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Engineering Methylotrophic Yeasts for Biotechnology Applications

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Abstract

Background: Because some yeast has evolved a methylotrophic lifestyle, they can use the single-carbon molecule methanol as a source of carbon and energy. Pichia pastoris (also known as Komagataella sp.) is one of them and is commonly employed for the generation of heterologous proteins as well as a model organism for organelle research. Our present understanding of the methylotrophic lifestyle is primarily based on extensive biochemical investigations that discovered numerous important methanol utilisation enzymes and their localization to the peroxisomes, including alcohol oxidase and dihydroxyacetone synthase. The pentose phosphate pathway is thought to be involved in C1 assimilation, but the specifics of these events are not yet understood.

Results: In this study, we compared the development of P. pastoris on a medium containing equal amounts of methanol and glycerol and glucose, as well as the regulation patterns of 5,354 genes, 575 proteins, 141 metabolites, and fluxes through 39 processes. We discovered that the whole methanol absorption mechanism is restricted to peroxisomes as opposed to using a portion of the cytosolic pentose phosphate pathway for xylulose-5-phosphate regeneration, as was previously thought. P. pastoris (and perhaps other methylotrophic yeasts) have developed a duplicated set of methanol-inducible enzymes that are specific to peroxisomes for this purpose. Sedoheptulose-1,7-bisphosphate is used as an intermediary in this compartmentalised cyclic C1 assimilation mechanism known as the xylose-monophosphate cycle. The high demand for their respective cofactors, riboflavin, thiamine, nicotinamide, and heme, caused by the strong induction of alcohol oxidase, dihydroxyacetone synthase, formaldehyde and formate dehydrogenase, and catalase, is reflected in the strong up-regulation of the corresponding synthesis pathways on methanol. Because of the high outflow towards methanol metabolic enzymes and their cofactors, methanol-grown cells contain more protein but fewer free amino acids. This illustrates an enhanced flow towards amino acid and protein synthesis and is also reflected in increased amounts of transcripts and/or proteins relevant to ribosome biogenesis and translation when taken in conjunction with up-regulation of several amino acid biosynthesis genes or proteins.

Conclusions: When taken as a whole, our study demonstrates how coordinated analysis of data from different systems biology levels can help reveal as-yet-unknown cellular pathways and completely change how we think about cellular biology.

Introduction

Methylotrophic yeasts can take carbon from a variety of sources. Similar efficiency is achieved when using multicarbon sources like sugars and sugar alcohols like glucose, glycerol, or mannitol as opposed to decreased C1-compounds like methanol. In addition to the cells being properly equipped with the enzymes required for substrate metabolism, their coordinated expression is a requirement for the efficient use of various carbon and energy sources. Several recombinant proteins are created using the methylotrophic yeast Pichia pastoris (syn. Komagataella sp.), and a growing number of biopharmaceuticals and industrial enzymes are also made using this method. Lately, P. pastoris has been used more frequently as a model organism for [1-5] the growth of peroxisomes and secretory organelles. Its development has been primarily driven by the methylotrophic lifestyle, which includes peroxisomes—specialized organelles—as well as strong, tightly controlled promoters for the production of recombinant genes. Peroxisomes are described as intracellular organelles that house the enzyme catalase, which breaks down hydrogen peroxide (H2O2) and produces its own. Moreover, these organelles house the P. pastoris fatty acid beta-oxidation pathway. Alcohols, fatty acids, D-amino acids, and primary amines are just a few examples of the atypical carbon and nitrogen sources that yeast peroxisomal oxidases are primarily involved in the metabolism of. In methylotrophic yeasts, peroxisomes are relatively abundant in methanol-grown cells but drastically drop in both number and volume upon catabolite repression. Peroxisomes include the first steps of the methanol utilisation pathway. Hansenula polymorpha, a different methylotrophic yeast, grows on glucose but only contains one tiny peroxisome, which can be used as a source for fission-based cell division when induction is initiated by switching the cells to methanol. The expression of genes associated to methanol utilisation is highly stimulated by methanol, in addition to genes encoding structural peroxisomal proteins. Alcohol oxidase (AOX) converts methanol to formaldehyde in the first phase of methanol assimilation, while dihydroxyacetone synthase, a unique transketolase, forms a C-C bond with the C1 molecule formaldehyde. These two enzymes' reactions and their distribution in peroxisomes have been extensively studied. Pentose phosphate reactions are thought to be a part of the subsequent cycle of the absorption of methanol, but the specifics are not yet fully understood. While numerous research have examined the cellular responses of P. pastoris to methanol induction in the context of the generation of recombinant proteins, it is mostly unknown how non-recombinant strains would react to the various carbon sources. As a result, we made the decision to look at how P. pastoris cells that aren't

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making recombinant proteins react to the two most common cultivation substrates, methanol and glucose, respectively. The methanol cultures were co-fed with glycerol to enable the same chemostat-controlled constant specific growth rates for direct comparison. A number of transcriptome regulatory studies of P. pastoris, examining the effects of growth rate, unfolded protein response (UPR) activation, oxygen availability, osmotic stress, or heterologous protein production, were possible due to the availability of whole genome sequences. While numerous research have examined the cellular responses of P. pastoris to methanol induction in the context of the generation of recombinant proteins, it is mostly unknown how non-recombinant strains would react to the various carbon sources. As a result, we made the decision to look at how P. pastoris cells that aren't making recombinant proteins react to the two most common cultivation substrates, methanol and glucose, respectively. The methanol cultures were co-fed with glycerol to enable the same chemostat-controlled constant specific growth rates for direct comparison. A number [5-10] of transcriptome regulatory studies of P. pastoris, examining the effects of growth rate, unfolded protein response (UPR) activation, oxygen availability, osmotic stress, or heterologous protein production, were possible due to the availability of whole genome sequences. Further information about the traits of P. pastoris cultivated at various temperatures, osmolarity, UPR induction, and oxygen supply was revealed by analyses of the host proteome. More recently, using 2D-DIGE and subsequent mass spectrometry identification of differentially abundant proteins, P. pastoris strains expressing an insulin precursor were examined for alterations in the cellular proteome as adaptive response to methanol induction during fed batch growth. Enzymes involved in the induction of the UPR and dissimilatory methanol metabolism were found in high abundance. Changes in metabolic fluxes brought on by the regulation of cellular enzyme concentrations will eventually modify the concentrations of free metabolites. Understanding metabolic networks requires quantitative measurement of intracellular fluxes. Methanol (co-)assimilation is connected to growth rate and has ramifications for the pentose phosphate pathway, according to the first genome-scale metabolic network models of P. pastoris and flux distributions of central carbon metabolism. The current study uses steady-state cultures of non-producing P. pastoris with a uniform specific growth rate and the carbon supply as the examined variable for transcriptomics, proteomics, metabolomics, and fluxomics investigations. This integrated systems level study made it possible to identify cellular functions like vitamin biosynthesis and amino acid metabolism that are co-regulated with methanol metabolism. These co-regulation patterns also served as the basis for deciphering the as-yet-unknown processes of sugar phosphate rearrangements recycling xylulose-5-phosphate for methanol fixation. Here, we offer a novel theory that describes the assimilation of methanol as a distinct, highly controlled mechanism that results from the duplication of the relevant genes.

Findings And Analysis

P. pastoris's growth characteristics vary greatly depending on the substrate

P. pastoris CBS7435 was grown in chemostats at a set specific growth rate of 0.1 h-1, or around 60% of the maximum growth rate on glucose. To prevent growth rate-dependent impacts during genome-scale analysis, constant growth is a must. A mixed feed strategy with glycerol-methanol co-feeding was used because the highest specific growth rate on pure methanol as a carbon source would be substantially lower and intracellular carbon fluxes could not be evaluated on methanol alone. Based on studies using P. pastoris, it was determined that a methanol-glycerol mixture of 8.5 g/L methanol and 49.0 g/L glycerol resulted in complete methanol consumption and full stimulation of the methylotrophic pathway. As stated in the Methods section, chemostats were operated in three biological replicates for each condition, and samples for transcriptomics, proteomics, and metabolite studies were collected in steady state after seven residence times. Separate chemostat cultivations using 13C-labeled substrates were carried out for metabolic flow investigation. HPLC was used to confirm that all cultures were substrate limited, meaning that there was no residual glucose or methanol/glycerol, respectively. Table 1 provides a summary of the growth metrics acquired from these cultures. Cells grown on methanol/glycerol had a 13% lower CO2 exchange rate than cells grown on glucose, but a 30% greater rate of oxygen uptake. The higher degree of reduction of methanol and glycerol relative to glucose can be used to explain the higher oxygen uptake rate of P. pastoris cultured in methanol/glycerol. An equimolar amount of oxygen is required just to move methanol into cellular metabolism because the oxidation of methanol to formaldehyde by AOX is an exothermic oxygen-consuming reaction. Cells grown on methanol/glycerol produced somewhat more biomass than cells grown on glucose, which is in good agreement with results from the literature. P. pastoris-specific DNA microarrays were used to study transcriptional regulation, and liquid chromatography-tandem mass spectrometry (LC/MS-MS) was employed for differential proteomics and metabolite quantification. Distribution of particular lipid classes was also examined. Using the 13C labelling patterns of proteinogenic amino acids, flux ratios were estimated.

Methods

Chemostat cultivation and strains

The 400 mL working volume of a 1.4-L bioreactor (DASGIP Parallel Bioreactor System, Germany) was used for the chemostat cultivations. In a nutshell, P. pastoris CBS7435 cryostock was inoculated into 100 mL of pre-culture media (10 g yeast extract, 20 g peptone, and 10 g glycerol per litre) and cultured at 28°C at 150 rpm overnight. The bioreactor was inoculated with this culture at an optical density (OD600) of 1.0. The cells were cultivated in carbon-limited chemostats with a dilution rate of 0.1 h1 for at least seven residence periods after a batch phase of about 24 hours before samples were taken.

Sampling and putting out

For transcriptomics, 4.5 mL of freshly made, pre-chilled (20°C) fixing solution (5% v/v phenol in ethanol abs.) and 9 mL of culture were combined, then 1.5 mL were aliquoted into ribolyzer tubes and centrifuged at 13,000 rpm for 1 min at 4°C. The tubes holding the fixed cell pellets were immediately kept at 80°C after the supernatant was discarded. 2 mL of the culture were centrifuged for protein measurement, and the cell pellet was kept at 80°C. Moreover, the supernatant was kept at 80°C for extracellular metabolite analyses. Using a pump, samples were quickly collected for intracellular metabolite measurement. 200 mL of 60% (v/v) methanol were used to quench 50 mL of fermentation broth at a temperature of 27°C. Using a vacuum pump, 2 mL of quenched cells were filtered with cellulose acetate filters (0.45 m, Satorius Biolab Products), yielding roughly 10 mg of biomass per filter.

Cold 60% (v/v) methanol was used to wash the cells once, and after that, the filter was stored on dry ice. Six samples were obtained from each chemostat culture using two filtering units (Polycarbonat Filter Holders, Satorius Lab Technologies Product).

Protein totals are calculated

Cell pellets from a 2 mL chemostat culture were resuspended in 1 mL of PBS after being washed with 0.9% NaCl (pH 7.0). According to Verduyn, the protein extraction was carried out by adding NaOH and incubating at 95°C. Centrifugation was used to remove cell debris after the addition of 0.8 M HCl and incubation. Bradford analysis was used to determine the total protein level in the supernatant. The yeast dry mass (%) was correlated with the total protein content.

Lipid evaluation

According to, lipid extraction from P. pastoris chemostat samples was carried out. Lipid extracts were loaded onto Silica Gel 60 plates (Macherey-Nagel, Düren, Germany) for the quantitative determination of non-polar lipids (TG, SE), free fatty acids, and free ergosterol. Chromatograms were developed in an ascending fashion using the solvent system light petroleum/diethyl ether/acetic acid (70:30:2; per vol.) for roughly the first third of the distance. Plates were then briefly dried, followed by additional development with the solvent system light petroleum/diethyl ether (49:1; per vol.) until the solvent front reached the top of the plate. Using an ergosterol standard, unesterified sterols and steryl esters were quantified densitometerically with a TLC scanner (Camag TLC Scanner 3) at 275 nm. By immersing the TLC plates into a charring solution (consisting of 0.63 g MnCl24H2O, 60 mL water, 60 mL methanol, and 4 mL concentrated sulfuric acid) and heating them at 100°C for 30 min, other lipids were permanently stained. At a wavelength of 400 nm, densitrometric scanning was carried out, and lipids were measured using triolein, oleic acid, or ergosterol as standards. Separate bands from the non-polar lipid analysis (see above) were used to estimate the overall quantities of glycerophospholipids. Glycerophospholipids were detected on plates by reversible iodine vapour staining, scraped off, and submitted to Broekhyuse technique quantification.

Conclusions

Methylotrophy is a special ability of microbes to survive on C1 molecules and requires effective routes to generate C-C bonds and oxidise C1 compounds via hazardous intermediates. The methylotrophic yeast P. pastoris has unique regulatory and metabolic characteristics, which are thoroughly explained by this systems level analysis. We were able to precisely pinpoint the probable pathway for XYL5P regeneration during methanol ingestion by co-regulating enzymes with AOX and DAS at the mRNA and protein level.

Author Contributions

The diagnosis and treatment of this cat were handled exclusively by Jennifer Weng and Harry Cridge. This report was written by Jennifer Weng, and Harry Cridge gave it a critical appraisal. The final draught of the manuscript has received the approval of both Jennifer Weng and Harry Cridge.

Conflict of Interest

According to the authors, there are no conflicts of interest that might be thought to compromise the objectivity of the research presented.

Ethics Statement

The case described in this report was handled as part of the regular clinical caseload at the university teaching hospital; an IACUC or other ethical approval was not necessary. All facets of this patient's care had the owner's consent.

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