *Pichia pastoris* (*Komagataella phaffii*) is one of the most commonly used expression systems for heterologous protein production. However the recombination machinery in *P. pastoris* is less effective in contrast to *Saccharomyces cerevisiae*, where efficient homologous recombination naturally facilitates genetic modifications. The lack of simple and efficient methods for gene disruption and specifically integrating cassettes has remained a bottleneck for strain engineering in *P. pastoris*. Therefore tools and methods for targeted genome modifications are of great interest.

Here we report the establishment of CRISPR/Cas9 technologies for *P. pastoris* and demonstrate targeting efficiencies approaching 100%. However there appeared to be a narrow window of optimal conditions required for efficient CRISPR/Cas9 function for this host. We systematically tested combinations of various codon optimized DNA sequences of *CAS9*, different gRNA sequences, RNA Polymerase III and RNA Polymerase II promoters in combination with ribozymes for the expression of the gRNAs and RNA Polymerase II promoters for the expression of *CAS9*. Only 6 out of 95 constructs were functional for efficient genome editing.

We used this optimized CRISPR/Cas9 system for gene disruption studies, to introduce multiplexed gene deletions and to test the targeted integration of homologous DNA cassettes. This system allows rapid, marker-less genome engineering in *P. pastoris* enabling unprecedented strain and metabolic engineering applications.

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1. Introduction

Pichia pastoris (*Komagataella phaffii*) is according to a recent literature survey the most commonly used eukaryotic expression host even surpassing *Saccharomyces cerevisiae* (Bill, 2014). It has been widely used for protein production in basic and applied research (Ahmad et al., 2014; Gasser et al., 2013). However, in contrast to the 'classic' yeast and model organism *S. cerevisiae*, it is considerably more difficult to achieve targeted genetic modifications, because the homologous recombination (HR) machinery in natural *P. pastoris* strains is less efficient (Higgins and Cregg, 1998; Li et al., 2007; Näätsaari et al., 2012). In contrast, in *S. cerevisiae* homologous overhangs of approximately 50 bp are sufficient to achieve targeting efficiencies close to 100%, while in *P. pastoris* even the addition of several hundred bp or more than a kbp of homologous overhangs usually results only in specific targeting efficiency of 0.1% to 30% (depending on the target locus and the size and the design of the donor fragment) (Higgins and Cregg, 1998; Li et al., 2007; Näätsaari et al., 2012). Hence so far the introduction of targeted genetic modifications has been highly challenging in *P. pastoris* and marker gene recycling (removal) causes additional workload. For example, in efforts to generate auxotrophic *P. pastoris* strains, only 5 out of 460 tested transformants showed correctly integrated expression cassettes (Nett and Gerngross, 2003). Deleting the gene coding for the *P. pastoris KU70* homolog, a keyplayer in the non-homologous-endjoining (NHEJ) repair, significantly increased homologous recombination efficiencies (Näätsaari et al., 2012). However in general NHEJ-defective organisms show lower transformation rates with linear integration cassettes, reduced growth rates and an increased sensitivity to radiation and DNA-damaging conditions (Carvalho et al., 2010; Näätsaari et al., 2012), thereby meeting demands for improved industrial strain engineering only partially.

Targeted single and double strand break induced DNA repair can be used to increase the HR frequency by several orders of magnitude (up to 4000–fold in *S. cerevisiae*) (Caldecott, 2008; Rouet et al., 1994; Smih et al., 1995; Storici et al., 2003). Therefore systems introducing breaks at programmable positions in the genome are of great interest. Recently, CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9) was reported to mediate targeted genome engineering in various pro- and eukaryotic hosts (DiCarlo et al., 2013; Jacobs et al., 2014; Jinek et al., 2012; Mali et al., 2013; Sander and Joung, 2014) and in principle there is no reason why this technology should not be applicable in any organism. It takes advantage of a nuclease, which is guided by a short RNA (guide RNA: gRNA/single guide RNA: sgRNA) to introduce a DNA strand break at regions complementary to the gRNA sequence. The breaks are sealed by the recruitment of the cellular repair machinery, allowing the introduction of various genomic modifications. Reprogramming CRISPR/Cas9 and thereby targeting different loci is performed by changing 20 bp of the gRNA, instead of cumbersomely engineering protein domains as required for comparable programmable TALEN strategies (Gaj et al., 2013; Kim and Kim, 2014; Weninger et al., 2015b). Due to its outstanding characteristics compared to other genome modification tools in terms of flexibility, to achieve multiplexing, the ease of retargeting and the potential to efficiently introduce site specific modifications, CRISPR/Cas9 is one of the most promising tools for targeted genome engineering (Gaj et al., 2013; Weninger et al., 2015b). However implementing CRISPR/Cas9 in a given organism requires the functional expression of *CAS9* and the gRNA, which is reliant on a set of interdependent features (Fig. 1) (Jinek et al., 2012; Mali et al., 2013). One frequently applied strategy is to express the *CAS9* gene in the cell. The gene has to be transcribed, translated and folded correctly in the heterologous organism, where genome targeting should be achieved. In order to target eukaryotic genomes the bacterial protein Cas9 needs to be fused to a nuclear localisation sequence (NLS) to allow import in the nucleus in eukaryotes. Using different NLSs can have a pronounced effect on nuclear targeting (Nelson and Silver, 1989; Weninger et al., 2015a). Furthermore cytotoxic effects of the large, overexpressed 160 kDa protein need to be minimized. The gRNA must remain in the nucleus and 5′ or 3′ RNA sequences, which might be added in the course of its transcription, must not have a negative influence on correct localization, folding and functionality. Cas9 and the gRNA have to assemble in the nucleus and the RNA-protein complex has to recognize the targeted homologous locus in the genome in order to introduce a strand break. Unspecified off-targeting and toxicity effects caused by overexpression must not have detrimental or lethal effects on the cells. Hence, efficient genome targeting can only be achieved, when all single components are correctly designed, expressed, produced and assembled in the host.

In this study, we aimed to develop a CRISPR/Cas9 system that enables specific and precise genome engineering in *P. pastoris* as a potent alternative to the currently applied genome engineering strategies.

2. Materials and methods

2.1. Chemicals

Enzymes were obtained from Thermo Fisher Scientific, Vienna, Austria. D(+)-biotin was obtained from Sigma-Aldrich, Vienna, Austria. Difco yeast nitrogen base w/o amino acids, Bacto tryptone and Bacto yeast extract were obtained from Becton Dickinson, Schwechat, Austria. Zeocin™ was obtained from Life Technologies, Carlsbad, CA, US and InvivoGen, France. Other chemicals were purchased from Carl Roth, Karlsruhe, Germany. Oligonucleotides were ordered from Integrated DNA Technologies, Leuven Belgium, see Supplementary Table S1 for the sequences.