Metabolic Engineering 41 (2017) 46–56

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

# Lipid engineering reveals regulatory roles for membrane fluidity in yeast flocculation and oxygen-limited growth

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ARTICLE INFO

*Keywords:*
Fatty acid unsaturation
Membrane fluidity
Yeast flocculation
Oxygen-limited growth
Hypoxia
Fermentation

ABSTRACT

Cells modulate lipid metabolism in order to maintain membrane homeostasis. Here we use a metabolic engineering approach to manipulate the stoichiometry of fatty acid unsaturation, a regulator of cell membrane fluidity, in *Saccharomyces cerevisiae*. Unexpectedly, reduced lipid unsaturation triggered cell-cell adhesion (flocculation), a phenomenon characteristic of industrial yeast but uncommon in laboratory strains. We find that ER lipid saturation sensors induce expression of *FLO1* – encoding a cell wall polysaccharide binding protein – independently of its canonical regulator. In wild-type cells, Flo1p-dependent flocculation occurs under oxygen-limited growth, which reduces unsaturated lipid synthesis and thus serves as the environmental trigger for flocculation. Transcriptional analysis shows that *FLO1* is one of the most highly induced genes in response to changes in lipid unsaturation, and that the set of membrane fluidity-sensitive genes is globally activated as part of the cell's long-term response to hypoxia during fermentation. Our results show how the lipid homeostasis machinery of budding yeast is adapted to carry out a broad response to an environmental stimulus important in biotechnology.

## 1. Introduction

The lipid composition of cellular compartments is thought to control the physicochemical properties of their membranes and thus could act as a broad regulator of membrane-localized cellular machinery. Lipid composition can vary tremendously between cells, organelles, and tissues, potentially reflecting differing constraints that molecular processes hosted by these membranes place (van Meer et al., 2008). For example, in budding yeast (*Saccharomyces cerevisiae*), global lipid analysis by mass spectrometry has shown that the cellular lipidome changes during growth and is dependent on carbon source (Klose et al., 2012). However, understanding functional roles for differences in lipid composition remains a challenge because a limited set of tools is available for studying lipid composition in vivo. Standard genetic approaches, e.g. gene knockouts, provide little functional information on essential lipid components, while chemical tools, such as media supplements (Bossie and Martin, 1989) and lipid chelators (Zidovetzki and Levitan, 2007), provide limited stoichiometric control over lipid composition.

An alternative strategy to chemical manipulation of lipid composition is to engineer cells in which the expression of lipid synthesis pathways is placed under experimental control. We sought to use this approach to investigate a central chemical parameter in determining the physical state of cell membranes: the proportion of fluidizing double bonds in phospholipid acyl chains (lipid unsaturation). Enzymatic desaturation generates *cis*-double bonds in acyl chains, whose geometry interferes with inter-lipid interactions, thereby reducing lipid packing and fluidizing the bilayer (Spector and Yorek, 1985). Membrane fluidity is thought to be maintained by cells in response to changes in temperature (Hazel, 1995) or solvents (Ingram, 1976) by modulating the fatty acid composition of membrane phospholipids. Changes in fatty acid unsaturation are also associated with human diseases, including the development of type-2 diabetes (Weijers, 2012) and tumor proliferation in cancer (Igal, 2010). However, the functional

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http://dx.doi.org/10.1016/j.ymben.2017.03.002
Received 20 December 2016; Received in revised form 10 February 2017; Accepted 8 March 2017
Available online 18 March 2017

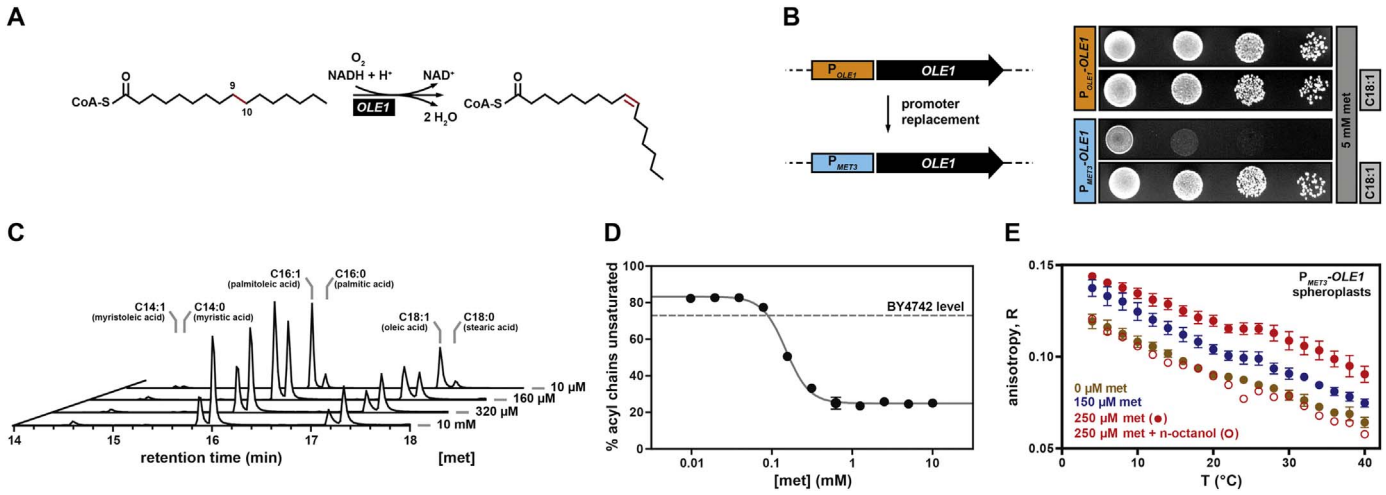


**Fig. 1. Engineering of *OLE1* expression modulates lipid unsaturation and membrane fluidity. (A)** Reaction scheme of oxygen- and NADH-dependent desaturation of fatty acid acyl chains catalyzed by Ole1p. **(B)** Replacement of the endogenous *OLE1* promoter ($P_{OLE1}$) by the methionine repressible *MET3* promoter ($P_{MET3}$) results in a yeast strain ($P_{MET3}$-*OLE1*) whose *OLE1* expression is under experimental control. Growth phenotype of $P_{MET3}$-*OLE1* cells is shown via spotting of a 10-fold dilution series on methionine containing media (5 mM) in the presence and absence of oleic acid. **(C)** GC-MS chromatograms of fatty acid profiles from $P_{MET3}$-*OLE1* cells at representative external methionine concentrations. **(D)** Percentage of total monounsaturated fatty acid species (% acyl chains unsaturated) at a given methionine concentration was determined from GC–MS data. A sigmoid function (y= a - (b - a)/(1+10^((log $C_{50}$−x)*m)) was fitted to data points. **(E)** Spectroscopic measurements of membrane fluidity taken of $P_{MET3}$-*OLE1* spheroplasts prepared from cells that were grown at different external methionine concentrations. Fluidity was measured at varying temperature by steady state fluorescence anisotropy of di-phenyl-hexatriene (DPH), which was added externally. Anisotropy values are presented as a unit-less ratio: R =($I_{||}$−$I_{\perp}$) /($I_{||}$ +2 $I_{\perp}$), where $I_{||}$ and $I_{\perp}$ are the emission (430 nm) intensities parallel and perpendicular, respectively, to the polarization of the excitation (360 nm). Higher anisotropy values reflect restricted mobility of the DPH probe and thus lower whole-cell membrane fluidity. Membrane fluidity was found to depend on the level of lipid unsaturation content, the presence of the membrane fluidizer *n*-octanol (0.02% w/v), as well as on the temperature. Values in the presence of *n*-octanol are shown as an average of two biological replicates. Error bars, SEM (n =3).

effects of these physical and chemical parameters on basic cellular physiology are still not fully understood. Higher eukaryotes feature a repertoire of desaturases with varying substrates and products (Hashimoto et al., 2008), including polyunsaturated species whose complex biophysical effects make identifying fluidity-regulated functions a challenge (Barelli and Antonny, 2016). In contrast, budding yeast features only a single lipid desaturase (Ole1p), which converts a wide range of coenzyme A-bound fatty acids into their corresponding Δ9-*cis* monounsaturated species (Stukey et al., 1989) (Fig. 1A).

Because bilayer fluidity is an important parameter for membrane-associated cellular processes, yeast has evolved mechanisms for modulating the level of *OLE1* expression in response to metabolic stages during growth (Casanovas et al., 2015) and to environmental stimuli, such as the supply of exogenous lipids. In budding yeast, a fatty acid regulatory (FAR) region in the *OLE1* promoter was first identified to be responsive to fatty acid metabolism genes (Choi et al., 1996). Later it was found that a pair of ER membrane-bound transcriptional activators – Spt23p and Mga2p (Hoppe et al., 2000), which were first identified as regulators of *TY* retrotransposon elements (Zhang et al., 1997) – mediate *OLE1*-specific lipid regulation. Biochemical and genetic experiments have led to a model in which membrane-anchored Spt23p and Mga2p are proteolytically processed in response to changes in fatty acid composition (Rape et al., 2001). The resulting active fragments, termed p90 domains, induce *OLE1* expression, though neither protein contains a recognizable DNA binding domain (Zhang et al., 1997; Burkett and Garfinkel, 1994; Dula and Holmes, 2000). Recent work has proposed a model in which proteolysis is driven by conformational changes in the transmembrane helices of Spt23p/Mga2p homodimers mediated by the unsaturated lipid content of the ER membrane (Covino et al., 2016). Several key aspects of this pathway are still under investigation, including the exact mechanism by which Spt23p/Mga2p or their interacting partners influence gene expression.

In this study we sought to systematically investigate the physiological effects of changes to lipid unsaturation by bypassing the cell's native lipid regulation pathway. We observed a surprising phenotype resulting from repression of the lipid desaturase-encoding *OLE1*: cell-cell adhesion, termed flocculation, which we find is a transcriptional response to low membrane fluidity. Flocculation is induced when oxygen availability for lipid desaturation reactions is restricted and is part of a wide-ranging transcriptional response to low membrane fluidity that is activated during microaerobic fermentation.

## 2. Materials and methods

### 2.1. Yeast strains

All strains (Table1) are available from The Joint BioEnergy Institute (JBEI) public Inventory of Composable Elements (ICE): https://public-registry.jbei.org/.

Detailed plasmid and strain construction methods are available in the Supplemental Information.

### 2.2. Growth and flocculation tests

Aerobic growth of *OLE1*-repressible strains was carried out in 24 well plates (Falcon) with 1 mL media at 30 °C using a microplate reader (BioTek Synergy 4; BioTek Instruments) with integrated temperature control and under continuous orbital shaking (slow shaking mode). The plates were sealed with a gas permeable adhesive seal (Thermo Fisher Scientific) to avoid evaporative losses of culture medium. These conditions were chosen to maintain consistent oxygenation of the cultures. Absorbance readings at 600 nm were used to characterize cell growth and detect flocculation via fluctuations in readings. Flocculation was also detected after 24 h of growth via 24 well plate imaging (UVP BioSpectrum Multispectral Imaging System) and confocal microscopy of cells in growth media stained with Calcofluor White at 23 °C. Micrographs were acquired on a LSM 710 (Zeiss) scanning confocal microscope with an oil immersion lens (100x magnification, 1.4 numerical aperture) using ZEN 2009 software (Zeiss).

Oxygen-limited fermentation growth of cells was carried out in a home-built apparatus based on 250 mL culture flasks fitted with rubber stopper. Caps were connected with tubing to a water bath, which allowed gas exflow via bubbling but restricted gas inflow. Flasks also featured syringe-fitted tubing for culture sampling at time points