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Culture Volume and Vessel Affect Long-Term Survival, Mutation Frequency, and Oxidative Stress of *Escherichia coli*

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Bacteria such as *Escherichia coli* are frequently studied during exponential- and stationary-phase growth. However, many strains can survive in long-term stationary phase (LTSP), without the addition of nutrients, from days to several years. During LTSP, cells experience a variety of stressors, including reactive oxidative species, nutrient depletion, and metabolic toxin buildup, that lead to physiological responses and changes in genetic stability. In this study, we monitored survival during LTSP, as well as reporters of genetic and physiological change, to determine how the physical environment affects *E. coli* during long-term batch culture. We demonstrate differences in yield during LTSP in cells incubated in LB medium in test tubes versus Erlenmeyer flasks, as well as growth in different volumes of medium. We determined that these differences are only partially due to differences in oxygen levels by incubating the cells in different volumes of media under anaerobic conditions. Since we hypothesized that differences in long-term survival are the result of changes in physiological outputs during the late log and early stationary phases, we monitored alkalization, mutation frequency, oxidative stress response, and glycation. Although initial cell yields are essentially equivalent under each condition tested, physiological responses vary greatly in response to culture environment. Incubation in lower-volume cultures leads to higher *oxyR* expression but lower mutation frequency and glycation levels, whereas incubation in high-volume cultures has the opposite effect. We show here that even under commonly used experimental conditions that are frequently treated as equivalent, the stresses experienced by cells can differ greatly, suggesting that culture vessel and incubation conditions should be carefully considered in the planning or analysis of experiments.

Escherichia coli can survive in batch culture for days, weeks, or even years without the addition of nutrients (1–4). While cells grown in laboratory culture are frequently examined during the first three phases of the bacterial life cycle (lag phase, exponential or logarithmic growth phase, and stationary phase), two additional phases can be observed when cells are incubated in long-term batch culture. After stationary phase, cells can enter the fourth phase, termed death phase, in which approximately 99% to 99.9% of the cells in the culture lose viability, depending on the strain and culture conditions. Cells that survive death phase may then enter long-term stationary phase (LTSP), in which the total number of cells remains roughly constant, accompanied by significant shifts in subpopulations within the culture (1, 3, 5, 6). Survival of cells better suited to their particular environment during LTSP leads to selection of beneficial alleles, called GASP (growth advantage in stationary phase) mutations, and therefore evolution of these populations (2–4, 6, 7).

Although the mechanisms controlling entry into death phase are not well understood, several stressors may contribute to cell death in long-term batch cultures. A major factor contributing to death phase is likely endogenous oxidative stress from normal cellular metabolism of growing cells (8–13). During the transition into stationary phase, oxidative stress response genes are often induced (11, 14). One mechanism by which oxidative stress contributes to cell death is the promotion of glycation, or nonenzymatic glycosylation, of proteins and nucleic acids in cells (15–18). It has been shown that death due to glycation can be abrogated by addition of the antiglycation agents carnosine and aminoguanidine (15, 19–21). Another factor modulating the survival of cells in batch culture is changes in pH (22–25). Typically, when *E. coli* is grown in Luria-Bertani (Lennox) medium (LB), cultures become slightly alkaline during log phase growth, with pH further increasing during early stationary phase. This increase in pH contributes to cell death and affects the timing of entry into long-term stationary phase (25–27).

Commonly, laboratory experiments may necessitate incubation of different volumes of cultures depending on the desired cell, protein, or plasmid yield, etc. In fact, growing cells in different culture volumes is routinely suggested as interchangeable in several commonly used reference handbooks (28–30). For example, in section 1.25 of *Molecular Cloning: a Laboratory Manual*, by Sambrook and Russell (28), it is suggested that for small-scale preparations of DNA, 2-ml cultures of *E. coli* should be grown in 15-ml test tubes (~1/10 volume), while for large-scale preparations, it is suggested in section 1.33 that cells be cultured in 525 ml of media in a 2-liter flask (~1/4 volume). In these cases, cultures may be scaled up from incubation in test tubes to flasks or even up to larger-scale bioreactors or fermentors. In fact, in common practice, the final cell yield (CFU/ml) appears to depend exclusively on the growth medium rather than the type of vessel used (e.g., 5 ml of cells grown in a test tube and 12.5 ml grown in a flask reach the same final density of $\sim 5 \times 10^9$ CFU/ml). However, in different vessels, cells likely experience different levels of oxidative or other stresses, which may lead to differences in survival and mutation frequency. We hypothesized that these differences can be measured and signatures of growth in specific environments could be revealed. Here we show that by measuring survival and physiological responses of cells grown in different vessels, while

Received 18 September 2013 **Accepted** 21 December 2013
Published ahead of print 27 December 2013
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doi:10.1128/AEM.03150-13

controlling for oxygen availability by incubating cells both in different culture volumes and anaerobically, we can identify factors affecting long-term survival.

MATERIALS AND METHODS

Bacterial strains and growth conditions. An *E. coli* K-12 lineage strain, MG1655 derivative PFM2 (31), was used in this study. Unless otherwise stated, cultures were initiated by transferring cells directly from a frozen 20% glycerol stock into 5 ml of Luria-Bertani (Lennox) medium (LB) (Difco) in an 18- by 150-mm borosilicate test tube and incubated overnight with aeration in a TC-7 rolling drum (New Brunswick Scientific, Edison, NJ) at 37°C. Cells were then diluted into LB, 1:1,000 (vol/vol), to initiate experiments. Cultures were incubated either in test tubes as described above or in 125-ml Erlenmeyer flasks on a shaking platform (200 rpm). For inoculation of cultures in multiple vessels at once, one large volume of LB was inoculated initially and then distributed into individual culture vessels.

For anaerobic growth experiments, different volumes of LB were distributed into screw-top 125-ml flasks, sealed with polypropanol caps with rubber septa (Qorpak), and then autoclaved. After sterilization, the air in the headspace of each flask was replaced with sterile nitrogen. Cells from an overnight culture were then inoculated 1:1,000 (vol/vol) by a needle through the septum. Samples for viable counts and pH analysis were removed by needle through the septum.

Monitoring of cell growth, survival, culture pH, and mutation frequency. To monitor cell growth and survival, viable cell counts were determined by serial dilution of cells sampled periodically from the cultures, followed by plating on LB agar (32). The limit of detection in all experiments was >1,000 CFU/ml (32). pH was monitored using pH paper with a range of 6.0 to 10.0 and increments of 0.3 to 0.5 pH unit (EMD Chemicals, La Jolla, CA).

Mutation frequency, as monitored by spontaneous resistance to rifampin (Rif^r), was determined by plating ~10^9 cells on LB agar supplemented with 100 μg/ml of rifampin (Sigma-Aldrich) (33). To determine mutation frequency, the number of Rif^r colonies was divided by the total number of CFU in each culture. This frequency is expressed as number of Rif^r colonies per 10^7 CFU in the culture.

β-Galactosidase assays. A plasmid expressing *lacZ* from the *E. coli oxyR* promoter, pAQ23 (31) (gift of B. Demple, Stony Brook University School of Medicine), was introduced into PFM2 by electroporation as described previously (28) and selected on LB agar plates supplemented with 150 μg/ml of ampicillin (Sigma-Aldrich). The plasmid was maintained by supplementing liquid cultures with ampicillin as appropriate. PFM2/pAQ23 (strain SF2562) was grown as described above, and cultures were sampled periodically during 1 to 4 days of incubation. β-Galactosidase activity was determined as described previously (29), but without the addition of chloroform. Briefly, 1.0 ml of cells was pelleted and resuspended in 1.0 ml of Z buffer (29). Then 100 μl of cells was mixed with 900 μl of Z buffer containing SDS at room temperature in order to lyse cells. Disrupted cells were mixed with 4 mg/ml of *o*-nitrophenyl-β-D-galactopyranoside (ONPG), incubated at room temperature for approximately 15 min, and centrifuged to remove debris. A_{420} and A_{550} readings were determined and modified Miller units were calculated based on the cell titer (CFU/ml) of each culture, rather than the optical density at 600 nm (OD_{600}); since some samples were taken post-death phase, the OD_{600} does not fully reflect the viable cell counts. Endogenous activity of LacZ in PFM2 is virtually undetectable (data not shown).

Isolation of *E. coli* protein. LB cultures of different volumes were grown for 16 to 20 h, and 1 ml from each flask was processed for protein isolation. Cells were pelleted by centrifugation at 13,000 rpm for 1 min, followed by resuspension in 10 mM Tris-HCl (pH 7.5) and 0.15 M NaCl. Cells were then sonicated, on ice, twice at the maximum setting at 1-min intervals (Bronson Scientific), followed by enzymatic digestion with DNase I (50 μg/ml), RNase A (50 μg/ml), and lysozyme (20 μg/ml) (Sigma-Aldrich) at 37°C for 1 h. Protein levels in the whole-cell lysate were quantified using the Quick Start Bradford dye reagent, as per the manufacturer's instructions (Bio-Rad).

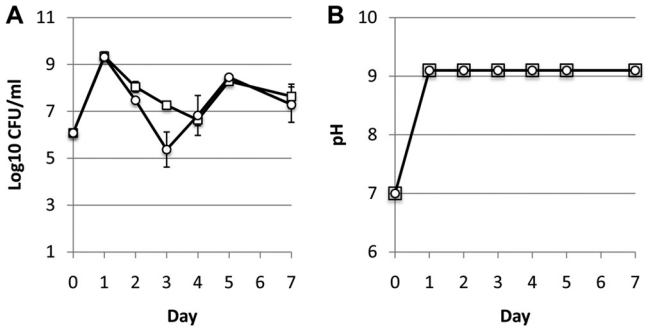

FIG 1 (A) Survival dynamics of PFM2 in tubes and flasks. Shown are growth curves of PFM2 in 5 ml in tubes (squares) and 12.5 ml in flasks (circles). Average data shown are from a representative experiment of three biological replicates. Error bars represent standard deviations. (B) pH of the same samples as in panel A.

Enzyme-linked immunosorbent assay (ELISA) to determine glycation levels. Fifty microliters of whole-cell lysate (protein concentration, 0.5 to 1.0 mg/ml) was loaded into standard polystyrene 96-well plates (Corning, Inc.) with 100 μl of sodium carbonate binding buffer and incubated overnight at 4°C. After 16 to 20 h, wells were washed twice with phosphate-buffered saline (PBS)–Tween 20 (0.05%), blocked with 200 μl of blocking solution (5% bovine serum albumin in PBS) for 1 h at 37°C, washed again three times in PBS–Tween 20, and incubated (1 h at 37°C) with 100 μl of horseradish peroxidase (HRP)-conjugated anti-carboxymethyl lysine (anti-CML) antibody (diluted 1:1,000 [vol/vol] in blocking solution) obtained from Cosmobio, Japan. After three additional washes (PBS–Tween 20) to remove excess antibody, 100 μl of color reagent (G Biosciences) was added for 10 min at room temperature. The color developed in the wells was quantified at 630 nm using a plate reader (BioTek ELx808) as specified by the manufacturer.

For antiglycation activity analysis, carnosine (Sigma-Aldrich) was added to LB at a final concentration of 50 mM (15).

RESULTS

The type of culture vessel affects long-term survival. With an interest in identifying how laboratory growth environments might affect the dynamics of long-term survival, we examined long-term batch culture under two typical laboratory conditions: 5-ml cultures grown in test tubes and 12.5-ml cultures grown in 125-ml Erlenmeyer flasks. Generation time during exponential growth and cell yields after overnight growth were the same in both culture vessels (data not shown and Fig. 1A). In tubes, PFM2 entered death phase before day 2, with more than 90% of cells losing viability. PFM2 titers continued to decrease to ~10^6 to 10^7 CFU/ml by day 4, where the viable cell counts "bottomed out." Between days 4 and 5, cell titers increased, stabilizing at ~1×10^8 CFU/ml. We refer to the extended death phase and recovery experienced by PFM2 as a "dip." In flasks, however, once cells entered death phase, viable counts decreased more rapidly and more severely than with cultures incubated in tubes (Fig. 1A). On day 3, the viable counts in flasks dropped more than 3 orders of magnitude, 10-fold more than cells cultured in test tubes. However, the cells cultured in flasks began to recover from death phase a day earlier than cells in tubes, and the viable counts were the same in both vessels by 4 days of incubation.

We monitored pH levels of the medium in these cultures. While the LTSP profiles of cultures in tubes and flasks varied in

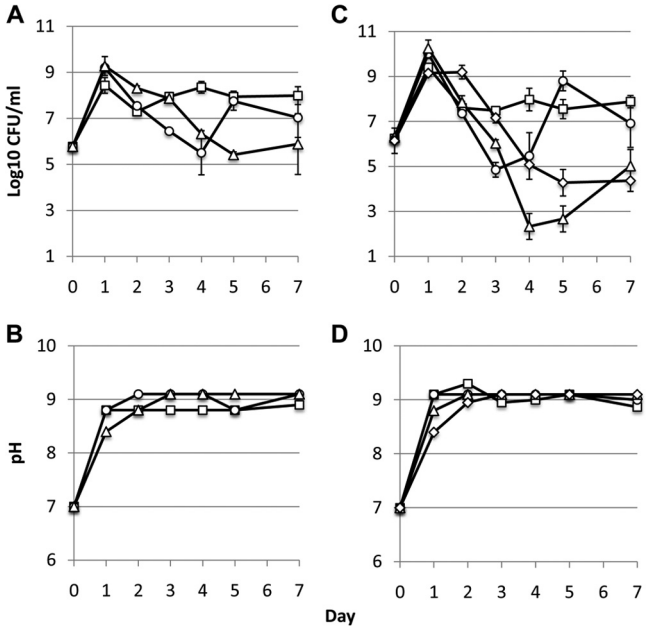

FIG 2 Survival dynamics of PFM2 in cultures of different volumes. Growth curves (A) and pH (B) of PFM2 incubated in 2 ml (squares), 5 ml (circles), or 12.5 ml (triangles) of media in test tubes are shown. Growth curves (C) and pH (D) of PFM2 cultured in 5 ml (squares), 12.5 ml (circles), 25 ml (triangles), or 50 ml (diamonds) of media in flasks are also shown. Data are the averages of three biological replicates; error bars represent standard deviations.

both the severity of death phase and the timing of recovery, pH did not differ between the two conditions at any time (Fig. 1B). Alkalization of bacterial cultures is frequently correlated with entry into, but not necessarily severity of, death phase (12, 25, 26).

Oxygen availability affects survival dynamics. The shape of the culture vessel can affect the dynamics of gas exchange between the medium and its environment, including the availability of oxygen (34). One difference between media in tubes and media in flasks is the surface area of the gas-liquid interface. This can affect the oxygen transfer rate (OTR), which correlates with the growth phase of a bacterial culture (35). We hypothesized that differences in OTR might also contribute to the dynamics of death phase. To determine if oxygen availability is a potential factor contributing to the observed differences in survival, we inoculated bacteria into tubes or flasks with different volumes of media (2, 5, or 12.5 ml in tubes and 5, 12.5, 25, or 50 ml in flasks). We found that the culture volume affected survival during LTSP in both tubes (Fig. 2A) and flasks (Fig. 2C).

In both tubes and flasks, cells incubated in the smallest tested volumes (2 ml in tubes and 5 ml in flasks) entered death phase between the first and second days of incubation but did not display the dip that is normally observed for PFM2 in standard 5-ml test tube long-term cultures. Instead, the counts remained stable, at ~10^7 to 10^8 CFU/ml, for the remainder of the experiment. In contrast, cells cultured in larger volumes (12.5 ml in tubes and 50 ml in flasks) showed a slower death phase (on the second day of culture, viable counts were 10 times higher than in other cultures), but ultimately death phase was just as severe. The slower death phase in higher-volume cultures correlated with a lower rate of alkalization (Fig. 2B and D). While smaller-volume cultures had a pH of ~9 after overnight incubation, the pH in larger-volume

TABLE 1 Average cell densities at peak and trough in each type of culture[a]

Vessel	Vol (ml)	Peak CFU/ml	Trough CFU/ml	Trough day
Tube	2	3.3×10^8	2.0×10^7	2
	5	1.6×10^9	9.1×10^5	4
	12.5	2.5×10^9	2.7×10^5	5.7
Flask	5	2.6×10^9	3.1×10^7	3
	12.5	1.1×10^{10}	8.3×10^4	3.3
	25	2.2×10^{10}	4.0×10^2	4.7
	50	1.3×10^9	1.2×10^5	5.3

[a] Data are averages of three independent cultures.

cultures was below 8.5. In contrast, the smaller volumes alkalized as quickly as the cultures with more standard volumes (5 ml in tubes and 12.5 ml in flasks; Fig. 2B and D). Twenty-five-milliliter flask cultures also had a slight delay in death phase compared to that of 12.5-ml flask cultures, correlating with a delay in pH increase (note the 10-fold-higher cell yield on the third day of incubation), but the extent of death phase was more severe than in any other volume, with minimum viable counts 1,000-fold lower than in 12.5-ml cultures. A comparison of the days at which the cultures reach their lowest viable counts (troughs) prior to recovery is summarized in Table 1.

Oxygen is not the only factor responsible for differences in survival under various culture conditions. To determine if differing oxygen levels were solely responsible for influencing the variation in LTSP survival, flask cultures with different volumes of media were incubated under anaerobic conditions (Fig. 3A). Cell growth dynamics in 5-, 12.5-, 25-, and 50-ml anaerobic cultures were much more similar to each other; the severe dip seen in aerobic cultures on days 2 to 5 was gone in all cultures, and viable counts remained above 1×10^8 CFU/ml throughout the experiment. However, there were still significant differences in the viable counts of cells at various points during incubation, particularly for cells incubated in 5 ml of culture media. These cells grew to a 10-fold-higher density than cells incubated in the other culture volumes after overnight incubation and remained 10-fold higher after 2 days of incubation. These results indicate that oxygen was not the only factor affecting LTSP survival in these cultures.

Differences among the culture conditions were more obvious

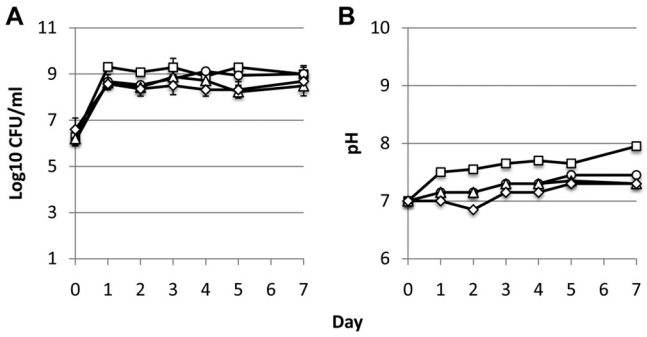

FIG 3 Survival dynamics of PFM2 in anaerobic cultures. Growth curves (A) and pH (B) of PFM2 in anaerobic cultures in flasks are shown for the following volumes: 5 ml (squares), 12.5 ml (circles), 25 ml (triangles), and 50 ml (diamonds). Data are the averages of three biological replicates; error bars represent standard deviations.

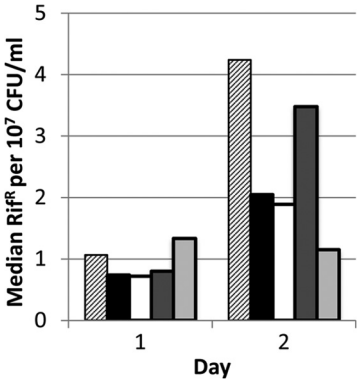

FIG 4 Spontaneous rifampin resistance mutations in different culture volumes. Median values ($n = 15$) for the number of rifampin-resistant mutants in different culture conditions are shown per 10^7 CFU/ml for 5 ml in tubes (hatched bar), 5 ml in flasks (black bar), 12.5 ml in flasks (white bar), 25 ml in flasks (dark gray bar), and 50 ml in flasks (light gray bar).

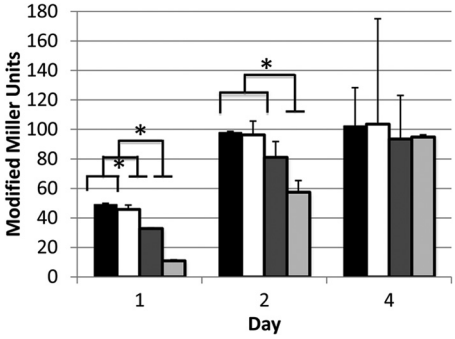

FIG 5 Expression of P_{oxyR} in different culture volumes in flasks. β-Galactosidase assays were performed on PFM2/pAQ23 (SF2562) during long-term growth in 5 ml (black bar), 12.5 ml (white bar), 25 ml (dark gray bar), and 50 ml (light gray bar). Bars represent average modified Miller units (see Materials and Methods) of duplicate samples. Error bars represent standard deviations. *, $P < 0.05$ using Student's *t* test.

when observing pH changes over time (Fig. 3B). While none of the cultures alkalized to the extent of aerobic cultures, pH values increased most rapidly in 5-ml cultures, while 50-ml cultures were the slowest to alkalize, similar to what was observed in aerobic cultures (Fig. 2D). Since oxygen was not available in any of these cultures, the differences in pH must have been due to another factor.

Survival in LTSP correlates with mutation frequency. The observed differences in the effect of oxygen tension led us to determine if the culture conditions described above led to differences in mutation frequency. By monitoring mutation frequency using spontaneous rifampin resistance as a reporter after 1 or 2 days under aerobic long-term culture conditions, we observed environment-specific differences in mutation frequency (Fig. 4). The median mutation frequencies on the first day of incubation were lowest in flasks with 5-ml and 12.5-ml cultures and highest in 5-ml cultures in tubes and 50-ml cultures in flasks (Fig. 4).

Compared with the mutation frequencies on day 1, all of the mutation frequencies increased at least 2-fold after 2 days of incubation, except in 50-ml cultures. For 50-ml cultures, the delay in change of mutation frequency correlated with the delay of entry into death phase (Fig. 2C).

Oxygen stress varies under different culture conditions. We hypothesized that there would be a higher mutation frequency in the cells experiencing the highest oxygen tension, but in fact the opposite was true. To determine whether cells were experiencing oxidative stress that correlated with the volume of media under the different culture conditions, we directly monitored *oxyR* expression using a transcriptional *lacZ* reporter plasmid (pAQ23) (36). β-Galactosidase levels were measured for cultures of strain SF2562 incubated in different volumes of LB in flasks during LTSP incubation over 4 days (Fig. 5). After 1 day of incubation, P_{oxyR} expression was highest for cells incubated in 5-ml and 12.5-ml cultures. Cells in 25-ml cultures had ~2-fold-lower activity and cells in 50-ml cultures had ~4-fold lower activity than smaller-volume cultures. These data confirm that cells in the different culture volumes were experiencing different levels of oxidative stress.

After 2 days of incubation, expression from P_{oxyR} increased ~2-fold in 5- and 12.5-ml cultures, more than 3-fold in 25-ml cultures, and ~6-fold in volumes of 50 ml. After 4 days of incubation, expression levels in all cultures were equivalent; however, while this represents only a small increase of expression in the 25-ml cultures, expression was almost doubled in the 50-ml cultures.

Culture volume affects glycation. Since cells experienced different amounts of oxidative stress as a function of culture volume, as well as differences in timing and degree of loss of viability, we wished to determine if glycation was affected. It has previously been shown that advanced glycation end products (AGEs) accumulate in *E. coli* during LTSP (15). We quantified the amount of carboxymethyl lysine (CML), a common glycation end product, using an ELISA with an anti-CML antibody for cells cultured in different volumes (Fig. 6). Glycation levels in 5-ml and 12.5-ml cultures were ~8-fold lower than in 50-ml cultures; cells cultured in 25 ml showed an intermediate amount of glycation, about three times higher than that observed for the smaller volumes.

Pepper et al. (15) demonstrated that the addition of the antiglycation agent carnosine to the culture medium significantly reduces the amount of CML while also increasing viability. Because cells cultured in different volumes experience different levels of glycation, we determined whether carnosine affects these cultures

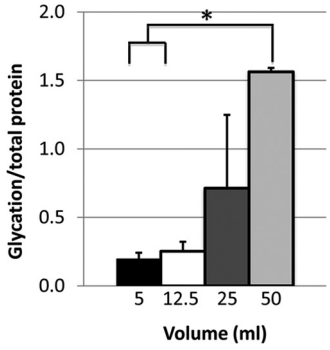

FIG 6 ELISA showing differences in glycation after overnight incubation using an anti-CML antibody. Assays were performed on total cellular protein from PFM2 during long-term growth in 5-ml (black bar), 12.5-ml (white bar), 25-ml (dark gray bar), or 50-ml (light gray bar) flask cultures. Bars represent average glycation/mg of protein of duplicate samples. Error bars represent standard deviations. *, $P < 0.05$ using Student's *t* test.

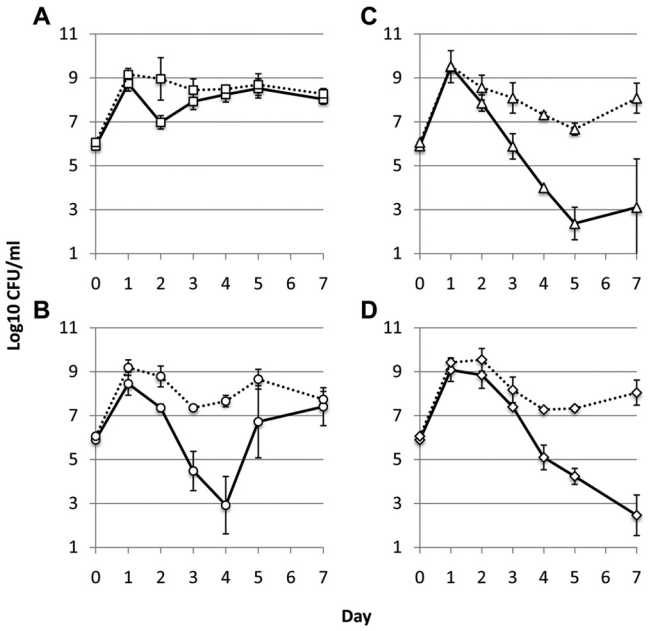

FIG 7 Carnosine partially rescues cells from death phase. Growth without (solid lines) or with (dotted lines) 50 mg/ml of carnosine in 5-ml (A; squares), 12.5-ml (B; circles), 25-ml (C; triangles), or 50-ml (D; diamonds) cultures in flasks is shown. Lines represent average of four biological replicates over two separate experiments; error bars represent standard deviations.

differentially. In fact, carnosine did little to change viable counts of cells in 5-ml cultures (Fig. 7A), which experienced the least viability loss overall, but dramatically changed survival in 12.5- and 25-ml cultures (Fig. 7B and C) and, especially, 50-ml cultures, in which the loss of viability was the greatest (Fig. 7D). Addition of carnosine to cultures did not alter pH (data not shown).

DISCUSSION

The type of culture vessel used to incubate cells affects long-term survival in *E. coli*. In *E. coli* K-12 strain PFM2, we see differences in both the timing of death phase and overall cell yields throughout the bacterial life cycle dependent on these factors. We chose to compare two very common laboratory conditions: growth in 5 ml of medium in test tubes and growth in 12.5 ml of culture medium in 125-ml flasks. The differences in survival between these two conditions, which are often considered interchangeable, were striking. The death phase of cultures in flasks was much more severe than in tubes, suggesting that these cells were experiencing a much greater level of stress in flasks.

We show that PFM2 causes an increase in culture pH during exponential-phase growth. Cultures alkalize during growth when amino acids are used as a carbon source because the catabolism of amino acids leads to excess nitrogen, which is converted to ammonia or other nitrogenous compounds and released. Therefore, in unbuffered media (such as LB), the pH of the culture increases; the pH in minimal medium cultures that are naturally buffered (such as M9 or M63) remains ~7.5 for days or even weeks in LTSP (25; S. Finkel, unpublished data). However, pH profiles are the same under both conditions, indicating that pH is not primarily responsible for the more severe death phase in cells cultured in flasks.

Since survival dynamics of the cells under these culture conditions are so different, what are the stressors experienced by the cells? It is important to understand what factors contribute to these differences in survival; therefore, we initially addressed the role of oxygen tension. We tested this in two ways: (i) by changing the headspace in the flask to affect mixing at the air-medium interface and (ii) by growing cells under anaerobic conditions. We found that oxygen is an important factor in survival during LTSP.

At smaller culture volumes (2 ml in tubes and 5 ml in flasks) cells enter death phase on the second day of incubation, as do cells under more standard culture conditions (5 ml in tubes and 12.5 ml in flasks), but then immediately level off and do not experience the dip seen under other culture conditions. We hypothesized that the number of cells that experience high oxygen tension at any given time is the greatest under these lower-volume conditions due to maximal mixing. Perhaps it is this exposure to oxygen, allowing more efficient respiration, which enables cells to recover from death phase so quickly. Another possibility is that volatile metabolic products are more efficiently released into the gaseous environment of the flask, therefore slowing the buildup of potential toxic products in the medium. This also suggests that the prolonged death phase observed under other culture conditions could be an effect of toxic by-products. One possible toxic by-product is ammonia, which we know accumulates in the medium and increases the pH. It has been shown that alkali stress can lead to induction of oxidative stress response proteins, as well as amino acid catabolism proteins that remove ammonia and generate acids (37). It is possible that increased exposure to oxygen in the medium from the headspace, due to the larger gas-liquid interface, may further induce alkali stress-responsive genes, which may be beneficial to the bacteria.

Cells cultured in larger volumes (12.5 ml in tubes and 50 ml in flasks) showed a delay of entry into death phase, which correlates with a delay in alkalization compared to that for cells cultured in intermediate volumes. In contrast, the pH in smaller-volume cultures (2 ml in tubes and 5 ml in flasks) does not correlate with survival, in that these cultures lack the dip seen in other cultures but do not have a difference in alkalization. These data suggest that while a change in pH affects the timing of entry into death phase, it does not determine the extent of survival during LTSP. In larger-culture-volume flasks, 50-ml cultures experience a less severe death phase than 25-ml cultures. This may reflect a less harsh environment in 50-ml cultures, since alkalization occurs more slowly. Alternatively, the delay in alkalization and entry into death phase may give cells more time to acclimate to their environment and therefore recover more quickly post-death phase.

While it is clear that oxygen availability is a major factor in survival, it is not the only important factor, as demonstrated by incubating cultures in different volumes of media in flasks under anaerobic conditions. The cells incubated anaerobically in 5-ml culture volumes still have higher viable counts than cells incubated anaerobically in larger culture volumes, even though the pH shows the greatest increase in 5-ml cultures. These data also support a hypothesis where the larger surface-to-headspace ratio allows these cells to better release a volatile toxic substance(s).

We hypothesized that the differences in survival seen during death phase are due to differences the cells experience as early as during the exponential growth and early stationary phases, even though cell counts at those times are equivalent. We tested this hypothesis by determining mutation frequencies in overnight and early-death-phase cultures. We expected that cells grown in 5-ml

cultures in flasks would have the highest mutation frequency, because we expected there to be more oxidative stress. However, we observed that cells in 50-ml cultures had the highest mutation frequency in overnight cultures. These data suggest either that the cells were not experiencing oxidative stress as we hypothesized or that oxidative stress does not directly correlate with mutation frequency. Shu et al. (38) observed an increase in mutation frequency at very low oxygen levels (<1%) compared to that at ambient levels (~20%) and suggested that this may be due to oxidative species generated under hypoxic conditions. Perhaps the cells in larger-volume cultures experience similar environments. Since specific types of mutations are more likely to occur due to oxidative agents (11), it would be interesting to determine if the spectrum of mutations occurring under the different culture conditions reveals the role oxygen plays in modulating the mutation frequency in these different cultures.

We also observed a difference in mutation frequency between cells cultured overnight in tubes versus flasks, the "interchangeable" conditions we first examined. From these data, it is clear that incubation under different culture conditions results in different genetic effects. Since basic science laboratories, as well as biotechnology researchers, frequently use larger culture vessels to scale up experiments, it is important to consider the possibility that the physiology of the cells is actually quite different in these environments. As these types of stresses may affect gene expression, protein production, growth, etc., all of which may affect the phenotype(s) being studied, it appears that these environments may not be interchangeable at all.

When we examined the mutation frequency on the second day of culture, we observed an increase in mutation frequency under all conditions studied, except in 50-ml flask cultures, in which there was also a delay in entrance to death phase.

Given these results, we wanted to determine if cells under different culture conditions were actually experiencing different levels of oxidative stress. We monitored expression of *oxyR* in these cells during long-term growth using a *lacZ*-based transcriptional reporter. OxyR is a major oxidative stress transcription factor and so is a good indicator of the level of oxidative stress cells may be experiencing. We hypothesized that there would be more mixing at the air-medium interface in smaller-volume cultures and that cells in those cultures would experience higher oxygen tension leading to increased levels of oxidative stress. In this study, our results were as hypothesized: during overnight growth, cells in 5-ml and 12.5-ml cultures in flasks had the highest level of P_{oxyR} expression, while expression by cells incubated in 25-ml and 50-ml cultures was lower. For cells grown in 50-ml cultures, P_{oxyR} expression was still low after 2 days of incubation. Levels of P_{oxyR} expression correlate with both pH and entry into death phase, and therefore, oxidative stress very likely plays a role in those phenotypes.

Given that P_{oxyR} expression is highest in 5-ml and 12.5-ml cultures, slightly lower in 25-ml cultures, and significantly lower in 50-ml cultures, it appears that the *oxyR* level in flasks is inversely correlated with mutation frequency. This may explain why the mutation frequency in 50-ml cultures did not increase after 2 days of incubation, as these cells experienced less oxidative stress than those in cultures of lower volumes. In fact, P_{oxyR} expression in all culture volumes correlated with the amount of death observed on day 2, with 5-ml and 12.5-ml cultures experiencing the greatest loss of viability, followed by an intermediate loss in 25-ml cultures and almost no loss of viability in 50-ml cultures (Fig. 2C).

Glycation is a nonenzymatic process in which proteins and nucleic acids are modified by reactive electrophiles generated during normal metabolism. Given that oxidative damage can lead to glycation of proteins, which, in turn, can lead to cell death (15–17), we monitored glycation levels under different culture conditions. We found that glycation levels correlated not with oxidative stress but instead with mutation frequency, with cells in 50-ml cultures having the most glycation end products.

Because mutation frequency and glycation correlate negatively with P_{oxyR} expression, we hypothesize that there may be a threshold of expression needed in order to protect against oxidative stress. It is possible that in 50-ml cultures, the amount of OxyR is too low during the first 2 days of incubation to activate the protective responses needed to lower glycation and mutation frequency, while cells in lower-volume cultures induce more OxyR earlier during incubation and therefore more of the protective responses it regulates. OxyR may also respond to, or protect against, pH stress, explaining why cells in 5-ml cultures survive better than those in larger volumes even though alkalization occurs more quickly.

To determine if glycation is a factor contributing directly to death, we added carnosine, which blocks glycation, to the different cultures. We found that addition of carnosine does in fact protect against death and is even more protective in the larger-volume cultures, correlating with higher glycation levels. Perhaps these cells have less protection against glycation from OxyR expression, which means that carnosine can have more of a protective effect. However, carnosine does not completely rescue cells from entering death phase, which indicates that glycation is not the only factor contributing to death in these cultures.

We have shown that there are significant changes of physiological responses and mutational responses due to culture conditions that are frequently used interchangeably in laboratory experiments. It is important to consider these factors when planning experiments or analyzing data. This is consistent with an analysis made by Fang (39) stating that results from experiments determining the effect of oxygen on antibiotic activity may diverge due to the differences in experimental conditions, namely, that some experiments were performed in 1/10 volume in flasks and some in 1/6 volumes in bottles; we have shown here definitively that oxidative stress can vary greatly with culture volume and vessel, which would likely lead to differential results in experiments depending on oxidative stress, It has also previously been shown that differences in culture volume can lead to changes in DNA supercoiling (40), which can lead to transcriptional and mutational changes. We know that differences in mutational and stress responses can lead to adaptive evolutionary events. Therefore, very simple changes in growth conditions may lead to complex evolutionary change and may leave specific genetic signatures of the environments in which the cells were adapted.

ACKNOWLEDGMENTS

We thank Lacey Westphal, Christopher Corzett, and Kavita Chavan for helpful suggestions and comments on the manuscript. We also thank Micheline Strand and Robert Kokoska for helpful suggestions regarding this project. We thank Patricia Foster and Ellen Popodi for providing strains used in this work.

This work was supported by U.S. Army Research Office grants W911NF1010444 and W911NF1210321.

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