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Iron, Glucose and Intrinsic Factors Alter Sphingolipid Composition as Yeast Cells Enter Stationary Phase

Robert L. Lester, Bradley R. Withers, Megan A. Schultz, and Robert C. Dickson¹

Department of Molecular and Cellular Biochemistry and the Lucille Markey Cancer Center, University of Kentucky College of Medicine, Lexington, Kentucky, 40536, USA

Abstract

Survival of *Saccharomyces cerevisiae* cells, like most microorganisms, requires switching from a rapidly dividing to a non-dividing or stationary state. To further understand how cells navigate this switch, we examined sphingolipids since they are key structural elements of membranes and also regulate signaling pathways vital for survival. During and after the switch to a non-dividing state there is a large increase in total free and sphingolipid-bound long chain-bases and an even larger increase in free and bound C20-long-chain bases, which are nearly undetectable in dividing cells. These changes are due to intrinsic factors including Orm1 and Orm2, ceramide synthase, Lcb4 kinase and the Tsc3 subunit of serine palmitoyltransferase as well as extrinsic factors including glucose and iron. Lowering the concentration of glucose, a form of calorie restriction, decreases the level of LCBs, which is consistent with the idea that reducing the level of some sphingolipids enhances lifespan. In contrast, iron deprivation increases LCB levels and decreases long term survival; however, these phenomena may not be related because iron deprivation disrupts many metabolic pathways. The correlation between increased LCBs and shorter lifespan is unsupported at this time. The physiological rise in LCBs that we observe may serve to modulate nutrient transporters and possibly other membrane phenomena that contribute to enhanced stress resistance and survival in stationary phase.

Keywords

Iron; sphingolipids; stationary phase; yeast; survival

1. Introduction

Microorganisms have many ways to cope with and survive numerous stresses including nutrient deprivation. Under some conditions, the budding yeast *Saccharomyces cerevisiae* negotiates nutrient deprivation by executing a multi-faceted transition from rapid cell division to a non-dividing or stationary state [1–5]. Many aspects of this transition are well characterized, but many are not and we sought to elucidate how sphingolipids might contribute to this transition that is crucial for survival. Sphingolipids including sphingosine (a long-chain base, LCB²), sphingosine-1-phosphate and ceramide control signal

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¹Corresponding author at: Department of Molecular and Cellular Biochemistry, University of Kentucky College of Medicine, 741 South Limestone, BBSRB room 173, Lexington, Kentucky, 40536-0509, USA. Tel: +1 859 323 6052; fax: +1 859 257 2283. bobb@uky.edu.

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transduction pathways that protect organisms against potentially life-threatening stresses of many sorts, in addition to their essential roles in membranes [6–9]. Both the complex sphingolipids in yeast membranes and the metabolic intermediates dihydrosphingosine (DHS) and phytosphingosine (PHS), collectively called long-chain bases (LCBs) which activate signal transduction pathways, have been shown to protect yeast cells from stresses. However, the majority of published studies have focused on dividing cells [10, 11]. To determine if sphingolipids also play protective roles in non-dividing cells, we examined yeast sphingolipids as cells exit the division cycle and enter stationary phase. There is a striking increase in total LCBs and the C20 species during this transition along with remodeling of the LCBs in complex sphingolipids. We identify several cellular factors that play roles in these changes. Finally, we show that the level of glucose and iron in culture medium influence LCBs and discuss the implications for cell survival.

Sphingolipid metabolism in *S. cerevisiae* (Fig. 1) begins by condensation of a fattyacyl-CoA derivative, typically C16:0 palmitoyl-CoA, with serine to yield 3-keto-DHS (3-ketosphinganine), a reaction catalyzed by serine palmitoyltransferase (SPT), followed by reduction to yield DHS. In *S. cerevisiae*, DHS is rapidly N-acylated with a C26 acyl group to give N-acylsphinganine (dihydroceramide) which is hydroxylated on C-4 to give phytoceramide. Alternatively, DHS is hydroxylated at C-4 to give PHS, which is acylated to produce phytoceramide. Ceramides in *S. cerevisiae* are converted to the three types of membrane-bound complex sphingolipids as indicated in Fig. 1 [12–15]

The initial indication that LCBs might control signal transduction pathways in *S. cerevisiae* cells came from studies of heat stress [16, 17]. These along with other studies have led to the view of LCBs as activators of the redundant protein kinases Pkh1 and Pkh2 (summarized in [18]), although some question this role for LCBs [19–21]. Pkh1/2 in turn phosphorylate and activate downstream protein kinases including Sch9, Ypk1 and Ypk2, Pkc1 and PKA, which control vital cellular processes, including survival during heat and nutrient stress, stationary phase and resistance to high levels of iron [3, 4, 10, 22–25].

Stationary phase in *S. cerevisiae* is reached by a complex but poorly characterized series of events. With glucose (2%) as the carbon source, cells produce energy by glycolytic conversion of glucose to ethanol. As the glucose level drops, cells undergo the diauxic shift, switching from the fermentative to the respiratory mode of energy production. This switch slows growth and, as ethanol and related compounds are consumed, cells pass through a ‘post-diauxic’ phase lasting 3–4 days [1, 2]. Finally, with ethanol exhausted cells enter true stationary phase, characterized by the accumulation of glycogen and trehalose, increased cell wall thickness and overall stress resistance and reduced metabolism. These changes sustain viability of quiescent yeast cells. Calorie restriction, imposed by reducing the concentration of glucose in the culture medium, increases lifespan by modulating many processes [26] including altering the timing of changes in metabolism and other cellular processes so they occur before the diauxic shift begins or earlier in the shift [27].

Most organisms require iron as a cofactor for iron-containing proteins that perform a large array of cellular functions. Studies using *S. cerevisiae* have advanced knowledge of organismal responses to iron deprivation [28, 29] and one key response is induction of the iron regulon, a set of genes controlled by the Aft1 transcription activator [30]. These genes promote uptake of extracellular iron, iron storage and other mechanisms that maintain iron homeostasis. Yeast also respond to low iron by reducing the need for iron-requiring proteins (summarized in [28, 29]), a response achieved by targeted decay of mRNAs encoding iron-requiring proteins [31].

Our results show that no single factor controls the increase in LCBs during the post-diauxic shift, rather many factors including nutrients, particularly calorie restriction and iron, and several yeast proteins play a part in the increase. A unique feature of this increase is an accumulation of C20-LCBs, which rise from a very low level in log phase cells to over fifty percent of total LCBs in stationary phase cells. A sizeable fraction of the C20-LCBs are incorporated into membrane-bound sphingolipids. The mechanisms regulating these changes are likely to be part of a large metabolic network containing a 'sphingolipid homeostasis subnetwork' [32, 33] that interfaces with other sub-networks governing stationary phase survival.

2. Materials and Methods

2.1. Strains, Plasmids, Media and Growth Conditions

Strains (Supplemental Information, Table S1) were cultured overnight at 30°C with shaking in 1–3 ml of synthetic medium, diluted into fresh medium (see figure legends) to give early log phase growth ($A_{600\text{nm}} \sim 1$) after overnight growth. Medium S [34] contained the L-amino acids (mg/L) tyrosine (30), leucine (400), aspartic acid (100), serine (400), threonine (200), tryptophan (80), glutamic acid (100), arginine (20), methionine (20), isoleucine (30), valine (150), phenylalanine (50), lysine (30), histidine (80), adenine (20), uracil (80), Yeast Nitrogen Base without amino acids and without ammonium sulfate (1.7 g/L), and ammonium sulfate (5 g/L). Medium SC contains all ingredients in Medium S plus 480 mg/L of leucine, lysine, tryptophan, uracil, and 500 mg/L of histidine. Medium SS: medium S was modified by replacing the Yeast Nitrogen Base (Difco) with 20 individual components (4 salts, 7 trace elements, and 9 vitamins). Iron-deficient Medium SSCX: except for vitamins, trace elements, calcium chloride, and magnesium chloride, all other components of medium SS as well as water, glucose, and succinate buffer were passed through Chelex-100 resin (BioRad, Hercules, CA) packed in a polypropylene column and stored in polystyrene flasks. All media were filter sterilized and, unless otherwise indicated, the carbon source was 2% glucose. For indicated experiments, media labeled S-B, SS-B, and SSCX-B were buffered with 0.2 M Na succinate (pH 4.5). To produce a quantity of cells for assaying LCBs and LCB-Ps, fatty acyl-CoAs, and SPT, S-B medium was inoculated from overnight cultures to an $A_{600\text{nm}}$ of approximately 0.007 and grown with shaking at 30°C with a flask volume to medium volume of 4–5:1.

2.2. Enzyme Assays

To prepare membranes for assay of SPT activity, ice was added to cultures to lower the temperature to about 5°C followed by centrifugation and washing twice with 0.1 M triethanolamine, pH 7.5. The cell pellet ($\sim 1000 A_{600\text{nm}}$ units) was suspended in 15 ml of breakage buffer (0.1 M triethanolamine, pH 7.5, 5 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride) containing 1 antiprotease tablet/10 ml (Roche Diagnostics, Indianapolis, IN). The sample was divided into $8 \times 15\text{ml}$ Corex glass tubes, one-half volume of 0.5 mm acid-washed glass beads was added to each tube, followed by vortexing at 4°C for 30 second intervals eight times, cooling with ice between vortexing. The supernatant was centrifuged at $1500 \times g$ and the resulting supernatants were centrifuged for 15 min at $100,000 \times g$ in a Beckman Optima centrifuge at 4°C. The membrane pellets were washed twice with 6 ml volumes of 0.1 M triethanolamine, pH 7.5, suspended in 3 ml storage of buffer (0.1 M triethanolamine, pH 7.5 and 20% glycerol) yielding about 4 mg protein/ml and stored at –20°C.

Assay conditions were similar to Pinto et. al. [35], except a different buffer and a higher concentration of serine were used. A reaction mixture contained 0.10 M triethanolamine, pH 7.5, 5 mM dithiothreitol, 2.5 mM EDTA, 50 μM pyridoxal phosphate, 25 mM L-serine,

fattyacyl-CoA (20–240 μ M), and 100 μ g membrane protein in a final volume of 100 μ L. After 20 minutes at 30°C, the reaction was stopped with 2 ml of a solution consisting of 100 parts n-butanol, 1 part glacial acetic acid, and 1.2 parts 0.05 mM C-20 dihydrosphingosine (internal standard) in ethanol. This solution was mixed vigorously with 0.5 ml 3.0 M NaCl, centrifuged, and the butanol layer was mixed with 0.5 ml water. After centrifugation, the butanol layer was removed, dried with a stream of nitrogen. The dried sample containing the product 3-keto-DHS was suspended in 200 μ L of 70.6 mM triethylamine in absolute ethanol and reacted with 40 μ L of 6-aminoquinolyl-n-hydroxysuccinimidyl carbamate reagent (AccQ Fluor Reagent Kit, Waters Corporation, Milford, MA). After 40 min incubation at room temperature, samples were centrifuged and 20 μ L were analyzed by reversed phase HPLC [36]. Elution was for 50 minutes isocratically at 1.0 ml/min flow rate with a solvent composed of 524.4 ml acetonitrile, 349.6 ml methanol, 114 ml water, 7 ml triethylamine, and 5 ml formic acid (88%).

To verify the identity of the products, some reaction mixtures (as described above) were terminated with 2 ml n-butanol followed by 50 μ L 10 M aqueous NaBH₄ to convert 3-keto-DHS to DHS. After 30 min at room temperature, 300 μ L of glacial acetic acid was added to destroy excess borohydride. Extraction of LCBs, formation of aminoquinolylcarbonyl derivatives and HPLC analysis were done as described above.

The measurement of ceramide synthase activity and fattyacyl CoA esters are described in Supplemental Information (Figs. S1 and S2).

2.3. Measurement of Free and Bound LCBs

Free LCBs and LCBPs were determined by using Method I or Method II as described in detail in Supplemental Information. Total LCBs were determined in HCl-methanol hydrolysates of whole cells and the fraction in complex sphingolipids (termed the bound LCBs) was calculated by subtracting the values of free LCBs (Method I).

2.4. Miscellaneous Procedures

Membrane protein concentration was measured by using the Coomassie Plus Protein Assay kit (Thermo Scientific, catalogue # 23236). Glucose was measured by using an Amplex Red Glucose Assay Kit (Invitrogen Corp., Carlsbad, CA). Cells for CLS assays (Fig. 5) were grown overnight at 30°C in SC medium and diluted into 25 ml (125 ml flask) of SC medium containing 3X iron (3.69 μ M Fe(NH₄)₂SO₄), 0.3 mM Ferrozine and 0.2 M succinate (pH 4.5) to give an initial A_{600nm} of 0.01. Cultures were incubated at 30°C in an air bath shaker (220 rpm) and cell viability assays were begun after 24 hrs (CLS day 1) by diluting and spreading cells on YPD plates. After 2–3 days of incubation at 30°C the colonies were counted and expressed as a fraction of the day 1 value. Statistical significance was determined by using the two-tailed Student's *t*-test.

3. Results

3.1. Changes in free and sphingolipid-bound LCBs as cells approach stationary phase

Free LCBs along with LCBPs were measured during and after the diauxic shift to explore the possibility that they influence properties of the plasma membrane and processes essential for exiting the cell division cycle and entering stationary phase in a state able to withstand nutritional deprivation and environmental stresses. Once cells deplete glucose and progress through the diauxic shift (Fig. 2A, around the 30 hr time point) the concentration of total free LCBs begins to rise and does so for at least 40 hrs to give a greater than 20-fold increase (Fig. 2A). This is surprising because it occurs near the end of the growth phase when most cells exit the cell division cycle (Fig. 2A, A_{600nm}). LCBPs also increase about

50% during this time-frame, but their level is dwarfed by the LCBs which are about forty times more abundant at the final time point (Fig. 2A).

Another unanticipated finding is an even larger increase in free C20-PHS, which rises from a few percent of the total LCBs in log phase cells to more than 50 percent in cells entering early stationary phase (Fig. 2B, 70 hrs). In contrast, the major LCB, C18-PHS, comprises about 70% of the free LCBs in log phase cells but only about 40% in stationary phase cells. We determined if any of the C20-PHS was incorporated into complex sphingolipids found primarily in the plasma membrane. Total LCBs were measured first by hydrolyzing cells with HCl-methanol to liberate LCBs bound to complex sphingolipids. The values for free-LCBs were then subtracted to give the concentration of bound-LCBs representing those in complex sphingolipids. From the 20 hr time point, when cells are in log phase, to the 70 hr time point, when they are in early stationary phase, there is about a 50% increase in the concentration of total bound LCBs (Fig. 2C). During this same period there is an even larger (~10-fold) increase in the bound-C20-PHS (Fig. 2B). Similar trends are seen in repeat experiments with DBY746 cells and in two other strain backgrounds, W303 (RCD408) and BY4742 (Table S2), indicating that the increase in free and bound-LCBs including C20-PHS are general phenomena.

3.2. Origin of the increase in Total and C20-LCBs

Two related proteins, Orm1 and Orm2, complex with SPT and negatively regulate enzyme activity to maintain sphingolipids in a physiological range [37–39]. The inhibitory action of Orm1 and Orm2 on SPT is controlled in response to sphingolipids levels in ways that are not entirely understood but involve phosphorylation by the sphingolipid-regulated Ypk1 protein kinase and the target of rapamycin complex 1 (TORC1) which modulates Orm1 phosphorylation [40]. If sphingolipid levels drop below normal, Orm1/2-mediated inhibition of SPT decreases thereby enhancing sphingolipid synthesis. In contrast, if sphingolipid levels rise above normal, SPT activity and the rate of LCB synthesis are down-regulated by Orm1/2. If Orm1/2 play roles in controlling the rise in LCBs that we observe as cells approach stationary phase, then there should be a higher fold-increase in LCBs in *orm* mutant than in wild-type cells. In wild-type cells (BY4742) total LCBs plus LCBPs increase between 1.5–2-fold during the time frame examined (Fig. 3A). The *orm1* and *orm2* single mutants show a 3-fold increase while the double mutant shows a fold-increase similar to wild-type (Fig. 3A). The C20-LCBs increase 7-fold in wild-type and double mutant cells and 15-fold in the single mutants (Fig. 3B), similar to changes found in other experiments (Table S2). The *orm1 orm2* double mutant cells clearly accumulate a higher absolute level of C18-LCBs and C20-LCBs than the single mutants, indicating that Orm1 and Orm2 play independent roles in controlling the increase in LCBs as cells progress from log to stationary phase.

Deletion of the major LCB lipid kinase gene, *LCB4*, increases the basal level of all LCBs in log phase cells and causes a larger than normal increase in all LCBs during a heat shock [41], indicating that phosphorylation of LCBs by Lcb4 plays a role in regulating LCB levels. Moreover, most of the Lcb4 protein is degraded by the end of diauxic shift [42] and this would be expected to contribute to the rise in LCBs that we observe. Using our growth conditions and strain DBY746, we find about a 60% decrease in LCB kinase activity after the diauxic shift (Fig. 3C). Thus, decreasing LCB kinase activity is a likely factor in LCB accumulation. In addition, an *lcb4Δ* mutant strain has a 9-fold higher level of total LCBs than WT cells at the 16 hr time point but the ratio drops to 2.5-fold at the 48 and 72 hrs time points (Fig. 3D). Total C20-LCBs are 7-fold higher in *lcb4Δ* cells at the 16 hr time point and 2-fold higher at 48 and 72 hr time points compared to WT cells (Fig. 3D). Thus, at all time points measured, total LCBs and C20-LCBs are higher in *lcb4Δ* cells, indicating that Lcb4 has a role in regulating LCB levels.

The increase in LCBs we observe might also be due to a reduction in ceramide synthase activity, since this enzyme consumes LCBs while making ceramides (Fig. 1). To quantify activity of this membrane-bound enzyme, we developed an *in vitro* assay that uses HPLC to measure the consumption of LCBs in a reaction containing yeast membranes. With C18-PHS as substrate there is about twice as much enzyme activity as with C18-DHS (Fig. S1). This assay is specific because omission of C26-acyl-CoA completely blocks product formation and addition of Fumonisin B1, a known inhibitor of yeast ceramide synthase activity [43], reduces activity by more than 90% with C18-PHS as substrate and by more than 70% with C18-DHS as substrate (Fig. S1). Using this assay we find a 60–70% decrease in enzyme activity during the diauxic shift (Fig. 4A), indicating that reduction in ceramide synthase activity contributes to the increase of LCBs and LCBPs (Fig. 4A).

Ceramide synthase contains two functionally redundant proteins, *Lag1* and *Lac1*, that complex with the *Lip1* protein to generate enzyme activity [44]. We examined the role *LAG1* and *LAC1* on ceramide synthase activity and LCB accumulation. Deleting either gene has no effect on the growth rate or final cell density but does reduce enzyme activity about 25% at the 19 hr and 45 hr time points (Fig. 4B). However, by 72 hrs the mutants contain the same amount of activity as wild-type cells. Deletion of either gene enhances accumulation of total LCBs and LCBPs with *lag1Δ* cells having the largest accumulation, about 2-fold above the level in wild-type cells (Fig. 4C). This mutant also has the highest accumulation of C20-LCBs and LCBPs while *lac1Δ* cells show slightly less accumulation but still more than wild-type cells (Fig. 4D). The most unexpected result is the 3-fold higher accumulation of C20-PHS in *lag1Δ* cells after the diauxic shift compared to wild-type or *lac1Δ* cells, which have a similar level (Fig. 4E). The data shown in Fig. 4 (panels A–E) support the idea that at least part of the increase in LCBs and LCBPs during and after the diauxic shift is due to a decrease in ceramide synthase activity. Moreover, *LAG1* is more prominent in promoting the incorporation of LCBs and C20-PHS into ceramides as its deletion causes the largest accumulation of LCBs.

Deletion of the *TSC3* gene, encoding a small peptide thought to stimulate SPT activity [45] reduces total LCB and C20-LCB levels induced by heat shock [46]. Similarly, we find an 85% reduction in total C20-LCBs in *tsc3Δ* cells compared to wild type cells after 70 hrs of growth while there is a smaller, 20%, reduction in total LCBs + LCBPs (Fig. 4F). Another alteration is the 1:1 mix of C20-PHS and C20-DHS in *tsc3Δ* cells compared to the 5:1 mix in wild-type cells. We conclude that Tsc3 promotes synthesis of C20-LCBs during and after the diauxic shift and adjusts the mix to favor formation of PHS over DHS.

We examined other intrinsic factors including C16:0-CoA and C18:0-CoA (Table S3), substrate specificity of SPT (Fig. S3A and S3B) and uptake of serine from the culture medium during the diauxic shift (Fig. S4). None of these factors appear to play a role in the increase in total LCBs or C20-LCBs that we observe. However, it is possible that C18:0-CoA is diverted from use in glycerophospholipid synthesis, which decreases during the time-frame of our experiments, into synthesis of C20-PHS and this contributes to their accumulation.

3.3. Caloric Restriction and LCB Accumulation

The increase in free LCBs after the diauxic shift may be due to growth being limited by decreasing nutrients, particularly glucose. We determined if simply limiting growth increased LCBs. Adding cycloheximide to log phase cells to limit protein synthesis or adding cerulenin to limit fatty acid synthesis did not cause total LCBs or C20-PHS to increase. Rather, it had the opposite effect and caused a decrease (data not shown).

On the other hand, limiting growth by reducing the concentration of glucose from 2 to 0.2%, a form of caloric restriction (CR), gave revealing results. Instead of causing LCBs to increase, the CR treatment represses the increase in total LCBs plus LCBPs and C20-PHS by 7-fold (Table 1). Because glucose consumption causes a drop in the pH of the culture medium, the medium for these experiments was buffered (Medium S-B, pH 4.5). Buffering had no effect on LCBs.

3.4. Iron Deprivation and LCB Accumulation

The effect of CR suggests that the accumulation of LCBs during the diauxic shift is triggered by nutrient limitation. In this case, CR reduces LCB accumulation simply by lowering biomass production thereby reducing or preventing nutrient limitation. We examined this proposal by determining if addition of excess nutrients to Medium S-B reduces LCB accumulation. The increase in LCBs is not reduced by a 5 to 10-fold excess of amino acids, uracil, adenine, salts, vitamins, or ammonium sulfate. However, a 10-fold excess of a mixture matching the seven trace elements in the Yeast Nitrogen Base component of Medium S sharply decreases LCB and LCBP accumulation. Individually adding a 20-fold excess of the seven trace elements shows that excess Fe reduces accumulation of LCBs and LCBPs (Table S4).

As another way to show that iron deprivation causes LCBs to accumulate, we reduced iron in culture medium by adding the iron chelator Ferrozine. Addition of Ferrozine to Medium S-B reduces growth in a concentration dependent manner and causes a large increase in LCBs (Table 2, Experiment 1). Ferrozine, however, triggers accumulation of both C18- and C20-DHS, not phytosphingosines. This decrease in hydroxylation is consistent with reduced synthesis of the Sur2/Syr2 protein catalyzing 4-hydroxylation of DHS to make PHS [47]. Sur2 is a member of the diiron family of proteins which require iron for functionality [48, 49] and iron deprivation, by repressing the iron regulon, ought to reduce synthesis of Sur2 and other non-essential iron-containing proteins [28].

In addition to binding Fe, Ferrozine could also increase LCBs by chelating other ions such as Co^{2+} and Cu^{2+} [50]. Therefore, we removed iron from the components of the medium by treatment with Chelex-100 resin (Medium SSCX). This iron deprivation strategy also attenuates growth ($A_{600\text{nm}}$ values) and causes a high accumulation of LCBs, primarily DHSs (Table 2, Experiment 2). Adding iron back to SSCX medium to give a normal concentration ($1.23 \mu\text{M}$) reduces LCB accumulation, showing that iron deprivation is primarily responsible for accumulation of DHSs. Adding Ferrozine or bathophenanthroline sulfonate, another widely used iron chelator, to Medium SSCX causes no further significant changes in LCB levels and adding $61.5 \mu\text{M}$ Fe in the presence of the iron chelators also reduces LCB accumulation (Table 2, Experiment 2). These data support the idea that iron deprivation is a key variable governing LCB accumulation.

Cells grown in Fe-depleted Medium SSCX-B begin to accumulate LCBs before glucose is consumed, further supporting the idea that Fe is the key nutrient responsible for LCB accumulation (Fig. S5). Adding Ferrozine or bathophenanthroline sulfonate to Medium SSCX slows growth significantly but does not further increase LCB levels (Fig. S5).

3.5. Fe Deprivation Reduces Viability

The most well documented response of yeast cells to iron deprivation is induction of the iron regulon and down-regulation of synthesis of non-essential iron-requiring proteins (summarized in [28, 29]). While these responses seem designed to protect cells from dying, the effect of iron deprivation on viability and long-term survival in stationary phase (chronological lifespan, CLS) has not been studied as far as we are aware. To determine how

iron deprivation affects CLS, we cultured cells in iron-deficient Medium SSCX-B with or without added Fe. Iron addition has only a small effect on glucose consumption but clearly increases mass yield as measured by $A_{600\text{nm}}$ measurements (Fig. 5A): increasing Fe to 61 μM does not increase mass yield beyond that obtained with 24.6 μM Fe. In medium without added Fe there is a rapid loss of viability as cells near the end of the diauxic shift (Fig. 5B, Day 2). Addition of Fe equal to the concentration in Yeast Nitrogen Base (1.23 μM) improves viability and 20-fold more Fe (24.6 μM) completely prevents any loss in viability over the 9 days of the assay (Fig. 5B). Most of the loss in viability occurs after glucose is consumed (near the end of the diauxic shift). These data demonstrate that cell growth is limited by Fe, even the concentration present in Yeast Nitrogen Base limits growth, at least under our culture conditions.

Total LCBs and LCBPs increase well into stationary phase in cultures with no added Fe or with 1.23 μM Fe (Fig. 5C) and are 10-fold higher than cultures with 24.6 μM Fe after 9 days of incubation, verifying the conclusion that Fe limitation causes an increase in the synthesis of LCBs and LCBPs. Finally, the LCB composition changes throughout the time-frame of this experiment with C20-LCBs becoming ever more abundant so that by day 9 they represent 60–70% of the total LCBs + LCBPs (Fig. 5D).

We next sought to reproduce the decrease in CLS caused by Fe deprivation in SSCX-B medium by using another culture medium, since it is difficult to reproducibly achieve a low level of Fe in SSCX-B medium. CLS experiments are often performed in defined culture medium (SDC) containing Yeast Nitrogen Base (1.23 μM Fe) which is lightly buffered (pH 6.0) with phosphate [51]. We modified this medium by replacing phosphate with the same concentration of succinate (200 μM , pH 4.5) as used in our other defined media and by using 3X Fe (3.68 μM). With this modified SDC medium there is a statistically significant reduction of CLS in DBY746 cells cultured in the presence of 0.6 mM Ferrozine (Fig. 5E). The loss of viability is due to iron deprivation since adding iron improves viability in a dose-dependent fashion.

4. Discussion

Here we describe several changes in sphingolipid metabolism that occur during and after the diauxic shift and suggest that some contribute to survival, although exact mechanisms, which likely involve responses produced by complex networks, will require further studies as discussed below. First, the concentration of free LCBs increases by more than 50-fold during and after the diauxic shift (Figs. 2, 4 and 5). Second, C20-PHS and C20-DHS are barely detectable in log phase cells but start to accumulate after the diauxic shift and comprise nearly half the LCBs in stationary phase cells. Third, more than a third of the newly made C20-PHS is incorporated into complex sphingolipids with much of this shift occurring during and after the last cell division. Fourth, the increase in LCBs during the diauxic shift is influenced by both cell-intrinsic and environmental or extrinsic factors. Fifth, the most notable extrinsic factor is iron which at reduced levels stimulates LCB accumulation. Finally, depriving cells of iron throughout the diauxic shift reduces CLS.

4.1. Origin of LCB accumulation and surge in C20-LCB synthesis

Our data implicate several contributors to the accumulation of LCBs and surge in C20-LCB synthesis during and after the diauxic shift as summarized in Fig. 6. SPT and ceramide synthase are key targets for intrinsic control of LCB accumulation and the increase in C20-LCB synthesis. We find that both Orm1 and Orm2 down-regulate SPT activity to maintain LCBs within a physiological range that continually rises as cells approach stationary phase (Fig. 3A and 3B). Recent studies using log phase cells show that inhibiting SPT by myriocin treatment activates the TORC2-Ypk1 pathway with Ypk1 phosphorylating Orm1 and Orm2.

Such phosphorylation promotes SPT activity and increases synthesis of LCBs and complex sphingolipid [37]. Another recent study found that TORC1 regulates Orm1 phosphorylation, at least in log phase cells where inhibition of TORC1 by rapamycin treatment reduces ceramide synthesis [40]. Further work is necessary to determine if TORC1 and TORC2 operate during the diauxic shift and stationary phase to control SPT activity and LCB accumulation.

We find a 60–70% reduction in ceramide synthase activity beginning in the diauxic shift and extending into the post-diauxic period (Fig. 4A). Since this enzyme consumes LCBs, a decrease in activity would enhance LCB and LCBP accumulation unless there was a corresponding decrease in SPT activity, which we did not observe. The two components of ceramide synthase, Lag1 and Lac1, which have overlapping actions, were studied to see if one or both contribute to the decrease in enzyme activity. There is a similar decrease in ceramide synthase activity in *lag1Δ* and *lac1Δ* mutant cells (Fig. 4B), indicating an equal contribution to the decrease in enzyme activity by each protein. On the other hand, they do not play equal roles in LCB accumulation because accumulation of total free LCBs (Fig. 4C) and free C20-LCBs (Figs. 4D and 4E) is higher in *lag1Δ* cells, indicating that Lag1 determines the ratio of C16-LCB to C18-LCB that will be used for ceramide synthesis while Lac1 does not do this.

The Lcb4 long-chain base kinase is likely also to contribute to LCB accumulation since its activity decreases about 40% during the diauxic shift (Fig. 3C) and deleting *LCB4* increases the level of total and C20-LCBs during and after the diauxic shift (Fig. 3D). It is hard to quantify the effect of Lcb4 on LCB accumulation without knowing the flux rate through this step in sphingolipid metabolism during the diauxic shift. Finally, the Tsc3 subunit of SPT is the most influential intrinsic factor we found for maintaining LCBs and LCBPs at wild-type levels in post-diauxic cells (Fig. 4F). Tsc3 is also the most prominent intrinsic factor in C20-PHS accumulation, since the level of this LCB species is reduced by 85% in post-diauxic *tsc3* mutant cells (Fig. 4F, 70 hr time point). The biochemical role of Tsc3 in sphingolipid synthesis remains an enigma. Suggested roles are to improve SPT substrate availability and specificity [46]. Further work is necessary to discover what controls Tsc3 activity and how this activity modulates LCB accumulation in post-diauxic cells.

Since the TORC2-Ypk2 signaling pathway and calcineurin have been implicated in regulating ceramide synthase activity [52] and sphingolipid metabolism [53–56], we determined if they are responsible of the reduction in activity seen during the diauxic shift. We found no indication of TORC2, Ypk2 or calcineurin controlling ceramide synthase activity or LCBs (Fig. S6). Our experiments focus on cells during and after the diauxic shift whereas previous studies examined log phase cells and this difference may explain why we find no involvement of TORC2, Ypk2 or calcineurin in down-regulation of ceramide synthase activity.

Other intrinsic factors that have been suggested to regulate sphingolipid synthesis and LCB levels are Rom2, a GTPase exchange factor for Rho1 [57] and casein kinase 2 [58]. How these proteins regulate sphingolipid synthesis and in what phase of growth remain to be determined.

Several of the changes in sphingolipids that we observe were predicted by mathematical modeling of yeast sphingolipid metabolism during the diauxic shift and verified by experimental measurements [59]. Because of differences in yeast strains, media and the longer time-frame over which we took samples, it is not possible to quantitatively compare the two data sets and our comments are based only on the trends. First, both data sets show an accumulation of LCBs and LCBPs during the diauxic shift, although the model did not

attempt to differentiate between different chain lengths. Second, both data sets show that these increases are due to many factors, not just a few. Third, the model predicted a small increase in Lcb4 kinase activity and a decrease in ceramide synthase activity. We find a decrease in ceramide synthase activity (Fig. 4), but instead of an increase Lcb4 kinase activity we find a decrease (Fig. 3C). Fourth, the model predicted an increase in serine uptake; however, we found a very large decrease in the rate of uptake during the diauxic shift (Fig. S4). Finally, the model predicted an increase in complex sphingolipids and this is consistent with our results.

Of the extrinsic factors, the concentration of iron in culture medium has a marked affect on LCBs and LCBPs with greater accumulation during iron deprivation (Tables 2 and S4, and Fig. 5C) in addition to the accumulation of non-hydroxylated DHSs rather than the hydroxylated PHSs. These results are in agreement with previous reports showing that iron deprivation triggers yeast cells to conserve iron by eliminating unessential iron-requiring proteins [28, 29] and, as part of this program, the level of the Sur2 protein goes down and non-hydroxylated DHSs accumulate [47]. Glucose also influences LCB and LCBP accumulation since their level drops nearly 7-fold when glucose is lowered from 2 to 0.2% (Table 1). Lowering the concentration of glucose reduces the mass of cells produced, and this in turn spares iron and avoids LCB accumulation caused by iron deprivation.

4.2. Functions of LCBs in stationary phase cells

The increase in free and bound C20-LCBs in stationary phase cells ought to affect membrane dynamics and membrane-mediated stress protection. Studies of large unilamellar vesicles made from dipalmitoylphosphatidylcholine and containing gangliosides with C18 or C20 LCBs (sphingosines) showed that increased LCB chain length promotes phase separation [60]. Another study showed an increase in micelle aggregation size with longer LCB chain length [61]. An increase in complex sphingolipids with a C20-LCB may also promote formation of a thicker, more compact and less permeable plasma membrane that provides greater stress protection [62], a well known characteristic of stationary phase cells [2].

The increase in free LCBs also has the potential to affect signal transduction pathways. LCBs are thought to activate Pkh1 and Pkh2 (reviewed in [63] and see also [18]) and this could activate downstream protein kinases including Pkc1, Sch9 and Ypk1/2, which modulate many cellular processes involved in stationary phase survival including cell wall metabolism.

The increase in LCBs we observe is likely to affect eisosomes [64], large complexes containing the Pil1 and Lsp1 proteins that bind the inner leaflet of the plasma membrane where they are essential for formation of furrow-like invaginations [65, 66]. Eisosomes also play roles in endocytosis and probably other processes including clustering of membrane lipids and proteins [67–70]. While we do not know exactly how LCBs affect Pil1 and eisosomes [64, 71, 72], it is clear that LCBs do have an influence and it seems likely that the increases we observe in LCBs during the diauxic shift may have physiologically significant influences on eisosomes and the processes they modulate.

Studies of sphingolipid and phosphoinositide metabolism suggested that increased LCBs might underlie the reduction of serine uptake seen in *sac1Δ* cells [73]. Sac1 dephosphorylates phosphatidylinositol-4-phosphate to generate phosphatidylinositol, the substrate which donates a phosphoinositol group to ceramide for synthesis of IPC. Deletion of *SAC1* reduces the rate of complex sphingolipid synthesis causing LCBs to accumulate, and this promotes permease breakdown, including those for serine transport. This hypothesis is based upon earlier work showing that increased LCBs promote degradation of some

permeases [74, 75]. These previous data along with the data presented here suggest that one function of the rise in LCBs during the diauxic shift is to trigger degradation of serine and perhaps other nutrient transporters, since nutrient demand is reduced in stationary phase cells. Sphingolipids are thought to play a similar role in mammals except ceramides rather than LCBs are the key sphingolipids [76].

Finally, the increase in LCBs, LCBPs and particularly C20-PHS during the diauxic shift represent, at least in part, a stress response because similar increases have been shown to occur during heat shock, although the increases are only transient [16, 17, 41, 46]. To show that C20-LCBs are actually important for stress resistance and survival in stationary phase will require understanding how they arise and then specifically blocking the mechanism.

4.3. Iron homeostasis and sphingolipids in stationary phase cells

S. cerevisiae cells mount multiple responses to iron deprivation to conserve and channel iron into essential iron-requiring proteins [28, 29]. These responses are at least partly responsible for alterations in glucose metabolism, energy production, amino acid biosynthesis, and ergosterol, fatty acid and sphingolipid metabolism observed in iron-deprived log phase yeast cells [47]. The main similarity to our results is the accumulation of DHS, rather than PHS. But in addition, we demonstrate an enormous increase in total LCBs and LCBPs in iron-deprived cells after the diauxic shift. How iron deprivation promotes such accumulation requires further study.

Many iron-requiring proteins are synthesized during the diauxic shift to up-regulate respiration and failure to make this change in ATP generation is deadly [77]. We determined if cells deprived of iron during their entire incubation time starting at a low cell density and continuing through the diauxic shift survive as well as cells incubated with a normal or higher than normal level of iron. CLS was greatly reduced by iron-deprivation (Figs. 5B and 5E) and, as far as we are aware, this is the first indication that iron deprivation reduces CLS in yeast. A previous report showed that iron deprivation caused a small decrease in the CLS of *isc1Δ* cells subjected to severe CR by incubation in water [78]. These authors also showed that *Isc1* plays a role in iron homeostasis during the diauxic shift. Whether there is a relationship between the increase in LCBs during the diauxic shift that we observe and *Isc1*-dependent iron homeostasis and turnover of complex sphingolipids, which was recently shown to play a role in CLS [79], requires further study.

Finally, the toxicity of a high level of iron was recently shown not to be due to the generation of reactive oxygen species that can damage macromolecules, but rather to accumulation of LCBs and LCBPs and activation of the *Pkh1-Ypk1* pathway [25]. Thus, high and low levels of iron promote LCB and LCBP accumulation in yeast cells and both extremes lead to cell death. The challenge is to determine if the mechanisms that promote LCB and LCBP accumulation and cell death are similar when cells confront an environment with a high or low level of iron.

4.4. Concluding remarks

We have identified some of the intrinsic and extrinsic factors that govern the increase in total C18-LCBs and free and sphingolipid-bound C20-LCBs during the diauxic shift, but even when these are combined with previous information there are still many gaps in our knowledge of sphingolipid metabolism. Our experiments have focused primarily on *de novo* synthesis, but turnover also has to be an essential component of sphingolipid metabolism and is likely to contribute to the increase C18-LCBs and LCBPs that we observe and perhaps to the increase in C20-LCBs. We favor the idea that C20-LCBs arise from *de novo* synthesis primarily because they are not detectable in some strains in log phase cells.

However, we cannot eliminate the possibility that C20-LCBs are preferentially degraded during log phase growth and accumulate late in the diauxic shift because of changes in the turnover pathway controlled by the Lcb4/5 kinase and Dpl1 lyase (Fig. 1). LCB and LCBP turnover pathways are important because blocking the Dpl1 lyase and Lcb3 phosphatase (Fig. 1) is lethal [80, 81], but the extent to which they contribute to the accumulations we observe is unclear. There is probably a basal level of turnover of complex sphingolipids during the diauxic shift [82–84] and this could contribute to an increase in C18-LCBs. Once C20-LCBs begin to be made and incorporated into complex sphingolipids, a reduction in turnover could help to set the rate of accumulation.

One implication of our data is that abnormally high LCB levels may reduce viability and CLS. For example, iron deprivation elevates LCBs and cells die (Table 2 and Fig. 5). Focusing on this correlation, however, misses the myriad of other effects that iron deprivation has on cells and which explain why they begin to die during the diauxic shift [77, 85, 86]. Several mutants we examined also accumulate abnormally high levels of LCBs. To our knowledge and with the exceptions noted below, no published data from studies of strains with mutations in sphingolipid metabolic genes support the idea that accumulation of LCBs above the normal physiological range reduces CLS. Exceptions are the *dpl1 lcb3* double mutant and mutants or drugs that block synthesis of complex sphingolipids or cause accumulation of a toxic metabolite [11, 22]. In all of these cases, viability loss occurs in log, not stationary phase cells and is not thought to be due to LCBs, but rather to other sphingolipids.

On the other hand, there is support for the idea that reducing the rate of sphingolipid synthesis, and thereby lowering the concentration of some LCBs and complex sphingolipids, does increase CLS. Calorie restriction is the most established way to increase CLS and we find an 85% reduction in total LCBs and LCBPs in calorie restricted cells (Table 1). Besides limiting iron depletion, the mechanisms by which calorie restriction reduces LCBs are unknown, but they are likely to be quite complex since calorie restriction influences many processes related to growth control and stress resistance [26, 87]. Recently we showed that reducing the rate of sphingolipid synthesis, which lowers the concentration of most LCBs and LCBPs, but only IPC and not other complex sphingolipids, does produce a significant increase in CLS [24]. The *tsa3Δ* mutant has low levels of LCBs (Fig. 4F) and we sought to determine if these cells have an increased CLS. CLS, however, cannot be measured because these cells grow slowly and faster growing variants take over the culture before the diauxic shift is completed. Other published data supporting roles for various sphingolipids have been discussed recently and most are consistent with the idea that reducing (not eliminating) the concentration of one of more sphingolipids increases CLS [24].

Overall, our data along with published data add support to the disposable soma theory of aging [88]. This theory argues that evolution selects for organisms that are most able to balance growth and reproduction with stress resistance. The normal concentration of sphingolipids in yeast cells represents the balance necessary to promote rapid growth and reproduction when nutrients are abundant and few stresses exist, while also being able to promote stress resistance and survival when nutrients are scarce and or other life-threatening stresses appear. Reducing the concentration of some sphingolipids, for example, by treatment with a low level of myriocin, shifts cells from a rapid growth and reproduction mode with low stress resistance to a slow growth and reproduction mode with increased stress resistance and longer CLS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CLS	chronological lifespan
CR	calorie restriction
CS	ceramide synthase
DHS	dihydrosphingosine
LCB	long-chain base
LCBP	long-chain base phosphate
PHS	phytosphingosine
SPT	serine palmitoyltransferase

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Highlights

- Sphingolipid long-chain bases rise 50-fold by early stationary phase
- C20-long-chain bases rise to more than 50% of the total in stationary phase cells
- More than 30% of the newly made C20-long-chain bases goes into complex sphingolipids
- Iron, glucose, and intrinsic factors regulate the rise in long-chain bases
- Long-chain bases they influence long term survival

Fig 1.
Outline of yeast sphingolipid metabolism. Metabolites discussed in the text are shown in bold lettering, enzyme names are in regular lettering and gene names are in italics. The Orm1 and Orm2 protein (boxed) act to down-regulate serine palmitoyltransferase activity.

Fig 2.

The concentration of free and bound LCBs and LCBPs increase during the diauxic shift. The concentration of LCBs bound in complex sphingolipids or unbound ('Free') is shown for DBY746 cells grown in Medium S. Values represent the average of duplicate cultures with the spread between the two cultures indicated by error bars. *A*, Total free LCBs and LCBPs along with the A_{600nm} of cells are shown. *B*, C20-PHS as a percent of free or bound C20-LCBs are shown. *C*, Total bound LCBs are shown.

Fig 3.

Factors influencing LCBs and LCBPs during the diauxic shift. *A*, The *Orm1* and *Orm2* proteins strongly affect LCBs. Total LCBs and LCBPs were measured in wild-type (WT, BY4741), *orm1* Δ (HXX1-7B), *orm2* Δ (RCD1060) and *orm1* Δ *orm2* Δ (HXX1-7D) at the indicated time points with the earliest point representing cells grown to an A_{600nm} of ~ 2.5 . *B*, Total C20- LCBs in the same samples as shown in panel *A*. *C*, A decrease in LCB kinase during the diauxic shift may contribute to the increase in LCBs, since LCB kinase activity in DBY746 cells decreases as shown. Values are the average of duplicate cultures, all of which differed by less than 20%. *D*, Deletion of *LCB4* causes total free LCBs and C20-LCBs to increase at all time points examined compared to WT cells.

Fig 4.

Decreasing ceramide synthase activity contributes to the increase in LCBs. Ceramide synthase activity (nmole ceramide/hr/mg protein) was quantified during the diauxic shift to determine if it contributes to the increase in LCBs. Values represent the average of duplicate cultures of DBY746 cells grown in Medium S with the spread between the two values indicated by error bars. *A*, Ceramide synthase activity and the total LCBs + LCBPs in DBY746 cells are shown. *B*, Ceramide synthase activity in RCD959 (*lag1::KAN*), RCD961 (*lac1::KAN*) and wild-type DBY746 cells is shown. *C*, Total free LCBs + LCBPs in RCD959 (*lag1::KAN*), RCD961 (*lac1::KAN*) and wild-type DBY746 cells is shown. *D*, Total free C20-LCBs + C20-LCBPs in RCD959 (*lag1::KAN*), RCD961 (*lac1::KAN*) and wild-type DBY746 cells is shown. *E*, The level of C20-PHS in RCD959 (*lag1::KAN*), RCD961 (*lac1::KAN*) and wild-type DBY746 cells is shown. *F*, The level of LCBs + LCBPs along with C20-LCBs (PHS + DHS) in *tsc3Δ* (RCD541) and wild-type DBY746 (transformed with pRS314) cells are shown. Values are the average $\pm$ S.D. of four cultures.

Fig 5.

Effect of iron deprivation on sphingolipids and chronological lifespan. The effect of iron deprivation on LCBs, LCBPs and lifespan was examined in DBY746 cells grown in SSCX-B medium with no Fe added or with 1.23 μM (1X) or 24.6 μM (20X) Fe added. Values in panels A – D represent the average of duplicate cultures with the spread between the two values indicated by error bars. *A*, The rate of glucose consumption and cell growth (A_{600nm}) are shown. *B*, Iron deprivation causes a loss of cell viability during stationary phase. *C*, Demonstration that iron deprivation promotes LCB and LCBP accumulation, which were quantified by HPLC Method II. *D*, Iron deprivation promotes accumulation of C20-LCBs. *E*, The effect of iron deprivation imposed by adding 0.6 mM Ferrozine (Fz) to cultures was examined. Cell survival in buffered SC medium containing 3.69 μM ferrous ammonium sulfate plus the indicated additional iron is shown. The viability of Ferrozine-treated (Fz) cells improves in a dose-dependent manner with increasing iron, demonstrating that iron deficiency shortens lifespan. Values are the average $\pm$ S.D. of three replicas (some error bars are hidden by a symbol).

Fig 6.

Diagram showing factors influencing the rise in LCBs during and after the diauxic shift. Intrinsic factors that influence the rise in LCBs during the diauxic shift are shown in bold font and enclosed in a rectangle. Orm1 and Orm2 maintain flux through SPT to preserve sphingolipid homeostasis and deleting either or both genes causes LCBs to rise above the normal level we observe. Tsc3 has roles in both the flux through SPT and the use of C18-CoA. Deleting *TSC3* reduces the level of LCBs produced during and after the diauxic shift, with the largest effect being a severe reduction in synthesis of C20-LCBs. Deletion of the *LCB4* gene, encoding the major kinase that phosphorylates DHS and PHS, causes LCBs to accumulate. Additionally, Lcb4 kinase activity drops during the time frame of our assays and this likely contributes to LCB accumulation. Ceramide synthase activity decreases

during and after the diauxic shift and this also contributes to the rise in LCBs. The Lag1 component of this enzyme modulates LCB accumulation, since cells lacking Lag1 accumulate about 2-fold more total LCBs and 2–3 times more C20-LCBs than do WT or *lac1* mutant cells. Iron deprivation and calorie restriction are extrinsic factors that cause an increase in total LCB accumulation. Sur2 (bold oval) activity is reduced by iron deprivation, leading to a higher than normal accumulation of LCBs, characterized by production of DHS at the expense of PHS.

Table 1
Caloric restriction, high levels of LCBs in stationary phase, and pH of the medium

Duplicate cultures of DBY746 cells were cultured for 46 hrs in Medium S-B at two concentration of glucose. LCBs were analysis by using HPLC Method I.

A600	pH	Glucose conc. (%)	Total LCBs & LCBPs pmoles/A600	% C20-PHS
11.89	4.5 ± 0.02	2.0	213 ± 23	43.5 ± 1.5
6.12	4.5 ± 0.02	0.20	31.2 ± 3.9	39.8 ± 0.8

Table 2
LCB accumulation in stationary phase with iron restriction by chelation

Cultures of strain DBY746 were grown in Medium S-B (1X YNB, 1.23 μ M iron) for 45 hours or in Medium SSCX-B (iron removed by Chelex treatment) for 47.2 hours containing various levels of Ferrozine (Fz) and bathophenanthroline sulfonate (BPS). Cells were processed for LCB analysis as described in Experimental Procedures (Experiment 1, HPLC Method I; Experiment 2, HPLC Method II).

	Medium	A600	LCBs & LCBPs (pmoles/A600)										Sum C20 LCBs+ LCBPs	Sum LCBs+ LCBPs	Sum C20 LCBs+ LCBPs
			C16-DHSP	C16-DHS	C18-PHSP	C18-PHS	C18-DHSP	C20-PHSP	C18-DHS	C20-PHS	C20-DHSP	C20-DHS			
Experiment 1 No Fz	S-B	12.5	0	1	0	65	0	2	8	54	2	1	133		59
0.30 mM Fz	S-B	5.3	0	0	0	28	2	1	211	45	1	64	353		111
0.90 mM Fz	S-B	0.62	1	1	5	34	16	1	1550	13	2	118	1740		134
Experiment 2 No Fe added	SSCX-B	2.56	0.3	3.3	1.1	97	5.4	1.3	525	24	1.5	31	690		58
0.23 μ M Fe	SSCX-B	7.32	0.2	0.3	1.2	45	0.5	0.2	14	43	0.4	2	107		45
2.4 μ M Fe	SSCX-B	8.52	0.3	0.2	0.95	51	0.4	0	12	49	0.4	1	116		51
61.5 μ M Fe	SSCX-B	7.92	0.3	0.2	0.9	40	0	0	6	28	0.3	1	76		29
No Fe + 0.1 mM Fz	SSCX-B	1.76	0.6	2	0.7	40	5	0.6	471	8	0.7	22	550		31
0.15 μ M Fe + 0.1 mM Fz	SSCX-B	8.44	0.3	0.2	0.1	48	0.4	0.2	11	46	0.4	2	108		48
No Fe + 0.1 mM BPS	SSCX-B	2.16	0.3	2	0.6	50	5	0.5	563	14	0.8	38	674		53
61.5 μ M Fe + 0.1 mM BPS	SSCX-B	8.36	0.4	0.2	0.9	42	0	0	6	26	0.3	0.6	76		27