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Research Article

In *Pichia pastoris*, growth rate regulates protein synthesis and secretion, mating and stress response

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Protein production in yeasts is related to the specific growth rate μ. To elucidate on this correlation, we studied the transcriptome of *Pichia pastoris* at different specific growth rates by cultivating a strain secreting human serum albumin at $\mu = 0.015$ to 0.15 h^{-1} in glucose-limited chemostats. Genome-wide regulation revealed that translation-related as well as mitochondrial genes were upregulated with increasing μ, while autophagy and other proteolytic processes, carbon source-responsive genes and other targets of the TOR pathway as well as many transcriptional regulators were downregulated at higher μ. Mating and sporulation genes were most active at intermediate μ of 0.05 and 0.075 h^{-1}. At very slow growth ($\mu = 0.015$ h^{-1}) gene regulation differs significantly, affecting many transporters and glucose sensing. Analysis of a subset of genes related to protein folding and secretion reveals that unfolded protein response targets such as translocation, endoplasmic reticulum genes, and cytosolic chaperones are upregulated with increasing growth rate while proteolytic degradation of secretory proteins is downregulated. We conclude that a high μ positively affects specific protein secretion rates by acting on multiple cellular processes.

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1 Introduction

The demand for recombinant industrial enzymes and pharmaceutical proteins is rapidly increasing. To further improve process efficiency it is of key importance to understand the relation between cellular growth and protein productivity.

Yeasts are a well-established platform for the production of recombinant proteins. It has been demonstrated that protein production is positively correlated with specific growth rate (μ) in *Saccharomyces cerevisiae* [1] and in *Pichia pastoris* [2]. Growth coupling of protein synthesis may be based on growth dependent regulation of the promoter used to express the recombinant gene. It can be anticipated that the transcriptional strength of glycolytic promoters (which are commonly used for protein production in yeasts [3]) is directly correlated with glycolytic flux and thus with specific growth rate. Indeed, transcript levels of the human serum albumin (HSA) gene in *P. pastoris* under control of the glyceraldehyde 3-phosphate dehydrogenase (GAP) promoter increased steadily with the same rate as actin transcript levels at increasing μ [4]. In contrast, HSA transcript levels under control of the translation elongation factor EF-1 (*TEF1*) promoter showed increased abundance relative to actin with increasing μ, indicating a strong positive correlation.

The majority of pharmaceutical proteins and many technical enzymes are produced by secretion into the culture supernatant. Thus, besides transcription and translation, the secretory pathway plays a major role in con-

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Abbreviations: BP, biological process; **ERAD,** ER associated degradation; **FC,** fold change; **GO,** gene ontology; **HSA,** human serum albumin; **RP,** ribosomal proteins; **TF,** transcription factor; **TOR,** target of rapamycin; **UPR,** unfolded protein response

trolling and potentially limiting productivity. The biological control of the secretory pathway in relation to growth is of great interest to increase our understanding of what triggers protein synthesis and secretion.

Therefore we have studied transcriptome regulation of a *P. pastoris* strain secreting HSA over a wide range of μ in chemostat cultures. Similar transcriptome studies were done with *S. cerevisiae* with different aims. Brauer et al. [5] compared cultures grown under limitation of different nutrients, investigating cell cycle control, stress response and metabolic activity. Castrillo et al. [6] compared cultures at four different nutrient limitations and three different growth rates at the levels of transcriptome, proteome, and metabolome, mainly to understand growth regulation at the different levels of gene expression and metabolic flux control. Induction of ribosomal and metabolic genes with increasing growth rate was related to control by the repressor/activator Rap1 and a positional effect of genes on the chromosomes near replication origins [7]. A factorial design of experiments was used to identify "general" growth regulated genes comparing carbon, nitrogen and oxygen limitation in chemostat [8].

While these studies provide ample information on cell growth control and stress regulation, there is only limited information concerning protein synthesis and secretion. The main general result is that *S. cerevisiae* upregulates translation-related genes with increasing growth rate which is not really surprising as the cells need a higher concentration of metabolic enzymes and have to reproduce their total cellular protein content at higher rate. More recently, correlation of recombinant protein synthesis with growth was investigated at the transcriptome level in *S. cerevisiae* [1] and in *Trichoderma reesei* [9]. Productivity of insulin precursor and α-amylase in *S. cerevisiae* increased with μ, accompanied by upregulation of unfolded protein response (UPR) and other stress related genes. In *T. reesei*, protein productivity correlated more with cell density than with specific growth rate.

In the present study we cover a very broad range of specific growth rates, ranging from 0.015 h^{-1} (the lowest setpoint we could maintain in chemostat) to 0.15 h^{-1} (nearly μ_{max}). As standard fed-batches reach very low μ at extended process times with high biomass concentrations [3] we were especially interested to gain understanding of the reaction of *P. pastoris* to such very low growth rates. Gene regulation at near-zero growth is not well understood. Boender et al. [10] studied transcriptome regulation of anaerobic *S. cerevisiae* cultures at μ below 0.01 h^{-1}, comparing them to higher growth rates. Main results were an upregulation of many mitochondrial genes at very slow growth, although cultures were grown anaerobically, and a decoupling of ribosomal genes (remaining active) from those involved in the translation process which are downregulated near zero growth.

By analyzing transcriptome regulation of *P. pastoris* cultures grown at a broad range of μ we aimed to understand growth regulated cellular processes in this yeast with a special emphasis on the protein folding and secretory pathway, including its quality control, and on peculiarities of very low specific growth rate.

2 Materials and methods

2.1 *Pichia pastoris* strain

The *P. pastoris* SMD1168H (Δ*pep4*) strain secreting HSA under control of the strong glycolytic GAP promoter employing the native HSA leader sequence for secretion was generated as described in [4]. The selected production clone was determined to have three copies of the expression cassette integrated in the genome.

2.2 Cultivations

Chemostat cultivations were carried out at a working volume of 1 L in a 3.5 L bench-top bioreactor (Minifors, Infors, CH). Cells were grown at dilution rates of 0.015, 0.025, 0.05, 0.075, 0.100, 0.125, and 0.150 h^{-1} (in triplicates). As preculture, a one liter shake flask containing 100 mL of YPG-Zeo medium (per liter: 10 g yeast extract, 10 g peptone, 10 g glycerol, 25 mg Zeocin) was inoculated with 1.2 mL cryostock of the *P. pastoris* strain and incubated for approximately 24 h at 25°C and 180 rpm. This culture was used to inoculate the bioreactor to an optical density (OD_{600}) of 1.0. Cultivation temperature was kept constant at 25°C, pH was controlled at 5.85 with 25% ammonia and the dissolved oxygen concentration was kept above 20% by controlling the stirrer speed between 600 and 1200 rpm at a constant airflow of 252 L h^{-1}. After batch end was reached (indicated by a sharp peak in dissolved oxygen concentration), continuous cultivation was initiated at a dilution rate of 0.1 h^{-1} for three resident times, followed by three different dilution rate setpoints. Samples were taken after at least five resident times when steady state conditions were attained. Viability was measured as described previously [11] on the Gallios™ flow cytometer (Beckman Coulter) using the Cell Viability Kit from BD Biosciences. Batch and chemostat media composition was described in ref. [2].

2.3 RNA extraction, microarray hybridization, and data analysis

RNA isolation, cRNA synthesis, hybridization to the *P. pastoris* DNA microarrays (Agilent platform) as well as scanning was done according to the Agilent protocol for 2-color expression arrays. The design and general processing of *P. pastoris* microarrays used in this study was described in ref. [12]. Samples were labeled in 2-color technical duplicates and hybridized against a reference pool generated of cells grown at various culture condi-

tions. For each dilution rate setpoint, log2 fold change (FC) values were calculated against the fastest specific growth rate of 0.15 h^{-1}, using the limma package of the R-project [13]. *p*-Value correction for multiple testing was done using the false discovery rate controlling method of Benjamini and Yekutieli [14]. To analyze the specific regulation at the lowest setpoint, comparisons of the FCs between 0.015 and 0.025 h^{-1} to those of 0.025 to 0.05 h^{-1} were performed.

2.4 Reporter metabolites

Reporter metabolites were calculated by the method published by Patil and Nielsen [15] using the BioMet toolbox [16]. For each condition, adjusted *p*-values out of the transcriptional comparison (each dilution rate setpoint against the highest specific growth rate of 0.15 h^{-1}) were used for the calculation of Z scores for each reporter metabolite. The published genome-scale metabolic model for *P. pastoris iLC915* [17] was applied as underlying metabolic model.

2.5 Cluster analysis and Gene Ontology (GO) term enrichment

Grouping of genes with similar expression patterns across different growth rates was performed with log2 FC values using *k*-means clustering analysis with Euclidian distance carried out with the Genesis software tool [18]. Criteria for the selection of regulated genes are described below in the Section 3. Gene Ontology (GO) term enrichment analysis for the obtained clusters was done using the GO term finder and Saccharomyces Genome Database (SGD) annotations [19]. The corrected *p*-value cut-off was set to 0.05 and a background list composed of annotated genes and genes with unknown function of *P. pastoris* was provided.

2.6 Analytical methods

HSA concentration was determined using the Human Albumin ELISA Quantitation Set (Cat. No. E80-129, Bethyl Laboratories, TX) as described in ref. [20].

For protein gel analysis of supernatants the NuPAGE® Novex® Bis–Tris system was used and carried out according to the manufacturer instructions. Proteins were either visualized by silver staining or transferred to a nitrocellulose membrane for western blot analysis. HSA was probed by using a 1:30 000 dilution in PBS with 0.1% Tween and 2% bovine serum albumin (BSA) of the HRP conjugated Human Albumin detection Antibody (A80-129P, Bethyl) and detection was performed using the SuperSignal™ West Pico Chemiluminescent Substrate (34079, Thermo Scientific).

3 Results and discussion

3.1 Product formation at different specific growth rates

To study the impact of growth rate on cell physiology, gene expression, and recombinant protein secretion, the HSA producing *P. pastoris* strain was grown in aerobic glucose limited chemostat cultures over a broad range of different dilution rates ($D = \mu$ = 0.015, 0.025, 0.05, 0.075, 0.1, 0.125, and 0.15 h^{-1}). Initially, cultivations were also attempted at a dilution rate of 0.175 h^{-1}, but cells were washed out before steady state was reached. Samples were analyzed for biomass concentration, cell viability, product concentration and quality as well as yield coefficients (Table 1). In accordance with a higher proportion of maintenance energy requirements on the overall energy budget, biomass yields decreased with slower growth [21]. The specific product formation rate (q_p), SDS–PAGE, and western blot analysis of supernatants are displayed in Fig. 1. A positive correlation between HSA productivity and μ could be observed over the full range of analyzed setpoints. We did not see any degradation of the secreted product at μ below 0.02 h^{-1}, different to a similar setup for production of an antibody Fab fragment [2].

3.2 Genome-wide transcriptional analysis at different specific growth rates

Changes in genome-wide gene expression were analyzed using in-house designed *P. pastoris* specific microarrays. Analysis of transcriptome data derived from different specific growth rates may be biased due to the fact that the majority of the so-called house-keeping genes have linearly increasing specific transcription rates with increasing μ (own unpublished data). To ensure that the expression values of these house-keeping genes do not change with μ [10], the same amounts of RNA were applied, leading to constant expression values of genes involved in glycolysis and other house-keeping functions, as illustrated in Fig. 2. Of 5354 ORFs represented on the microarrays, 2886 genes were differentially regulated at least at one growth rate when compared to the highest μ of 0.15 h^{-1} (adjusted *p*-value < 0.02). No minimal log2 FC was applied since also small changes in gene expression were considered important in order to obtain a global view on transcriptional regulation. Growth rate responsive genes were grouped into 12 different clusters according to their expression profile by *k*-means cluster analysis (see Fig. 3 and Supporting information, Data S1). Of the 2886 differentially regulated genes, expression of 1226 genes was positively correlated to growth rate (clusters 6, 11, and 12) and 1080 genes showed a negative correlation (clusters 3, 5, 7, and 9). Transcription of the remaining 579 genes was regulated in a more complex way, showing up- or down-regulation only at very low growth rates (clusters 1, 4, and

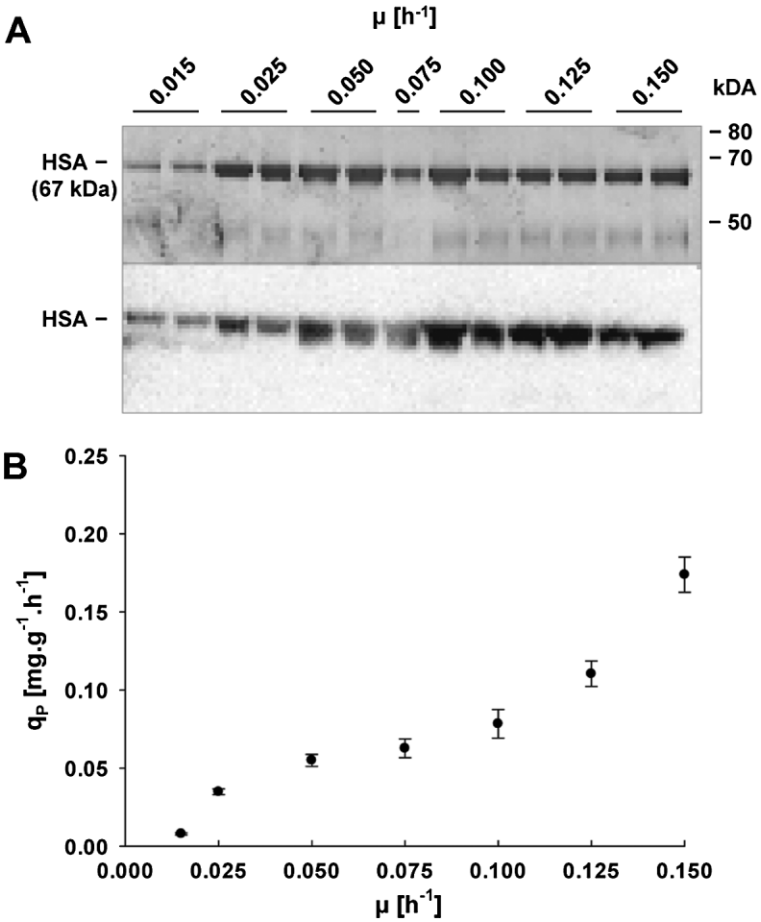


Figure 1. Recombinant HSA production by *P. pastoris* grown at different specific growth rates in glucose-limited chemostat cultures. (**A**) Representative SDS–PAGE (top) and corresponding anti-HSA western blot (bottom) under reducing conditions. Equal volumes of undiluted culture supernatants were loaded and visualized by silver staining or transferred to a nitrocellulose membrane for western blot analysis developed with HRP conjugated Human Albumin detection Antibody (A80-129P, Bethyl). For each growth rate setpoint (except for $\mu = 0.075$ h^{-1}) two samples from individual cultivations were analyzed. (**B**) Specific HSA secretion rate q_P plotted against specific growth rate μ. The specific HSA secretion rate was calculated using mean product concentrations and yeast dry mass from three independent chemostat cultivations. All samples were analyzed in technical duplicates. Error bars represent standard error of the mean.

10) or peaking in expression between μ of 0.05 and 0.075 h^{-1} (clusters 2 and 8).

Significantly enriched GO terms for the obtained clusters were determined using the GO Term Finder web tool and SGD annotations [19]. A comprehensive list of all determined GO terms for the categories biological process (BP), molecular function (MF), and cellular component (CC) can be found in Supporting information, Data S1. Enriched GO terms for the category BP are listed in Table 2.

For all clusters displaying an expression pattern positively correlated to growth rate (clusters 6, 11, and 12), genes involved in the BPs of "gene expression," "translation," "biosynthetic processes," or related terms were significantly enriched. For example, out of 280 genes associated with the term "ribosome biogenesis," 196 genes could be found in one of the respective clusters, showing that faster growing cells cover their increased demand in novel cell material by boosting their translational capacity. Similar findings have been described for *S. cerevisiae*

Table 1. Overview of growth and product formation parameters at the different dilution rates studied

D (h^{-1})	YDM (g L^{-1})	Product (mg L^{-1})	Y_{XS} (g g^{-1})	Y_{PS} (mg g^{-1})	Y_{PX} (mg g^{-1})	Viability (%)
0.015	18.25 ± 0.35	9.69 ± 1.05	0.37 ± 0.007	0.19 ± 0.021	0.53 ± 0.048	94.1
0.025	20.43 ± 0.27	8.44 ± 1.39	0.41 ± 0.005	0.57 ± 0.028	1.39 ± 0.074	95.9
0.05	24.27 ± 0.55	26.70 ± 2.07	0.49 ± 0.011	0.53 ± 0.041	1.10 ± 0.076	99.5
0.075	25.74 ± 0.47	21.57 ± 2.44	0.51 ± 0.009	0.43 ± 0.049	0.84 ± 0.081	n.a
0.1	26.56 ± 0.98	20.65 ± 1.64	0.53 ± 0.020	0.41 ± 0.033	0.78 ± 0.091	99.0
0.125	26.29 ± 0.27	23.25 ± 1.97	0.53 ± 0.005	0.46 ± 0.039	0.88 ± 0.065	98.8
0.15	25.95 ± 0.52	30.04 ± 1.77	0.52 ± 0.010	0.60 ± 0.035	1.16 ± 0.075	98.7

Values represent the means of three cultivations ± standard error of the mean. YDM, yeast dry mass; Y_{XS}, biomass per substrate yield; Y_{PS}, product per substrate yield; Y_{PX}, product per biomass yield.

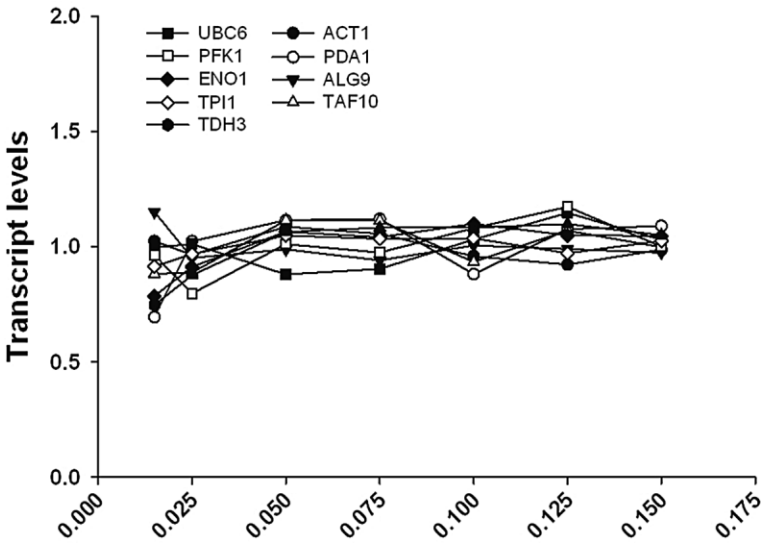


Figure 2. Expression levels of house-keeping genes in *P. pastoris* grown at different specific growth rates in glucose-limited chemostat cultures. House-keeping genes were selected according to ref. [10] and supplemented with genes encoding glycolytic enzymes. Gene expression values were normalized by dividing by the respective average expression values of a given gene. For every growth rate setpoint samples were collected from three individual cultivations and microarray experiments were performed in 2-color technical duplicates for every sample. *UBC6*, ubiquitin conjugating enzyme; *PFK1*, subunit of phosphofrucokinase; *ENO1*, enolase I; *TPI1*, triose phosphate isomerase; *TDH3*, glyceraldehyde-3-phosphate dehydrogenase; *ACT1*, actin; *PDA1*, subunit of pyruvate dehydrogenase; *ALG9*, mannosyltransferase; *TAF10*, subunit of *TFIID* and SAGA complexes.

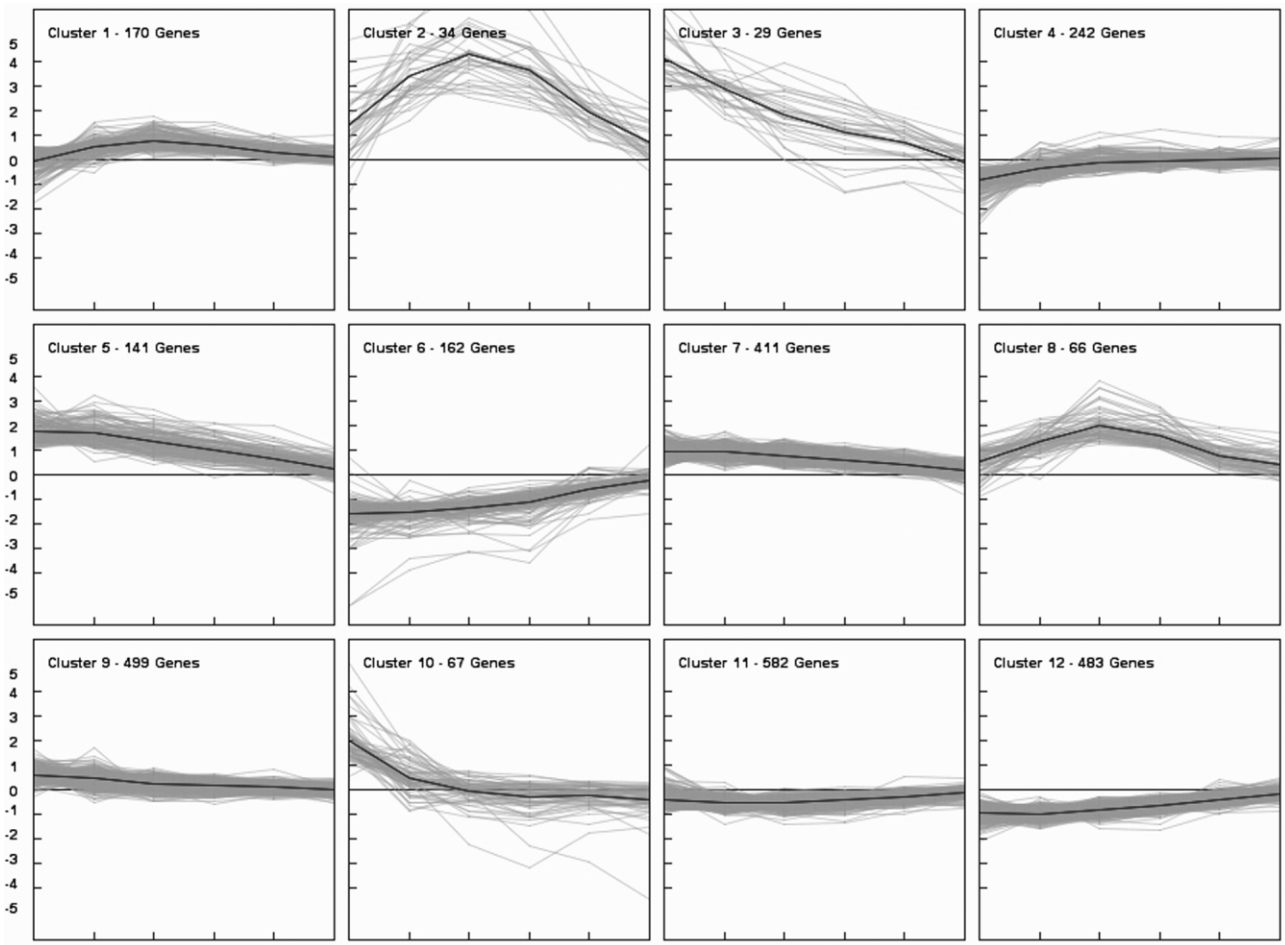


Figure 3. Global gene expression profiles in *P. pastoris* grown at different specific growth rates in glucose-limited chemostat cultures. Genes that were differentially expressed when compared to the highest μ of 0.15 h^{-1} at least at one growth rate setpoint (adjusted *p*-value < 0.02, see Section 2) were grouped into 12 clusters using the *k*-means clustering algorithm. Relative expression levels (log2 scale, *y*-axis) are displayed for each gene at the different growth rate setpoints (*x*-axis) as well as the mean FC (black line). For every growth rate setpoint samples were collected from three individual cultivations and microarray experiments were performed in 2-color technical duplicates for every sample.

Table 2. Enriched GO terms for the category "biological process" of clusters including all regulated genes

Cluster number	Number of genes without a homolog in *S. cerevisiae* among total number of genes within cluster	GO term	Corrected *p*-value
1	69 of 170	Carbohydrate metabolic process	2.67E-02
2	19 of 34	Cellular process involved in reproduction	2.35E-08
		Response to pheromone	1.67E-07
		Reproduction	2.99E-07
		Multi-organism cellular process	4.68E-07
		Multi-organism process	1.02E-06
		Cytogamy	1.42E-06
		Cell surface receptor signaling pathway	3.86E-03
		Cellular developmental process	2.38E-02
3	22 of 29	Unknown	6.75E-04
4	93 of 242	–	–
5	59 of 141	–	–
6	35 of 162	rRNA metabolic process	9.51E-19
		Ribonucleoprotein complex biogenesis	1.18E-17
		ncRNA metabolic process	1.61E-13
		Gene expression	4.10E-11
		Cytoplasmic translation	8.08E-10
		Translation	3.92E-08
		RNA phosphodiester bond hydrolysis	6.36E-06
		Cellular biosynthetic process	1.65E-05
		Oganic substance metabolic process	2.74E-05
		Cellular metabolic process	5.22E-05
		Primary metabolic process	5.41E-05
		Metabolic process	2.39E-04
		Macromolecule metabolic process	1.56E-03
		Nitrogen compound metabolic process	5.23E-03
		RNA 5′-end processing	1.00E-02
		Cellular amino acid biosynthetic process	2.31E-02
		Biosynthetic process	2.63E-02
		Methylation	2.65E-02
		Cellular process	3.27E-02
7	149 of 411	Cell communication	1.96E-07
		Response to stimulus	4.85E-05
		Signaling	6.25E-04
		Peroxisome organization	4.84E-03
		Response to chemical stimulus	5.05E-03
		Mitochondrion degradation	1.28E-02
		Catabolic process	1.33E-02
8	39 of 66	Unknown	2.41E-02
9	159 of 499	Response to stimulus	4.69E-02
10	42 of 67	Unknown	1.73E-03
		Amino acid transmembrane transport	9.52E-03
		Glutamine family amino acid catabolic process	3.93E-02
11	153 of 582	Gene expression	5.85E-06
		Mitochondrion organization	1.30E-05
		Biosynthetic process	1.84E-05
		Cellular metabolic process	1.99E-05
		Organic substance biosynthetic process	2.42E-05
		Cellular process	2.54E-05
		tRNA metabolic process	5.38E-05
		Metabolic process	2.27E-04
		Translation	2.36E-04

Table 2. Enriched GO terms for the category "biological process" of clusters including all regulated genes (continued)

Cluster number	Number of genes without a homolog in *S. cerevisiae* among total number of genes within cluster	GO term	Corrected *p*-value
11	153 of 582	Organic substance metabolic process	6.44E-04
		Amino acid activation	5.65E-03
		Macromolecule metabolic process	6.45E-03
		Primary metabolic process	3.56E-02
12	83 of 483	Ribosome biogenesis	3.01E-40
		ncRNA metabolic process	2.59E-36
		Gene expression	2.89E-35
		Cellular metabolic process	8.87E-23
		Primary metabolic process	2.96E-22
		Organic substance metabolic process	1.61E-21
		Metabolic process	4.99E-20
		Macromolecule metabolic process	5.89E-18
		Cytoplasmic translation	5.63E-16
		Cellular process	9.97E-16
		Cellular biosynthetic process	1.30E-14
		Biosynthetic process	2.24E-13
		Nitrogen compound metabolic process	3.04E-13
		RNA phosphodiester bond hydrolysis	1.84E-10
		Alpha-amino acid biosynthetic process	4.40E-10
		Cellular aromatic compound metabolic process	6.14E-10
		Cellular component organization or biogenesis	2.12E-09
		Cellular nitrogen compound metabolic process	2.68E-09
		Heterocycle metabolic process	3.35E-09
		Mitochondrial translation	3.55E-09
		RNA 5′-end processing	5.88E-09
		Organic cyclic compound metabolic process	8.86E-09
		rRNA 5′-end processing	3.96E-08
		Protein metabolic process	9.16E-06
		Nucleic acid phosphodiester bond hydrolysis	2.84E-04
		Regulation of translation	8.81E-04
		Single-organism biosynthetic process	9.94E-04
		Nuclear export	2.18E-02
		Methylation	3.12E-02

Redundant GO terms were excluded using the web-based tool REVIGO [51].

[5–7] and other organisms like *Escherichia coli* [22], so that upregulation of translation can be regarded as a conserved process in response to growth. Using the common TF algorithm of Genomatix MatInspector software [23], Rap1 binding sites were identified in the promoters of 68 out of 77 annotated ribosomal protein (RP) genes, additionally all RP genes contain binding sites for the ribosomal RNA processing element (RRPE)-binding protein Stb3 in their promoter regions.

For clusters 11 and 12 a large fraction of genes was related to mitochondrial functions, including not only "mitochondrial translation" but also several members of the TOM and TIM complexes as well as genes important for cytochrome c oxidase activity. Upregulation of mitochondrial genes with increasing carbon-limited growth rate has been described for *S. cerevisiae* as well [5, 24]. These results likely reflect increased energy requirements and therefore elevated respiratory activity at faster growth.

In cluster 6, the genes with the highest downregulation at low growth rate can be found: Flo5-2, a lectin-like cell wall protein involved in flocculation and the hypothetical gene PAS_chr2-1_0002.

Genes that show lower expression levels at higher growth rates can be found in clusters 3, 5, 7 and 9, albeit with different degrees of regulation (Fig. 3), and will thus be discussed together. In particular a large number of transcription factors (TFs) and transcriptional regulators is present in cluster 9 (42 TFs; corresponding to more than 8% of total genes), and to a lesser extend also in clusters 5

and 7 (9 and 27 TFs, respectively, corresponding to approximately 6.5% of total genes present in the clusters). Most of these transcriptional regulators are involved in stress response, response to nutrient levels and cell organization based on their function in *S. cerevisiae*. Out of 22 TFs without homologs in *S. cerevisiae*, only one TF has been assigned a function (Mpp1 is an activator of peroxisome biogenesis and function in *Hansenula polmorpha* [25]). The regulatory targets of the other TFs are unidentified yet.

Important enriched GO terms for all genes negatively correlated to increasing growth rate (cluster 3, 5, 7, and 9 together) are "cellular catabolic processes," "response to external stimulus," "response to nutrient levels," "intracellular signal transduction" and "biological regulation." Many genes with a role in cellular catabolic processes are involved in autophagy as well as transport to the peroxisome and mitochondrial degradation, which have been reported to be under control of target of rapamycin (TOR) signaling [26] and are similarly regulated in response to growth rate in *S. cerevisiae* [6, 7]. Additionally, proteolysis related processes such as transport to the vacuole, ER-associated degradation (ERAD), proteasomal degradation, and a high number of genes involved in ubiquitination are among the downregulated genes. However, the regulated gene set is distinct from the one observed in *S. cerevisiae* by Fazio et al. [8]. Some of the genes involved in ubiquitination are also responsible for cell cycle regulation, in particular G1/S transition of the mitotic cell cycle and the anaphase promoting complex (APC). No clear correlation of subunits of the proteasome could be determined, although the TF that stimulates expression of proteasome genes, Rpn4, is part of cluster 7 (see also discussion below). Many of the transcriptional regulators are involved in transcriptional control of carbon-source responsive genes. For example Aca1, Azf1, Cat8-2, Hap5, Mig1-1, and associated regulatory proteins such as Grr1 and Reg1 play a role in glucose repression and induction. A homolog of Adr1 clusters in this group which activates transcription of several glucose-repressed genes including peroxisomal proteins and genes involved in methanol utilization [27]. Furthermore Gal4/Lac9 is found here which probably regulates a different set of target genes, as none of the other GAL genes (*GAL1-3*, *GAL7*, and *GAL80*) is present in *P. pastoris*.

Interestingly, although we monitored gene expression changes in relation to growth rate in carbon-limited conditions, also nitrogen-responsive regulators and their corresponding transcription targets are downregulated with increasing growth rate (see Supporting information, Data S1). As this behavior was not observed in the studies employing *S. cerevisiae* upon carbon limitation [6, 24], differences in the regulation of nutrient responsive regulons between the two yeasts can be concluded.

In this respect, TOR complex subunits are among the growth rate regulated genes in our study, contrary to the data presented for *S. cerevisiae* by Castrillo et al. [6]. It should be noted that *P. pastoris* (similar to most other fungi and higher eukaryotes) has just one TOR gene named *TOR2* (compared to the two highly similar TOR genes *TOR1* and *TOR2* in *S. cerevisiae*) [26], which probably acts in both TOR complexes. While TORC1 which consists of Tor1/2, Kog1, Lst8, and Tco89 is a key regulator of cell growth in response to nutrient availabilty, TORC2 is built of Tor2, Avo1, Avo2, Tsc11, Lst8, Bit61, and Slm1/2 and responsible for regulation of cell wall integrity, actin cytoskeleton polarization and receptor endocytosis [28]. Along with *TOR2, KOG1*, *TSC11*, and *AVO2* are downregulated with increasing growth rate. No homologs of Bit61 and Slm2 can be found in the *P. pastoris* genome.

While TOR signaling usually exhibits its regulatory function by affecting the cellular localization of transcription regulatory proteins, we also see significant transcriptional control of TOR responsive TFs in *P. pastoris*. A high degree of transcriptional regulation in *P. pastoris* as compared to *S. cerevisiae* has been described previously for regulation of glycolytic genes and seems to be related to the high affinity glucose uptake system of the Crabtree negative yeast [29].

We see transcriptional induction of glyoxlate shunt enzymes at low growth rate (corresponding to lower glucose concentration), similar to the observations by Regenberg et al. [7] in *S. cerevisiae*, although the growth rates employed in our study are significantly lower and respiratory growth is maintained throughout all growth rates in *P. pastoris*. Although it might be assumed that this is due to the lower glucose concentrations at lower growth rate, we do not observe glucose repression of *ICL1* and *MLS1* when analyzing promoter activity (our unpublished data). As also described for *S. cerevisiae* in Gutteridge et al. [24], genes responsible for the degradation of the storage carbohydrates glycogen and trehalose have lower expression levels at increasing growth rate, while glycogen and trehalose biosynthetic genes do not show a growth dependent regulation pattern (see Supporting information, Data S1). Regarding sugar transporters [30], the gene encoding the single plasma membrane glucose sensor *SNF2* is downregulated with increasing growth rate, along with transporters of alternative carbon sources such as glycerol and maltose. Only one of the two *P. pastoris* high affinity glucose transporters (PAS_chr3_0023) is upregulated at low growth rates, while expression of the second one (*GHT1*) seems not to be affected by growth rate. *P. pastoris* low affinity glucose transporters and hexokinase *HXK1* are downregulated at low specific growth rate, which leads to the induction of the alcohol oxidase genes *AOX1* and *AOX2* as reported by Zhang et al. [31]. None of the other genes involved in the methanol utilization pathway are found among the growth regulated genes in our study.

TOR signaling also influences the expression of nitrogen responsive genes through the GATA transcription

activators Gat1 and Gln3 in reponse to different nitrogen sources [32], however, the correlation to growth rate was not reported previously. Although upstream activators of TORC signaling are largely unknown, glutamate and glutamine have been established to be important indicators of nutrient status [33]. In this respect, glutamate is one of the highest scoring reporter metabolites (see Supporting information, Table S3) at low growth rates in our study which indicates high transcriptional changes of metabolic genes around this metabolite.

TORC1 signaling also couples the highly energy consuming step of ribosome biogenesis with nutrient availability by controlling the transcription of RP genes and other components of the translation machinery through the localization of RP specific TFs [34]. Under good nutrient conditions, TOR inhibits catabolic processes such as autophagy [26].

Other TFs with expression negatively correlated to growth rate are involved in stress response (e.g. general stress response regulator Msn4; Aft2, and Cth1 involved in iron homeostasis, oxidative stress response TF Yap1, zinc-responsive TF Zap1, stress regulatory proteins Sko1 and Skn7; Rfx1 regulating genes in reponse to DNA damage) or regulation of cell wall organization in response to various stimuli (e.g. regulators of cell wall integrity pathway Rlm1 and Ssd1; Rim101 and Nrg1 involved in pH response and cell wall reorganization; Mit1, Hms1, and Flo8 conferring the distinction between pseudohyphal and vegetative growth and the expression of cell surface flocculins), some of which have been shown to physically interact with the TOR complexes. It should be highlighted that many of the other genes present in clusters 3, 5, 7, and 9 are indeed under transcriptional control of the above-mentioned transcriptional regulators (see Supporting information, Data S1).

Additionally, the MCM2-7 complex that binds chromosomal replication origins and assembles as part of the prereplicative complex during the G1 phase of the cell cycle is enriched in the clusters 7 and 9.

Genes with a bell shaped expression pattern peaking at an intermediate growth rate of 0.05 h^{-1} were grouped into the clusters 2 and 8. GO analysis for the category BP showed that a high proportion of genes from cluster 2 corresponds to mating related terms like "cellular process involved in reproduction" and "response to pheromone" (among them genes encoding the pheromone receptors Ste2 and Ste3, the G-protein α subunit Gpa1 and the scaffold protein Far1). In case of cluster 8 only genes with unknown function were significantly enriched. However, several further key elements of the mating and fusion pathway (*STE4*, *STE12*, and *FUS3-1*) as well as other mating related genes were located in this cluster.

A common strategy of yeasts to survive unfavorable growth conditions is the formation of spores. While natural isolates of *S. cerevisiae* usually occur in a diploid or polyploid state [35], *P. pastoris* is most stable in its vegetative haploid form and needs to mate in order to allow sporulation [36]. Contrary to *S. cerevisiae*, which mates spontaneously in rich media, *P. pastoris* (like *Schizosaccharomyces pombe* and *Kluyveromyces lactis*) enters its sexual life cycle only under certain conditions such as nitrogen starvation [37]. It is therefore not surprising that the mating pathway is induced in this yeast when nutrients become more limiting. The drop in expression levels below specific growth rates of 0.05 h^{-1} may reflect a change in the survival strategy of *P. pastoris*, as the risk of being unable to complete the sexual life cycle may become too high at scarce nutrient supply.

For *K. lactis* it has been shown that the TF RME1 plays a important role in the regulation of mating genes in response to nutrional signals [38] and for fission yeast the TF Ste11 (not to be confused with its *S. cerevisae* homonym) has been described as a key element in the activation of mating in response to starvation (reviewed in [37]). Expression of *RME1* in *P. pastoris* shows a comparable pattern to the genes found in clusters 2 and 8 but is less strongly regulated (cluster 1). However, no putative RME1 binding motif was identified when the promotor regions of 22 genes connected to mating were analyzed (data not shown) and there seems to be no homolog to *S. pombe* Ste11 in the *P. pastoris* genome, leaving the question unanswered how *P. pastoris* concerts nutritional and mating signaling.

Cluster 1 is comprised of genes which are upregulated at intermediate growth rates between 0.025 and 0.075 h^{-1} (but to a lesser extent than clusters 2 and 8) and partially downregulated at the lowest μ of 0.015 h^{-1}. Genes significantly enriched in this cluster are related to "carbohydrate metabolic processes," playing a role in storage carbohydrate synthesis, cell wall remodeling, lipid metabolism, and central carbon metabolism, among them *TDH3*, the native gene controlled by the GAP promoter. For cluster 4 no significantly enriched GO terms were identified, however the HSA gene fell into this almost unregulated cluster. Both *TDH3* and HSA were unregulated except for decreased transcription at the lowest growth rate.

Cluster 10 consists of genes which are upregulated at very slow growth and are discussed below in Section 3.5.

During our study, we could also observe that at growth rates below 0.1 h^{-1}, a considerable fraction of cells changed their morphological appearance. At a growth rate of 0.015 h^{-1} most of the cells had an elongated shape and formed ocassionally branched pseudohyphae (Supporting information, Fig. S1). Interestingly, if the growth rate was increased again above 0.075 h^{-1}, some cells remained in the elongated state. In *S. cerevisiae*, expression of Flo11, a GPI-anchored cell surface glycoprotein (flocculin) required for filamentous growth, is subjected to epigenetic regulation and the epigenetic state of *FLO11* is stable over several generations [39, 40]. It is therefore possible, that also in *P. pastoris* the transition between the

yeast and the filamentous form is under epigenetic control.

Filamentous growth in yeast is triggered by different extracellular stimuli, nutrient limitation being the most common. In *S. cerevisae* at least four signaling cascades have been decribed which regulate filamentous growth: the mitogen activated protein kinase (MAPK) pathway, the rat sarcoma/protein kinase A (RAS/PKA) pathway, the TOR pathway, and the sucrose nonfermentable (SNF) pathway (extensively reviewed by [41]). However, how these different pathways exactly work together in order to adminstrate filamentous growth is a subject of current research. Homologs of most genes taking part in regulation of filamentous growth were identified in *P. pastoris*. Although their mode of action is mainly based on kinase activity, we observed growth rate dependent transcriptional regulation of many of the respective genes (Supporting information, Data S1), showing mostly stronger expression at more limiting conditions.

3.3 Core set of growth regulated genes

By comparing their data with previous studies Fazio et al. [8] have identified a core set of growth regulated genes of *S. cerevisiae*. The 21 common upregulated genes include 11 involved in translation (mainly ribosomal) and 3 related to sphingolipid synthesis. Among the 10 common downregulated genes 4 are involved in regulation of fructose 1,6-bisphosphatase, the key regulatory enzyme of gluconeogenesis. Of these 31 core growth regulated genes 26 have homologs in *P. pastoris*, and of these 21 (81%) were regulated in the same manner (Supporting information, Tables S1 and S2). It is not surprising that ribosomal genes are a majority among the common upregulated genes. Another common regulatory pattern seems to be directed towards upregulation of gluconeogenesis at faster growth. In *S. cerevisiae*, Pfk26 (6-phosphofructo-2-kinase) leads to inhibition of gluconeogenesis, so that its downregulation with increasing growth will enable an upregulation of gluconeogenesis. Interestingly *P. pastoris* has two homologs of *PFK26* which are reversely regulated. The major form, *PFK26-1* is downregulated with increasing growth rate as in *S. cerevisae*. The core growth regulated genes include a second regulatory pathway of gluconeogenesis, involving the glucose induced degradation (GID) complex which is responsible for 1,6-fructose bisphosphatase (Fbp1) degradation. Thus downregulation of VID genes as parts of the GID complex will result in higher Fbp1 activity which will thus trigger more gluconeogenesis at faster growth. We observe the same pattern of gluconeogenesis upregulation in both yeasts, with less representation of VID gene regulation in *P. pastoris*, but a more complex *PFK26* regulation.

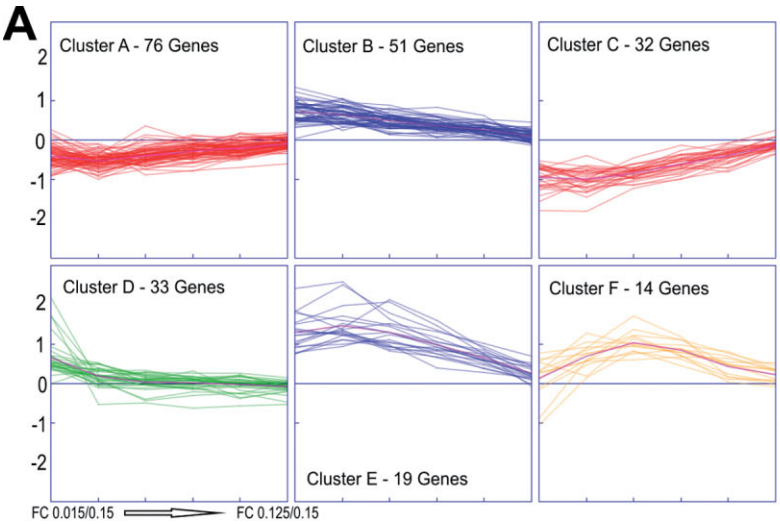
This large overlap of conserved growth regulated genes across the species border is certainly surprising, considering the evolutionary distance between these two yeasts and their different physiology [42]. We can thus postulate that these genes represent something like an evolutionary conserved core set of genes directly regulated together with growth rate.

3.4 Protein folding and secretion

Apart from the observed upregulation of genes associated to biosynthesis, translation, and ribosomes in particular, it is of major importance to understand how processes related to protein folding and secretion are regulated with growth. We have therefore analyzed a subset of genes related to these processes in more detail. Recently, we have investigated the genomic setup of eight yeast species (including *P. pastoris*) concerning the secretory pathway [43]. Genes were identified by reciprocal BLAST search of *S. cerevisiae* genes annotated to ER, protein folding, glycosylation, ERAD, Golgi, SNAREs, and vesicle-mediated transport, backed up by manual curation. This list was further supplemented with genes for vacuolar proteins, yielding a final list of 542 *P. pastoris* genes, about 10% of the genome. All of these genes excluding the unregulated ones (adjusted p-value < 0.02) were grouped according to their log2 FC transcription values comparing each μ setpoint with the highest setpoint by k-means clustering into six clusters (Supporting information, Data S2). The genes fall essentially into four categories (Fig. 4A): steadily upregulated (cluster A and C), steadily downregulated (cluster B and E), upregulated only at the lowest μ setpoint (cluster D), and genes with a more complex regulation pattern with a maximum at intermediate μ around 0.05 h^{-1} (cluster F). These clusters were then mapped on the folding and secretion pathway to visualize gene regulation (Fig. 4B). Genes related to translocation, glycosylation, and protein folding and the proteasome are mainly upregulated at higher μ, while about 20% of regulated genes related to ERAD and the vacuole are downregulated with increasing μ. Among the downregulated vacuolar genes 10 are related to autophagy. Genes related to vesicular transport show a more complex regulation pattern. Figure 4C indicates tabularly which BPs of folding and secretion are highly regulated.

Among the genes showing upregulation with increasing μ we found many UPR regulated genes of translocation, protein folding, and glycosylation as well as cytosolic chaperones, which have been shown to be induced upon overexpression of the UPR regulator *HAC1* [12]. In *P. pastoris* UPR is triggered by induction of *HAC1* transcription rather than by posttranscriptional splicing of the *HAC1* mRNA [44]. As *HAC1* expression is upregulated with μ in our experiment (cluster 6 in Fig. 2) as are its primary targets, we can conclude that UPR induction with increasing specific growth rate is one major regulatory reaction to increasing cellular proliferation.

Positive correlation of recombinant protein secretion rate may thus be inferred to increasing efficiency of pro-



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Biological process	Total gene number	Regulated genes	up w ith μ	dow n w ith μ	not regulated
Translocation	25	68%	56%	8%	32%
Glycosylation	46	54%	30%	9%	46%
Protein folding	74	50%	39%	7%	50%
ERAD	27	37%	11%	19%	63%
Proteasome	34	24%	18%	3%	76%
Vesicular transport	187	34%	17%	7%	66%
Related to vacuole	145	43%	8%	19%	57%

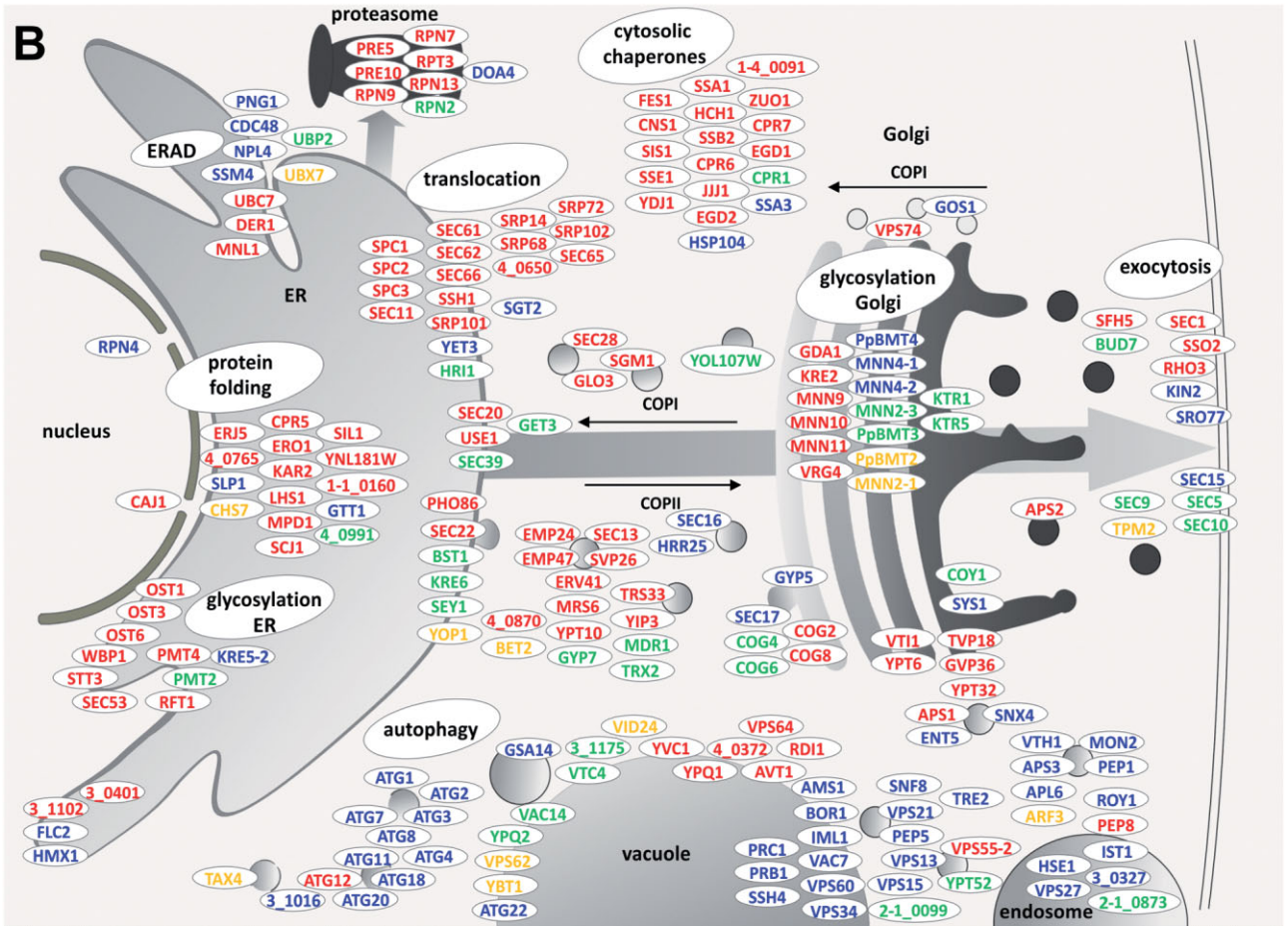

Figure 4. Transcriptional regulation of genes encoding proteins with a role in protein folding and secretion in *P. pastoris* grown at different specific growth rates in glucose-limited chemostat cultures. (**A**) Genes related to the secretory pathway that were at least at one growth rate setpoint differentially expressed (adjusted *p*-value < 0.02, see Section 2) when compared to the highest μ of 0.15 h^{-1} were grouped into six clusters using the *k*-means clustering algorithm. Relative expression levels (log2 scale, *y*-axis) are displayed for each gene at the different growth rate setpoints (*x*-axis) as well as the mean FC (magenta line). For every growth rate setpoint samples were collected from three individual cultivations and microarray experiments were performed in 2-color technical duplicates for every sample. (**B**) Genes of the clusters in (A) were mapped on the secretory pathway, using the color code of the clusters. For genes without a homolog in *S. cerevisiae* or a different yeast, the *P. pastoris* GS115 ORF number was assigned. If *P. pastoris* contained more than one ortholog, a number was added, e.g. MNN4-1, MNN4-2. (**C**) Overview of total numbers of genes allocated to different biological processes related to the secretory pathway, and relative numbers of regulated genes. Color intensities reflect the degree of regulation of the respective groups.

tein translocation to the ER, and to enhanced folding assistance in the ER. The observed upregulation of glycosylation per se is not connected to secretion. At closer sight this relates mainly to early *N*-glycosylation steps in the ER, which have important functions in protein quality control via the calnexin cycle [45]. It should be noted, however, that HSA is not glycosylated so that this folding assistance is not effective for this product.

Intracellular proteolytic degradation has been described to be a considerable sink for recombinant protein in *P. pastoris* [46, 47]. While we observe mainly upregulation of proteasomal genes, more ERAD genes are downregulated than upregulated with increasing μ. Faster growth causes a need for proteasomal activity to turn over cell cycle regulators and other regulatory proteins [48], while ERAD – although connected to proteasomal degradation – is devoted to degrade misfolded secretory proteins, so that these two functions diverge at fast growth. The yeast vacuole is the second main intracellular container for protein disposal. Twenty percent of all genes related to the vacuole were downregulated with increasing growth, indicating that *P. pastoris* reduces the need for disposal (and turnover) of cellular proteins at increasing μ. The downregulation of 9 out of 11 ATG (autophagy related) genes with increasing μ shows clearly a decrease of protein turnover at faster growth, which may come to the benefit of recombinant protein production as well.

Vesicular transport genes are less regulated, which may reflect the fact that only part of the proteins entering the secretory pathway are dedicated to leave the cell. In more detail, intracellular vesicle transport (COPI and COPII vesicles) are rather upregulated than downregulated, while genes related to exocytosis are mainly downregulated. This may constitute a bottleneck for (recombinant) protein secretion at least at higher growth rates. Overexpression of *KIN2* (which is downregulated at faster growth) increased the secretion of a recombinant protein in *P. pastoris*, while overexpression of *SSO2* (upregulated at high growth rate) did not [49], supporting the hypothesis of an exocytosis bottleneck.

3.5 Gene regulation at very slow growth

To get a better understanding of transcriptional regulation in response to very slow growth, we compared pairwise the growth rate setpoints 0.015 and 0.025 h^{-1} (comparison A) as well as 0.025 and 0.05 h^{-1} (comparison B) and identified 732 genes which were at least in one comparison differentially expressed (adjusted p-value < 0.05). The quotient between FC values of regulated genes was calculated in order to discriminate between genes that showed a similar trend in expression between comparison A and B and those with a divergent expression profile. Genes with a quotient between 1.5 and 0.67 were excluded and the remaining 494 genes were ranked in four different groups (Supporting information, Data S3): Group 1 contained 226 genes which were downregulated in comparison A and less downregulated, not regulated or upregulated in comparison B. Group 2 was composed of 238 genes which showed the opposite expressional trend. Group 3 (11 genes) was comprised of genes which were stronger downregulated in comparison B than in comparison A and group 4 of 19 genes with stronger upregulation in comparison B than in comparison A. The gene sets of the groups were analyzed for enriched GO terms (Table 3).

In case of group 1, genes enriched for the category BP were either related to "filamentous growth" or to "sexual reproduction" and neighboring terms emphasizing again that cells refrain from mating and sporulation as a survival strategy when conditions become very scarce. The most significantly enriched term in the category MF was "nucleic acid binding TF activity." While some genes related to this term (*HMLALPHA*, *KAR4*, and *RME1*) are involved in the sexual life cycle, others play a role in the regulation of filamentous growth (*HMS1*, *MGA1*, *NRG1*, and *PDH1*). Group 1 was also enriched for genes with a transmembrane transport activity being around half of the genes involved in amino acid and polyamine transport.

For group 2, three terms for the category BP were enriched – "arginine metabolic process," "arginine biosynthetic process," and "single-organism metabolic process." In *S. cerevisiae* transcription of genes related to arginine biosynthesis is regulated by the repressor Arg81 [50], and its *P. pastoris* homolog is downregulated with increasing growth rate. At μ = 0.015 h^{-1}, however, a subset of arginine synthesis genes is upregulated compared to the next higher setpoints, and this does not coincide with *ARG81* regulation. A closer look at the pathway revealed that specifically all genes involved in the urea cycle (*CPA1*, *ARG3*, *ARG1*, *ARG4*, and *CAR1*) are upregulated at the lowest growth rate, contrary to the majority of the upstream synthesis process from glutamate to ornithine (*ARG2*, *ARG7*, and *ARG8*). This regulation pattern is also reflected in the reporter metabolites (Supporting information, Table S3). For the comparison 0.015–0.025 h^{-1} metabolic genes which convert all the intermediates of the urea cycle (ornithine, citrulline, argininosuccinate, fumarate, and arginine) are subjected to regulation, contrary to the comparison of the next two setpoints, 0.025–0.05 h^{-1}. We conclude that arginine synthesis follows an Arg81 driven upregulation with increasing growth rate, while at very slow growth another regulatory pathway induces the urea cycle. In mammalian cells the urea cycle serves the disposal of surplus ammonium into urea, but its activity has not been discussed in yeasts. Under normal growth conditions it appears not reasonable for a single cellular organism to spend energy on the removal of a nitrogen source. It will be interesting in future to investigate whether very slow growth leads to a surplus of ammonium which may be caused, e.g. by amino acid degradation.

Table 3. Enriched GO terms for the categories biological process (BP), molecular function (MF), and cellular component (CC) of genes regulated at very low μ

Group	Number of genes without a homolog in *S. cerevisiae* among total number of genes within cluster	GO term	Corrected *p*-value
1	79 of 224	BP sexual reproduction	3.14E-04
		BP multi-organism cellular process	1.75E-02
		BP cellular process involved in reproduction	1.85E-02
		BP filamentous growth of a population of unicellular organisms	1.86E-02
		BP filamentous growth	2.82E-02
		BP multi-organism process	4.41E-02
		BP reproductive process	4.57E-02
		MF nucleic acid binding transcription factor activity	4.67E-03
		MF sequence-specific DNA binding transcription factor activity	4.67E-03
		MF amino acid transmembrane transporter activity	1.78E-02
		MF cation transmembrane transporter activity	1.82E-02
		MF ion transmembrane transporter activity	2.65E-02
		MF substrate-specific transmembrane transporter activity	3.95E-02
		CC plasma membrane	1.03E-05
		CC cell periphery	2.33E-04
		CC plasma membrane part	2.32E-02
2	92 of 239	BP arginine metabolic process	2.18E-02
		BP arginine biosynthetic process	3.43E-02
		BP single-organism metabolic process	4.92E-02
		CC plasma membrane	3.56E-04
		CC cell periphery	5.23E-03
3	6 of 11	MF hydrolase activity, hydrolyzing *O*-glycosyl compounds	9.07E-03
		MF hydrolase activity, acting on glycosyl bonds	1.32E-02
		CC intrinsic to plasma membrane	1.58E-02

4 Concluding remarks

We have studied the genome-wide transcriptional regulation of *P. pastoris* from very slow growth to almost μ_{max} with special attention to protein folding and secretion. Upregulation of ribosomal genes and other genes involved in translation appears as a general pattern as observed also in other organisms. Also mitochondrial genes are generally upregulated in *P. pastoris* – even stronger than in *S. cerevisiae*, which is probably due to the fact that *P. pastoris* as a Crabtree negative yeast maintains a fully respiratory metabolism under all studied conditions. Transcriptional upregulation at lower μ pertains a large number of transcriptional regulators, indicating that *P. pastoris* reacts to different growth rates by tuning the expression levels of many TFs, like those regulating stress response as well as carbon source and nitrogen responsive genes. Apparently *P. pastoris* represses mating and sporulation both at very slow and fast growth for different reasons. At fast growth, equivalent to rich nutrient supply, there is no need for sexual reproduction which increases genetic variability and is thus advantageous in less favorable conditions. At very low growth rates, indicative of scarce nutrient supply, the cells seem to avoid the risk not to be able to finish the sexual cycle.

A closer look on the regulation of the secretory pathway reveals that genes related to protein translocation into the ER and to folding in the cytosol and ER are mostly upregulated at higher growth rates, implying that these processes are necessary for higher protein turnover rates at fast growth. Downregulation of vacuolar genes indicates that proteolytic degradation is less prevalent at higher growth rates. Most of the proteins entering the secretory pathway in yeasts fulfill a function inside the cell rather than being secreted outside. This may explain why genes related to vesicular transport and exocytosis are not clearly upregulated at fast growth. We conclude that higher growth rates bring benefits for the production of secreted recombinant proteins, which must be balanced however with process parameters like oxygen and heat transfer, total process time and productivity.

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