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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 bioprocesses 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