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The *Saccharomyces cerevisiae* pheromone-response is a metabolically active stationary phase for bio-production

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ABSTRACT

The growth characteristics and underlying metabolism of microbial production hosts are critical to the productivity of metabolically engineered pathways. Production in parallel with growth often leads to biomass/bio-product competition for carbon. The growth arrest phenotype associated with the *Saccharomyces cerevisiae* pheromone-response is potentially an attractive production phase because it offers the possibility of decoupling production from population growth. However, little is known about the metabolic phenotype associated with the pheromone-response, which has not been tested for suitability as a production phase. Analysis of extracellular metabolite fluxes, available transcriptomic data, and heterologous compound production (para-hydroxybenzoic acid) demonstrate that a highly active and distinct metabolism underlies the pheromone-response. These results indicate that the pheromone-response is a suitable production phase, and that it may be useful for informing synthetic biology design principles for engineering productive stationary phase phenotypes.

1. Introduction

Microorganisms can be used to manufacture products ranging from therapeutic proteins and industrial enzymes through to metabolites for the replacement of existing petrochemicals and fossil fuels (Woolston et al., 2013). A major challenge for all bio-processes is balancing resources between biomass accumulation and product formation, as both outcomes require the same cellular resources such as carbon precursors, energy in the form of ATP, and reducing power in the form of NADH and NADPH. Biomass accumulation is essential to achieve the volumetric productivity required for commercial processes; however, excess biomass accumulation limits product yields. Moreover, the product or its intermediates may be toxic to the host organism, again limiting biomass production. Dynamic regulatory systems can be used to trigger the expression of a production pathway after the completion of a growth phase (Venayak et al., 2015). However most non-growth associated phenotypes are poor production phases due to the depletion of available resources and the subsequent induction of stress response mechanisms (Albers et al., 2007; Chubukov and Sauer, 2014).

The yeast *Saccharomyces cerevisiae* is a widely used industrial host microbe, and has growth characteristics that typify the limitations of normal growth based physiology in industrial microorganisms. *S. cerevisiae* populations undergo an exponential growth phase where carbon and nitrogen resources are rapidly consumed until they limit biomass production. During exponential growth, approximately 90% of cellular energy is directed towards ribosome biogenesis (Warner et al., 2001). Carbon- or nitrogen-limited populations cease rapid growth and enter a 'stationary phase', which is characterised by the induction of stress survival mechanisms and a drastic reduction in the overall rate of protein synthesis relative to the exponential phase (Werner-Washburne et al., 1993). In the case of carbon starvation, there is no substrate left for conversion into product; and under nitrogen starvation, stress signalling severely limits metabolic productivity even in the presence of excess carbon (Albers et al., 2007). An ideal scenario for bio-production would involve a rapid growth phase where biomass (or 'catalyst') accumulates to a level that enables high volumetric productivity, before switching to a metabolically active stationary phase. This phase would then be maintained even in the presence of high concentrations of cellular resources such as carbon and nitrogen. With cells metabolically active but not growing and dividing, a much greater proportion of carbon could be directed towards target metabolites. Such a strategy would also

Abbreviations: PHBA, para-hydroxybenzoic acid; GFP, Green Fluorescent Protein; MAPK, Mitogen Activated Protein Kinase

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open up the possibility of implementing growth limiting genetic modifications such as the silencing of essential genes using dynamic regulatory mechanisms (Williams et al., 2015a, 2015b). Stationary phase production is also very attractive because it enables the formation of products that are normally toxic to growth, and therefore limiting to production (Holtz and Keasling, 2010; Keasling, 2008).

The cell-cycle arrest phenotype of the yeast mating system represents a unique phase in the life-cycle of *S. cerevisiae*, which could be useful as a production phase for metabolic engineering where metabolic productivity is decoupled from growth-based physiology. The mating system has evolved to facilitate the synchronisation of the cell cycle and the fusion of two cells of opposite mating type to form a diploid. Briefly, haploid yeast cells of each mating type (a or α) secrete specific peptide pheromones that they use to detect the proximity of a potential mating partner of the opposite mating type. Binding of pheromone to specific G-protein coupled membrane receptors triggers an intracellular mitogen activated protein kinase signalling event which results in the de-repression of the Ste12p transcription factor and the initiation of the pheromone-response (Bardwell, 2005). Activation of the mating phenotype results in polarized growth, remodelling of cellular morphology and global transcription patterns, and arrest of growth in the G1 phase of the cell cycle (Bardwell, 2005), similarly to entry into stationary phase during carbon or nitrogen starvation. This phenotype can also be triggered via the addition of purified mating peptide to laboratory yeast cultures.

The *S. cerevisiae* mating system has become a cornerstone of eukaryotic synthetic biology (Furukawa and Hohmann, 2013). The pheromone communication system has been utilised for synthetic quorum sensing (Williams et al., 2015a, 2013), signal amplification (Groß et al., 2011), intercellular and interspecies communication (Hennig et al., 2015; Jahn et al., 2013), and biological computation (Regot et al., 2011). Furthermore, the depth of knowledge surrounding the mitogen activated protein kinase (MAPK) signal transduction machinery has enabled the construction and fine-tuning of a multitude of synthetic regulatory circuits (Bashor et al., 2008; Ingolia and Murray, 2007; O'Shaughnessy et al., 2011; Tanaka and Yi, 2009). In addition to relevance as a potential production phase, knowledge of the pheromone-response metabolism will be invaluable for future design of MAPK related synthetic regulatory systems. However, despite extensive utilisation of the mating system in synthetic biology, almost nothing is known about aspects of the phenotype that are not specifically related to mating.

Activation of the pheromone-response could result in a number of different scenarios with respect to metabolic engineering outcomes for a specific product. These include: an unproductive phenotype similar to the G1 arrest of the carbon- or nitrogen-limited stationary phases; higher productivity due to the limitation of carbon flux towards biomass; or no overall effect on cellular productivity due to the diversion of cellular resources towards the mating phenotype. In addition to considerations of general metabolic productivity, it is also important to identify any fundamental differences in metabolism, as they can help to decide which heterologous products will be favoured by the natural fluxes in the network. For example, specific anabolic pathways could be up-regulated in response to mating pheromone, suggesting that industrial products which are derived from these pathways would have higher yields during the pheromone-response.

The concept of limiting biomass formation to enhance cellular productivity has received some attention in the field of therapeutic protein production in mammalian cell cultures (Kumar et al., 2007). In particular, the manipulation of the eukaryotic cell cycle to induce a growth arrest phenotype has been successfully used to improve heterologous protein production. For example, the overexpression of the cyclin dependent kinase inhibitor p21and its inducer C/EBPα in a Chinese Hamster Ovary cell line resulted in stable cell-cycle arrest in the G1 phase and a 10–15 fold higher protein productivity per cell (Fussenegger et al., 1998). Similarly, the overexpression of the p21 cyclin inhibitor in an NS0 mouse myeloma cell line increased protein productivity ~4 fold (Watanabe et al., 2002). The increased productivity due to p21 mediated cell-cycle arrest has been attributed to higher mitochondrial membrane potential providing more ATP for peptide bond formation, and increased ribosomal biogenesis (Bi et al., 2004; Khoo and Al-Rubeai, 2009). It is possible that the cell cycle arrest phenotype of the *S. cerevisiae* pheromone-response could result in similar productivity improvements.

In this work, we have investigated the pheromone-response in *S. cerevisiae* as a growth arrest phase for metabolic engineering and synthetic biology applications. Fundamental metabolic differences in pheromone-treated populations were identified by comparing external metabolite fluxes, metabolic and global gene expression patterns, and the production capacity of a heterologous compound of industrial importance, para-hydroxybenzoic acid (PHBA).

2. Materials and methods

2.1. Growth media

Strains were grown in chemically defined CBS medium with 5 g/L ammonium sulfate, 20 g/L glucose, vitamins and trace elements (Verduyn et al., 1992) solidified with 20 g/L agar when solid medium was required. During strain construction auxotrophies were complemented with purified amino acids (Sigma) in CBS agar plates, while YPD (Yeast extract 10 g/L, Bacteriological Peptone 20 g/L, glucose 20 g/L) or YPG (galactose in place of glucose) supplemented with appropriate antibiotics was used during gene

Table 1
Plasmids.

Name	Details	Origin
pRS406	*URA3* integrating vector	(Sikorski and Hieter, 1989), Euroscarf
yEGFPCLN2PEST-pRS406	Destabilized *GFP* gene in pRS406	(Williams et al., 2013)
pSF019	p*TEF1* driven *lacZ* expression	(Partow et al., 2010)
pTEF1-yEGFPCLN2PEST	*TEF1* promoter driven *GFP* expression	This Study
pUG6	Contains geneticin resistance marker gene	(Güldener et al., 1996), Euroscarf
pUG66	Contains phleomycin resistance marker gene	(Güldener et al., 1996), Euroscarf
pTCW022	*pFUS1J2-UBiC-CYC1t-pFUS1J2-ARO4-CYC1t-pFUS1J2-TKL1-CYC1t*-pRS406	(Williams et al., 2015a)
PHBA01	*pTEF1-UBiC-CYC1t*-pRS406	This study
PHBA02	*pTEF1-UBiC-CYC1t-pTEF1-ARO4* ^{K229L} *-CYC1t*-pRS406	This study
PHBA03	*pTEF1-UBiC-CYC1t-pTEF1-ARO4* ^{K229L} *-CYC1t-pTEF1-TKL1-CYC1t*-pRS406	This study

Table 2
S. cerevisiae strains.

Name	Genotype	Notes	Origin
CEN.PK113-5D	MATa; *ura3-52*; *MAL2-8^{C}; SUC2*	Haploid MATa lab strain	Euroscarf
PSP01	CEN.PK113-5D, *bar1::phleo*	*BAR1* gene deleted	This study
PSP02	CEN.PK113-5D, *bar1::phleo*, *fus1::KanMX*	*BAR1* and *FUS1* deleted	This study
PSP03	CEN.PK113-5D, *bar1::phleo*, *fus1::KanMX*, *ura3-52::pTEF1-GFPCLN2PEST-ADH1t*-pRS406	PSP02 + constitutive destabilized *GFP* expression	This study
PSP04	CEN.PK113-5D, *bar1::phleo*, *fus1::KanMX*, *ura3-52*::pRS406	Prototrophic control strain	This study
PSP05	CEN.PK113-5D, *bar1::phleo*, *fus1::KanMX*, *ura3-52: :pTEF1-UBiC-CYC1t-pTEF1-ARO4 ^{K229L} -CYC1t-pTEF1-TKL1-CYC1t*-pRS406	para-hydroxybenzoic acid producing strain	This study

deletion procedures. *E. coli* DH5α was used for plasmid propagation/storage and was grown in LB medium with appropriate antibiotics.

2.2. Strains and plasmids

Primers, plasmids, and strains used in this study are shown in Tables S1, 1, and 2 respectively. DNA manipulation and propagation were carried out using standard techniques (Sambrook and Russell, 2001) unless stated otherwise. All *S. cerevisiae* transformations were carried out using the lithium acetate method (Gietz and Schiestl, 2007). Yeast cells can die if the pheromone-response is induced and a mating partner is not found, and this effect can be mitigated by deleting the *FUS1* gene (Zhang et al., 2006). Deletion of the *FUS1* gene was performed with the reusable LoxP-KanMX-LoxP cassette as described previously (Güldener et al., 1996) using the FUS1KOF and FUS1KOR primers to amplify the geneticin deletion cassette from pUG6 (Gueldener et al., 2002) and the FUS1DCF and FUS1DCR primers to check the chromosomal locus for deletion. Strains transformed with yeast integrating plasmids were screened for correct integration using PCR as previously described (Stansfield and Stark, 2007). In order to reduce the capacity of yeast cells to become desensitized to mating pheromone we deleted the *BAR1* gene which encodes for a secreted alpha-pheromone protease (Chan and Otte, 1982). The *BAR1* ORF was replaced with the phleomycin resistance cassette from pUG66 using the same method as for *FUS1* except with the *BAR1* primers.

The *pTEF1-yEGFPCLN2PEST*-416 plasmid was made by inserting the *TEF1* promoter amplified from pSF-019 (Partow et al., 2010) using primers 9/10 into *yEGFPCLN2PEST*-pRS406 (Williams et al., 2013) digested with *XhoI*/*EcoRI*. The gene – *CYC1* terminators for *UBiC*, *ARO4^{K229L}*, and *TKL1* were amplified from the pTCW022 (Williams et al., 2015a) plasmid using primers 13/14, 17/18, and 20/21. *TEF1* promoters were used to control the expression of *UBiC*, *ARO4^{K229L}*, and *TKL1* genes. The *TEF1* promoter region was PCR amplified from the pSF019 plasmid using primers that have 5′ extensions to create 40 bp homologous overlap junctions for each gene - *CYC1* terminator cassette using primers 11/12 (*UBiC*), 15/16 (*ARO4*), and 19/20 (*ARO4^{K229L}*). Overlap extension PCR (Horton et al., 1989) was used to assemble the *TEF1* promoters 5′ of their respective ORF-*CYC1* terminators. The *pTEF1-UBiC-CYC1t* cassette from the overlap assembly process was then PCR-amplified using primer pair 23/24 and inserted into pRS406 (Sikorski and Hieter, 1989) using *XhoI* and *EcoRI* to make plasmid PHBA01. Similarly the *ARO4* expression cassette was amplified (primers 25/26) and inserted into PHBA01 using *EcoRI* and *NotI* to make PHBA02. The *TKL1* expression cassette generated by overlap assembly was amplified with primer pair 27/28 and inserted into PHBA02 using *NotI* cut sites at both ends to make PHBA03. All constructs were sequenced to check for PCR errors.

2.3. Growth conditions

Shake-flask fermentations were carried out at 30 °C, 200 rpm with aluminium foil used to cover flask tops and medium making up 10% of the baffled flask volume. Single colonies from solid CBS agar plates that had been streaked with glycerol-stocked strains were used to inoculate 10 mL of liquid CBS medium. After 24 h growth, cells were passaged into a second pre-culture (25 mL) and grown to mid to late log phase ($OD_{660\ nm}$ of 1–5) prior to inoculation of the experimental culture (50 mL) at an $OD_{660\ nm}$ of 0.4. Synthetic alpha-pheromone (Genscript, Piscataway, NJ, USA) was added to flasks at a final concentration of 1 μM at indicated time points. Samples for analysis of extracellular metabolites were obtained by centrifugation of 1 mL of culture at 13,000 × *g* for 7 min at 4 °C and storing the supernatant at −20 °C until analysis. Population density was measured using absorbance at 660 nm ($OD660_{nm}$) on a spectrophotometer (LibraS4, Biochrom UK). $OD_{660\ nm}$ values were converted to biomass using a conversion factor of 0.243 g dry cell weight per 1 OD unit (determined using exponentially growing CEN.PK113-5D populations).

2.4. Analytics

Extracellular glucose, ethanol, glycerol, and acetate concentrations were determined using HPLC as previously described (Dietmair et al., 2010). Metabolite concentration were normalised to carbon moles along with biomass, and these values were used to estimate CO_2 production and O_2 consumption as described previously (Stephanopoulos et al., 1998). PHBA concentrations were measured in extracellular supernatants as previously described (Williams et al., 2015a). GFP measurement was carried out as described previously (Williams et al., 2013).

2.5. Transcriptome analysis

Although transcriptomics has previously been carried out on yeast populations responding to pheromone (Roberts et al., 2000), the data analyses and interpretation were oriented specifically towards mating related processes. As we were interested other changes that are more peripheral to the canonical pheromone-response, the existing transcriptome data were re-analysed with known mating related genes excluded. The log normalised fold changes in gene expression and corresponding p values for populations treated with 50 nM alpha pheromone for 2 h were obtained from a previous study (Roberts et al., 2000). The gene names were assigned to gene ontology (GO) terms using the *Saccharomyces* Genome Database (SGD) GO slim mapper (http://www.yeastgenome.org/cgi-bin/GO/goSlimMapper.pl), and any genes under the categories of ‘sexual reproduction’ and/or ‘conjugation’ were removed from the data set. Genes with p values ≤ 0.01 and fold changes ≥ 2 were then used for GO term analysis. For central carbon metabolic gene analysis, transcripts with p values ≤ 0.05 and fold changes ≤ -1.5 and $\geq +1.5$ were

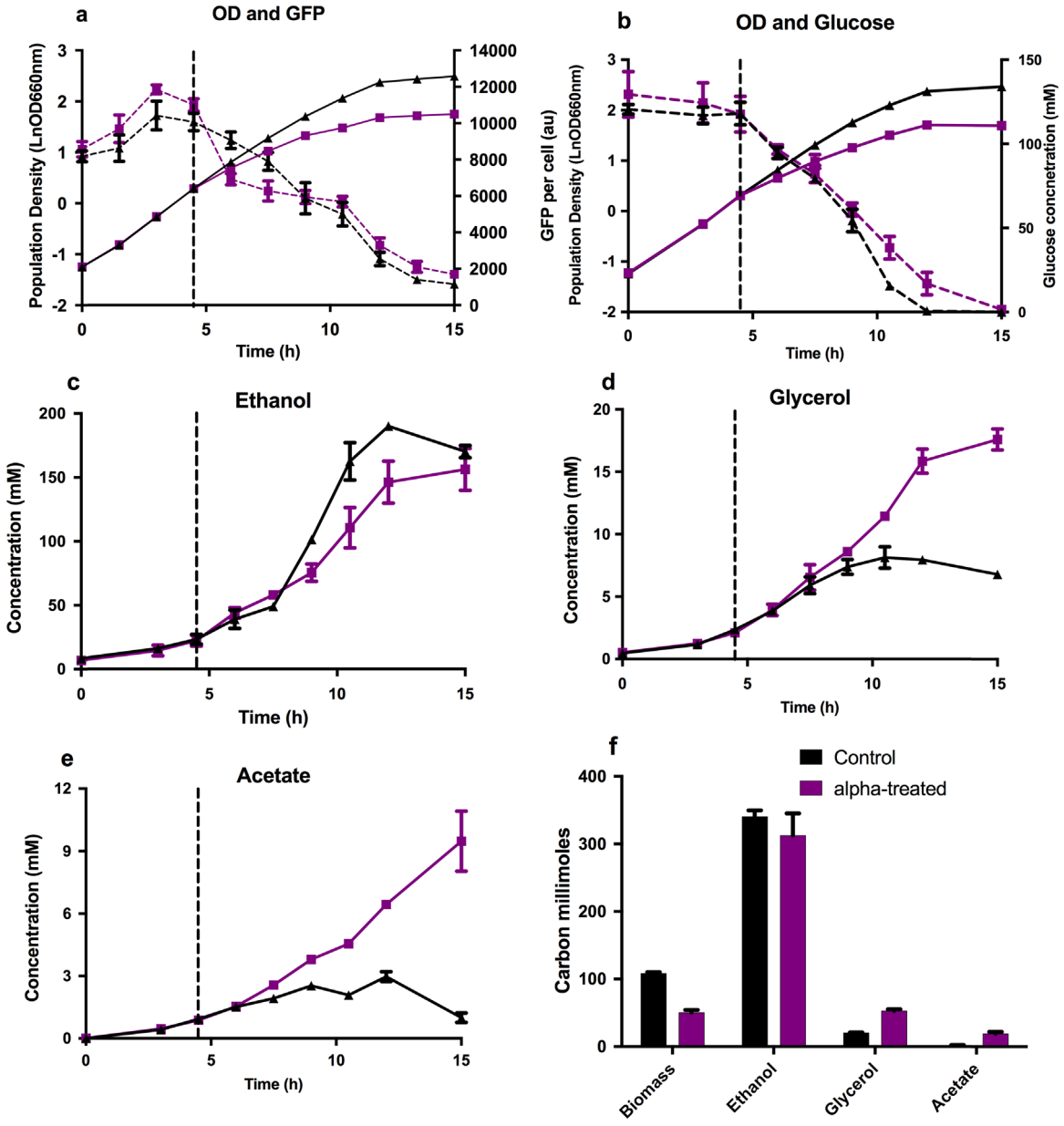

Fig. 1. A strain with constitutive expression of *GFP* (PSP03, **a**), and a strain with no *GFP* expression (PSP04, **b-e**) were grown for 15 h in shake-flasks with (squares, purple lines) and without (triangles, black lines) 1 μM alpha pheromone added at 4.5 h (vertical dashed lines). *GFP* expression per cell (dashed lines) and population density (solid lines) were measured (**a**) along with extracellular glucose (dashed lines in **b**) and extracellular metabolite concentrations (**b-e**). Relative carbon concentrations were calculated as described in (Stephanopoulos et al., 1998) after 15 h of growth (**f**), where an *S. cerevisiae* carbon content of 24.6 g/C-mol with 7% ash (Stephanopoulos et al., 1998) was used along with a conversion factor of 0.243 to convert $OD_{660\ nm}$ to grams dry cell weight. All measurements were carried out in biological triplicate with error bars representing ±1 standard deviation.

considered, and manually mapped to central carbon metabolism using an adaptation of the metabolic map presented in (Oliveira et al., 2012). Figures were constructed using Adobe Illustrator (Adobe Systems Software Ireland Ltd).

2.6. Statistical analysis

All experiments were conducted in biological triplicate (precultures initiated using separate colonies from solid media). Means, standard deviations, and p-values were calculated in GraphPad Prism 6 using two-sided student's *t*-tests with equal variance. Biomass and extracellular metabolite rates were calculated using linear regression (LINEST equation in Microsoft Excel) individually for each biological replicate, only using data points after the addition of pheromone to cultures at 4.5 h. In the case of biomass, data were natural log transformed (Ln) prior to regression analysis. The mean rates from triplicate experiments were reported along with the pooled standard errors.

3. Results and discussion

3.1. Gene expression capacity and growth characteristics of the pheromone-response

An ideal bio-production phase has a high general capacity for gene expression such that heterologous enzymes can be expressed to a high level. A production phase should also have high metabolic activity through central carbon metabolism to allow efficient supply of carbon precursors to products. These factors are

Table 3
Summary of external metabolite flux rates.

	μ (h^{-1})*	Glucose uptake (mmol g^{-1} h^{-1})	Ethanol (mmol g^{-1} h^{-1})	Glycerol* (mmol g^{-1} h^{-1})	Acetate* (mmol g^{-1} h^{-1})	Respiratory quotient
Control	0.26 ± 0.03	12.55 ± 1.47	20.52 ± 2.58	0.53 ± 0.17	0.16 ± 0.06	4
Treated	0.17 ± 0.02	15.40 ± 1.52	19.65 ± 2.85	2.17 ± 0.29	0.89 ± 0.11	2

Average growth and extracellular product secretion rates are shown for cultures treated with alpha-pheromone (treated) and without (control). All rates were calculated using data points after the addition of pheromone at 4.5 h, including in the control populations. Mean values of biological triplicates are presented with errors representing ± standard error. * denotes significant difference between control and pheromone treated groups (two sided students *t*-test with equal variance with $p \leq 0.05$).

particularly important to assess for the pheromone-response because all of the other known types of growth arrest in yeast result in a 'stationary phase' which is characterised by low gene expression and metabolic activity (Albers et al., 2007; Herman, 2002; Werner-Washburne et al., 1993).

To address the question of gene expression capacity of the cell-cycle arrest associated with the pheromone-response, *GFP* expression levels between cultures treated with and without synthetic α-pheromone were compared (strain PSP03, Table 2). The *TEF1* promoter was used to control *GFP* expression because the promoter is constitutively active (Da Silva and Srikrishnan, 2012) and *TEF1* mRNA levels are unaffected by pheromone treatment (Roberts et al., 2000). Consequently, the level of *GFP* should reflect the overall gene expression capacity of the populations.

To induce the mating response, alpha-pheromone was added to one set of parallel cultures at 4.5 h. A rapid reduction in growth rate was observed within two hours relative to the non-pheromone-treated control, consistent with the cell cycle arrest phenotype of the pheromone-response (Bardwell, 2005) (Fig. 1a). *GFP* fluorescence showed a similar pattern in both treatments throughout the experiment (Fig. 1a). Fluorescence initially increased up until 4 h, then declined over the remainder of the culture. The decreasing expression from the *TEF1* promoter is consistent with our recent findings (Peng et al., 2015; Williams et al., 2015b), and is explained by the use of a highly destabilized version of *GFP* with a 20-min half-life (Mateus and Avery, 2000). This destabilized *GFP* is highly sensitive to decreases in expression, which may have been masked by the stability (7 h half-life) of the *GFP* protein used in previous analyses of the *TEF1* promoter in yeast (Partow et al., 2010; Sun et al., 2012). Although this decline occurred after pheromone addition in the treated culture, the fluorescence response was the same in the untreated culture, indicating that this is a generic pattern during cultivation rather than being pheromone-specific. The declining *GFP* expression rate in the treated strain (-782 ± 104 au/cell/hr) was very similar to the control strain (-934 ± 54 au/cell/hr) over the remainder of the cultivation time following initiation of culture growth arrest. These data suggest that gene expression capacity remains as active during G1 phase growth arrest as it is in exponentially growing populations, thus demonstrating the potential for this phase to be useful for production.

In addition to gene expression capacity, another principle requirement for metabolic engineering applications is to have an active central carbon metabolic network. This can be investigated generally by examining biomass accumulation in parallel with carbon source consumption and the accumulation of the predominant extracellular metabolites produced by *S. cerevisiae*. Parallel measurements of glucose uptake, along with biomass, acetate, ethanol, and glycerol production throughout 15 h of shake-flask cultivation clearly demonstrated that populations treated with pheromone were at least as metabolically active as the non-treated control populations (Fig. 1b-h). As with the *GFP* expression strains, pheromone addition at 4.5 h resulted in a characteristic decrease in growth rate (Fig. 1b), but surprisingly a very similar glucose consumption profile (Fig. 1c) with rates of 15.40 ± 1.52 mmol g^{-1} h^{-1} and 12.55 ± 1.47 mmol g^{-1} h^{-1} for pheromone treated and non-treated respectively (Table 3). In contrast to the relatively small differences in glucose consumption and ethanol production, glycerol and acetate production were markedly increased in pheromone-treated cultures (Fig. 1, Table 3).

When summarized with end-point metabolite concentration values converted to carbon moles (Fig. 1f) it is clear that the carbon not used for biomass production is directed towards side-product formation in the form of glycerol, acetate, and CO_2 (calculated as 296 and 273 carbon moles for pheromone treated and non-treated respectively). When carbon balance values were used to infer CO_2 and O_2 production and consumption rates, the respiratory quotients obtained (Table 3) strongly suggest that the pheromone-response entails a shift towards a more respiratory metabolism compared to the fermentative metabolism of the control cultures. The current understanding of the shift between fermentative and respiratory metabolism in yeast is that a reduced glucose uptake rate results in de-repression of TCA cycle enzymes and a corresponding increase in TCA cycle flux, oxidative phosphorylation, and a decrease in fermentative flux towards ethanol (Blank and Sauer, 2004; Dijken et al., 1993; Heyland et al., 2009). The slightly reduced ethanol production rate of pheromone treated populations is consistent with this understanding, but it is interesting that the specific glucose uptake rates are not significantly different between the groups. These data support the concept that cell cycle arrest in response to pheromone results in an active and distinct metabolic phenotype, as compared to 'standard' carbon/nitrogen-limited stationary phases (Albers et al., 2007).

3.2. Transcriptome analysis

In addition to assessing the general gene expression capacity and external metabolic fluxes during the pheromone-response, it is also important to consider global changes in gene expression. Most starvation based stationary phases are characterised by the induction of stress resistance modules at the transcriptional level, and the hypothesis that the pheromone-response growth arrest phenotype is distinct from these phases can be tested using transcriptomics. Global changes in *S. cerevisiae* gene expression in response to pheromone treatment have previously been reported (Roberts et al., 2000). This study elegantly demonstrated the complexity of the pheromone-response pathway and the degree to which its signalling components are related to other MAPK modules. However, the data interpretation/analysis did not include transcriptional changes outside of the signalling and effector components of the pheromone-response and related MAPK modules. To gain insight into other changes of relevance to metabolic engineering, the data were re-analysed after excluding any genes primarily involved in the pheromone-response (GO terms 'sexual reproduction' and 'conjugation'). A 99% confidence interval and minimum 2-fold change were used as selection criteria to identify up-regulated (Table S2) and down-regulated (Table S3) genes.

Structural processes, including cell wall and cytoskeletal organization, were up-regulated in pheromone-treated populations.

This likely relates to the characteristic 'shmoo' cell morphology of the mating phenotype. Many up-regulated genes were assigned to categories associated with control of the cell cycle, mitosis, budding, and cytokinesis. These genes regulate the characteristic G1 phase cell-cycle arrest of the mating phenotype. The same categories involving cell-cycle related genes that were up-regulated also featured in the down-regulated gene GO terms (Table S3). This reflects the complexity of regulation required to arrest cell division, with the coordinated up- and down-regulation of a multitude of genes needed to elicit such fine control.

The GO term 'Transposition' refers to the movement of DNA between non-homologous sites and includes many retrotransposon genes. It has previously been shown that Ty3 retrotransposons are up-regulated in mating populations (Kinsey and Sandmeyer, 1995), and it was interesting to see that genes more generally involved in transposition were significantly up-regulated (Table S2). Transposition during the pheromone-response might provide a mechanism to increase genetic variation in the population prior to mating and, given that the process appears to be regulated by the host (not the transposons), could serve as an example of symbiotic retrotransposition.

The most notable down-regulated genes were involved in ribosomal RNA biogenesis and processing. This is consistent with the fact that much of the cellular resources of an exponentially growing population are directed towards ribosome synthesis (Warner et al., 2001), and demonstrates a down-regulation of this process during the pheromone-response phase. Although a decrease in ribosome biogenesis could be thought of as limiting the protein expression capacity of the cell, there are still very high expression levels of genes which are switched on during the pheromone-response (Bardwell, 2005), and we have previously demonstrated sustained induction of a pheromone regulated metabolic pathway (Williams et al., 2015a). Given the drain that ribosome biogenesis imposes on ATP supply (Warner et al., 2001), and the fact that pheromone regulated genes can still be highly expressed, a reduction in ribosome synthesis can be viewed as a principle requirement for a 'productive stationary phase'. Many down-regulated genes were involved in DNA replication/repair and chromosome segregation, again reflecting the arrested state of cell division. It is interesting to note that these processes are also typical of the starvation responses associated with stress induced stationary phases (Wu et al., 2004), but that the yeast which were used for the pheromone-response transcriptome analysis were cultured in rich YPD media, and were not starving (Roberts et al., 2000). This observation highlights the unique nature of the pheromone mediated growth-arrest, and validates the idea of attempting to use it as a production phase where flux towards biomass is limited while nutrients are still abundant.

Changes in the expression levels of metabolic genes are of direct relevance to the metabolic component of the pheromone-response. Therefore in addition to analysing global transcriptional changes, central carbon metabolism-specific changes were analysed by mapping the expression levels of significantly changed (95% confidence intervals) metabolic genes along with the reactions they encode (Fig. 2). Significant increases in the transcript levels of a multitude of metabolic enzymes were observed, further suggesting that the mating phenotype has a distinct and active metabolism. In particular, expression levels of genes involved trehalose and glycerol synthesis, the TCA cycle, and the pentose phosphate pathway were significantly up-regulated (Fig. 2). The high expression levels observed for multiple central carbon metabolic genes, along with the active secretion of metabolic side-products (Fig. 1) act as a strong indication that central carbon metabolism is active during the growth-arrest phenotype. There were also a number of interesting trends in regards to specific metabolic processes that are worth speculating on.

Trehalose acts as a major storage carbohydrate in yeast, and increased synthesis has been associated with exposure to thermal, osmotic, and ethanol stress (Pereira et al., 2001). Given that these experiments were carried out at 30 °C (Roberts et al., 2000) and considering that the hyper osmolarity glycerol (HOG) response to osmotic stress and the pheromone-response are insulated from one another (O'Rourke and Herskowitz, 1998) it is possible that ethanol stress during the pheromone-response may be linked to the observed up-regulation of trehalose synthesis genes (Fig. 2). It has previously been reported that trehalose synthesis is required to enable endocytosis at relatively low ethanol concentrations (Lucero et al., 2000). Endocytosis is a process where cells internalise their plasma membrane proteins from the extracellular environment, and is integral to the pheromone-response in yeast where pheromone bound membrane receptor proteins are internalised (Marsh et al., 1991). Consistent with this idea, genes involved in endocytosis were highly up-regulated in response to pheromone (Table S2). It is therefore possible that the up-regulation of trehalose biosynthetic genes in response to pheromone evolved as a mechanism to protect cells from ethanol stress during pheromone bound receptor endocytosis. An alternative explanation is that there is actually a low level of osmotic stress associated with the morphological changes that occur during the pheromone-response, which is responsible for the up-regulation of storage carbohydrate synthesis (see below).

Up-regulation of glycerol synthesis genes (Fig. 2) is consistent with the much higher levels of glycerol accumulation observed in pheromone-treated cells (Fig. 1). It has been proposed that mating yeast cells require a precise osmotic balance prior to cell wall degradation and membrane fusion, and that this balance is achieved with the export of glycerol from the cell via the FPS1 transporter (Philips and Herskowitz, 1997). Recent work has further demonstrated the capacity of yeast responding to pheromone to excrete glycerol, demonstrating that the HOG pathway is actually partially activated by the pheromone-response (Baltanas et al., 2013). The results presented here strongly support these findings, and support the role of trehalose synthesis as an osmoprotectant rather than solely to enable endocytosis under ethanol stress.

Respiratory metabolism requires a greater flux through the TCA cycle for the generation of reducing power to drive oxidative phosphorylation through the electron transport chain. The strong up-regulation of TCA cycle genes suggests that pheromone treatment results in a more highly respiratory metabolism. The extracellular metabolite accumulation rates and respiratory quotients independently support this finding (Table 3). The oxidative branch of the pentose phosphate pathway (PPP) is initiated by glucose-6-phosphate dehydrogenase (ZWF1) in an irreversible step (Nogae and Johnston, 1990). The PPP is responsible for producing NADPH, a critical source of reduction potential required by many anabolic pathways (Minard et al., 1998). The PPP also plays a major role in mitigating the effects of oxidative stress by supplying NADPH to glutathione- and thioredoxin-dependent enzymes (Slekar et al., 1996). The strong up-regulation of PPP genes (ZWF1, GND2, RPE1) in response to pheromone (Fig. 2) could occur as a consequence of the increased rate of respiration initiated by pheromone (Table 3) and the subsequent increase in oxygen radicals. In concordance with this idea was the up-regulation of a multitude of genes involved in both oxidative stress and respiration (Table S2).

It is important to note that the cultures which were used for RNA extraction in the original transcriptome study (Roberts et al., 2000) were carried out under different conditions than the cultures used here to calculate extracellular flux rates (rich YPD rather than minimal medium, Fig. 1, Table 3). Therefore the trends in metabolic gene expression levels which correspond to the flux data should be considered as independent, but consistent observations.

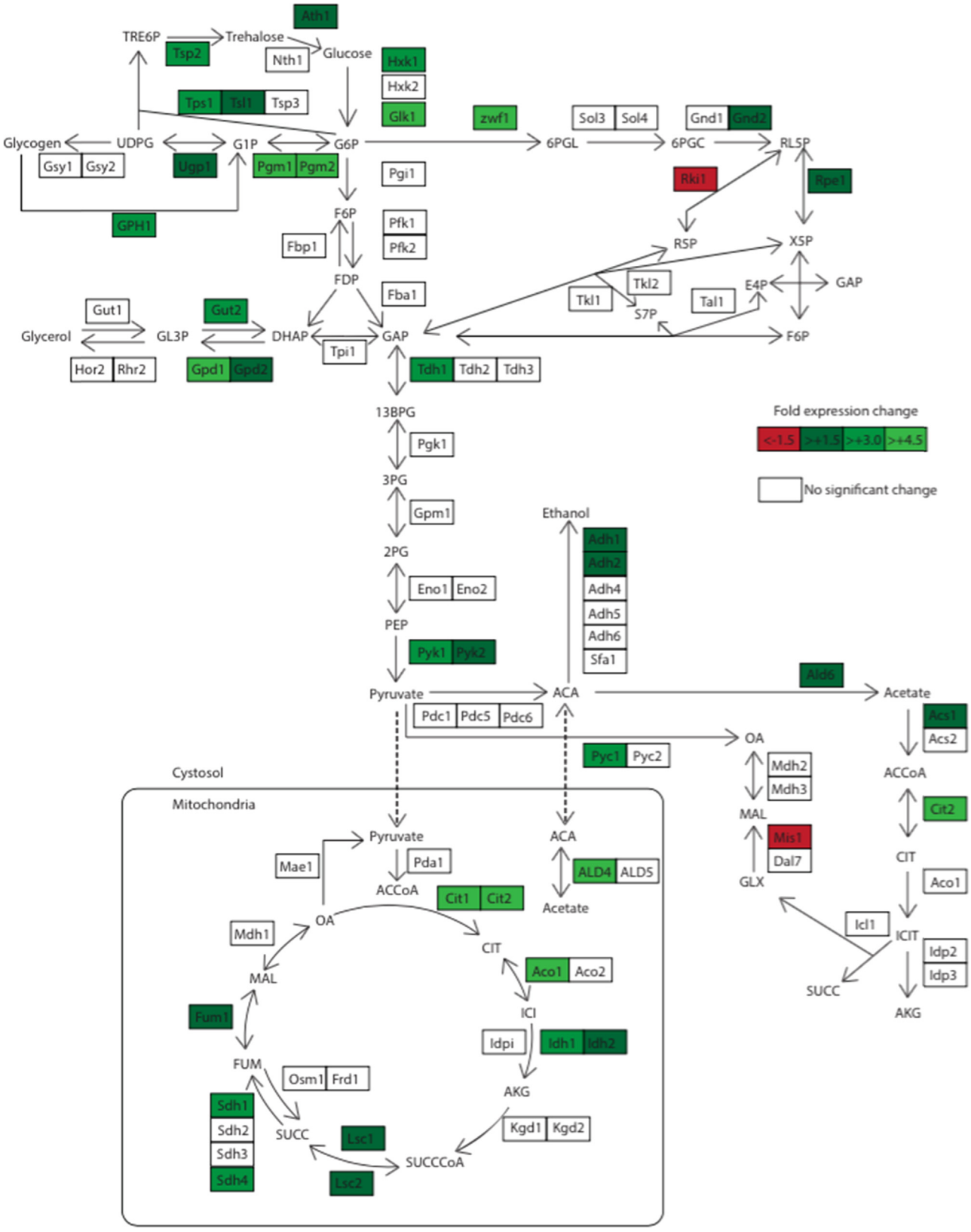

Fig. 2. Transcript levels for genes encoding central metabolic enzymes are shown with up-regulation in green and down-regulation in red. Metabolites are connected by arrows representing reactions catalysed by enzymes (gene names in boxes). Single headed arrows represent one way reactions and double headed denote reversible reactions. The metabolic map and abbreviations were adapted from Oliveira et al. (2012).

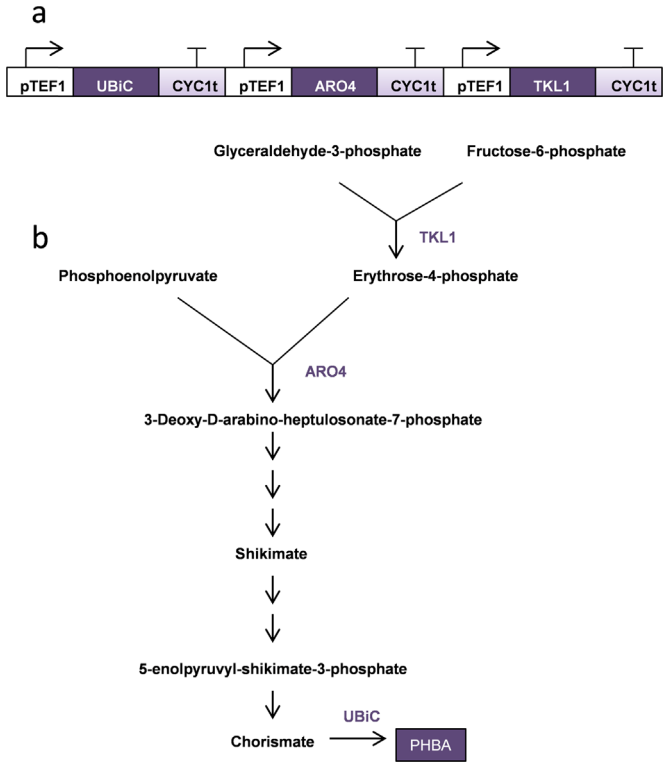

Fig. 3. Engineered PHBA production pathway. Gene expression constructs (a) and the role of the expressed enzymes (*TKL1*, *ARO4*, *UBiC*) are shown for PHBA production from the shikimate pathway (b).

3.3. Engineered pathway productivity during the pheromone-response

To directly test the hypothesis that the pheromone-response is suitable as a production phase, heterologous compound titers from a strain engineered to produce PHBA ± pheromone were compared. PHBA is an important industrial chemical used in liquid crystal polymers (Krömer et al., 2012) that can be derived from the shikimate pathway (Averesch and Krömer, 2014; Winter et al., 2014), which is now also being developed for the synthesis of important products such as codeine and morphine (DeLoache et al., 2015; Galanie et al., 2015; Thodey et al., 2014). PHBA productivity under pheromone exposure was here used to assess the suitability of the mating phenotype as a production phase. A minimally engineered PHBA producing strain was constructed as in Fig. 3 using manipulations previously shown to be effective at increasing shikimate pathway flux (Curran et al., 2013). The constitutive *TEF1* promoter was used to drive heterologous expression of the *UBiC* gene and over-expression of both the *TKL1* and the feedback resistant *ARO4*^{K229L} (Luttik et al., 2008) genes in the engineered pathway; this ensured that gene expression levels would be consistent between treated and untreated populations.

The PHBA producing strain (PSP05) was grown with and without 1 μM alpha pheromone treatment in early log phase ($OD_{660\ nm}$ ~1, 4 h). As expected, growth rate slowed significantly after α-pheromone treatment, indicating that the pheromone-response had initiated (Fig. 4a). After 48 h, population densities were significantly different, with the control group at an $OD_{660\ nm}$ of 12.93 ± 0.40, and the pheromone-treated group at 10.13 ± 0.40. PHBA concentrations were measured during 48 h of fermentation (Fig. 4b), with no significant difference ($p=0.11$) between the two groups after 48 h (control = 274 ± 16 μM, alpha = 217 ± 46 μM). In order to more directly assess the capacity for metabolic productivity, the biomass-specific PHBA production yields and rates between pheromone-mediated growth-arrest at 6 h and glucose exhaustion at 48 h were compared with the control population rate and yield during the glucose consumption phase between 0 and 24 h. There was a significant decrease in the biomass specific PHBA production rate during the glucose consumption phase with the control group producing 9.4 ± 1.95 μM g^{-1} h^{-1} and the pheromone treated group producing 3.35 ± 0.58 μM g^{-1} h^{-1}. Interestingly, the biomass specific PHBA yield was significantly higher in the pheromone treated group during this phase at 93.2 ± 16.2 μM gDCW^{-1} compared to 62.3 ± 1.3 μM gDCW^{-1} ($p=0.03$). Pheromone treatment also resulted in a significantly higher glucose specific PHBA yield during the respective glucose consumption phases (1.64 ± 0.11 $\mu M_{PHBA}\ mM^{-1}_{glu\ cos\ e}$ compared to 1.19 ± 0.05 $\mu M_{PHBA}\ mM^{-1}_{glu\ cos\ e}$ $p=0.003$). Although the PHBA production rate was lower during pheromone-mediated growth-arrest, both the biomass- and glucose-specific PHBA yields were higher. This suggests that the growth arrest phenotype of the *S. cerevisiae* pheromone response system results in a more productive glucose consumption phase where carbon otherwise directed towards cell growth becomes available for metabolite production. The titer was not greater in the pheromone treated group due to the lower amount of biomass, and the fact that the growth-arrest phenotype was not initiated until the mid-exponential growth phase.

3.4. Practical considerations for growth-arrested metabolic productivity

The current data are promising in that we demonstrate the pheromone mediated cell-cycle arrest phenotype is an active phase, but further optimisation of the system would be required to move towards the idealised 'productive stationary phase' that was outlined in the introduction. Cells responding to pheromone expend resources executing the mating program, which involves extensive cell-wall remodelling and cell morphology changes. Minimising this energy/resource drain through further metabolic engineering might improve overall pheromone-phase productivity of specific engineered pathways. Moreover, use of a fed-batch approach along with titration of pheromone induction time might demonstrate greater improvements in the pheromone-response productivity compared to the control. A potential issue with using the pheromone-response as a production phase is the capacity for cells to become desensitized to pheromone after prolonged exposure through a variety of well-understood mechanisms (Bardwell, 2005). This phenomenon was not observed during the timeframe of these experiments (population density was still reduced after 48 h of cultivation; Fig. 4), but may need to be prevented through engineering for any longer-term applications. Another consideration is that although it is possible to use the mating phenotype as a production phase, adding purified pheromone to a large-scale fermentation is likely to be prohibitively expensive, depending on the value of the fermentation product. To circumvent this limitation, we recently developed a synthetic quorum sensing circuit where cells produce and respond to their own pheromone in a population density dependent manner (Williams et al., 2013). This quorum sensing network has since been coupled to a recently developed yeast RNA interference module (Crook et al., 2014; Drinnenberg et al., 2009; Si et al., 2014; Williams et al., 2015b) to successfully dynamically regulate the production of PHBA (Williams et al., 2015a). This auto-response system could be applied to ensure sufficient pheromone is available during cultivation.

There are many other methods for arresting the cell cycle under nutrient rich conditions, and it is possible that they could be used as inducers of stationary phase metabolic productivity. For

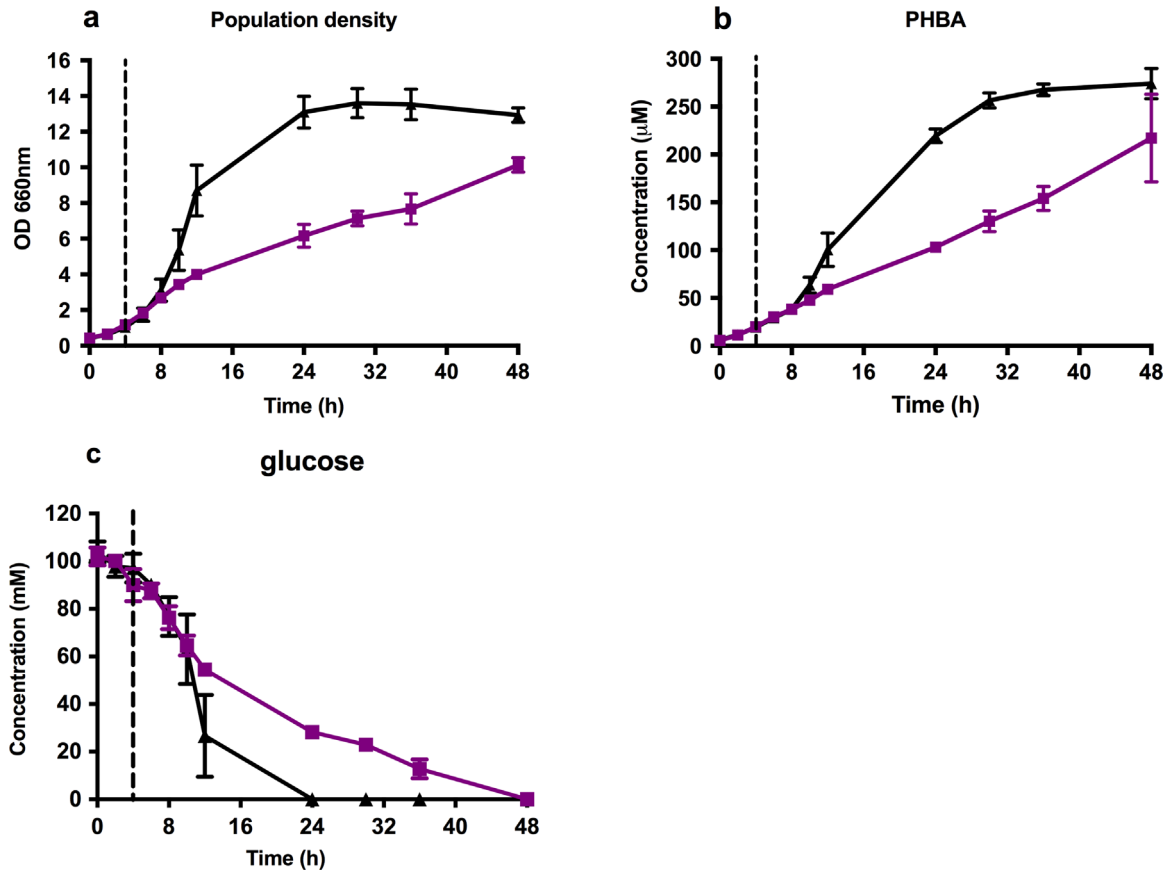

Fig. 4. PHBA production in pheromone treated cultures. A strain minimally engineered to produce PHBA (strain PHBA03) was grown with (purple lines, squares) and without (black lines, triangles) 1 μM alpha pheromone treatment at early exponential phase (OD of 1, at 4 h, indicated by vertical dashed lines). (a) Population density ($OD_{660\ nm}$), (b) extracellular PHBA concentration, and (c) extracellular glucose concentrations were measured for shake-flask growth with and without pheromone over 48 h.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

example hydroxyurea can be used to inhibit dNTP synthesis and arrest the cell cycle in S phase (Koç et al., 2004), and nocodazole to disrupt microtubule polymerisation and arrest cells in metaphase (Arber, 2000). While these methods have proved invaluable in elucidating cell-cycle related processes in basic research, they are yet to be explored as inducers of productive growth-arrest phenotypes in biotechnology. These inducers would have the advantage of not involving the initiation of a complex mating phenotype and the associated energetic cost. However, they indirectly induce stress response mechanisms that are likely to impose their own limitations on metabolic productivity. Furthermore, unlike with our synthetic pheromone quorum-sensing circuits (Williams et al., 2015a, 2013) there are no available mechanisms for auto-induction using chemical inducers such as hydroxyurea and nocodazole. In an excellent recent example of chemically induced growth-limitation, pantothenate (vitamin B12) was used to regulate growth and farnesene (jet-fuel) productivity in yeast (Sandoval et al., 2014). In this system the removal of pantothenate from the growth medium of a producer strain resulted in a 70% reduction in farnesene yield and a concomitant increase in growth rate. This enabled pantothenate to be used as an inducer whereby a rapid biomass formation phase in the absence of pantothenate was followed by a switch to a growth-limited production mode upon pantothenate addition to the medium. There are likely to be many analogous systems for other engineered pathways and metabolic networks that hold great promise as inducers of stationary phase metabolic productivity.

It is possible that the growth arrest induced by pheromone in *S. cerevisiae* could also be a useful production phase in other yeast species that use sexual pheromones to coordinate mating. However, although some species such as *Schizosaccharomyces pombe* use pheromones, they only mate under nutrient starvation conditions (Yamamoto et al., 1997). Clearly the growth arrest phenotype would not be effective as a production phase under these conditions. Given the drawbacks of using a naturally evolved mating phenotype for production, a much grander solution is to try and reverse engineer the principle features of a 'productive stationary phase'. In this scenario a synthetic regulatory circuit could be employed as a 'master controller' that can simultaneously arrest the cell cycle, reduce ribosomal biogenesis, and switch on the expression of production pathway enzymes while maintaining high central carbon metabolism fluxes. The fact that growth fluxes are no longer required in this scenario opens up the possibility of silencing the expression of 'essential' genes, and producing metabolites or proteins that are normally highly toxic to growth. Although the specific mechanisms required to implement such a dramatic regulatory and metabolic shift would differ between organisms, it is possible that the general features are universal.

4. Conclusions

The *S. cerevisiae* pheromone-response leads to a distinct and active metabolic phenotype that is suitable for the production of PHBA, and for bioprocesses in general. The key metabolic differences which result from pheromone treatment were identified as a glucose uptake rate that is comparable to exponentially growing populations, increased by-product formation, up-regulation of

storage carbohydrate synthesis genes associated with osmolarity and oxidative stress responses, and increased respiratory activity. It is conceivable that the pheromone-response could be used with particularly good effect to increase flux towards metabolites of interest that are associated with the metabolic changes that underlie the phenotype.

Although the pheromone-response shows promise as a production phase, it is associated with a complex mating phenotype. The concept of using a growth-arrest as a production phase would be far more effective if it could be reverse engineered and streamlined so that it is decoupled from the unnecessary aspects of the mating phenotype. The ability to engineer a switch from rapid growth, to growth-arrested production in the presence of abundant carbon and nitrogen sources would become an essential design feature of industrial microorganisms. Further investigation of the *S. cerevisiae* pheromone-response may provide a means for understanding how to coordinate and engineer such a powerful synthetic biology module.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.meteno.2016.05.001.

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