lac-1 and lag-1 with ras-1 affect aging and the biological clock in Neurospora crassa

John K. Brunson¹ | James Griffith²,³ | Daneisha Bowles³ | Mary E. Case³ | Jonathan Arnold³

¹Center for Marine Biotechnology and Biomedicine, J. Craig Venter Institute—West Coast Campus, University of California San Diego Scripps Institution of Oceanography, La Jolla, CA, USA
²College of Agricultural and Environmental Sciences, University of Georgia, Athens, GA, USA
³Genetics Department University of Georgia, Athens, GA, USA

Correspondence
Jonathan Arnold, Genetics Department University of Georgia, Athens, GA, USA. Email: arnold@uga.edu

Abstract
Using an automated cell counting technique developed previously (Case et al., Ecology and Evolution 2014; 4: 3494), we explore the lifespan effects of lac-1, a ceramide synthase gene paralogous to lag-1 in Neurospora crassa in conjunction with the band bd (ras-1) gene. We find that the replicative lifespan of a lac-1KO bd double mutants is short, about one race tube cycle, and this double mutant lacks a strong ~21-hr clock cycle as shown by race tube and fluorometer analysis of fluorescent strains including lac-1KO. This short replicative lifespan phenotype is contrasted with a very long estimated chronological lifespan for lac-1KO bd double mutants from 247 to 462 days based on our regression analyses on log viability, and for the single mutant lac-1KO, 161 days. Both of these estimated lifespans are much higher than that of previously studied WT and bd single mutant strains. In a lac-1 rescue and induction experiment, the expression of lac-1+ as driven by a quinic acid-dependent promoter actually decreases the median chronological lifespan of cells down to only 7 days, much lower than the 34-day median lifespan found in control bd conidia also grown on quinic acid media, which we interpret as an effect of balancing selection acting on ceramide levels based on previous findings from the literature. Prior work has shown phytoceramides can act as a signal for apoptosis in stressed N. crassa cells. To test this hypothesis of balancing selection on phytoceramide levels, we examine the viability of WT, lag-1KO bd, and lac-1KO bd strains following the dual stresses of heat and glycolysis inhibition, along with phytoceramide treatments of different dosages. We find that the phytoceramide dosage–response curve is altered in the lag-1KO bd mutant, but not in the lac-1KO bd mutant. We conclude that phytoceramide production is responsible for the previously reported longevity effects in the lag-1KO bd mutant, but a different ceramide may be responsible for the longevity effect observed in the lac-1KO bd mutant.

KEYWORDS
aging, balancing selection, biological clock, ceramide, circadian rhythms, lac-1, lag-1, Neurospora crassa
1 | INTRODUCTION

Our previous publication revealed that the longevity assurance gene (lag-1), a gene encoding a ceramide synthase, not only is a longevity gene but also exerts control over the biological clock in the model organism *Neurospora crassa* (Case et al., 2014). Circadian rhythms and biological clocks play important roles in diverse cellular processes across the tree of life (Bell-Pederson et al. 2005). Recently, transcriptomics studies screening a wide variety of marine bacteria have demonstrated widespread diel synchronization of the biota inhabiting the world's oceans (Oetson et al., 2014). At the organismal level, transcriptomic studies using microarray technologies have shown that nearly twenty-five percent of the genes in the *N. crassa* have a circadian response, revealing that the biological clock has evolved as an important mechanism for controlling many biochemical processes (Dong et al., 2008).

These same transcriptional studies revealed a transient response in transcription levels of lag-1 under knockdown of white collar-1 (wc-1), an important *N. crassa* clock gene (Case et al., 2014). These findings spurred our interest in studying the longevity and clock effects of lag-1 knockouts, along with our current study on knockouts of the lag-1 paralog, longevity assurance cognate-1 (lac-1).

Ceramides represent a family of diverse yet closely related signaling sphingolipids with over 200 structural variants found in mammals alone (Hannun & Obeid, 2011). Phytochemical is one of the two major classes of ceramides found from fungal populations; the other class includes the monohexose-modified ceramides (Merrill, 2002) (Warnecke & Heinz, 2003). Phytochemical has been demonstrated to play an important role in mediating and possibly decreasing instances of stress-induced cell death, but the decrease in stress-related cell death only occurs at specific dosages, beyond which phytochemical can have a lethal effect (Plesofsky, Lavery, Castle, & Brambl, 2008). This is partial evidence for balancing selection on a quantitative trait, namely the level of phytochemical synthesis. The evolution of balancing selection on quantitative traits is an extensive subdiscipline unto itself and may provide a useful framework to examine the evolved thresholds of phytochemical synthesis (Schulter, 1988) (Stinchcombe, Agrawal, Hohenlohe, Arnold, & Blows, 2008) (Lerner, 1954).

Both lag-1 and lac-1 have been demonstrated to play important roles in ceramide synthesis in yeast, acting as components of ceramide synthases responsible for converting dihydrosphingosine or phytosphingosine into ceramides (Guillas et al., 2001). From our recent publication, we found that a lag-1 knockout combined with a band (bd) mutation in *N. crassa* (more recently identified with the mammalian protooncogene ras-1, Belden et al., 2007) seemed to halt the proper functioning of the clock, as revealed by race tube experiments and 48 hour expression profiling of the *frq* clock oscillator (Case et al., 2014). However, this is not the first known instance of a gene implicated in lipid metabolism exerting a unique effect on clock function. For example, mutants in the lipid metabolism genes, chain elongation (cel) and *choline requirer* (chol-1), have both been shown to create lipid deficiencies and subsequently maintain circadian rhythms in double mutants, such as chol-1, wc-1 lacking a critical clock gene (e.g., wc-1) in *N. crassa* (Lakin-Thomas & Brody, 2000). Subsequent work using a luciferase (luc) reporter under the control of *frq* promoter (frqP) found 22-hr periodicity for the luciferase tracings in the bd, chol-1, csp-1, frqP: luc mutant under choline starvation while the banding pattern displayed a long period characteristic of chol-1 (Shi, Larrondo, Loros, & Dunlap, 2007). This raised the question of whether or not the metabolic oscillator tied to chol-1 might be independent of the FRQ-WCC oscillator. With regard to viability, a double knockout in LAG-1 and LAC-1 has been demonstrated to cause lethality in yeast. However, the same study found that expression of LAG-1, and therefore the rate of sphingolipid and ceramide metabolism, has a nuanced effect on yeast longevity. A moderate increase in LAG-1 expression increases longevity, but too much LAG-1 transcript has a negative effect on viability (Jiang, Kirchman, Allen, & Jazwinski, 2004). This is further evidence for balancing selection on ceramide synthesis. However, the lipid metabolism hypothesis for the link between aging and the clock is not the only model in existence. Other models have demonstrated a possible role played by reactive oxygen species in both aging and clock function (Gyongyosi and Kaldi 2013) and more recently in the Ras-Erk-ETS signaling pathway (Slack et al., 2015). A variety of metabolic linkages may exist between the clock and aging (Judge, Griffith, & Arnold, 2017). The lag-1 gene is particularly interesting because it seems to affect directly both aging and the clock, suggesting a possible connection between the two processes.

We are therefore interested to see whether the lac-1 gene has similar effects on longevity or the clock to those exerted by lag-1. Aging as defined from an evolutionary perspective is the persistent decline in “fitness components due to internal physiological deterioration” (Rose, 1991). Do conidia age? If they were dormant, one might argue they cannot age. Recent single cell work, however, reveals that conidia are metabolically active and have a working single cell oscillator (Deng et al., 2014, 2016). They are not dormant. The internal physiological deterioration of conidia under stress has been linked to the apoptotic signal of phytochemicals in *N. crassa*, a metabolic product of lac-1 (Plesofsky et al., 2008). We will measure a fitness component through viability curves. It is then reasonable to presume that conidia do age, if viability curves of conidia decline much as those do for *S. cerevisiae* cells.

Lifespan can be measured in two ways (Bitterman, Medvedik, & Sinclair, 2003; Case et al., 2014). Replicative lifespan is simply how many serial transfers from one race tube to the next can be carried out. Chronological lifespan is how long a conidium lives in a particular medium. We have been using an automated cell counting method in order to assess conidial chronological lifespan in *N. crassa* mutants as an alternative to the traditional plating method used for over 50 years (Berkes, Chan, Wilkinson, & Paradis, 2012). This automated cell counting method is less laborious and more accurate than previously used methods that involve plating conidial suspensions, counting colonies, and then estimating the total number of viable cells per ml in the solution based on the number of colonies formed on plates (Munkres & Furtek, 1984b). Estimates of chronological lifespan can be extracted from measurements of conidial viability (Table 2) (Munkres & Furtek, 1984a). Based on our comparison of the two methods, we find that the automated cell counting method is five times more precise than the plating method in estimating viability (Case et al., 2014).
Here, we further evaluate the recently developed automated cell counting method by testing the single gene effect of the ceramide synthase encoding gene lac-1 using a quinic acid-inducible promoter. We also use race tubes and an mCherry fluorescent reporter downstream of a clock-controlled gene promoter to explore the replicative lifespan and clock phenotypes of the lac-1KO bd strain. We utilize a regression model for determining the significance of the effect lac-1 induction has on chronological lifespan. Finally, we examine a dosage gradient of the sphingolipid biosynthesis links aging and circadian rhythms in a common stress response (Case et al., 2014).

### 2 | MATERIALS AND METHODS

#### 2.1 | Strains and media

The strains used in our chronological lifespan and replicative lifespan studies include wild-type WT OR74A (FGSC 987), lac-1KOa (NCU 02468 and FGSC 13903, Colot et al., 2006), frqKO A (FGSC 15070), lac-1KO (PB66), lac-1KO bd (PB42), lac-1KO bd his-3 (PB47), bd A (FGSC1858), and lac-1KO bd (PB20i) with a functional copy of lac-1 under the control of a quinic acid-dependent promoter (Table 1). Strains PB66, PB42, and PB47 were all generated from a genetic cross between MFNC9 (FGSC 10626, Castro-Longoria, Ferry, Bartnicki-Garcia, Hasty, & Brody, 2010), MFNC9 × 87-84-10A, and strain PB20i was created through a spheroplast transformation of MFNC9 (PB40). Strain PB40 was created from a cross between MFNC9 and knockout of frq, frqKOa, (FGSC13903) and 87-84-10A (PB42). Strain PB40 was created from a cross between MFNC9 and knockout of frq, frqKOa, (FGSC13903) and 87-84-10A (PB42). The use of fluorescent recorders (mCherry) to assay the effect of lac-1 on the clock fluorescent strains used in this study include MFNC9 (FGSC 10626, Castro-Longoria, Ferry, Bartnicki-Garcia, Hasty, & Brody, 2010), frqKO A (FGSC 15070), lac-1KO (PB66), lac-1KO bd (PB42), lac-1KO bd his-3 (PB47), bd A (FGSC1858), and lac-1KO bd (PB20i) with a functional copy of lac-1 under the control of a quinic acid-dependent promoter (Table 1).

Fluorescent strains used in this study include MFNC9 (FGSC 10626, Castro-Longoria, Ferry, Bartnicki-Garcia, Hasty, & Brody, 2010), frq51, and lac-1KO, MFNC9 (PB40). Strain PB40 was created from a cross between MFNC9 and lac-1KOa. The frqKO, MFNC9 (frq51) strain was created by a cross between MFNC9 and knockout of frequency (frq), frqKOa.

Media used for these experiments include 1.5% glucose media, 0.001 M quinic acid media, corn meal agar media for performing crosses, minimal carbon media for fluorescence experiments (3% sorbose, 1 M sorbitol, 0.0125% glucose, 0.0125% fructose, 1× Vogels), and sorbose + fructose + glucose (SFG) media for plating spores from crosses (Davis & de Serres, 1970).

#### 2.2 | Replicative lifespan: race tube experiments

All race tube media were prepared as Vogel’s + 1.5% agar + 0.5% arginine using either 0.001 M QA or 1.5% glucose as the carbon source. The carbon source QA was chosen to mimic better natural conditions. Initial race tubes were started by adding 20 μl of ~107 cells/ml conidial solution and filtered through cotton. The growth fronts in the tubes were marked at the same time every day under red light. Once the samples had grown to the end of the tube, their pictures were taken and digitized. In addition, the daily growth fronts and any bands in the samples had grown to the end of the tube, their pictures were taken and digitized. In addition, the daily growth fronts and any banding activity were measured relative to the point of inoculation and entered into a FORTRAN-IV program to calculate growth rate, period, and phase shift data (Dharmananda, 1980).

#### 2.3 | The use of fluorescent recorders (mCherry) to assay the effect of lac-1 on the clock

Fluorescent mCherry strains (MFNC9; lac-1KO, MFNC9 (PB40); frqKO, MFNC9 (frq51)) were grown on 1.5% glucose media for 72 hr at 25°C in the dark and were allowed to conidiate on the benchtop for an

| Strain | Genotype | FGSC no. | Genetic background |
|--------|----------|----------|-------------------|
| WT OR74A | WT A | 987 | NA |
| lac-1KOa | lac-1KO a | 13903 | NA |
| frqKO A | frqKOa | 15070 | NA |
| 87-84-10A | bd his-3 A | NA | NA |
| bd A | bd A | 1858 | NA |
| PB66 | lac-1KO | NA | lac-1KOa × 87-84-10A |
| PB42 | lac-1KO bd | NA | lac-1KOa × 87-84-10A |
| PB47 | lac-1KO bd his-3 | NA | lac-1KOa × 87-84-10A |
| PB20i | lac-1KO bd (qa-2; lac-1*) | NA | PB47 (transformed) × 87-84-10A |
| MFNC9 | ccg-2p:mCherry | 10626 | NA |
| PB40 | lac-1KO ccg-2p:mCherry | NA | MFNC9 × lac-1KOa |
| frq51 | frqKO ccg-2p:mCherry | NA | MFNC9 × frqKOa |

| Strain | WT | lac-1KO | lac-1KO.bd |
|--------|----|---------|------------|
| Death rate (b) | -0.0258 | -0.0043 | -0.0015 |
| Standard error (SE) | 0.0035 | 0.0035 | 0.0035 |
| Expected average lifespan (~1/b) | 38.75969 | 232.5581 | 663.6667 |
| Expected median lifespan (~ln 2)/b | 26.86617 | 161.197 | 462.0981 |
additional 96 hrs. Strains were then transferred to minimal media (1.5% sorbose + 0.001 M quinic acid in 1× Vogels media, filter-sterilized) and filtered through cotton to select conidia. Concentration was adjusted to 5–6 × 10⁶ cells/ml when possible. 200 μl of cellular suspension was added to each well in the 96-well deep-well plate (Marsh Biomedical Products, Inc., Rochester, NY, USA), which was then allowed to synchronize under light at room temperature (−25°C) for 26 hr. The loaded, light-synchronized cells inserted into the fluorometer (DTX 800/88 Beckman Coulter, Inc., Fullerton, CA 92834, USA), and an experiment was run in the dark for 48 hr with measurements being taken every 30 min using 535-nm excitation and 625-nm emission filter using the fluorescence plate reader. All fluorescent strains were analyzed in triplicate.

Data analysis for the fluorometry experiments was performed by averaging fluorescent intensity across each triplicate set for each time point. These averages were then graphed to represent the entire 48-hour profile for each strain. In all cases, a detrending was required to remove the effects of conidial growth from the fluorometry profile in order to make possible a spectral analysis (Bloomfield, 1976). For example, a periodogram presumes the underlying time series is stationary. There are a variety of standard ways for detrending circadian rhythm data (e.g., Izumo, Sato, Straume, & Johnson, 2006), but since the growth is nearly perfectly linear (see Section 3), the results are not going to differ from the simplest approach of subtracting a trend line. We have calculated the effects of detrending on the periodogram (Deng et al., 2016). This detrended data set was then subjected to spectral decomposition and a Fisher periodicity test for departures from white noise (Bloomfield, 1976).

### 2.4 Cellometer experiments for measuring conidial chronological lifespan

Cellometer experiments using the Cellometer Auto2000 (Nexclom Lawrence, MA, USA 01843) were performed using the protocol and settings for counting macroconidia only as described in the previous publication for counting macroconidia only (Case et al., 2014). Inducible strains were either grown on quinic acid agar media or on 1.5% glucose agar media for two days (30°C in the dark) and then allowed to conidiate overnight under a light at room temperature (−25°C). Conidial suspensions were created by washing cultures with sterile ddH₂O and were equilibrated to ~3.0 × 10⁶ cells/ml using the cellometer. The suspensions were then kept in the dark for the length of the experiment.

Statistical analysis of the cellometer experiments was conducted using linear models applied to log viabilities described in the previous publication (Case et al., 2014). A separate model was utilized to analyze the effect of lac-1 induction using the quinic acid-dependent promoter. The model for viability as a function of the media and strain is a minor modification of a linear model described previously (Case et al., 2014) to accommodate a slightly different experimental design. The framework of the general linear model has been extensively used to analyze selection components (Endler, 1986). In 1990, we created a more flexible and general framework for selection component analysis known as the generalized linear model for selection component experiments (Williams, Anderson, & Arnold, 1990), which included such classic analyses as those of Christiansen and Frydenberg (1976) using mother–offspring combinations and the general linear model (Endler, 1986). The advantage of this generalized linear model is being able to test a sequence of nested hypotheses about selection (Christiansen & Frydenberg, 1976; see Tables 3 and 4), solutions to overdispersion in viability counts, and resistant fitting methods (Williams et al., 1990).

Here, we use a special case of the generalized linear model. The survivorship counts of cells are large (~10³–10⁴) so that the viability estimates are likely to be normal by the Central Limit Theorem. As a result, a simple regression is likely to approximate a life-table analysis, and in our past experience it has worked (Case et al., 2014). For strain i at time X_{ijk} for day j on media k, the model is for the log viability (Y_{ijk}):

$$Y_{ijk} = \beta_{ik}X_{ijk} + \epsilon_{ijk}$$

where ɛ_{ijk} is the measurement error. The measurement errors are assumed to be independently and identically distributed with a normal distribution having mean of 0 and variance σ². The regression coefficients β_{ik} capture the linear relationship of log viability to time X_{ijk} for the ith strain in media k. The fitting of this model and the analysis of variance (ANOVA) used to test nested hypotheses about selection are described previously (Case et al., 2014).

The model for the experiment without a media variable is also a linear model of the same form, removing the media factor, and letting i = 1, 2, or 3 to index strains compared.

### 2.5 Plasmid construction

Plasmids were constructed using pDE3dBHqα-2 as a template (Cheng, Yang, & Liu, 2001) in Escherichia coli DH10α. The plasmid pDE3dBHqα-2 contains a carbamicillin resistance cassette, a functional copy of

| Description | Degrees of freedom (df) | Sums of squares (SS) | Estimated mean square (EMS) | F | Significance probability (p) |
|-------------|------------------------|----------------------|-----------------------------|---|----------------------------|
| Between strains versus one viability | 2 | 0.0493 | 0.0247 | 13.61 | <.005 |
| Viability | 1 | 0.0466 | 0.0466 | 25.67 | <.001 |
| Error | 20 | 0.0363 | 0.0018 |
| Corrected total | 23 | 0.1322 | |

Analysis of variance (ANOVA) of the log viability regression model for the three strains in Table 2, described in Section 2.
the histidine-3 (his-3) gene, and a quinic acid-inducible promoter. The gene lac-1<sup>+</sup> was amplified from genomic OR74A. N. crassa DNA using primers that would incorporate an EcoRI restriction site at the 5′ end of the amplicon and a Clai restriction site at the 3′ end of the amplicon for each gene target. We treated the plasmid and the amplicons with a double digest of EcoRI and Clai restriction enzymes (FastDigest, Thermo Fisher Scientific, Inc. Waltham, MA, USA 02451). Following ligation of the amplicons with the linearized plasmids using T4 DNA Ligase (New England Biolabs, Inc. Ipswich, MA USA), we transformed chemically competent E. coli cells using the newly constructed plasmids using standard protocol. Transformed cells were plated on carbenicillin-containing LB agar and incubated overnight at 37°C. Resulting colonies were then grown up for an additional 24 hours in carbenicillin-containing LB liquid media. Plasmids were extracted using the High-Pure Plasmid Isolation Kit (Roche, Inc. Indianapolis, IN, USA) and were confirmed to contain lac-1 by sequencing the region around the insertion. This yielded the plasmid: pDE3dBHqa-2: lac-1<sup>+</sup>.

### 2.6 Creation of inducible strains

The new plasmid pDE3dBHqa-2: lac-1<sup>+</sup> was transformed into PB47 (lac-1<sup>KO</sup> bd his-3) using a spheroplasting method (Case, Schweizer, Kushner, & Giles, 1979). The resulting transformant was made homokaryotic by crossing to 87-84-10A (bd his-3). PB20i was selected among the progeny of this cross.

### 2.7 qRT-PCR

To analyze the effects of quinic acid induction on the quinic acid-dependent promoter in the inducible strain PB20i, we grew two liquid cultures of PB20i and two liquid cultures of WT OR74A in 1.5% glucose growth media for 48 hours in the dark (25°C, 200 rpm). The fungal tissue was isolated from the liquid media using vacuum filtration followed by rinsing with sterile ddH<sub>2</sub>O. PB20i and the WT strain were then either transferred to quinic acid liquid media or returned to the 25°C shaker to grow for another 4 hr at 200 rpm. After 4 hr, the tissue was isolated from growth media using vacuum filtration and ddH<sub>2</sub>O rinsing, dried, and then frozen in a −80°C freezer. The tissue was then ground in liquid nitrogen, and mRNA was extracted using the Spectrum Plant Total RNA kit 50 (Sigma Aldrich, Inc. St. Louis, MO, USA). Synthesis of cDNA from the extracted mRNA was performed using a SuperScript III 1st Strand cDNA Synthesis Kit (Invitrogen, Inc. Grand Island, NY USA 18080-051).

Reverse transcriptase quantitative PCR (RT-qPCR) was then performed on the cDNA samples in triplicate using the ABI-Prism 7500 with Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent Technologies, Santa Clara, CA, 95051). We used primers designed to detect lac-1<sup>+</sup>, as well as ubiquitin primers as an endogenous control (primers are in Brunson, 2015; Table 4). The relative quantity (RQ) of lac-1<sup>+</sup> mRNA was measured relative to the endogenous control and the WT sample grown in quinic acid media for 4 hours. We measure the transcription of ubiquitin as an endogenous control to determine the relative quantification of transcripts expressed by the cell (Al-Omari et al., 2015).

### 2.8 Phytoceramide dosage-dependent stress-response assay

We used a protocol we have modified from a previous study on ceramide stress signaling in N. crassa (Plesofsky et al., 2008). We have modified the protocol to test conidial response to an increasing gradient of phytoceramide in WT, lag-1<sup>KO</sup> bd (MC31), and lac-1<sup>KO</sup> bd (PB42) strains of N. crassa grown in liquid culture.

Conidia were grown on solid 1.5% glucose agar media for 48 hr in the dark at 25°C. Cells were then shifted into the light for 24 hr to ensure conidiation. Conidia were then transferred to liquid Vogel’s medium with 0.05% glucose and were allowed to grow for 2 hr at 30°C (shaken at 200 rpm). Following the 2-hr growth phase, conidial concentration was adjusted to ~1.0 × 10<sup>6</sup> cells/ml and 2-DG was added to 0.015% from a stock solution (200 mg/ml). Concentration following the 2-hr growth period was measured using the Cellometer Auto2000 (Nexcelom). From these, 2–3 ml aliquots of conidia from each strain were taken for each phytoceramide treatment. Phytoceramide was added to the aliquots from a 20 mmol/L stock solution in ethanol, resulting in

### TABLE 4

| Description | Degrees of freedom (df) | Sums of squares (SS) | Estimated mean squares (EMS) | F | Significance probability (p) |
|-------------|------------------------|----------------------|----------------------------|---|-----------------------------|
| Difference between induced and noninduced with media effect | 1 | 0.5615 | 0.5615 | 25.18 | <.0001 |
| Media effect and construct effect | 1 | 0.0085 | 0.0085 | 0.38 | .54 |
| Media effect only | 1 | 0.0677 | 0.0677 | 3.04 | .09 |
| Viability effect only | 1 | 0.7478 | 0.7478 | 33.53 | <.0001 |
| Error | 27 | 0.6021 | 0.0223 | |
| Corrected total | 31 | 1.9876 | | |

ANOVA of the log viability regression models used to analyze the effects of inducing lac-1<sup>+</sup> transcription using the quinic acid-dependent promoter (see Section 2).
aliquots containing 0, 1, 5, 10, 20, 30, and 50 μmol/L concentrations of phytoceramide. Cells in liquid media were then transferred to a 45°C shaker (200 rpm) and grown for 44 hr. For both strains, we included an "unstressed" control grown for 44 hr at 25°C in liquid Vogel’s media containing 0.05% glucose, 0.05% fructose, and 1% sorbose.

After 44 hr, all cells were stained with propidium iodide and counted using the Cellometer Auto2000 protocol described in previous publication (Case et al., 2014).

3 | RESULTS

3.1 | Phenotypes of lac-1KObd

The double mutant lac-1KO bd exhibits very slow growth on race tubes, regardless of whether quinic acid or glucose media was used. Average growth rates of 4.29 and 5.202 mm/day were observed for strains grown on glucose and quinic acid, respectively. Interestingly, the lac-1KO bd strain exhibited signs of short replicative lifespan, such as decreased growth rate, by the end of the first race tube cycle in both the initial banding phenotyping experiment and the subsequent growth rate experiment (Figure 1a,b). In contrast, the control bd strain (Figure 1a) has no difficulty reaching the end of the tube in 7 days (with the time between bands being about 22 hr).

We also used race tubes to get an initial phenotype of clock function from the lac-1KO bd strain. Although the initial banding phenotyping experiment suggests that the double mutant does have some sort of regular clock oscillations (Figure 1a), subsequent experiments revealed a very irregular banding pattern with periods ranging from 67.6 to 90.9 hr with an average of 82 hr on a different carbon source (Figure 1b). This may represent a highly senescent clock phenotype, in which replicative lifespan is no more than one race tube transfer. Other examples include strains with Kalilo elements in N. crassa (Griffiths, 1992) and strains of Podospora anserina (Osiewacz, 2002).

We have observed two altered banding phenotypes associated with lag-1KO bd double mutants, suggesting disrupted clock function. In addition to the previously described “no banding” phenotype observed in the MC31 strain of lag-1KO bd, we have since found a “double banding” phenotype that we have recently confirmed in two separate strains of lag-1KO bd (Case et al., 2014) (Figure 1c).

We also examined by fluorometry the expression of clock-controlled gene-2 (ccg-2) in a lac-1KO background over 48 hr (Figure 1d) with both positive (MFNC9) and negative controls (WT, frequency (fra) deletion with mCherry recorder under the control of a ccg-2 promoter (fraq51)) all on the same plate. The gene ccg-2 was selected because it is one of the best characterized outputs of the clock (Bell-Pedersen, Dunlap, & Loros, 1992) and has high expression (Castro-Longoria et al., 2010; Dong et al., 2008). This ccg-2 promoter region has been shown to have two upstream promoter elements, one region associated with clock function and another region associated with developmental regulation (Bell-Pedersen, Dunlap, & Loros, 1996). The reference strain (MFNC9) was engineered to lack much of the promoter element associated with developmental regulation (Castro-Longoria et al., 2010). We verified this by sequencing the ends of the ccg-2 promoter region from both ends. As a consequence, MFNC9 is engineered only to give a readout from the clock.

For WT, the mean fluorescence (over three replicates, see Section 2) never exceeded 200,000 fluorescence counts, while the lac-1 (PB40) mutant’s mean fluorescence never dropped below 250,000. The series in Figure 1d were detrended with an R^2 of 0.58 for MFNC9 and R^2 > 0.95 for remaining strains (See Section 2). The positive control (MFNC9) shows pronounced periodicity of at least 21 hr following spectral decomposition, but the remaining mutants showed no such periodicity. (The positive control was replicated in triplicate on the luminometer, and fluorescent counts displayed a strong peak in the periodogram at 22.5 hr.) For example, if one were to fit a sinusoid, A cos(ωt + ϕ), where A is amplitude, ω is frequency, and ϕ is phase,

![FIGURE 1](image-url)  The double mutant lac-1KObd affects replicative lifespan and clock function as assayed through a ccg-2 promoter with a downstream fluorescent reporter gene. (a) Initial banding phenotyping of lac-1KObd, grown on glucose with a bd (FGSC 1858) control showing banding and growth to end of race tube. (b) Subsequent growth rate experiment of lac-1KObd, grown on quinic acid. (c) Various phenotypes of lag-1KObd, grown on quinic acid. One of the double bands is marked with an arrow. (d) Loss of clock function of lac-1KO as assayed through a fluorescent recorder downstream of ccg-2 promoter. Periods (±2 SE) of MFNC9, WT, FRO51, LAC40, and LAC36 were estimated by fitting a sinusoid A cos(ωt + ϕ) and found to be 32 ± 2, 41 ± 8 hr, 41 ± 2, 41 ± 2, 20 ± 2, and 20 ± 2. The period of replicate 2 of MFNC9 was 28 ± 2 hr. Race tubes used glucose (GL) or quinic acid (QA) as described in Section 2.
as a function of time \( t \), to the fluorescent time series on WT or FRQ51 as in Correa et al. (2003), one would obtain an extremely long period estimate of 41 hr with a standard error of 4 and 1, respectively. Such a long period is indistinguishable from a trend and hence indicative of loss of periodicity. For example, the lac-1 (PB40) mutant behaved like a frq knockout with the fluorescent ccg-2 recorder or the WT without any recorder. The amplitude of the fluctuations for lac-1 is similar in magnitude to that of WT (Figure 1d). So, even if lac-1 were periodic, it would do so with much reduced amplitude relative to the reference magnitude to that of WT (Figure 1d). So, even if a recorder. The amplitude of the fluctuations for

### 3.2 Chronic lifespan of lac-1KObd: single gene effect

After examining the replicative lifespan and clock phenotypes of lac-1KO bd, we wanted to see how the mutations affected the viability of individual conidia. The viability of the strain was assayed with the Cellometer Auto2000 (Nexcelom) (see Section 2). We compared the viabilities of the lac-1KO bd strain (PB42) to WT (OR74A) and the single mutant lac-1KO (PB66) over the course of a 7-day experiment with viability readings taken on the cellometer once each day at the same time of day (Figure 2).

Statistical regression analysis of the viability curves in Figure 2 revealed the average and median lifespans reported in Table 2. The expected median lifespan for WT N. crassa was 26.87 days, similar to a previously reported observation of 24 days (Munkres & Furtek, 1984a). However, the expected median lifespan for the lac-1KO single mutant was much higher at 161.2 days, and the expected median lifespan for the double mutant was even longer at 462.1 days. We confirmed that cells in water at room temperature were still viable after 60 days. This indicates an increase in viability and in chronological lifespan, which stands in contrast with the short replicative lifespan observed in the race tubes. An ANOVA for testing viability differences between these three strains is summarized in Table 3.

### 3.3 Induction experiment

To verify the phenotypic effect of lac-1 knockout on chronological lifespan, we cloned the lac-1KO gene from WT OR74A genomic DNA and placed it in under the control of a quinic acid-dependent promoter located on the plasmid pDE3dBHqa-2 (Cheng et al., 2001). We then transformed this plasmid into a lac-1KO bd his-3 strain and crossed with a bd his-3 strain to make the transformant homokaryotic. We tested the inducible promoters in the progeny by performing qRT-PCR on WT strains and construct-containing transformants following growth on either glucose media or quinic acid media (Figure 3). Expression of lac-1+, transcript was two orders of magnitude higher in the WT strain grown on quinic acid than it was on glucose, which was not surprising considering that quinic acid is a limited carbon source and a starvation response may repress the expression of certain genes (data not shown). However, we were able to detect an induction effect in the progeny strain PB20i, noting that the level of transcription as driven by the quinic acid promoter is not quite as high as the transcription being driven by the natural promoter of lac-1 as seen in the WT strain, yet still higher than the noninduced knockout as grown on glucose.

We then performed a viability experiment using the Cellometer Auto2000 (Nexcelom) on the bd strain (FGSC 1858A) and the inducible progeny PB20i, both strains grown on glucose and quinic acid for a total of four treatments (Figure 3). Selecting the bd strain as the control was made because it is in the double mutant lac-1, bd that there is the most pronounced viability effect. To analyze this data, we used a modified version of the linear model described in the Section 2 to account for media effect, construct effect, and the effect of inducing transcription at the quinic acid-dependent promoter. The analysis is summarized in Table 4.

![Figure 2](image_url) The double mutant lac-1KObd substantially increases chronological lifespan relative to wild type (WT). Viabilities of WT, lac-1KO, and lac-1KO bd strains are presented as a function of time, yielding chronological lifespan estimates (Table 2). Viability is measured as a proportion of living cells to total cells, with dead cells counted using propidium iodide fluorescence measured on the Cellometer Auto2000 (Nexcelom).
We found that induction of lac-1 had a highly significant effect on viability (p < .0001), while the introduction of the construct from the earlier transformation did not have a significant effect (p = .54). We would expect a significant construct effect if the plasmid had integrated into an important gene or region of the genome, but this does not seem to be the case for the transformation of pDE3dBHqa-2: lac-1 into lac-1KO bd his-3. The media effect alone was just barely insignificant (p = .09), and in a similar experiment on an inducible lag-1 strain the media effect was not significant (unpublished results).

It is worth noting that the experiments summarized in both Tables 3 and 4 have R² values comparable to chronological lifespan analyses on Cellometer data from our previous publication (Case et al., 2014).

Our estimated death rates revealed a strong decrease in viability after the induction of lac-1 using the quinic acid-dependent promoter (Table 5). The inducible strain PB20i had a very similar viability to the lac-1KO bd strain previously analyzed (Tables 2 and 5), confirming the viability effect of the double mutant, but induction of lac-1 shortened the estimated median chronological lifespan to just 7.5 days. While there may be other explanations for this sharp decrease in viability, such as interference between the induced lac-1 transcript and other transcriptional units, there is interesting evidence that the expression of ceramide synthases, such as lag-1 or lac-1, and the level of phytoceramide production may have been optimized by natural selection to a specific level over time, and any other level of ceramide production may be deleterious (Plesofsky et al., 2008). Jiang et al., 2004.

### 3.4 Ceramide experiment

Our findings from the lac-1 inducible experiment prompted us to explore the effects of phytoceramide dosage on WT, the lac-1KO bd double mutant PB42, and a lag-1KO bd double mutant (MC31) described in our previous publication (Case et al., 2014). Previous studies have suggested that phytoceramide is expressed at an optimum level within the conidia of *N. crassa* (Plesofsky et al., 2008). We predict that the optimum level of phytoceramide production shifts based on the activity of phytoceramide-producing genes. In the event that genes responsible for the synthesis of phytoceramide are knocked out, the optimum conidial viability should shift to a higher dosage of phytoceramides, since phytoceramide is being produced at a lower level, or not at all, within the ceramide synthase knockout mutants. To test this hypothesis, we designed an experiment akin to a previous study on the effects of phytoceramide dosage and stress response, except here we have incorporated the use of the Cellometer Auto2000 for viability measurements instead of using standard plating protocol (Plesofsky et al., 2008). Results of this experiment are summarized in Figure 4.

While all three genotypes have absolute maximum viabilities at ~10 μmol/L phytoceramide, the lag-1KO bd double mutant approaches a second maximum at a phytoceramide concentration of ~30 μmol/L. This dosage effect represents interesting additional evidence for balancing...
selection acting on phytoceramide concentration, since a stabilizing effect can be witnessed even in the absence of an important ceramide synthase and is distinguishable from the stabilizing effect observed in a WT strain treated with the same dosage gradient of phytoceramide. The dosage–response curve lac-1KO bd double mutant closely mirrors the curve for the WT strain, so we cannot conclude that the absence or lack of phytoceramide production is creating the longevity effect observed in lac-1KO bd mutant. The dosage response of lac-1KO bd, for example, is:

\[ \text{Log viability} = 53.52(\pm 1.5807) + 8.1198(\pm 4.298)X - 4463(\pm 0.0236)X^2 \]

+ .0057(\pm 0.003)X^3 (with \( R^2 > .99 \)),

where \( X \) is the phytoceramide concentration. The fit is nearly perfect, and all of the regression coefficients (e.g., -0.4463) on phytoceramide concentration and its powers are highly significant. The intercepts of the other two dosage–response curves are similar in shape, but the intercepts are quite different with WT and lag-1KO bd being 4.1030 ± 0.0985 and 64.57 ± 7.86, respectively. So, clearly the mutant curves are significantly different from WT, although they mirror each other.

4 | DISCUSSION

Case et al. (2014) provided one line of evidence that aging and circadian rhythms as stress responses are linked through \( lag-1 \); its homolog \( LAG1 \) in \( S. \) \textit{cerevisiae} was the first longevity gene to be characterized in yeast (D’mello et al., 1994). The homologs to \( LAG1 \) in \textit{Caenorhabditis elegans}, \textit{Homo sapiens}, and \textit{N. crassa} were later shown to complement \( LAG1 \) (Case et al., 2014; Jiang, Kirchman, Zagulski, Hunt, & Jazwinski, 1998), and so \( LAG1 \) is likely to have a highly conserved role in aging in eukaryotes. The double mutant, \( LAG1, LAC1 \), appears not to produce any sphingolipids, such as ceramides (Schorling, Valle, Barz, Riezman, & Oesterhelt, 2001).

Here, \( lag-1^{KO} \) \textit{bd} and \( lac-1^{KO} \) \textit{bd} have substantial effects on chronological lifespan and clock phenotype in \textit{N. crassa}, consistent with the hypothesis that sphingolipid metabolism links aging and circadian rhythms in a common stress response. In both genotypes, we find that chronological lifespan is significantly increased versus the wild type as measured using automated cell counting protocol (Berkes et al., 2012). Unexpectedly, the \( lac-1^{KO} \) \textit{bd} double mutant seems to increase chronological lifespan (Table 2), while at the same time decreasing replicative lifespan (Figure 1). The \( lag-1^{KO} \) \textit{bd} double mutant has similar phenotypes with regards to chronological and replicative lifespan (Case et al., 2014). This is not without precedent. The \( RAS1 \) paralog, \( RAS2 \), in \textit{S. cerevisiae} also simultaneously increases chronological lifespan and decreases replicative lifespan (Fabrizio et al., 2003; Sun, Kale, Childress, Pinswasdi, & Jazwinski, 1994). Chronological aging and replicative aging, however, are not the same traits. In fact, in \textit{S. cerevisiae} they appear to be under the control of distinct suites of genes (Stumpferl et al., 2012) with a handful of genes affecting both (Fabrizio et al., 2003; Sun et al., 1994).

We also conclude that the concentration of phytoceramide is responsible, at least in part, for the altered longevity phenotype in the \( lag-1^{KO} \) \textit{bd} strain. In the WT control strain, we observe a different optimal concentration than what has been reported previously (Plesofsky et al., 2008). This is not unexpected, since phytoceramide is being administered in two different ways in the two experiments: In our experiment we expose conidia to phytoceramide in liquid culture as opposed to on agar plates. In addition, our protocol for estimating chronological lifespan using automated cell counting is specific to viability of macroconidia, whereas a plating method would not employ such specificity. However, regardless of the method used, we are still able to observe the optimization of phytoceramide in \textit{N. crassa} conidia, confirming previous observations as well as allowing us to recognize subtle shifts in viability profiles as a result of genetic knockouts (Figure 4). This demonstrates that our automated cell counter-based estimates of chronological lifespan could be reasonably adapted to a wide variety of assays and experiments, especially considering we have already demonstrated its accuracy in viability estimates (Case et al., 2014).
While we have not identified the specific biochemical cause of increased chronological longevity among \textit{lac-1}^{KO} bd mutants, we have shown that the phytoceramide-producing property of its homolog \textit{lag}-1 is an important contributor to the longevity effect seen in \textit{lag-1}^{KO} bd. Since \textit{lag-1}^{KO} bd also has an altered clock phenotype, phytoceramide might play a role in regulating clock function in \textit{N. crassa} (Case et al., 2014). A separate ceramide synthesized by \textit{lac-1} may account for the increased chronological lifespan of \textit{lac-1}^{KO} bd strains and could be identified using similar dosage gradients used in the phytoceramide experiments above (Figure 4).

Pinpointing the role phytoceramide might play in clock regulation will require several important considerations. For example, is cell-to-cell communication required for proper clock functioning and circadian synchronization? If this is the case, then phytoceramide or other signaling molecules may be important for clock function, and situations in which individual conidia have no other cells to communicate with may require media supplementation with the proper signaling molecule. This is a good rationale for developing microfluidics devices for measuring the clock and longevity (Deng et al., 2016). Using microfluidics, cells could be separated into droplets of media containing differing numbers of conidia. If cellular signaling plays a role in either longevity or the clock, then droplets containing one or two conidia should display longevity and clock phenotypes distinct from droplets containing many conidia. Furthermore, if phytoceramide were the compound responsible for synchronization of clock oscillation, a dosage scheme similar to the one employed in Figure 4 would prove useful in designing a microfluidics experiment for assaying the clock.

Microfluidics technologies would also solve some of the issues with our current cellometer-based longevity assays (Deng et al., 2014, 2016; Lee, Lee, & Hong, 2013). In our development of a protocol for assaying cell survivability in suspensions of approximately $3.0 \times 10^6$ cells/ml, we found that the number of viable cells decreased dramatically over the course of experimental day 0, but returned to a viability percentage consistent with a typical linear regression model by the 24-hr time point was taken (as described in Section 2). Our day zero analyses have shown that conidia producing hyphae are most likely to die at the start of the experiment, contributing significantly to the decline in overall viability of the population (unpublished data). While we have not posited a mechanism for the subsequent rise in viability leading up to day 1, we imagine that this mechanism might continue to play a role throughout the course of the experiment, especially if the rise in viability is linked to the utilization of organic matter released from dead cells. Cell-sorting microfluidics could circumvent this issue by segregating cells into droplets containing one or only a few conidia and would remove substantial noise from the data in addition to improving measurements for clock oscillations in \textit{N. crassa} mutants. These mutants could theoretically contribute either to basic clock functioning or to cell-to-cell signaling important for synchronization of individual biological clocks.

**ACKNOWLEDGMENTS**

We would like to thank Dr. David Hall for the use of the Beckman-Coulter fluorometer and for review of manuscripts. We would also like to thank Dr. Richard Meagher for various materials and advice on fluorometer and qRT-PCR experimental design. We would also like to thank Dr. Sydney Kushner and Dr. Bijoy Mohanty for assistance in troubleshooting \textit{E. coli} transformation protocols.

**CONFLICT OF INTEREST**

None declared.

**REFERENCES**

Al-Omari, A., Griffith, J., Judge, M., Taha, T., Arnold, J., & Schuttler, H.-B. (2015). Discovering regulatory network topologies using ensemble methods on GPGPUs with special reference to the biological clock of \textit{Neurospora crassa}. \textit{IEEE Access}, 3, 27–42. doi: 10.1109/ACCESS.2015.2399854

Belden, W. J., Larrondo, L. F., Froehlich, A. C., Shi, M., Chen, C. H., Horos, J. J., et al. (2007). The band mutation in \textit{Neurospora crassa} is a dominant allele of \textit{ras-1} implicating RAS signaling in circadian output. \textit{Genes & Development}, 21, 1494–1505.

Bell-Pedersen, D., Dunlap, J. C., & Horos, J. J. (1992). The \textit{Neurospora} circadian clock-controlled gene, ccg-2, is allelic to \textit{ras} and encodes a fungal hydrophobin required for formation of the conidial rosette layer. \textit{Genes & Development}, 6, 2382–2394.

Bell-Pedersen, D., Dunlap, J. C., & Horos, J. J. (1996). Distinct cis-acting elements mediate clock, light, and developmental regulation of the \textit{Neurospora} crassa \textit{ccg-2} gene. \textit{Molecular and Cellular Biology}, 16, 513–521.

Bell-Pedersen, D., Cassone, V. M., Earnest, D. J., Golden, S. S., Hardin, P. E., Thomas, T. L., Zoran, M. J. (2005). Circadian rhythms from diverse organisms. \textit{Nat. Rev. Genet.} 6 (7): 544-556.

Berkes, C. A., Chan, L. L.-Y., Wilkinson, A., & Paradis, B. (2012). Rapid quantification of pathogenic fungi by Cellometer image-based cytometry. \textit{J. Microbiol. Methods}, 91, 468–476.

Bitterman, K. J., Medvedik, O., & Sinclair, D. A. (2003). Longevity regulation in \textit{Saccharomyces cerevisiae}: Linking metabolism, genome stability, and heterochromatin. \textit{Microbiology and Molecular Biology Reviews}, 67, 376–379.

Bloomfield, P. (1976). \textit{Fourier analysis of time series}: An introduction. New York, NY: Wiley.

Brunson, J. K. (2015). \textit{Sphingolipid synthesis and the aging biological clock in \textit{Neurospora crassa}} (p. 20). University of Georgia Honors Dissertation, Athens, GA: University of Georgia.

Case, M. E., Griffith, J., Dong, W., Tigner, I. L., Gaines, K., Jiang, J. C., ... Arnold, J. (2014). The aging biological clock in \textit{Neurospora crassa}. \textit{Ecology and Evolution}, 4(17), 3494–3507.

Case, M. E., Schweizer, M., Kushner, S. R., & Giles, N. H. (1979). Efficient transformation of \textit{Neurospora crassa} by utilizing hybrid plasmid DNA. \textit{PNAS USA}, 76, 5259–5263.

Castro-Longoria, E., Ferry, M., Bartnicki-Garcia, S. N., Hasty, J., & Brody, S. (2010). Circadian rhythms in \textit{Neurospora crassa}: Dynamics of the clock component frequency visualized using a fluorescent reporter. \textit{Fungal Genetics and Biology}, 47, 332–341.

Cheng, P., Yang, Y., & Liu, Y. (2001). Interlocked feedback loops contribute to the robustness of the \textit{Neurospora} circadian clock. \textit{Proceedings of the National Academy of Sciences of the United States of America}, 98, 7408–7413.

Christiansen, F. B., & Frydenberg, O. (1976). Selection component analysis of natural polymorphisms using population samples including mother-offspring combinations. \textit{Theor. Pop. Biol.}, 4, 425–445.

Colot, H. V., Park, G., Turner, G. E., Ringelberg, C., Crew, C. M., Litvinkova, L., ... Dunlap, J. C. (2006). "A high-throughput gene knockout procedure for \textit{Neurospora} reveals functions for multiple transcription factors" (vol 103, pg 10352, 2006). \textit{Proceedings of the National Academy of Sciences of the United States of America}, 103, 10352–10357.
