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A novel chimaeric flocculation protein enhances flocculation in *Saccharomyces cerevisiae*

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ABSTRACT

Yeast flocculation is the reversible formation of multicellular complexes mediated by lectin-like cell wall proteins binding to neighbouring cells. Strong flocculation can improve the inhibitor tolerance and fermentation performance of yeast cells in second generation bioethanol production. The strength of flocculation increases with the size of the flocculation protein and is strain dependent. However, the large number of internal repeats in the sequence of *FLO1* from *Saccharomyces cerevisiae* S288c makes it difficult to recombinantly express the gene to its full length. In the search for novel flocculation genes resulting in strong flocculation, we discovered a DNA sequence, *FLONF*, that gives NewFlo phenotype flocculation in *S. cerevisiae* CEN.PK 113-7D. The nucleotide sequence of the internal repeats of *FLONF* differed from those of *FLO1*. We hypothesized that a chimaeric flocculation gene made up of a *FLO1* variant derived from *S. cerevisiae* S288c and additional repeats from *FLONF* from *S. cerevisiae* CCUG 53310 would be more stable and easier to amplify by PCR. The constructed gene, *FLOw*, had 22 internal repeats compared to 18 in *FLO1*. Expression of *FLOw* in otherwise non-flocculating strains led to strong flocculation. Despite the length of the gene, the cassette containing *FLOw* could be easily amplified and transformed into yeast strains of different genetic background, leading to strong flocculation in all cases tested. The developed gene can be used as a self-immobilization technique or to obtain rapidly sedimenting cells for application in *e.g.* sequential batches without need for centrifugation.

1. Introduction

Flocculation is a natural way for yeast cells to adhere to each other and increase the survival chance of the collective by formation of multicellular agglomerates. It is used mainly as a rapid and cheap way of separating cells from the broth in fermentation processes (Verstrepen et al., 2003). Additionally, we have shown that in the special case of fermentation of toxic lignocellulose hydrolysates, flocculation can increase the inhibitor tolerance of the cells and result in faster and more complete fermentation (Westman et al., 2014).

Flocculation is caused by lectin-like cell wall proteins, flocculins, that bind to mannoproteins in the cell wall of neighbouring cells (Goossens and Willaert, 2010). In *Saccharomyces cerevisiae*, the flocculation genes are situated in the sub-telomeric regions of the genome (Teunissen and Steensma, 1995). Furthermore, the flocculation genes contain tandem repeats in the middle of the genes. The number of repeats determines the strength of the flocculation, and to some extent also the sugar specificity of the flocculation proteins (Liu et al., 2007; Verstrepen et al., 2005; Westman et al., 2014). Recombination events can easily alter the number of repeats and the flocculation characteristics can, therefore, change rapidly in response to *e.g.* environmental stress (Verstrepen et al., 2005). For strong flocculation of recombinant flocculating strains, a stable construct with a long flocculation gene, *i.e.* with many tandem repeats, is required. This has proven difficult to achieve due to the nature of the flocculation genes. In PCR and in amplification in *Escherichia coli*, the long sequences of internal repeats can lead to recombination events that shorten the gene by changing the length of the internal region (Teunissen et al., 1993; Westman et al., 2014). It has therefore been difficult to recombinantly express the entire *FLO1* gene (Watari et al., 1994; Westman et al., 2014).

An alternative to recombinant expression of the known *FLO1* is to

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search for novel flocculation genes in strongly flocculating *S. cerevisiae* strains (Zhao et al., 2011). Due to the rapid evolution of flocculation genes it is expected that certain strains can harbour genes resulting in a stronger and more robust flocculation than that obtained by *FLO1* from S288c (Verstrepen et al., 2005).

The aim of the current work was to design a flocculation gene resulting in improved flocculation characteristics when expressed in otherwise non-flocculating strains. To this end, a DNA sequence (*FLONF*) was isolated from the constitutively strongly flocculating *S. cerevisiae* CCUG 53310 (Westman et al., 2012), using primers designed for *FLO1* from S288c. Expression in CEN.PK 113-7D did not lead to the expected Flo1 phenotype flocculation, inhibited only by mannose. Instead the resulting strain displayed NewFlo phenotype flocculation that was inhibited by several sugar types. Since the internal repeat region of *FLONF* differed from that of *FLO1*, we hypothesized that a more stable flocculation gene could be created by merging a partial *FLO1* gene with a part of the internal repeat region of *FLONF*. Construction of a gene with a mix of different repeats would make it more stable in the strain, and easier to transfer between different strains to obtain rapidly sedimenting cells for application in *e.g.* sequential batches without need for centrifugation. With this in mind, we created a chimaeric gene that resulted in strong constitutive flocculation upon expression in otherwise non-flocculating yeast strains.

2. Materials and methods

2.1. Yeast strains and medium

The naturally constitutively flocculating strain *S. cerevisiae* CCUG 53310 was used for isolation of *FLONF*. The recombinant strains used in this study originated from *S. cerevisiae* CEN.PK 113-7D (MATa, MAL2–8C, SUC2) (van Dijken et al., 2000) which was used as reference strain, as well as from KE6–12.A (Tomás-Pejó et al., 2014) and IBB10B05 (Novy et al., 2014). Inoculations of pre-cultures were done by picking a colony from fresh YPD agar plates (10 g/l yeast extract, 20 g/l soy peptone, 20 g/l glucose, and 20 g/l agar), prepared from a frozen glycerol stock.

Aerobic cultures for cell propagation were grown in 250 ml cotton-plugged conical flasks in a shaker (125–200 rpm) at 30 °C. Liquid YPD medium containing 20 g/l of glucose was used for propagation of cells.

2.2. Isolation of a flocculation gene from S. cerevisiae CCUG 53310 and construction of a recombinant flocculating yeast strain

Using the primers *FLO1*-FW and *FLO1-HO*-RV (Table 1), giving a region homologous to the *HO* locus in *S. cerevisiae* CEN.PK 113-7D, a gene sequence from *S. cerevisiae* CCUG 53310 was amplified. The *TDH3* promoter (*TDH3*p) was amplified from genomic DNA of *S. cerevisiae* CEN.PK 113-7D by PCR using the primers EcoRV-*TDH3*p-FW and SpeI-*TDH3*p-RV (Table 1) and cloned in the pUG6 vector (Güldener et al., 1996). The resulting vector was used as template for amplification of the KanMX-*TDH3*p cassette using the primers SphI-KAN-FW and SalI-*TDH3*p-RV (Table 1). The cassette was cloned in YIplac211 (Gietz and Akio, 1988) and amplified by PCR using the primers *TDH3*p-*FLO1*-RV and *HO*-KAN-FW (Table 1), giving flanking ends homologous to the *HO* locus in *S. cerevisiae* CEN.PK 113-7D, and to the DNA sequence isolated from *S. cerevisiae* CCUG 53310, respectively. By fusion PCR the KanMX-*TDH3*p cassette and the DNA sequence were merged together and amplified using the primers *HO*-FW and *HO*-RV (Table 1) in two PCR reactions. In the first step the flocculation gene and the KanMX-*TDH3*p cassette were mixed in a single PCR reaction for PCR-based fusion, using the Phusion polymerase (Thermo Scientific, Lafayette, CO, USA). The DNA fragment resulting from the fusion was used as template for the second PCR reaction, with the primers *HO*-FW and *HO*-RV. This yielded a PCR product with flanking regions homologous to the *HO* locus that was used for homologous recombination in CEN.PK 113-7D using the lithium acetate based transformation method (Gietz and Woods, 2002). Transformants were selected on YPD plates containing 200 µg/ml G418 (Sigma-Aldrich, Steinheim, Germany) and tested for flocculation ability in liquid YPD-medium, shaking overnight. The correct integration into the *HO* locus was confirmed by PCR using the primers SapI-KAN-RV and *HO*-upstream-2 (Table 1). The correctness of the sequences and of the integration was verified by sequencing (Eurofins MWG Operon, Ebersberg, Germany). The strain was named CEN.PK FLONF.

2.3. Inverse PCR

The flanking regions of *FLONF* were investigated by inverse PCR (Ochman et al., 1988). Genomic DNA was digested by 10 different restriction enzymes (AatII, BlpI, XbaI, HindIII, MluI, NotI, PstI, SalI, BstXI and XhoI) creating sticky ends. The digested DNA was ligated at a DNA concentration of 1 µg/ml, to favour self-ligation. After phenol:-chloroform extraction, the DNA was used as template for inverse PCR reactions using the primers IPCR-FW, IPCR-RV1 and IPCR-RV2. The samples from the inverse PCR were analysed on an agarose gel and interesting bands excised, purified and sequenced (Eurofins MWG Operon, Ebersberg, Germany). The sequences were investigated using EMBOSS Needle to assess the similarity to the flanking sequences of the flocculation genes in *S. cerevisiae* S288c.

2.4. Ploidy test

The ploidy of *S. cerevisiae* CCUG 53310 was investigated by flow cytometry of cells stained with Sytox green. Exponentially growing cells were harvested and fixated in cold 70% ethanol. The cells were washed in 10 mM EDTA, pH 8.0, followed by treatment with RNase A, 0.1 mg/ml at 37 °C for 2 h. Sytox green was added to a final concentration of 1 µM and the cells were analysed with a guava easyCyte 8HT flow cytometry system. The haploid strain *S. cerevisiae* BY4741, the diploid *S. cerevisiae* CEN.PK 122 MDS and the tetraploid strain *S. cerevisiae* G26 were used as references.

2.5. Construction of FLOw

The cassette with flanking regions of the *HO* locus containing KanMX, the *TDH3* promoter and the strongly flocculating *FLO1* variant was amplified in two parts from the genomic DNA of *S. cerevisiae* CEN.PK 113-7D strongly flocculating mutant (Westman et al., 2014). The 5′ part of the 'strongly flocculating' *FLO1* (Genbank accession number KM366095) variant cassette was amplified with *HO*-upstream and *FLO1*-N-term-RV (Table 1). This amplicon contained the part of the flocculation gene encoding the carbohydrate binding N-terminal of the protein. The amplicon ended after the first (5′ to 3′) of the eleven internal repeats in the gene, according to a domain analysis by Pfam (Punta et al., 2012). The 3′ part of the 'strongly flocculating' *FLO1* was amplified with *FLO1*-reps-FW and *HO*-downstream (Table 1). This amplicon contained the remaining, 3′ part, of the flocculation gene, including the last ten (5′ to 3′) repeats.

Repeats from the *FLONF* flocculation gene (Genbank accession number KJ716851.1) of *S. cerevisiae* CCUG 53310 were amplified using the primers *FLONF*-reps-FW and *FLONF*-reps-RV (Table 1) from genomic DNA of CEN.PK FLONF. This gave regions homologous to the ends created by primers *FLO1*-N-term_RV and *FLO1*-reps_FW in the two fragments amplified from *S. cerevisiae* CEN.PK 113-7D strongly flocculating mutant. The PCR product was purified from an agarose gel.

By fusion PCR, the three fragments were assembled and the entire cassette was amplified using the nested primers *HO*-upstream-2 and *HO*-downstream-2 (Table 1) in two PCR reactions. Phusion polymerase