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RESEARCH ARTICLE

Identification of superior cellulase secretion phenotypes in haploids derived from natural *Saccharomyces cerevisiae* isolates

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One sentence summary: Screening for superior cellulase secretion in haploids derived from natural *Saccharomyces cerevisiae* isolates.
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ABSTRACT

The yeast *Saccharomyces cerevisiae* is considered an important host for consolidated bioprocessing and the production of high titres of recombinant cellulases is required for efficient hydrolysis of lignocellulosic substrates to fermentable sugars. Since recombinant protein secretion profiles vary highly among different strain backgrounds, careful selection of robust strains with optimal secretion profiles is of crucial importance. Here, we construct and screen sets of haploid derivatives, derived from natural strain isolates YI13, FINI and YI59, for improved general cellulase secretion. This report details a novel approach that combines secretion profiles of strains and phenotypic responses to stresses known to influence the secretion pathway for the development of a phenotypic screen to isolate strains with improved secretory capacities. A clear distinction was observed between the YI13 haploid derivatives and industrial and laboratory counterparts, Ethanol Red and S288c, respectively. By using sub-lethal concentrations of the secretion stressor tunicamycin and cell wall stressor Congo Red, YI13 haploid derivative strains demonstrated tolerance profiles related to their heterologous secretion profiles. Our results demonstrated that a new screening technique combined with a targeted mating approach could produce a pool of novel strains capable of high cellulase secretion.

Keywords: second generation bioethanol; recombinant yeast; heterologous protein secretion; cellulases; cellobiohydrolase; screen

INTRODUCTION

During consolidated bioprocessing (CBP), the production of heterologous cellulolytic enzymes, including exoglucanases i.e. cellobiohydrolases (CBHs), endoglucanases (EGs) and β-glucosidases (BGLs) from a fermentative host like *Saccharomyces cerevisiae*, is required for the hydrolysis of recalcitrant lignocellulosic biomass. Several techno-economic analysis reports suggest cellulolytic enzyme production to be the limiting step in CBP biomass conversion technologies (Wingren, Galbe and Zacchi 2003; Zhuang *et al.* 2007; Khajeeram and Unrean 2017; Olofsson *et al.* 2017). This highlights the importance of studying heterologous cellulolytic enzyme secretion, which is highly dependent on genetic background of the host yeast strain as well as highly protein specific in nature (Ilmén *et al.* 2011; Den Haan *et al.* 2013; Kroukamp *et al.* 2013; Davison, den Haan and van Zyl 2016; Van Zyl, Den Haan and Van Zyl 2016). Therefore, selection of novel hybrid strains with increased secretion

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profiles is crucial for the development of suitable industrial CBP yeasts.

Recent studies have broadened the search to include trait analysis of strains of *S. cerevisiae*, which have demonstrated high phenotypic variance in different yeast subgroups (Van Dijken *et al.* 2000; Garay-Arroyo *et al.* 2004; Warringer *et al.* 2011). Other industrially relevant traits from natural strain isolates have also been selected for including tolerance to high temperatures (Mukherjee *et al.* 2014; Ruyters *et al.* 2014), microbial inhibitory compounds (Mukherjee *et al.* 2014; Ruyters *et al.* 2014; Davison, den Haan and van Zyl 2016; Jansen *et al.* 2018) as well as high ethanol productivity, concentrations and yields (Jin *et al.* 2013; Ruyters *et al.* 2014; Jansen *et al.* 2018). These results described the relationship between phenotype and genetic background, which provided insights into how genetic variants are known to influence phenotypes and functional variances occur in different strains of the same species. In particular, past research suggested that natural isolates outcompeted laboratory and industrial derivatives for secretory capacity of heterologous cellulolytic enzymes (Warringer *et al.* 2011; Davison, den Haan and van Zyl 2016).

As with any industrially relevant trait, improving the phenotype of heterologous protein secretion in yeast is not straightforward. Firstly, this phenotype is complex and shares the common features of quantitative traits, i.e. polygenic control and environmental influence (Liu *et al.* 2014; Kroukamp *et al.* 2017). Due to this complexity of protein secretion, rational engineering methods for strain development have yielded limited success to improve general protein secretion capacities. Therefore, the genetic basis of superior recombinant protein secretion must be investigated as a complex architecture of genes that affects this phenotype through direct and interactive effects (Steinmetz *et al.* 2002). Approaches that generate genetic variation in a non-targeted fashion, for instance intra-species hybridisation of genetically stable haploid strains, have proven successful for other desired characteristics (as demonstrated in Jansen *et al.* 2018 and Kroukamp *et al.* 2018). The approach of sexual hybridisation strategies using phenotypically characterised haploid segregants of carefully selected parental strains (cell-to-cell mating) is thus an appealing strategy.

While large sets of strains have been previously generated for the genetic mapping of other industrially relevant traits from glycerol yield to ethanol tolerance and yield (Deutschbauer and Davis 2005; Cubillos, Louis and Liti 2009; Cubillos *et al.* 2011; Parts *et al.* 2011; Liti and Louis 2012; Salinas *et al.* 2012; Meijnen *et al.* 2016; Coi *et al.* 2018), there is a lack of stable haploid strains for research on heterologous enzyme secretion. One of the most important factors in determining the success of these studies is the availability of an easy screen to identify the few superior cells among a large pool of inferior variants. However, quantifying the recombinant protein secretory capacity of strains, especially in different genetic backgrounds, can be challenging. While studies have attempted to overcome this challenge through the use of small, easy-to-screen enzymes (Liu *et al.* 2013; Huang *et al.* 2015), screening strains for a range of recombinant cellulolytic enzymes have proven to be laborious, expensive and time-consuming (Den Haan *et al.* 2015). Thus, an easy, indirect method to evaluate innate secretion capacity is desired and can be achieved through analysis of secretion stress.

In the last decade, it has become obvious that many heterologous protein products exert severe stress on the host cells when being overexpressed, limiting the potential secretion yield. Likewise, the intimate connection of unfolded protein response (UPR) to recombinant protein production (Mattanovich *et al.* 2004), cell wall integrity (CWI; Verna *et al.* 1997) and membrane lipid balance is well documented (Travers *et al.* 2000). For instance, cells treated with tunicamycin are known to elicit endoplasmic reticulum (ER) stress and activate the conserved UPR pathway that drives transcription of genes such as chaperones and folding enzymes, which are intimately linked to the secretion pathway (Kuo and Lampen 1974; Travers *et al.* 2000). In eukaryotes, this *N*-acetylglucosamine antibiotic prevents the first step of N-linked glycosylation of proteins that causes extensive protein misfolding, leading to the activation of UPR. Past research suggested that innate higher protein folding capacity, could alleviate ER stress, lessening the impact of UPR, which may enable higher levels *T.e.*Cel7A secretion in *S. cerevisiae* (Ilmén *et al.* 2011; Davison, den Haan and van Zyl 2016). As such, tunicamycin may be used to screen haploid segregants for superior secretory capacities of certain reporter proteins and possibly also tolerance to other stresses. A survey of the published literature failed to return any previous studies on phenotypic screening using a combination of stress tolerances for the isolation of strains with innate, distinct secretory capacity. The current study, therefore, proposed to investigate the diversity of secretion stress and cell wall tolerance among different strains of *S. cerevisiae* to determine whether it could serve as a useful selection agent to evaluate strains' capacity for recombinant protein secretion.

Here, we developed a novel approach that combines secretion profiles of strains and phenotypic responses to stresses known to influence the secretion pathway for the development of a phenotypic screen to isolate strains with improved secretory capacities. We describe the technical approaches used for the generation of genetically stable haploid derivatives from three *S. cerevisiae* natural strain isolates ranging in secretory phenotypes (namely high, medium and low) in order to understand the phenotypic differences linked to a range of secretory capacities. Our efforts resulted in several hybrids showing hybrid vigour (also known as heterosis) for heterologous protein secretion. Considering the peculiarities and complexities of the enhanced secretion protein phenotype, the construction of haploid strains representative of a range of secretion phenotypes that are amendable to genetic studies and phenotypic studies is of great value.

MATERIALS AND METHODS

Yeast strains and media

In this study, we screened three diploid, homothallic strain isolates described previously (Davison, den Haan and van Zyl 2016; Table 1), as well as their haploid derivatives shown in Table 2. Strain isolates were obtained from the Agricultural Research Council (ARC) Infruitec—Nietvoorbij Wine Research Centre (Van der Westhuizen, Augustyn and Pretorius 2000) and deposited in the Culture Collection of the Plant Protection Research Institute (PPRI-ARC, Queenswood, Pretoria). The industrial strain derivatives of Ethanol Red were obtained from the Thevelein Lab at KU Leuven and laboratory strains namely S288c (ATCC 204508) and Y294 (ATCC 20116) were included in this study for comparison of relatively diverse backgrounds. The native *Saccharomyces* strains were identified at the species level by sequencing the variable D1/D2 portion of the eukaryotic 26S rDNA as described previously in Davison, den Haan and van Zyl (2016). After species identification, sequences were aligned using MUSCLE version 3.70 (Edgar 2004). The phylogenetic dendorgram was prepared

Table 1. List of strains and plasmids used in this study.

Strain or plasmid	Relevant features	Reference
Strains		
E.coli DH5α	*deoR endA1 gyrA96 hsdR17 6[lac]U169 recA1 supE44 thi-1 [q80 lacZ6M15]*	Life Technologies, Rockville, Md.
Superior secreting *S. cerevisiae*		
YI13	*MATa/MATα*	KX428528
YI13_HO	*MATa/α;HO::GALp kanMX4*	This work
Moderate secreting *S. cerevisiae*		
FIN1	*MATa/MATα*	KX428522
FIN1_HO	*MATa/α;HO::GALp kanMX4*	This work
Low secreting *S. cerevisiae*		
YI59	*MATa/MATα*	(Davison *et al.* 2016)
YI59_HO	*MATa/α;GALp HO::kanMX4*	This work
Industrial *S. cerevisiae*		
ER12	*MATα*	JT 22739.12
ER16	*MATα*	JT 22739.16
ER17	*MATa*	JT 22739.17
ER19	*MATa*	JT 22739.19
ER19 × 12	*MATa/MATα*	This work
ER17 × 12	*MATa/MATα*	This work
Reference *S. cerevisiae*		
S288c **a**/α	*MATa/α mal gal2 sal1-1 MKT1-30D CAT5-91I MIP1-661A-UCC1166*	This work
S288c a	*MATa mal gal2 sal1-1 MKT1-30D CAT5-91I MIP1-661A-UCC1166*	ATCC204508
Y294	*MATα leu2-3112 ura3-52 his3 trp1-289*	ATCC201160
Hoeg	Brewing strain	KX428523.1
MH1000	Industrial strain	KX428525
Plasmids		
pHK301.1	*bla URA3 HO site:GALpKanMX4tef1t*	This study
pHKHO.gal	*bla URA3 HO-GALpkanMX4*	This study
pHK212_SD1	*bla URA3 PGKp-T.e.cel7A-PGKt;NatMX4*	This work
pHK212_SD2	*bla URA3 PGKp-T.r.cel5A-PGKt;NatMX4*	This work
pHK212_SD3	*bla URA3 PGKp-S.f.cel3A-PGKt;NatMX4*	This work

using Molecular Evolutionary Genetics Analysis software version 5.0 (Tamura *et al.* 2011).

Yeast cells were routinely cultivated at 30°C in YPD medium (1% yeast extract, 2% peptone, 2% glucose; Merck, Darmstadt, Germany) and synthetic complete (SC) media (1.7% yeast nitrogen base; Becton, Dickinson and Company, New Jersey, United States of America), 2% glucose, complete amino acid mix, ammonium sulphate (Merck) supplemented with the appropriate antibiotics and solidified with 2% agar (Merck) to make plates. For long-term storage, strains were stored at −80°C in 15% glycerol-based standard storage medium. Putative strain lines were screened in 1 mL antibiotic-selective YPD or SC medium cultured in 96 deep well culture plates (Greiner bio-one, Kremsmunster, Austria), covered with AeraSeal breathable film (Sigma-Aldrich, Missouri, USA) in an adapted Titramax 101 incubator 1000 (Heidolph, Schwabach, Germany) at 30°C, shaking at 600 rpm. Unless otherwise stated, confirmation screening rounds were performed in 10 mL SC medium cultivated in 100 mL Erlenmeyer flasks shaking at 200 rpm at 30°C. *Escherichia coli* DH5α was grown in either Terrific Broth (1.2% tryptone, 2.4% yeast extract and 0.4% glycerol; Merck) or on Luria-Bertani agar plates (0.5% yeast extract, 1% tryptone, 1% sodium chloride and 2% agar; Merck) supplemented with appropriate antibiotics for selecting plasmid-containing cells.

Strain construction, sporulation and transformation

A flow diagram of the steps followed in this work is shown in Fig. 1. Stable, haploid strains were constructed by disrupting at least one *HO* gene allele from native yeast strains with the KanMX4 marker under the control of galactose-inducing promoter to allow genetic stability in haploid state, followed by sporulation to identify heterothallic, haploid progeny (Van Zyl, Lodolo and Gericke 1993). The deletion cassette containing the KanMX4 marker was amplified from plasmid pHK301.3 (Table 1) by PCR primers listed in Table 3 and using KAPA Biosystems polymerase (Roche, Basel, Switzerland) following manufacturers' instructions. The PCR product was isolated on a gel, extracted using Zymogen Gel cleanup kit (Zymo Research, California, USA) and used to transform the strain isolates YI13, FINI and YI59, using the protocol described in Davison, den Haan and van Zyl (2016). Confirmation of *HO* gene deletion was performed by PCR using *Taq* DNA polymerase (New England Biolabs, Massachusetts, USA), using primers (Table 3), which amplify the 3 kb entire *HO* gene including upstream and downstream regulatory elements, under manufacturer's instructions. Sporulation was induced on minimal sporulation agar medium (1% potassium acetate, 2% agar; Merck) supplemented with the appropriate antibiotics. Asci were digested with a solution of 5% lyticase (Melford, Ipswich, UK) for 8 min, and spores

Table 2. Constructed *S. cerevisiae* haploid derivative strains.

Strain	Derivative strain	Genotype
YI13	YI13_C2	*MAT***a**; *ho::GALp kanMX4*
	YI13_6C	*MAT***a**; *ho::GALp kanMX4*
	YI13_E1	*MAT***a**; *ho::GALp kanMX4*
	YI13_9D	*MATα*; *ho::GALp kanMX4*
	YI13_G1	*MATα*; *ho::GALp kanMX4*
	YI13_9B	*MATα*; *ho::GALp kanMX4*
	YI13_8B	*MAT***a**; *ho::GALp kanMX4*
	YI13_D2	*MAT***a**; *ho::GALp kanMX4*
	YI13_E4	*MAT***a**; *ho::GALp kanMX4*
	YI13_3A	*MATα*; *ho::GALp kanMX4*
	YI13_A1	*MATα*; *ho::GALp kanMX4*
	YI13_D3	*MATα*; *ho::GALp kanMX4*
	YI13_E10	*MAT***a**; *ho::GALp kanMX4*
	YI13_J1	*MAT***a**; *ho::GALp kanMX4*
	YI13_7C	*MAT***a**; *ho::GALp kanMX4*
	YI13_9D	*MAT***a**; *ho::GALp kanMX4*
YI59	YI59_E1	*MAT***a**; *ho::GALp kanMX4*
	YI59_F2	*MAT***a**; *ho::GALp kanMX4*
	YI59_E3	*MAT***a**; *ho::GALp kanMX4*
	YI59_D4	*MAT***a**; *ho::GALp kanMX4*
	YI59_L4	*MAT***a**; *ho::GALp kanMX4*
	YI59_G4	*MATα*; *ho::GALp kanMX4*
	YI59_G2	*MATα*; *ho::GALp kanMX4*
	YI59_D2	*MAT***a**; *ho::GALp kanMX4*
	YI59_L1	*MATα*; *ho::GALp kanMX4*
	YI59_D3	*MATα*; *ho::GALp kanMX4*
	YI59_F4	*MAT***a**; *ho::GALp kanMX4*
FIN1	FINI_E3	*MATα*; *ho::GALp kanMX4*
	FINI_B4	*MATα*; *ho::GALp kanMX4*
	FINI_H3	*MATα*; *ho::GALp kanMX4*
	FINI_G4	*MATα*; *ho::GALp kanMX4*
	FINI_B3	*MATα*; *ho::GALp kanMX4*
	FINI_G1	*MATα*; *ho::GALp kanMX4*
	FINI_C1	*MAT***a**; *ho::GALp kanMX4*
	FINI_C3	*MAT***a**; *ho::GALp kanMX4*
	FINI_C2	*MAT***a**; *ho::GALp kanMX4*
	FINI_C59	*MAT***a**; *ho::GALp kanMX4*
	FINI_G3	*MAT***a**; *ho::GALp kanMX4*
	FINI_B2	*MAT***a**; *ho::GALp kanMX4*
	FINI_E1	*MAT***a**; *ho::GALp kanMX4*

were separated using a micromanipulator (MSM300 Singer Instrument, Watchet, UK). MAT**a** and MATα were genotyped by PCR and confirmed by a mating test.

Plasmid constructions were done according to standard molecular biology techniques previously described by Sambrook and Russel (2001). Initial PCR products were amplified using *Taq* DNA polymerase (New England Biolab) as instructed by manufacturer, using forward and reverse primers listed in Table 3. All kits and enzymes were used as recommended by manufacturers. Restriction endonucleases and T4 DNA ligases were purchased from New England Biolabs. PCR products and DNA fragments were routinely separated on 1% agarose (Lonza, Rockland, ME, USA) gels and fragments of appropriate sizes were isolated using the Zymoclean Gel DNA Recovery kit (Zymo Research). All plasmids constructed and utilised in this study are summarised in Table 1.

Preliminary screening was performed with the reporter cellobiohydrolase protein *T.e.*Cel7A on a low copy CEN6 containing plasmid, pHK212_SD1, before further screening with low copy plasmids pHK212_SD2 and pHK212_SD3 expressing *T.r.cel5A* (the EGII) and *S.f.cel3A* (the BGLI), respectively. For the construction of the plasmids pHK_SD1/2/3, open reading frames of cellulase genes namely *T.e.cel7A*, *T.r.cel5A* and *S.f.cel3A* were extracted from their respective plasmids pMUSD3, 2 and 1 by digesting with *Pac*I and *Asc*I, and subsequently inserting the individual fragments into corresponding restriction sites on the yeast expression vector pHK212. In this way, three distinct plasmids encoding different reporter proteins were constructed under the control of *PGK1* promoter and terminator (listed in Table 1). Plasmid isolations were carried out using cetyltrimethylammonium bromide method (Sambrook and Russel 2001). Yeast transformations were carried out according to a previously described protocol (Davison, den Haan and van Zyl 2016). The presence of cellulase genes in strains was confirmed through PCR using primers specified in Table 3.

Determination of efficiency of transformation, mating and sporulation

To determine transformation efficiency, cells were grown to mid-logarithmic growth phase at 30°C in YPD medium (OD_{600} = ~0.4) and transformed with 1 μg of plasmid DNA by electroporation (Sambrook and Russel 2001). The transformants were serially diluted and plated onto YPD agar media supplemented with the appropriate antibiotic marker. After 24, 48, 72 and 96 h incubation, the colonies were counted, and colony-forming units per micrograms of plasmid (CFU.μg^{-1}) were calculated. Cell matings were performed according to a protocol focused on stimulation of **a**/α-factor expression and optimised cell–cell contact during mating (Soellick *et al.* 2001). Mating and sporulation efficiency were determined as described in Chinen *et al.* (2011). The spore viability for each cross was scored after each tetrad dissection.

Phenotypic screening

To obtain a global view of the landscape of stress tolerance profiles in *Saccharomyces cerevisiae*, we selected three strain isolates ranging in secretory phenotypes, namely YI13, FIN1 and YI59, based on innate general secretion capacities (Davison, den Haan and van Zyl 2016). These three homothallic, diploid strain isolates, with an identified range of secretory capacities (Davison, den Haan and van Zyl 2016), namely YI13 (high-), FIN1 (medium-) and YI59 (low secretor), serve as potential strains for future genetic studies (Table 1). This study proposed a phenotypic characterisation technique, with secretion and industrial stress tolerance as underlying factors, to assist with the screening of a set of strains for their applicability in a mating study towards improved heterologous cellulase secretion.

Characterisation of every strain was estimated as an average growth in YPD medium either on agar plate or in liquid culture with measurements taken every 24 h (Kvitek, Will and Gasch 2008). In our previous study, such an approach was used to characterise a collection of *S. cerevisiae* strain isolates, by cultivation on YPD agar plates supplemented with the appropriate chemicals (Davison, den Haan and van Zyl 2016).

Environmental and secretion stresses

Environmental stresses assayed include a range in temperatures (30°C, 37°C and 40°C), ethanol concentrations (7.8%–9% w/v) and a range of inhibitory cocktail concentrations (25%–40%), as previously described in Davison, den Haan and van Zyl (2016). Strains evaluated include the three strain isolate lines, namely

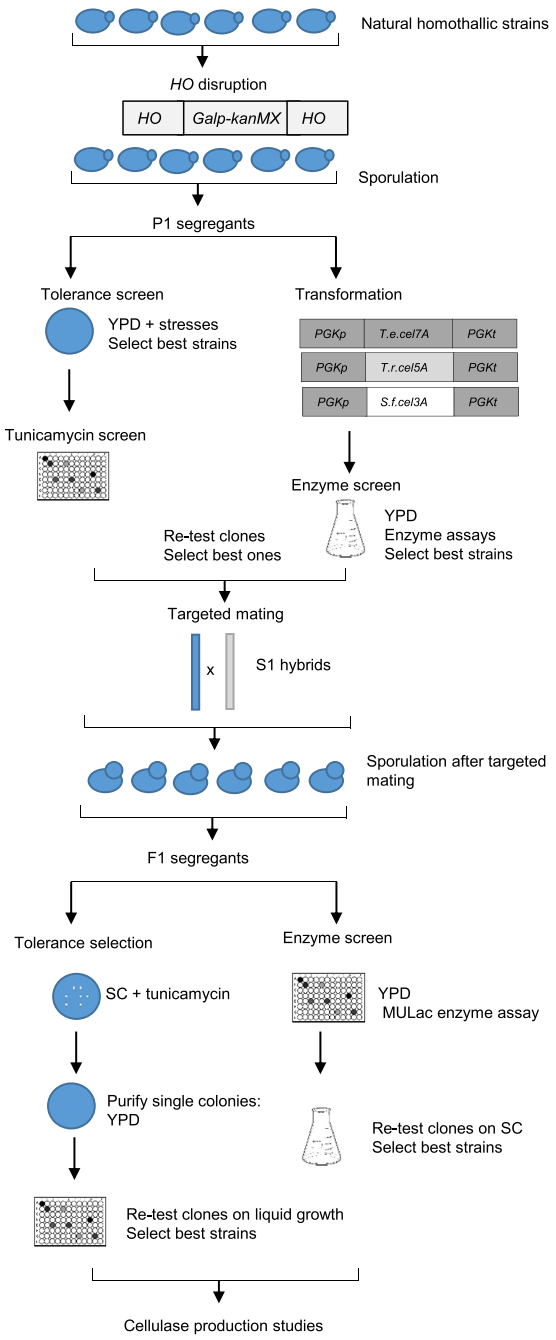

Figure 1. Overview of haploid and diploid strain generations. A diagram illustrating the steps followed for the generation of strains containing *HO* deletion, *T.e.cel7A*, *T.r.cel5A* and *S.f.cel3A*, is shown. The KanMX4 cassette inserted (light grey box) is flanked by 40 bp *HO* homologous sites and under the control of a galactose inducible promoter. Strains were disrupted for *HO* gene and sporulated. Four viable spores were obtained from tetrad dissection, and haploid spores were selected on geneticin (G418) and galactose-supplemented plates. MAT**a** and MATα were genotyped by PCR and confirmed by a mating test. The haploid strains were transformed with pCEN6/ARS4 plasmids individually expressing *T.e.cel7A*, *T.r.cel5A* and *S.f.cel3A* gene cassettes (dark grey boxes). Alternatively, segregants were screened for survival in multiple stresses, including secretion stressor tunicamycin. In parallel to these two approaches, we also performed targeted mating by outcrossing the best performing segregant with an Ethanol Red strain as well as inbreeding the best preforming isolates to produce novel hybrid strains. These hybrid strains were sporulated to produce F1 segregants utilised for the next round of screening. See 'Methods' for more details about these procedures.

FIN1, YI13 and YI59, and reference strains, namely Ethanol Red derivatives ER12, ER16, ER17 and ER19, and laboratory reference strains S288c and Y294. To evaluate direct stresses found in secretory pathway, compounds such as tunicamycin (0.5–2.0 μg.mL^{-1}; Sigma), sodium orthovanadate (0.4–1.0 mg.mL^{-1}; Sigma) and Congo Red (400–800 μg.mL^{-1}; Sigma) were utilised.

For these stress experiments, cells were grown to mid-exponential phase at 30°C, collected and spotted onto to fresh solid medium containing the corresponding stress as described previously (Davison, den Haan and van Zyl 2016). Spot plates were made by diluting the cultures to $OD_{600} = 0.5$ and spotting (3 μL) 10 fold serial dilutions. Unless indicated, colony growth was inspected after 2–4 days of incubation at 30°C. Inhibition (%) assay was performed by inoculating the cultures to $OD_{600} = 0.5$ in YPD or SC medium (96 deep well plate filled with 250 μL/well) supplemented with tunicamycin (range of 1.0–3.0 μg.mL^{-1}) and cell growth (OD_{600}) was measured at 24 h intervals at an incubation at 30°C for a period of 48 h. Control assays containing 1 μL.mL^{-1} of 100% dimethyl sulfoxide (DMSO) were included to discard the specific effect of the solvent.

Enzyme assays

Enzyme activity assays were performed as described in Davison, den Haan and van Zyl (2016), whereby cellobiohydrolase activity from transformants expressing *T.e.cel7A* was evaluated using methyllumberiferyl-β-lactopyranoside (MULac; Sigma) as a substrate. To evaluate EG activity from transformants expressing *T.r.cel5A*, enzyme assays were performed using the carboxylmethyl cellulose/3,5 dinitrosalicylic acid (CMC/DNS) method. Finally, to evaluate BGL activity from *S.f.cel3A* expressing transformants, enzyme assays were performed with *p*-nitrophenyl β-D-glucopyranoside (Sigma).

Ploidy determination

Flow cytometry was used to verify the ploidy of the top performing strains in terms of *T.e.*Cel7A secretion, namely YI13 and its derivatives namely C2, E4 and 3B as well as parental reference control strain ER12. Protocols for ploidy determination were described in Davison, den Haan and van Zyl (2016). DNA histograms were recorded with a FACS Diva Version 6.1.3 flow cytometer (BD BioSciences, Franklin Lakes, New Jersey, USA). The DNA histograms were analysed with a mathematical model to determine the distribution of cells in the various ploidy classes.

Targeted mating, mass sporulation and selection strategies

To create an F1 pool with targeted mating, each parental strain was sporulated and screened individually (see earlier). As shown in Fig. 1, transformation with either *T.e.cel7A*, *T.r.cel5A* and *S.f.cel3A* expressing pCEN6/ARS4 plasmids allowed the identification of top performing isolate, which in turn was outcrossed to an Ethanol Red strain and inbred to another top performing haploid isolate to produce hybrid strains. For the mating, each parental haploid containing pHK212_SD1 was utilised and we confirmed the plasmids were largely retained after sporulation and germination (data not shown). After the targeted mating, the hybrid strains were sporulated individually, and the tetrads were digested using random spore isolation (Ausubel *et al.* 1987).

We sub-cultured F1 hybrid segregants from the outcrossed (n = 200) and inbred lines (n = 60) in liquid medium YPD (96 deep well plate filled with 1 mL/well) and incubated the cells

Table 3. PCR primers used to construct and confirm haploid strains.

Primer	Sequence 5′-3′	Reference
Mating type determination		
MAT**a**-L	ACTCCACTTCAAGTAAGAGTTTG	(Davison *et al.* 2016)
MATα-L	GCACGGAATATGGGACTACTTCG	
MATlocus-R	GCACGGAATATGGGACTACTTCG	
HO knockout		
galG418HO-L	GTGCGCAGATGGCTCCGCTG	This study
galG418HO-R	CCGCCACATTTTATACACTCTGGTCC	
HO confirmation		
HO-L	AGAAAGGGTTCGCAAGTCCT	This study
HO-R	CTACGTTGCCTCCATCGAAC	
T. emersonii cel7A determination		
CCBH-L	GACTTTAATTAAAATGCTAAGAAGAGCTTTACTATTG	(Ilmén *et al.* 2011)
CCBH-R	GACTGGCGCGCCTTACAAACATTGAGAGTAGTATGGG	
T. reesei cel5A determination		
EGII-L	GTTAACAACAATTTGGGTGG	(Davison *et al.* 2016)
EGII-R	CAATGGAGAAAAAGCACC	
S. fibuligera cel3A determination		
BGL-L	GACTCGCGAGTCCCAATTCAAAACTATACC	(Davison *et al.* 2016)
BGL-R	CCGCTCGAGCGGTCAAATAGTAAACAGGACAGATG	

for 48 h before enzyme quantification assays. After quantification, hybrid pools (n = 20) containing F1 outcrossed segregants were selected based on their distinct secretory capacities and cultured for 72 h in 10 mL minimal media in 100 mL Erlenmeyer flasks at a starting $OD_{600} = 0.1$ for enzyme quantification assays. In parallel, inhibition (%) assays were performed on the hybrid pools.

Statistical analysis

The significance of the differences in physiological properties of yeast strains was assessed by one-way ANOVA, unpaired *t*-tests and Mann–Whitney U test ($P > 0.05$).

RESULTS AND DISCUSSION

Large-scale screening of *Saccharomyces* strain lines for stresses impacting secretory pathway

This study exploited the natural variation that exists among strain isolates to create novel hybrids with enhanced innate secretory capacity. Previously, we had screened three natural strain isolates identified from inland and coastal winery regions of Western Cape, South Africa (Davison, den Haan and van Zyl 2016). We hypothesised that mating genetically divergent strains increases the chance of obtaining hybrids demonstrating hybrid vigour (heterosis) for industrial traits due to the fact that each strain contributes different beneficial genetic components. To estimate the evolutionary relatedness of different strains to commercially available wine, brewing and industrial strains, we aligned the D1/D2 region of the rDNA of selected strains and constructed a phylogenetic tree. MH1000 (industrial strain) and HOEG (brewing strain) as well as laboratory reference strain S288c (Fig. S1, Supporting Information). We found that the commercial strain MH1000 and laboratory strain S288c appear to be closely related to the YI1, V3, MF15, YI59, YI19 and FIN1 isolates, whereas brewing strain HOEG appears to be closely related to YI13 isolate, suggesting that the isolated strain lines potentially evolved from commercial contaminants. Three genetically divergent, homothallic strains showing distinct secretory capacities namely YI13 (high secretor), FINI (median secretor) and YI59 (low secretor; Davison, den Haan and van Zyl 2016) and industrial derivative Ethanol Red strains (ER12, ER19, ER17 and ER16) were chosen for further analysis. Given its widespread application in bioethanol industries, Ethanol Red is used as a reference strain throughout this paper.

Since many natural and industrial *Saccharomyces cerevisiae* strains are known to demonstrate poor sexual reproduction and transformation efficiency (often hindrances in studies involving heterologous protein production and hybrid generation), we tested the strains' capacity for genetic transformation and the ability to generate viable spores. The strain isolates demonstrated a range of transformation efficiencies from 2.0×10^3 to 1.6×10^5 (Fig. S2a, Supporting Information) lower than reported transformation efficiencies of laboratory strains (Yu *et al.* 2016); however, the strains of importance, namely YI13, YI59, FIN1 and Ethanol Red strains, retained sufficient transformation, as well as mating and sporulation efficiencies required for mating studies (Fig. S2, Supporting Information). Strain isolates demonstrated median sporulation capabilities (Fig. S2b, Supporting Information), with prominent strains YI13, FIN1 and YI59, demonstrating tetrad (%) values of 46.6%, 54.4% and 50.0%, respectively. Low sporulation efficiency in both wine and laboratory strains are notorious as reported by Gerke, Chen and Cohen (2006), encumbering mating analysis. As a result, cross breeding strain isolates with highly sporulating Ethanol Red strains, which produced more than 80% viable tetrads (Fig. S2b, Supporting Information), are desired. All strain isolates were confirmed to be homothallic (data not shown); therefore, to obtain stable *MAT***a** and *MAT*α haploid derivatives, a single *HO* gene knockout was performed in the strains FINI, YI13 and YI59. After these strains were sporulated, only tetrads with four viable spores were taken into account (listed in Table 2). Strains were confirmed to be genetically stable after multiple rounds of sub-cultivation (data not shown). The mating type ratios' of segregants derived from YI13, FIN1 and YI59 did not significantly deviate from the expected 2**a**:2α segregation ratio of the mating type locus, indicative of true diploid yeast strains (Table 2).

We hypothesised that the impact of uniform stress responses linked to the secretory pathway can lead to various reactions

in different genetic backgrounds, namely YI13, FIN1 and YI59 as well as their haploid derivatives, reference strains S288cα, S288cα/**a**, Y294 and Ethanol Red strains ER19, ER19, ER17, ER12 and constructed diploid strains ER19 × 12 and ER17 × 12. A range of phenotypic variance was observed between the strains (Fig. 2a and b), as expected when haploids were compared with heterozygotes (Szafraniec *et al.* 2003; Wu, Benjamin and Thibault 2014). Interestingly, natural isolates and their derivatives displayed a wider phenotypic variance for specific traits including the tolerance profiles of ethanol, inhibitor and sodium orthovanadate when compared to other stresses (Fig. 2a). This supports the claim that background-specific effects can create phenotypic expression variation as summarised by Fournier and Schacherer (2017). As a result, even uniform stress responses can lead to various reactions, especially in different genetic backgrounds. This is supported by Szafraniec *et al.* (2003), who suggested that heterozygous genetic backgrounds could have small, interfering effects resulting in the displayed significant variation of the trait phenotype.

In contrast, more homogenous behaviour was observed between isolate derivatives for the traits of Congo Red, temperature and tunicamycin (Fig. 2a). Congo Red sensitivity was shown here to be a feature of diploid strain isolates, with the haploid derivatives demonstrating higher tolerance (Fig. 2a). This is potentially associated with the presence of higher chitin content at increased ploidy states of cells (Schekman and Brawley 1979). Unexpectedly, the Ethanol Red derivative lines also displayed extreme sensitivity to Congo Red compared to the other strain isolate derivatives (Fig. 2a), with growth inhibited at 400 μg.mL^{-1} concentration (data not shown). In contrast to the Congo Red phenotypes, an inverse relationship was observed between tunicamycin tolerance and ploidy, whereby the diploid strains' tolerance was similar or outranked the strain isolate derivatives (Fig. 2a). Haploid derivatives had pointedly reduced tunicamycin tolerance compared to the diploid strain isolates (namely YI13, FIN1 and YI59). One possible explanation is a multi-gene phenotype, which, when halved unmasks weaker tolerance (or whereby loci can interact antagonistically), resulting in a multiplicative action of effects (Szafraniec *et al.* 2003). Diploidy provides the immediate benefit of masking deleterious mutations if only their negative effects are reduced in heterozygotes. However, this suggested feature of haploid derivatives warrants further investigation. This not only highlights the degree of phenotypic behaviour that exist between derivatives of different genetic backgrounds and the allelic distribution of genes for different tolerance profiles, but also the highly interrelated yeast stress response pathways (as reviewed by Kawakami *et al.* 2016).

It is worthy to note that the high secretor strain YI13 demonstrated high tolerance to cell wall stressor namely Congo Red, cell membrane stressors namely high ethanol concentrations, high temperature and inhibitory compounds found in lignocellulosic hydrolysates and the secretion stressor tunicamycin (Fig. 2a). It has been hypothesised that a regulatory relationship exists between the CWI and the secretion pathway (Chen *et al.* 2005; Scrimale *et al.* 2010; Torres-Quiroz *et al.* 2010), although further evidence of this needs to be provided. Some evidence of this was observed in literature, with both Scrimale *et al.* (2010) and Torres-Quiroz *et al.* (2010) suggesting that tunicamycin, a chemical that activates UPR (Wimalasena *et al.* 2008), can also activate the yeast mitogen-activated protein (MAP) kinase Mpk1p (1, 4), an important component of the CWI. Chen *et al.* (2005) proved that MAP kinases signalling pathways were activated under ER stress, which was further regulated in a manner independent of the *IRE1/HAC1* pathway. More recently, Tang *et al.* (2016) demonstrated that strains with deletions of key Golgi mannosyltransferases genes could up-regulate components in the secretory pathway and affect the CWI. Taking into account the results of this study, the pathways elicited by tunicamycin and Congo Red are not independent of each other, and a potential regulatory relationship may exist between the stress responses of the secretion pathway and CWI.

Growth tolerance in the presence of chemical stresses connected to secretion performance and ploidy state

During a previous study, we analysed the tolerance profiles of a variety of natural *S. cerevisiae* strain isolates to chemical stressor, tunicamycin and, in the process, identified a top performing secretory strain YI13 (Davison, den Haan and van Zyl 2016). In this paper, we explored the connection between secretion capacity and the ability to grow in the presence of low concentrations of the secretion stressor tunicamycin, as well as the connection between ploidy state of a strain and the ability to tolerate high concentrations of the cell wall stressor, Congo Red, as these traits would be easier to select for. We initially measured growth capacity of each strain on solid YPD medium supplemented with different concentrations of the chemical stressors, and subsequently tested tunicamycin tolerance in a more sensitive liquid medium.

Here, we completed the analysis of tunicamycin tolerance of three yeast strain lines, each with ranging secretory phenotypes by allowing the parallel measurement of the growth response (OD_{600}) for both diploid and haploid derivatives under a chosen stress condition (Fig. 2b). Using this chemical, we demonstrated that various forms of secretion stress could potentially be used to identify strains with higher secretory capacity. Initially, at lower concentrations of tunicamycin (1.0–1.5 μg.mL^{-1}), the majority of the haploid strains demonstrated high inhibition (%) compared to the diploid strains (Fig. 2b), similar to the results displayed in Fig. 2a. From Fig. 2b, the superior secretor derivatives, namely YI13 derivatives, demonstrated significantly lower inhibition (%) at tunicamycin concentrations of 3.0 μg.mL^{-1} compared to other strain isolate derivatives (P value = 8.7E-5), with all stains inhibited at 3.5 μg.mL^{-1} (data not shown). Only the YI13 haploid derivatives survived concentrations up to 3.0 μg.mL^{-1} (Fig. 2b), which resulted in a clear distinction being observed between YI13 haploid derivatives and other strain isolate derivatives.

In parallel to the tunicamycin tolerance assay, the secretion phenotypes were evaluated using a low copy centromeric plasmid containing *Talaromyces emersonii cel7A* (CBHI) with a carbohydrate-binding module from *Trichoderma reesei* Cel7A (Ilmén *et al.* 2011). The YI13 derivative strains, Ethanol Red strains and laboratory strain S288c were transformed. A range of secretory capacities was demonstrated between the strains (Fig. 3a) reinforcing the hypothesis that differences in genetic background of host yeast strains have profound effects on expression on recombinant proteins (Szafraniec *et al.* 2003), especially with respect to heterologous protein secretion levels. The YI13 constructs had the highest activity per dry cell weight (DCW) for the time monitored, achieving up to 1.28 U.mgDCW^{-1} (Fig. 3a). Furthermore, a range of activity levels were observed among the YI13 segregants, ranging from 0.21 to 1.10 U.mgDCW^{-1} (Fig. 3a) with the best segregant displaying activity that was 10-fold higher than the best performing Ethanol Red derivative. In particular, the six YI13 haploid transformants

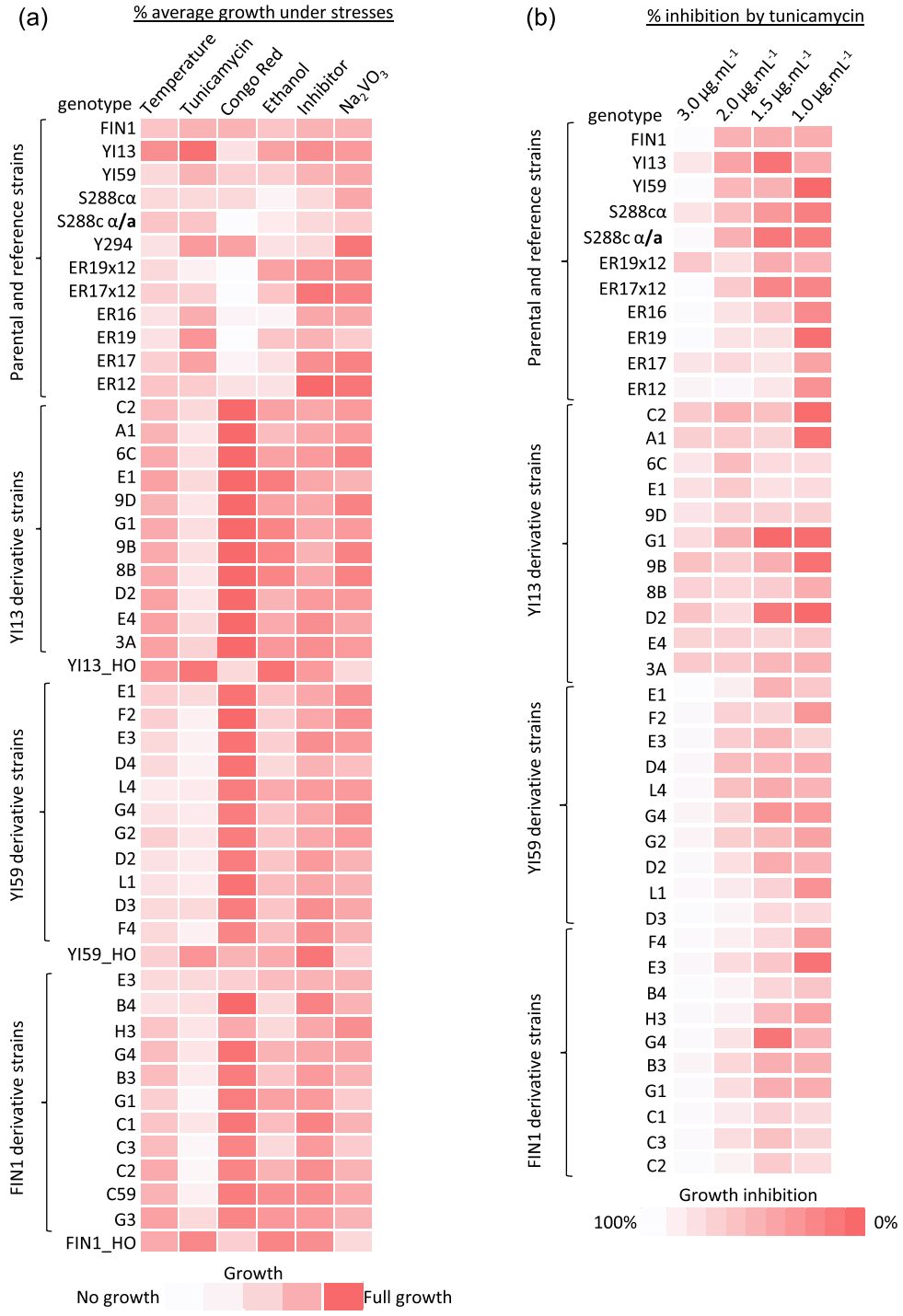

Figure 2. Summarised phenotypic response of three yeast strain lines, namely YI13, FIN1 and YI59, as well as parental and reference strains, to specific stresses known to influence the secretory stress response system. This includes (**a**) average growth of strains spotted onto YPD plates in a 10-fold serial dilution range starting at OD_{600} = 0.5. Plates were either supplemented with or incubated under specific environmental stresses prominent in a CBP industrial context including temperature (30°C–40°C), ethanol concentrations (7.8%–9.0% w/v) and a range of inhibitor cocktail concentrations (25%–40%) or direct stresses found in secretory pathways including tunicamycin (0.5–2.0 μg.mL^{-1}), sodium orthovanadate (0.4–1.0 mg.mL^{-1}) and Congo Red (400–800 μg.mL^{-1}). (**b**) Inhibition (%) by ER stressor, tunicamycin, was determined by average growth score of each strain cultivated under stressed and non-stressed conditions. Cultures were incubated at 30°C in 250 μl YPD medium in 96 deep well culture plates with measurements taken after 48 h, with the control supplemented with 1 μL.mL^{-1}100% DMSO. Each row on both plots represents a different strain or derivative and each column indicates a given environment according to the keys shown at the bottom. Colour-scale boxes beneath the respective figures represent the average growth score of each strain.

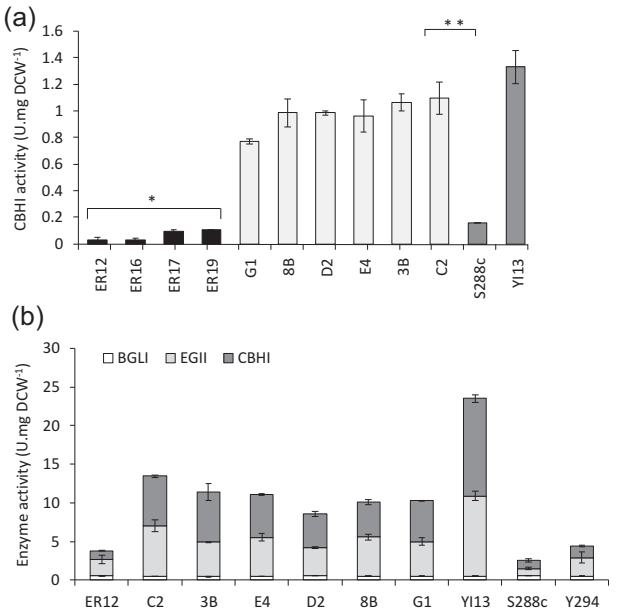

Figure 3. Relative enzyme activity of YI13 derivatives displaying a range of secretion profiles (**a**) expressing *T.e.cel7A* (CBHI) cultivated in 1 mL YPD media in 96 deep wells, or expressing (**b**) *T.e.cel7A* (CBHI; dark grey), *T.r.cel5A* (EGII; light grey) and *S.f.cel3A* (BGLI; white), respectively, when cultivated in 10 mL YPD media in 100 mL Erlenmeyer flasks. All values represent the mean of three repeats and error bar indicates standard deviation from the mean. ANOVA test: *ns, not significant; $P \leq 0.01$. Unpaired *t*-tests: **$P \leq 0.05$.

secreted significantly more *T.e.*Cel7A (CBHI) compared to the respective S288c (*P* value = 0.004) and Ethanol Red transformants (*P* value = 0.008; Fig. 3a). No significant difference was observed in *T.e.*Cel7A activities between the Ethanol Red derivative strains (*P* value = 0.85; Fig. 3a). Upscaling the experiments to flasks demonstrated no difference in the ranking of the strains (Fig. 3a and b). The strain ER12 was chosen for mating analysis because previous studies identified weak strains as contributors for beneficial genetic variation that is often absent from superior strains (Hu *et al.* 2007; Swinnen, Thevelein and Nevoigt 2012).

In addition to selection for higher secretory capacity, we also explored a selection strategy based on survival in medium containing low levels of the secretion stressor, tunicamycin. Interestingly, the tunicamycin tolerance ranking of the strains (Fig. 2b) remained similar to that exhibited by secretion profiles of the *T.e.*Cel7A (CBHI; Fig. 3a). Furthermore, a large range of activities (2.1–10.4 U.mgDCW^{-1}) was observed between the strains expressing another reporter cellulase gene namely *T.r.cel5A* (EGII; Fig. 3b). In this case, a 3.1-fold difference existed between control ER12 and the highest performing haploid YI13 derivative for *T.e.*Cel7A activity, namely C2 (Fig. 3b). This may be indicative of a potential 'general secretion' enhancing effect being observed in the overall highest performing haploid, namely C2. However, the lack of variance between the haploids expressing *S.f.cel3A* (BGLI; Fig. 3b), suggested preferential pathways for specific heterologous reporter proteins. Comparing the highly glycosylated protein of *S.f.*Cel3A with the physically smaller cellulases *T.e.*Cel7A and *T.r.*Cel5A, this enzyme was secreted at poor extracellular activity levels across all strain backgrounds (Fig. 3b). The observed variations in Fig. 3b can be attributed to general protein properties such as protein size, number of di-sulphide bonds, protein hydrophobicity etc. (as reviewed by Kroukamp *et al.* 2017). Furthermore, the cell wall-associated nature of *S.f.*Cel3A (as described by Gurgu *et al.* 2011) may explain the discrepancies regarding the extracellular activity levels displayed among the strains. As a result, the *T.e.*Cel7A was used as a reporter protein for subsequent screening of the F1 hybrids.

Targeted mating generates improved F1 hybrids

The parental reference strains for hybridisation were chosen based on the clear distinction in *T.e.*Cel7A activity observed between the best performing YI13 haploid derivatives C2 and 3B, and parental reference haploid strain ER12 (Fig. 3a and b). In addition to outcrossing the C2 segregant to ER12, we inbred the best performing segregants C2 and 3B using a targeted mating approach. Flow cytometric analysis was performed on the top performing YI13 derivatives, in respect to *T.e.cel7A* and *T.r.cel5A* secretion levels, namely C2, E4 and 3B, and confirmed the strains to be true haploids containing half the DNA content of diploid strains (Fig. S3, Supporting Information). The F1 hybrids from both crosses incorporated two initial genomes, with F1 segregants demonstrating a mosaic of genomes. We obtained 60 outcrossed and inbred F1 pools of hybrid segregants and assayed the extracellular *T.e.*Cel7A activity of these pools (Fig. 4a). An interesting result was that the average secreted *T.e.*Cel7A of outcrossed segregants was significantly higher than that of the inbreds (*P* value = 0.0006; Fig. 4a). This can partly be explained by the specific genetic combination of parents that may provide a superior genetic basis, and that inbreeding can often result in weaker hybrids as demonstrated by hybridisation studies to improve ethanol tolerance by Snoek *et al.* (2015). It is important to note that we focus on alleles that have been exposed to natural selection, and while it may increase the chance of introducing alleles that are beneficial for the desired trait, it could potentially be detrimental for other phenotypes of interest. Another factor to consider is epistasis, whereby two alleles that are beneficial in a specific genetic background can have a disadvantageous effects when combined in the same strain. Therefore, the effect of epistasis is more pronounced in the outbred pools due to more complex, mosaic genetic backgrounds with alleles combined from different strains.

Based on the previous analyses, we further sporulated and isolated 210 segregants from the outcrossing of C2 with ER12 segregants (known as H7 hybrids) and screened for extracellular *T.e.*Cel7A activity (Fig. 4b). Interestingly, some of the transformants showed peculiar behaviour as they secreted *T.e.*Cel7A poorly or not at all, similar to Ethanol Red strain lines and reference laboratory strains (Fig. 4b). The secretory performance in the mosaic segregants was higher than that in the parental haploid (C2) and hybrid strain (H7) for 20 out the 210 strains (Fig. 4b). Approximately 9.0%–9.5% of the total haploid derivatives screened displayed extracellular *T.e.*Cel7A activity less than the ER12 parental strain and more than C2 parental strain (Fig. 4b). As a next step, we re-tested pools of outbred F1 segregants that showed distinct secretory capacities i.e. lowest and highest, in comparison to a pool of randomly selected segregants in 10 mL minimal media in Erlenmeyer flasks. We found significant differences between the 'high' (*P* value = 7.4E-06) and 'low' pools (*P* value = 1.7E-05) compared to the 'random' pool, as a result confirming our screening approaches (Fig. 4c). Ten of the best performing haploid derivatives were re-cultivated in 10 mL YPD media in Erlenmeyer flasks and assayed. Under these conditions, the E7.1 haploid had the best performance for extracellular *T.e.*Cel7A activity compared to the other transformants, significantly outperforming the parental diploid strain YI13 by 1.7-fold (*P* value = 3.1E-06) and ER12 parental haploid strain by

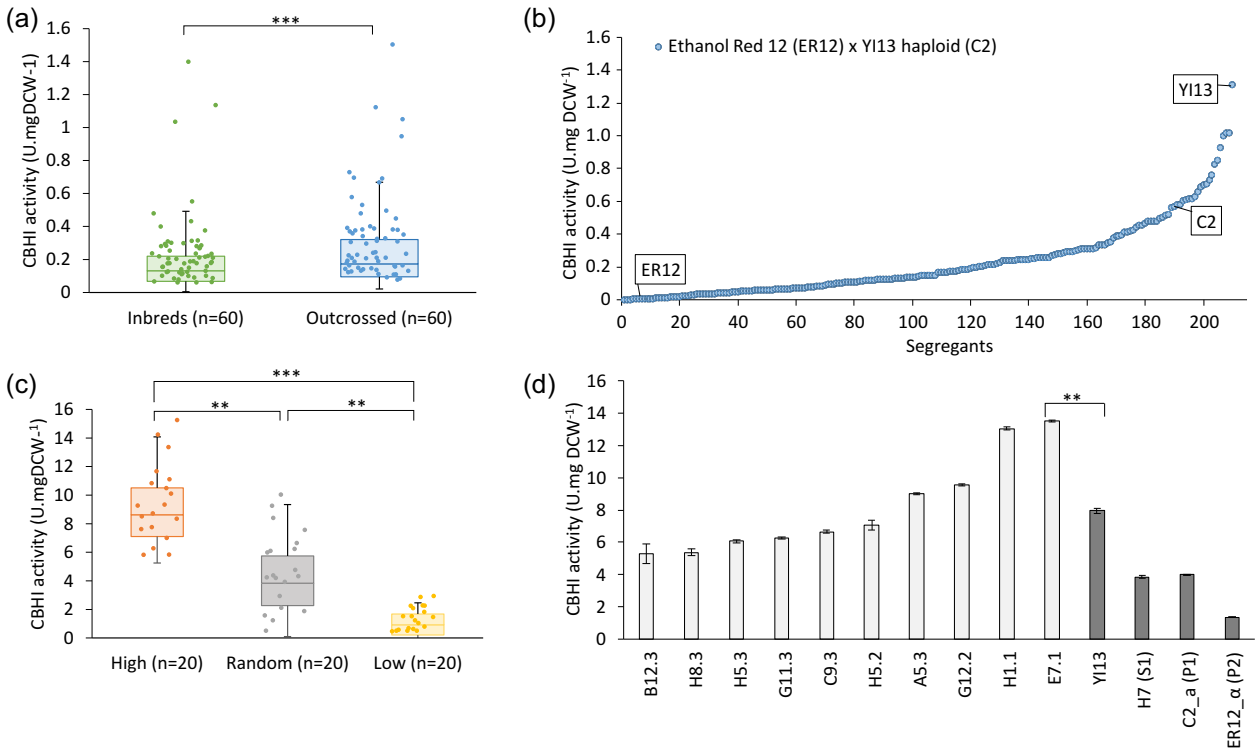

Figure 4. Targeted mating yields hybrids with increased *T.e.*Cel7A secretion capability. (**a**) Outcrossed F1 hybrids display higher yields of extracellular *T.e.*Cel7A than F1 inbreds cultivated in 1 mL YPD media in 96 deep well plates (Mann–Whitney test: $^{***}P \leq 0.05$). (**b**) Outcrossed F1 segregants display a gradual increase in extracellular *T.e.*Cel7A activity in 1 mL YPD in 96 deep well plates. (**c**) Distinct pools of isolated F1 outcrossed segregants demonstrate a range of significant secretory capacities cultivated in 10 mL minimal media in 100 mL Erlenmeyer flasks (Mann–Whitney test: $^{***}P \leq 0.05$, unpaired *t*-test: $^{**}P \leq 0.05$). (**d**) Relative enzyme activity profiles of top performing F1 generation hybrids displaying a range of secretion profiles cultivated in 10 mL YPD media in 100 mL Erlenmeyer flasks (unpaired *t*-test: $^{**}P \leq 0.05$).

4.9-fold (*P* value = 2.6E-05; Fig. 4d). Such extremes of either very high or very low phenotype, as well as the gradual increase in secreted activity (i.e. continuous and normally distributed) suggested a Gaussian-distributed trait and distinctly typifies this as a quantitative trait with multiple alleles being responsible for high *T.e.*Cel7A secretion. This further demonstrated that more than one allele is conferring the high secretion phenotype. Furthermore, past research suggested that multiple alleles are responsible for the high secretion phenotype and that small enhancements combined can contribute to a superior secretion phenotype as described by Brookfield (1996), and specifically hypothesised for *T.e.*Cel7A secretion by Kroukamp *et al.* (2017).

To confirm that some of the hybrids obtained in our outcrossed hybridisation strategy outperformed the commonly used bioethanol strain and initial parental strains, we re-tested 10 hybrids from the high secretion pool in triplicate and upscaled to Erlenmeyer flasks. The results show that some selected outcrossed F1 segregants demonstrated heterosis and outperformed both their parental strains (Fig. 4d). This superior performance of F1 generation has been demonstrated in yeast previously. Heterosis has been was demonstrated for cellulase production before by Kroukamp *et al.* (2017). While the precise mechanisms underlying heterosis remain unknown, future work to characterise the novel strains generated in this study with increased secretion capacity at a genetic and phenotypic level would be of interest, for example using a quantitative trait loci (QTL) mapping approach as described by Hubmann *et al.* (2013). In addition to selection for superior secretory capacities, we also explored a selection strategy based on survival in minimal medium containing a range of concentrations of tunicamycin. To ascertain the degree of secretion stress variation between outcrossed F1 segregants, we performed stress tolerance analysis on the selected strains from pools of segregants. In this way, we exploit strain differences that may physiologically activate the UPR. We found that the stress tolerance distribution of the F1 segregants was less broad than that of the parental strains (Fig. 5). Nevertheless, we found that some of the segregants in the 'low' pool i.e. displaying low extracellular *T.e.*Cel7A activity, performed worse in tunicamycin-induced stress compared to the 'high' pool i.e. displaying high extracellular *T.e.*Cel7A activity (Fig. 5). This evidence correlates to the hypothesis that a highly reactive secretory stress response exhibited by these strains potentially results in superior protein-folding capabilities, which had been suggested by previous studies (Ilmén *et al.* 2011; Davison, den Haan and van Zyl 2016).

Nevertheless, strains that cultivate well in the presence of tunicamycin are not always the best secretor strains, which was also demonstrated by the parental and hybrid strains: the best performing strain out of the collection, namely E7.1 (Fig. 4d), grows well in the presence of tunicamycin, but not as good as some of the segregants in the 'low' pool (Fig. 5). Therefore, it is important note that this method is only suitable when scoring the ultimate strain is not a necessity. However, when screening large numbers of recombinant strains, this method presents a way to screen the first selection of candidates likely resulting in a much smaller pool of recombinant strains to analyse. Hence, the presented method is a suitable way of making the first selection of high secreting strains, but a second test remains necessary to identify the best performing strain. Furthermore, the diversity

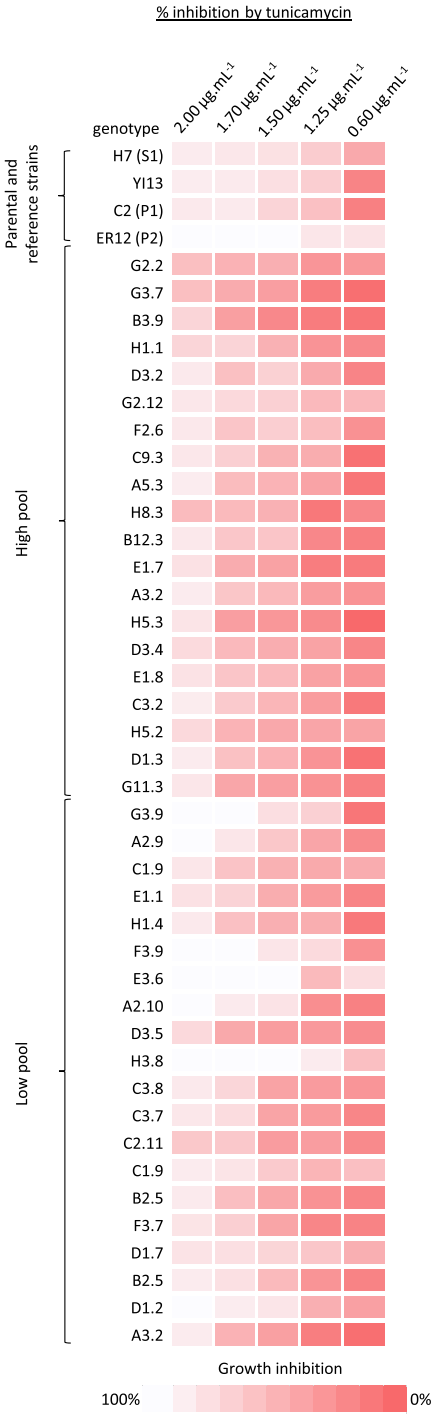

Figure 5. Summarised phenotypic response of yeast strain lines to tunicamycin. Each row represents growth in medium with different concentrations of chemical stressor tunicamycin for F1 segregants from the outcrossed hybrid population, as well as the parental strains. Colour boxes represent the inhibition (%) determined by average growth score of each strain cultivated under stresses and non-stresses conditions in triplicate repeats, according to the key shown at the bottom. Cultures were incubated at 30°C in 250 μl minimal medium in 96 deep well culture plates with measurements taken every 24 h for 48 h. Values represent the average of three repeats.

of functions contributing to ER integrity presents an obstacle in our efforts to use this stress as a selection tool due to complexity surrounding how unexpected factors function together to support protein folding in the ER (Jonikas *et al.* 2009). The advantage in a selectable trait such as tolerance to chemical stresses, including tunicamycin, is the ability to screen large numbers of segregants, which should increase the chances of selecting the best candidate for ER stress and, indirectly, secretion stress. It is recommended to include a combination of chemical stresses in a comprehensive screen followed by systematic analysis of secretory phenotype in order to fully understand a complex cellular process such as heterologous protein secretion.

CONCLUSIONS

By analysing phenotypic traits directly and indirectly linked to secretory stress, we were able to set up a novel standardised screening procedure suitable for recombinant protein production and potentially other traits including inhibitor tolerance. A main factor in large-scale segregant screening required for a genetic and mating studies involves the large amounts of material, costs, time and effort needed. We have developed a screening protocol applicable for large-scale haploid screening, through the use of antimicrobial compounds tunicamycin and Congo Red to substantially reduce the number of strains that have to be assayed quantitatively. This protocol is appropriate for multi-parallel screenings at relatively low cost and effort and without the need for highly specialised equipment. With further development, this method could be a basis for an automated screening procedure to facilitate the high-throughput applications needed to analyse QTLs linked to a desired trait. In conclusion, evaluating and characterising the natural, intra-species hybridisation of genetically stable haploid strains allows for the selection of improved hybrids for industrial traits, as well as allowing for the easy genetic manipulation required for research on heterologous enzyme secretion.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSYR* online.

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