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Determination of Growth-Phase Dependent Influences Exerted by Prions on Yeast Lipid Content Using HPTLC–Densitometry

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Summary

Prions of the baker's yeast *Saccharomyces cerevisiae* allow for the inheritance of complex traits based solely on the acquisition of cytoplasmic protein aggregates and confer distinctive phenotypes to the cells which harbor them, creating heterogeneity within an otherwise clonal cell population. These phenotypes typically arise from a loss-of-function of the prion-forming protein that is unable to perform its normal cellular function(s) while sequestered in prion amyloid aggregates, but the specific biochemical consequences of prion infection are poorly understood. To begin to address this issue, we initiated a direct investigation into the potential control that yeast prions exert over fungal lipid content by utilizing the prions [*URE3*] and [*PSI⁺*], the first two prions discovered in yeast. We utilized silica gel high-performance thin-layer chromatography (HPTLC)–densitometry to conduct pair-wise quantifications of the relative levels of free sterols, free fatty acids, and triacylglycerols [petroleum ether–diethyl ether–acetic acid (80:20:1) mobile phase, phosphomolybdic acid (PMA) detection reagent]; sterol esters and squalene (hexane–petroleum ether–diethyl ether–acetic acid (50:20:5:1), PMA); and phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol (chloroform–diethyl ether–acetic acid (65:25:4.5), cupric sulfate–phosphoric acid) in otherwise clonal prion-infected ([*PSI⁺*] or [*URE3*]) and prion-free ([*psi⁻*] or [*ure-o*]) cells in two growth phases: log-phase and stationary phase. Our analysis revealed multiple statistically significant differences ($p < 0.00625$) between prion-infected and prion-free cells. Interestingly, prion-induced changes varied dramatically by growth phase, indicating that prions exert differential influences on cell physiology between log and stationary growth. Further experimental replication and extension of the analysis to other prions is expected to resolve additional physiological effects of prion infection. This investigation demonstrates that HPTLC–densitometry is an effective method for studying prion-induced alterations in lipid content in yeast.

Keywords

yeast; prion; lipid; thin-layer chromatography

Introduction

Numerous proteins of the baker's yeast *Saccharomyces cerevisiae* have evolved the ability to form heritable, self-perpetuating amyloid aggregates now known as "yeast prions" [1–3]. Because they confer phenotypes and are heritable, yeast prions allow for the inheritance of complex traits based solely on the acquisition of a cytoplasmic protein aggregate [4]. One central characteristic of all prions is that they are self-templating: once formed, the prion aggregate recruits additional monomeric protein into the growing fiber [5]. Because yeast prions are formed of aggregates of normally functional proteins, the sequestration of the prion-forming protein within these aggregates typically results in a loss-of-function phenotype as the result of prion infection. Interestingly, in many cases, this loss of protein function causes complex pleiotropic effects as a consequence because the prion-forming protein is often a regulator of transcription or translation [4, 6, 7]. One particularly striking example is the prion-forming protein Swi1, which normally functions as part of the canonical *Swi/Snf* chromatin remodeling complex, responsible for regulating the expression of ~6% of all yeast genes [8, 9]. In the presence of the [*SWI⁺*] prion, Swi1 is sequestered in cytosolic aggregates creating deficiencies in *Swi/Snf* function [2, 9, 10]. Thus, the aggregation state of a singular protein can manifest a larger change in gene regulation, resulting in numerous changes in cellular biochemistry.

Given their potential complexity, the specific biochemical consequences of prion infection are difficult to predict de novo; in contrast, direct empirical measurement may be more successful in identifying distinct biochemical changes, but given the recent discovery of most yeast prions, the majority of these potential effects remain unidentified. To begin to address this issue, we initiated a direct investigation into the potential control that two prions, called [*URE3*] and [*PSI⁺*] [1, 11, 12], exert over fungal lipid content. Like [*SWI⁺*], both [*PSI⁺*] and [*URE3*] affect the proteome of yeast cells which harbor them. [*PSI⁺*] causes widespread nonsense suppression (ribosomal read-through of stop codons) whereas [*URE3*] is formed from the Ure2 protein, which regulates the expression of genes involved in nitrogen metabolism [13]. Here, we present the results of a side-by-side analysis of genetically identical pairs of yeast cell populations with or without two prions individually and in two distinct growth states: logarithmic growth (log phase) and stationary phase. We utilized high-performance thin-layer chromatography (HPTLC)–densitometry to quantify the levels of eight different lipids. The approach yielded a number of statistically significant differences between prion-infected and prion-free strains that, intriguingly, depended strongly on yeast growth phase, demonstrating that HPTLC–densitometry is an effective method to study prion-induced alterations in lipid content in yeast.

Experimental

Chemicals

Unless otherwise stated, all chemicals including solvents were reagent grade and purchased from Sigma-Aldrich, St. Louis, MO, USA. Water was deionized (DI) and biological impurities removed by a departmental purification system. Yeast extract–peptone–dextrose–adenine (YPDA) medium was prepared by dissolving 10 g of yeast extract (No. BP9727, Fisher Scientific, Pittsburgh, PA, USA), 20 g of peptone (No. BP1422, Fisher Scientific),

and 20 g of D-glucose (No. AC41095, Acros Organics, part of Fisher Scientific) in 1 L of water. Eighty milligrams of adenine (No. A8626, Sigma-Aldrich) was then added following sterilization of the medium by autoclaving.

Sample Preparation

S. cerevisiae cells of the W303 genetic background were used throughout this investigation [14]. Two pairs of strains, differing only by prion status ($[PSI^+]$ vs. $[psi^-]$ and $[URE3]$ vs. $[ure-o]$) were used, and samples were collected from 7 to 8 separate cultures of each strain. Strain Y1981 ($[rng^-]$ $[PSI^+]^{STR}$ $[ure-o]$ *ade1-14 ADE2 trp1-1 ura3-1 leu2-3,112 his3-11,15 can1-100 GAL2⁺ met2-1 lys2-2*) harbors the strong variant of $[PSI^+]$ termed $[PSI^+]^{STR}$ [15, 16]. Y2172 is genetically identical to Y1981 [17] and was created by culturing Y1981 in the presence of 4mM GdnHCl which cures yeast prions by inhibition of chaper-one protein activity that is essential to prion propagation [18]. Similarly, strain Y2086 ($[RNQ^+]$ $[psi^-]$ $[URE3]$ *ade2-1 trp1-1 URA3 ura2 :kanMX leu2-3,112 his3-11,15 dal5 ::PDAL5-ADE2*) harbors a strong variant of the $[URE3]$ prion, and strain Y2104 is genetically identical, but lacking both $[URE3]$ and $[RNQ^+]$ prions, likewise being created by GdnHCl curing of Y2086. For logarithmic phase cultures, cells were grown continuously in log phase for at least six generations by subculturing in liquid YPDA medium with constant agitation (200 rpm) at 30 °C prior to collection. The optical density (OD) of the cell culture was measured by optical absorbance at 600 nm using a Genesys 20 spectrophotometer (Thermo Scientific, Madison, WI, USA). Log phase cells were collected at OD = 0.8. For stationary phase cultures, cells were grown for at least 18 h without subculture in liquid YPDA medium with constant agitation (200 rpm) at 30 °C and collected when cell densities reached 7.5 ± 0.5 OD. For each independent culture, 120 OD units of cells were collected by centrifugation using a bench-top centrifuge (Model 228, Fisher Scientific) at 3300 revolutions min⁻¹ for 3 min, washed with DI water, and recollected. The wet cell weight of each pellet was recorded as the sample weight. The samples were flash frozen in liquid nitrogen and stored at -20 °C until extraction.

Lipid Extraction

Lipid extraction followed procedures previously described [19]. Cells were lysed by bead-beating. Each sample was separated into four 2 mL centrifuge tubes. Five hundred microliters of methanol and approximately 0.5 g of 0.5 mm silica beads (No. 11079105, Biospec Products, Bartlesville, OK, USA) were added to each tube. The tubes were then disrupted using a cell disruptor (Scientific Industries, Inc., Bohemia, NY, USA) for 30 s followed by 30 s rest periods, repeated for 5 min total. Four tubes of the same sample were then combined in a 250-mL beaker, and 8 mL of methanol and 20 mL of chloroform were added. The mixture was magnetically stirred at room temperature for 1 h. Lysate was filtered using a sintered glass funnel (No. CLS3606060M, Sigma-Aldrich) and washed twice in a 125 mL separatory funnel with Folch solution (0.88% KCl) as previously described [20]. The volume of Folch solution added was approximately 25% of the chloroform-methanol (2:1, v/v) volume. The lipophilic layer was collected and evaporated using a rotary evaporator with a 125-mL round bottom flask. The lipid film was then dissolved in 6 mL of chloroform-methanol (2:1, v/v) and collected in Teflon lined vials (No. 03-339-22D, Fisher

Scientific). The solvent was evaporated just to dryness in a warm water bath (approximately 40 °C) with N₂ gas. Samples were reconstituted in 1.00 mL chloroform–methanol (2:1, v/v).

HPTLC–Densitometry Analysis

Nonpolar Lipid Mix No. 1130 (Matreya Inc., Pleasant Gap, PA, USA) containing a total of 25 mg of cholesterol, oleic acid, triolein, methyl oleate, and cholesteryl oleate in equal amounts (20% each) as standards for the free sterol (FS), free fatty acid (FFA), triacylglycerol (TAG), methyl ester, and steryl ester (SE) fractions, respectively, was used for neutral lipid analysis. The standard solution was prepared at 0.200 µg µL⁻¹ for each lipid by diluting the 1.00 mL standard with chloroform–methanol (2:1, v/v) in a 25-mL volumetric flask. In addition to the mixed standard, a squalene standard (No. 53626, Sigma-Aldrich) was prepared at a concentration of 0.200 µg µL⁻¹ in chloroform–methanol (2:1, v/v). Ergosterol is the major FS and ergosteryl oleate the major SE in yeast [21]; the cholesterol and cholesteryl oleate standards can be used for their quantification, respectively, because the *R_F* values of both sterols and both steryl esters are the same on silica gel, and their molecular weights are essentially equal for the purpose of our analyses (molecular weight ratios both 1.02).

The standard for phospholipid analysis was Polar Lipid Mix No. 117 (Matreya Inc.) containing 25% each of cholesterol, phosphatidylcholine (PC), phosphatidylethanolamine (PE), and lysolecithin in a total of 25 mg in 1.00 mL. The standard solution was prepared at 0.125 µg µL⁻¹ of each component by dissolving the 1.00 mL standard with chloroform–methanol (2:1, v/v) in a 50-mL volumetric flask. In addition to the mixed standard, a phosphatidylinositol (PI) standard (No. 1048, Matreya, Inc.) was prepared at a concentration of 0.125 µg µL⁻¹ in chloroform–methanol (2:1, v/v).

Analyses were performed on 10 × 20 cm HPTLC–HLF silica gel plates (Analtech Inc., Newark, DE, USA) that contained 19 scored lanes and a preadsorbent zone spotting area (Catalog No. 61927). Before use, plates were precleaned by development to the top with dichloromethane–methanol (1:1, v/v), dried with a stream of air in a fume hood, and activated for 30 min on a CAMAG (Wilmington, NC, USA) plate heater at 120 °C. The lanes and preadsorbent zone facilitate reproducible manual application of sample and standard initial zones in order to obtain the preferred band shape upon development, and the lanes facilitate initial line up of densitometer source slit for densitometric scanning.

Standard and reconstituted sample solutions were applied in 2.00, 4.00, 8.00, and 16.0 µL aliquots (0.200 to 3.20 µg for neutral lipids and 0.250 to 2.00 µg for polar lipids) to the preadsorbent zone of separate lanes on the HPTLC plates using a 10-µL Drummond (Broomall, PA, USA) digital microdispenser. A gentle stream of air was directed across the preadsorbent zone during application of the sample to quickly dry the standards and samples as they were applied. Plates were developed to a distance of 8 cm beyond the preadsorbent zone–silica gel interface with 50 mL of the Mangold mobile phase, petroleum ether (boiling range 30–60 °C)–diethyl ether–glacial acetic acid (80:20:1, v/v/v), for determination of FSs, FFAs, and TAGs; the Smith mobile phase, *n*-hexane–petroleum ether (30–60 °C)–diethyl ether–glacial acetic acid (50:20:5:1, v/v/v/v), for SEs and squalene [22]; or the Wagner mobile phase, chloroform–methanol–water (65:25:4.5, v/v/v), for phospholipids. Ascending,

one-dimensional development was carried out in an HPTLC twin-trough chamber (CAMAG) containing a saturation pad (Analtech Inc.). Prior to insertion of the spotted plate for development, the chamber was equilibrated with the mobile phase vapors for 30 min. Development required approximately 10–15 min.

After development, neutral lipid plates were dried with a stream of cool air from a hair dryer in a fume hood for 10 min, sprayed with 5% ethanolic phosphomolybdic acid (PMA) solution, and heated on a CAMAG plate heater at 115 °C for 10 min to detect neutral lipids as blue bands on a yellow background. After development, dried plates containing phospholipids were sprayed with 10% cupric sulfate in 8% phosphoric acid and heated at 140 °C for approximately 30 min to detect brown-black bands on a white background.

Neutral and polar lipids were quantified by slit-scanning densitometry in the absorbance–reflectance mode using a CAMAG TLC Scanner 3 with slit dimensions 4.00×0.45 mm micro, scanning rate of 20 mm s^{-1} , and distance between band centers of 10.2 mm to match the center to center distances between scored lanes. The tungsten light source, set at a wavelength of 610 nm, was used for neutral lipid determination and the deuterium light source, set at 370 nm, was used for the polar lipids. The winCATS software automatically generated polynomial regression calibration curve (standard zone weights versus peak areas) and interpolated sample zone weights based on their peak areas. The percentage by weight of lipid in each wet cell sample was calculated using the equation:

$$\text{Percent lipid} = 100 * (w) * (R) / \text{sample weight in } \mu\text{g}$$

where w = lipid mass (μg) of sample interpolated from the calibration curve and R = reconstitution volume (μL)/spotted volume (μL). If the area of more than one sample aliquot was bracketed within the calibration curve, the weight of the aliquot giving a scan area closest to the average area of the two middle standards was used.

Statistical Analysis

For each lipid fraction, an F -test was conducted using Microsoft Excel to determine whether the variance of data from two yeast strains were equal and an equal- or unequal-variance two-tailed t -test was then conducted accordingly. The Bonferroni correction for multiple comparisons was applied to correct for the false-positive rate ($n = 8$, $\alpha = 0.00625$); under these conditions, only differences with $p < 0.00625$ were considered statistically significant at the 95% confidence interval.

Results

HPTLC–Densitometry Analysis

Lipid fractions were identified by agreement of R_F values between standard and sample zones. In the Mangold mobile phase, the R_F values of standards were cholesterol, 0.16; oleic acid, 0.35; and triolein, 0.56. In the Smith mobile phase, the R_F values of standards were cholesteryl oleate, 0.63; and squalene, 0.94. In the Wagner mobile phase, the R_F values of standards were PE, 0.30; PC, 0.50; and PI, 0.23. The Scanner 3 automatically carried out

polynomial regression to create calibration curves from standard zones (regression coefficient was at least 0.997 for each analyte) and interpolated weights of bracketed sample zones. Example plates with standard and sample chromatograms used for lipid quantification are shown in Figs. 1–3.

$$[PSI^+] \text{ vs. } [psi^-] \text{ cells}$$

In logarithmic phase $[PSI^+]$ cells, elevated levels of FSs (60% increase) and the phospholipids PC (40% increase) and PE (73% increase) were observed when compared to $[psi^-]$ control (Table I). In sharp contrast, in stationary phase, yeast cells harboring $[PSI^+]$ exhibited no significant difference in lipid profile when compared to cells lacking the prion (Table I).

$$[URE3] \text{ vs. } [ure-o] \text{ cells}$$

In logarithmic phase, yeast cells with the $[URE3]$ prion exhibited only one statistically significant difference, which was modestly higher levels of the sterol precursor squalene (20% increase) (Table II). Surprisingly, in stationary phase, $[URE3]$ cells exhibited numerous statistically significant differences in the lipid profile compared to cells without prion. Specifically, there were large increases in level of FSs (127% increase), squalene (300% increase), and PI (70% increase) (Table II). In contrast, the levels of TAGs, PC, and PE were decreased by 30%, 47%, and 37%, respectively, in cells that harbored the prion.

Discussion

Our investigation not only revealed a number of significant differences in lipid content between prion-infected and prion-free cells, but we found a surprisingly large dependence of these differences on the growth phase examined. There are innumerable physiological distinctions between growth phases that may explain these differences. For example, ergosterol synthesis is active during logarithmic phase growth but halted under anaerobic conditions because oxygen is required for some enzymes of the ergosterol biosynthetic pathway [23, 24]. Future investigations may explore additional growth conditions including alterations of temperature, media type, and the presence of cell stressors.

Although we found distinctions from prion-free cells for both prions examined, we found the most distinctions for the $[URE3]$ prion, specifically in stationary phase cells. Three factors complicate the analysis of the cells bearing $[URE3]$ used in this study. The first is that the strain used (Y2086) harbors not only $[URE3]$ but also the $[RNQ^+]$ prion, for which no physiological phenotypes are known beyond increasing the rate of the formation of other prions [25]. It is an intriguing possibility that one or more of the biochemical changes identified in this study are due to the $[RNQ^+]$ prion and may represent a physiological phenotype. The second complication is that, in order to detect the $[URE3]$ prion, this strain has a genetically engineered detection system where the *DAL5* gene, normally encoding an allantoin uptake protein, has been replaced by the *ADE2* gene, which encodes a component enzyme of the adenine biosynthetic pathway [26]. Consequently, our strains are additionally

prototrophic for adenine when [*URE3*] is present and auxotrophic when [*ure-o*] [27]. We mitigated this issue by culturing cells in rich media supplemented with additional adenine which should render this distinction irrelevant in subcultured cells growing in log phase; however, this distinction may have had a more significant effect in stationary phase cells growing in depleted media; as such, this distinction may be partially responsible for some of the differences observed in stationary phase [*URE3*] cells. Finally, Dal5 protein expression would normally be an expected biochemical consequence of the presence of [*URE3*], but this protein is absent in our experimental strains. How its expression might alter the experimental outcomes could be one of many subjects for future investigations that might use “wild” [*URE3*] and [*ure-o*] strains that lack the detection system and the [*RNQ⁺*] prion, or strains that only contain [*RNQ⁺*], as these would disentangle these potentially confounding variables in the future.

This study serves as a proof-of-principle that HPTLC–densitometry is a feasible method for analyzing the consequences of prion infection in yeast and has demonstrated that growth phase is a critically important variable in such an analysis that must be carefully controlled. In addition to advancing our understanding of a novel biological mechanism of inheritance and biodiversity, investigations of this kind may have potential practical value. Specifically, knowledge of prion-based alterations in lipid content may be of interest to anyone interested in isolating biological lipids en masse from living cells. Factors which could enhance the production of the lipid of interest, or reduce the production of difficult to eliminate contaminants, may be of significant interest. Likewise, currently unappreciated variations in membrane composition due to the presence of prions may affect drug uptake kinetics, cell viability, and growth rates in yeast disease models; information of this type may therefore be consequential to early stage drug screening or target identification assays in yeast.

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Fig. 1.
Example HPTLC plate for the analysis of phospholipids using the Wagner mobile phase. Lanes 1–4 are mixed phospholipid standard solution applied in 2.00, 4.00, 8.00, and 16.0 μL aliquots, respectively. Lanes 5–8 are phosphatidylinositol standard solution applied in 2.00, 4.00, 8.00, and 16.0 μL aliquots, respectively. Lanes 9–12 and 13–16 are two typical reconstituted sample solutions applied, respectively, in 2.00, 4.00, 8.00, and 16.0 μL aliquots each

Fig. 2.

Example HPTLC plate for the analysis of neutral lipids using the Mangold mobile phase.

Lanes 1–4 are mixed nonpolar lipid standard solution applied in 2.00, 4.00, 8.00, and 16.0

µL aliquots, respectively. Lanes 5–8, 9–12, and 13–16 are three typical reconstituted sample

solutions applied, respectively, in 2.00, 4.00, 8.00, and 16.0 µL each

Fig. 3. Example HPTLC plate for the analysis of SEs and squalene (S) using the Smith mobile phase. Lanes 1–4 are mixed nonpolar lipid standard solution applied in 2.00, 4.00, 8.00, and 16.0 μ L aliquots, respectively. Lanes 5–8 are squalene standard solution applied in 2.00, 4.00, 8.00, and 16.0 μ L aliquots, respectively. Lanes 9–12 and 13–16 are two typical reconstituted sample solutions applied, respectively, in 2.00, 4.00, 8.00, and 16.0 μ L aliquots each

Table I

Lipid content in $[PSI^+]$ vs. $[PSI^-]$ cells

Lipid fraction	Log phase			Stationary phase		
	$[PSI^+]$ ($n = 8$)	$[PSI^-]$ ($n = 8$)	p -Value	$[PSI^+]$ ($n = 7$)	$[PSI^-]$ ($n = 7$)	p -Value
FSs	0.024 ± 0.004	0.015 ± 0.003	2.7×10^{-4}	0.030 ± 0.001	0.029 ± 0.008	0.81
FFAs	0.03 ± 0.01	0.021 ± 0.009	0.15	0.018 ± 0.007	0.03 ± 0.01	0.012
TAGs	0.015 ± 0.008	0.02 ± 0.01	0.65	0.07 ± 0.06	0.06 ± 0.06	0.84
Squalene	0.008 ± 0.004	0.008 ± 0.002	0.93	0.007 ± 0.002	0.01 ± 0.002	0.051
SEs	0.04 ± 0.01	0.05 ± 0.01	0.80	0.09 ± 0.01	0.10 ± 0.02	0.56
PC	0.052 ± 0.007	0.04 ± 0.01	0.0041	0.08 ± 0.02	0.08 ± 0.02	0.98
PE	0.052 ± 0.008	0.03 ± 0.01	0.0039	0.044 ± 0.006	0.05 ± 0.01	0.85
PI	0.021 ± 0.006	0.020 ± 0.008	0.67	0.05 ± 0.02	0.06 ± 0.02	0.62

Table II

Lipid content in [URE3] vs. [ure-o] cells

Lipid fraction	Log phase			Stationary phase		
	[URE3] (<i>n</i> = 8)	[ure-o] (<i>n</i> = 8)	<i>p</i> -Value	[URE3] (<i>n</i> = 8)	[ure-o] (<i>n</i> = 8)	<i>p</i> -Value
FSs	0.042 ± 0.006	0.044 ± 0.005	0.37	0.034 ± 0.009	0.015 ± 0.002	6.0 × 10 ⁻⁵
FFAs	0.11 ± 0.02	0.10 ± 0.02	0.44	0.14 ± 0.04	0.12 ± 0.03	0.21
TAGs	0.049 ± 0.007	0.050 ± 0.006	0.77	0.22 ± 0.08	0.33 ± 0.06	0.0095
Squalene	0.006 ± 0.001	0.0050 ± 0.0004	0.0018	0.003 ± 0.001	0.0010 ± 0.0002	2.1 × 10 ⁻⁴
SEs	0.060 ± 0.01	0.054 ± 0.007	0.18	0.30 ± 0.06	0.32 ± 0.06	0.53
PC	0.027 ± 0.007	0.025 ± 0.005	0.43	0.032 ± 0.003	0.06 ± 0.01	2.7 × 10 ⁻⁶
PE	0.018 ± 0.005	0.024 ± 0.005	0.018	0.017 ± 0.001	0.027 ± 0.001	4.1 × 10 ⁻¹⁰
PI	0.05 ± 0.01	0.045 ± 0.007	0.33	0.051 ± 0.007	0.030 ± 0.008	1.3 × 10 ⁻⁴