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The Effect of Media and Filtration in Inducing the Oxidative Stress Response in *Escherichia coli*

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by

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ABSTRACT

Escherichia coli grown in complex medium (LB), but not defined medium (DGC), arrest DNA replication when collected on membrane filters and resuspended in fresh medium. The arrest is similar to that observed when cells are challenged with hydrogen peroxide. Yet, the reason behind this arrest is unknown.

I hypothesized that the arrest in replication in complex medium after filtering might be due to oxidative shock, and therefore cells grown in complex medium and filtered should induce an oxidative stress response similar to cells treated with hydrogen peroxide. Utilizing the indicator dye H_2DCFDA, which fluoresces in response to reactive oxygen species, I established an assay to measure levels of oxidative stress in *E.coli* cultures treated with hydrogen peroxide and compared them to cells grown in complex medium and filtered. Using a deletion mutant in *oxyR*, which is sensitive to oxidative stress and unable to induce a cellular response to hydrogen peroxide, I assessed the fraction of fluorescent-positive *oxyR* cells grown in complex or defined media and filtered, to *oxyR* cells treated with hydrogen peroxide. Contrary to what I hypothesized, I found that filtration of cells grown in complex medium does not significantly induce oxidative stress. I discuss alternative reasons as to what may be causing replication to arrest, such as the lack of divalent metals like iron and manganese, osmotic stress and dehydration, the sensitivity of H_2DCFDA, and the difference in medium makeup.

INTRODUCTION

Reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, and hydroxyl radicals are produced in organisms from all three domains of life (11, 15, 23, 25, 26). They can be generated as a byproduct of a cellular response to stressors such as bacteria and cytokines, or of aerobic metabolism (9, 16, 28). Cells encode multiple defense mechanisms to process excess reactive oxygen species. However, when a cell is unable to maintain homeostasis due to excess ROS, oxidative stress occurs (11, 25, 27, 28). Excess ROS occurs from endogenous sources or environmental exposure, and can damage proteins, lipids, and nucleic acids. Damage to DNA can result in a range of base and sugar modification products which negatively affects replication (15, 23, 25, 26). ROS have been linked to many age-associated diseases in humans such as mitochondrial dysfunction, cancer, cardiovascular diseases, and neurodegenerative diseases (10, 21, 23, 27, 29).

Reactive oxygen species also affect replication in bacteria such as *Escherichia coli*. Hydrogen peroxide can damage the integrity of the bacterial chromosome by oxidizing bases, causing lesions including 8-oxo-guanine, which mispair during replication or transcription, and thymine glycol, which block some RNA and DNA polymerases and can result in lethality (1, 2, 3, 14, 22). Such oxidized bases are normally repaired via the base excision repair pathway, which is initialized by 8-oxoguanine DNA glycosylase, endonuclease III, exonuclease III, or endonuclease IV (3, 14, 22).

In *E.coli*, the *oxyR* regulator is induced in the presence of certain reactive oxygen species such as free radicals. Another regulator, induced in the presence of superoxide free radicals, is the SoxR/S regulator (9, 11, 16). There is relatively little functional overlap between the stress

response pathways regulated by these two genes, though their respective stressors are chemically related. When activated by oxidative stress, these regulators induce transcription of several genes associated with DNA repair and other protective roles in removing and limiting oxidative damage in the cell (9, 11). Ferric Uptake Regulator, encoded by *fur*, is one gene that is activated by OxyR and SoxS. Fur is a transcription factor which responds to iron availability to control iron metabolism. OxyR binds to the *fur* promotor and SoxS to the upstream *fldA* promotor, to induce Fur expression and limit damage caused by the interaction of iron with hydrogen peroxide (9, 32, 35).

Iron acts as a cofactor for many enzymes involved in multiple metabolic pathways within the cell. It is vital for processes such as metabolism and ribonucleotide biosynthesis (6, 18, 25, 30, 31). However, iron is toxic in excess, due to its propensity to react with hydrogen peroxide via the Fenton reaction, the byproduct of which can severely damage cellular DNA (6, 18, 19, 24, 25, 30, 31). In contrast, manganese is capable of ameliorating the toxic effects of iron, since it is not readily oxidized by hydrogen peroxide and is able to remetallate a subset of iron-dependent enzymes following hydrogen peroxide-induced oxidative stress (5, 6, 18, 31). In addition, *E.coli* encodes several functionally redundant manganese-dependent enzymes that can catalyze iron-cofactored reactions inactivated by hydrogen peroxide (5, 6, 31).

In *E.coli*, oxidative stress induced by hydrogen peroxide treatment transiently inhibits cellular replication. Yet, resumption of replication was observed even in the absence of lesion removal (17, 36). It was observed that restoration of replication relies on the presence of manganese in the growth medium. This demonstrates that the physical disruption to the DNA caused by hydrogen peroxide is not the cause of cellular arrest. Rather, replication is enzymatically inactivated, likely by oxidation of an iron-dependent protein, which requires

manganese to restore synthesis (17). It has also been observed that cell cultures supplemented with manganese recovered DNA synthesis at a faster rate than those supplemented with iron or nonsupplemented (18).

A transient arrest in replication has also been observed in filtered cells grown in complex medium, but not defined medium (Wang, Hackert, Courcelle, unpublished data). This behavior is similar to what is observed following hydrogen peroxide treatment, however, whether filtration is causing oxidative stress and inducing this arrest is unknown. To determine whether filtration of complex medium-grown cells arrest replication as a result of oxidative stress, I established an assay to determine whether the fluorogenic dye, H_2DCFDA, could be used to detect oxidative stress. I then conducted an experiment where I grew cells in complex or defined medium, filtered or mock-treated them, and visualized their levels of fluorescence. I found that filtering cultures grown in complex medium did not significantly increase the percentage of H_2DCFDA-positive cells in the presence of oxidative stress, suggesting the arrest of replication following filtration is not due to the induction of oxidative stress.

MATERIALS & METHODS

Media

Luria-Bertani (LB) was used as the complex medium, and phosphate-buffered Davis medium supplemented with 0.4% glucose and 0.2% casamino acids (DGC), as the defined medium.

Bacterial strains

E.coli K-12 strain BW25113 and its *oxyR* mutant derivative from the Keio collection were used in this study (8).

H_2DCFDA staining following hydrogen peroxide or filtration treatment

Fresh overnight cultures were diluted 1:100 in DGC and grown at 37°C to mid-log. 1-ml aliquots were removed from each culture as mock-treated controls, and the remaining cells were treated with 10mM hydrogen peroxide for 30 minutes. At the end of this treatment time, 1-ml aliquots were removed from the hydrogen peroxide-exposed cultures. Cells from each treatment were collected by centrifugation for 2 minutes at 14,000 rpm, washed once in 1-ml of phosphate-buffered saline (PBS: 137 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and 2.7 mM KCl), and then resuspended in 1-ml mixture of PBS supplemented with 10 mM H_2DCFDA. Cells were incubated with dye in a 37°C water bath, shaking in the dark for 30 minutes. 0.5-ml aliquots were removed, and cells were collected by centrifugation. Cell pellets were washed once in 1-ml PBS, before resuspension in 100-µl PBS. 5-µl aliquots were removed and spotted on agar pads for visualization by microscopy. Cells were observed under 40X magnification, H_2DCFDA fluorescence was observed using a blue light filter (515 nm emittance/ 410-490 nm excitation).

For filtration treatment, fresh overnight cultures were diluted 1:100 in DGC or LB media and grown at 37°C to mid-log. 1-ml aliquots were removed from each culture for mock-treated

controls, while 5-ml of each remaining culture was filtered on 0.45-μm nitrocellulose membranes and resuspended in 5-ml of the appropriate fresh medium. As a control for H_2DCFDA staining, cells grown in DGC medium were also treated with 10mM hydrogen peroxide for 30 minutes. 1-ml aliquots were collected from the filtered and hydrogen peroxide-treated cultures. Cells from all treatments were collected by centrifugation, then processed and visualized as described above.

RESULTS

H_2DCFDA as an indicator of oxidative stress

Arrest in DNA replication was previously observed in *E.coli* grown in complex medium and filtered (Wang, Hackert, Courcelle, unpublished data). The arrest was similar to that observed when cells were challenged with hydrogen peroxide. I hypothesized that this arrest in replication may be due to oxidative shock. To test this, I developed an assay using H_2DCFDA, an indicator of oxidative stress, to help visualize stress caused by hydrogen peroxide. H_2DCFDA readily diffuses into cells and is oxidized by reactive oxygen species into 2', 7'-dichlorohydrofluorescein (DCF) which is highly fluorescent (4, 10). Wild-type and *oxyR* mutant cells were grown in defined medium to mid-log, treated with 10 mM hydrogen peroxide for 30 minutes, and then stained with H_2DCFDA. The *oxyR* strain lacks the master regulator of the oxidative stress response in *E.coli*. Therefore, *oxyR* cells treated with hydrogen peroxide and dyed with H_2DCFDA should have a high ratio of fluorescing to non-fluorescing cells.

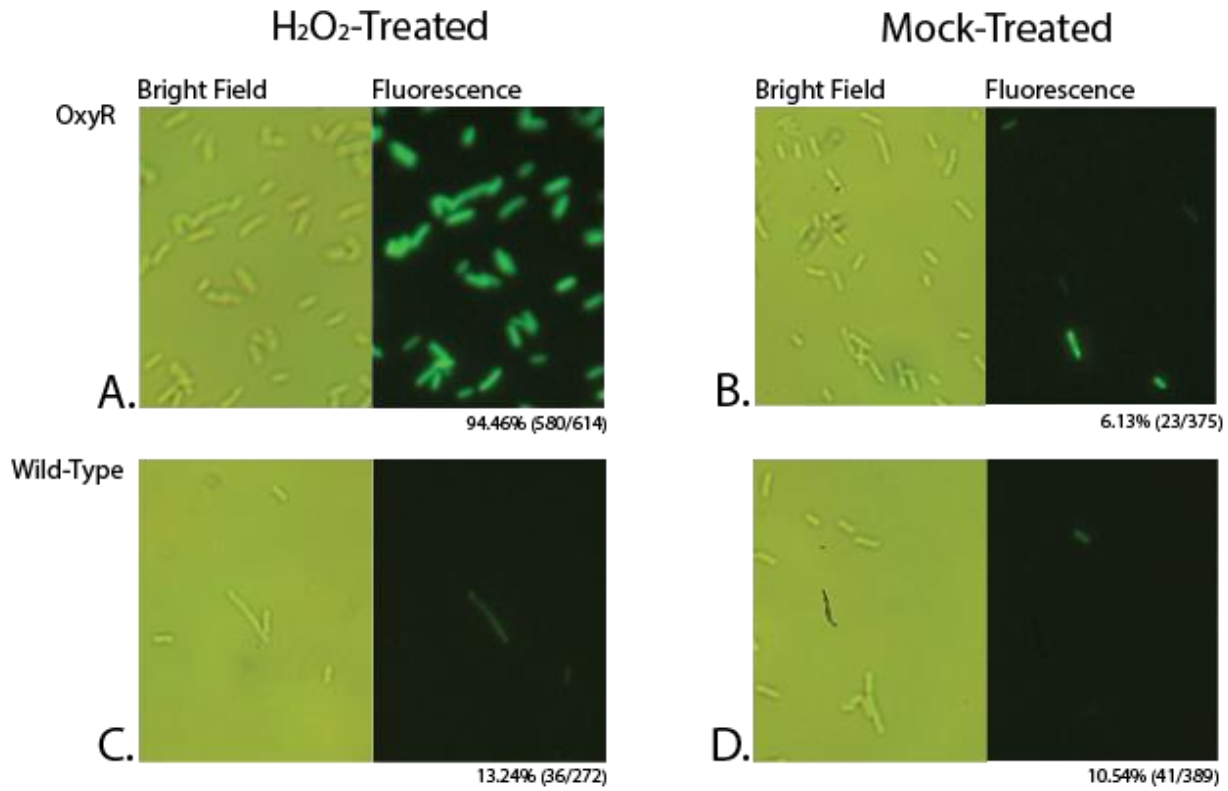

Figure 1: H_2DCFDA as an indicator of oxidative stress. Wild-type and *oxyR* cells were grown to mid-log and either treated with 10mM hydrogen peroxide for 30 min or mock-treated. Cells were collected, stained with H_2DCFDA and visualized by bright field and fluorescence microscopy at 40X magnification. Representative images from hydrogen peroxide- and mock-treated wild-type and *oxyR* cultures are shown. A minimum of 270 cells were evaluated for fluorescence staining. The percentage of fluorescing cells in each population was determined and is shown in the bottom right corner of each fluorescence micrograph.

When mock-treated, 10.54% of wild-type cells were found to fluoresce (Figure 1D). When treated with hydrogen peroxide, 13.24% of wild-type cells were found to fluoresce (Figure 1C). This is expected, because wild-type cells contain the master regulator of the oxidative stress response and therefore are able to process hydrogen peroxide. In contrast, 6.13% of mock-treated, *oxyR* cells were found to fluoresce (Figure 1B). When treated with hydrogen peroxide, 94.46% of *oxyR* cells were found to fluoresce (Figure 1A), more than an eight-fold increase. Therefore, H_2DCFDA is a suitable dye for observing oxidative stress.

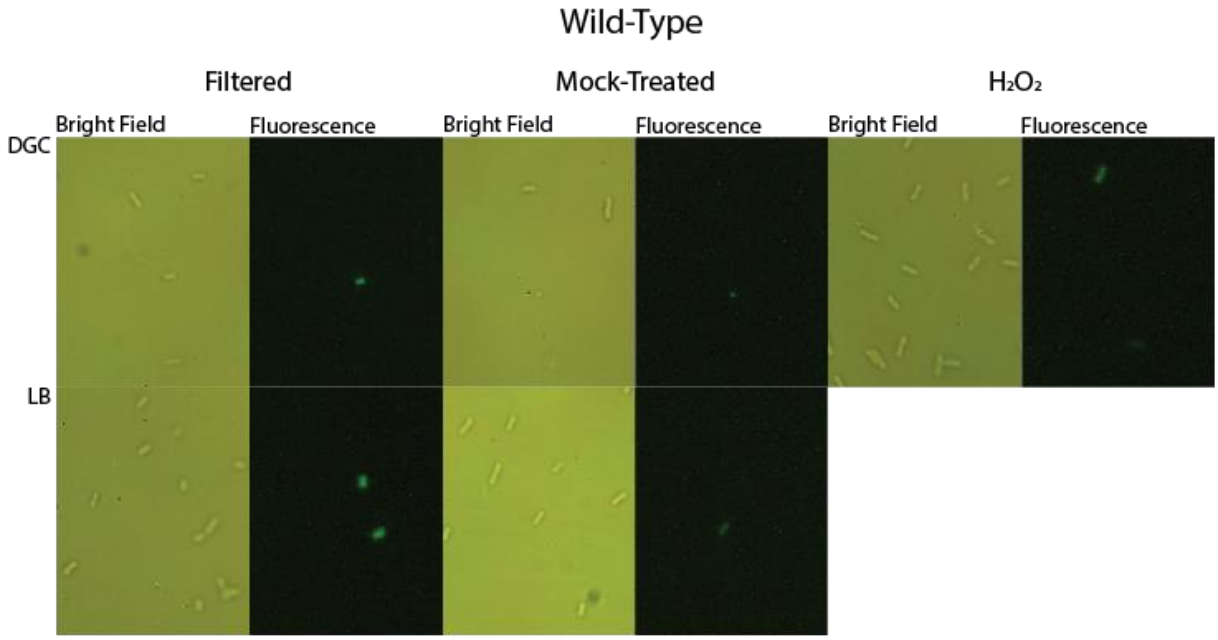

Figure 2A

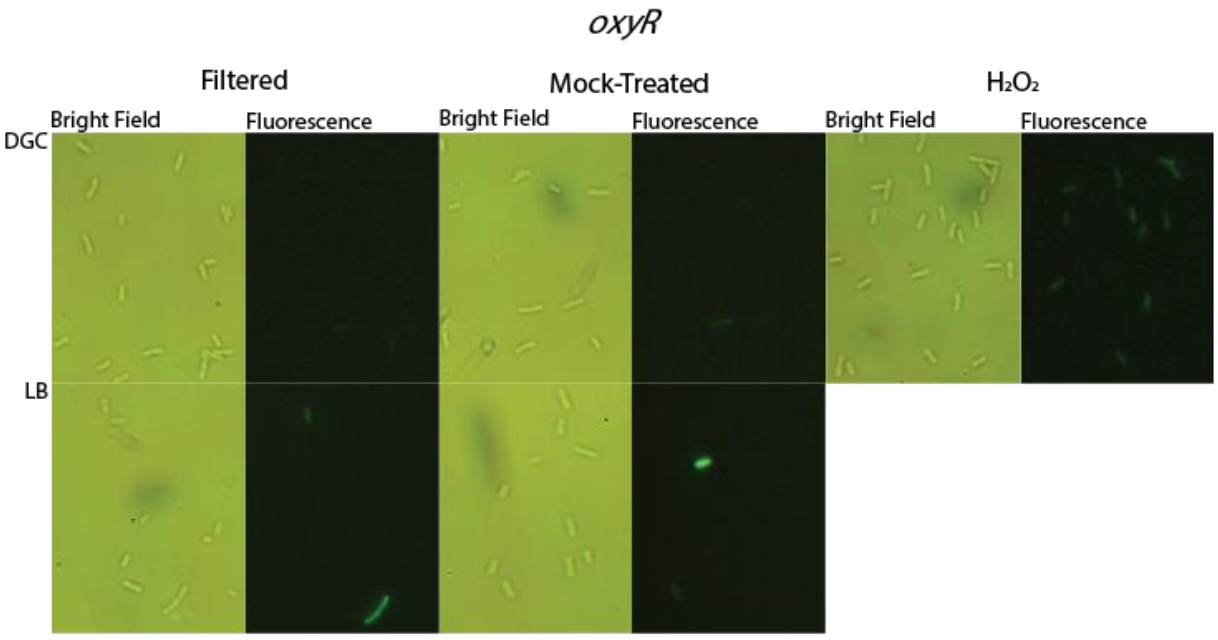

Figure 2B

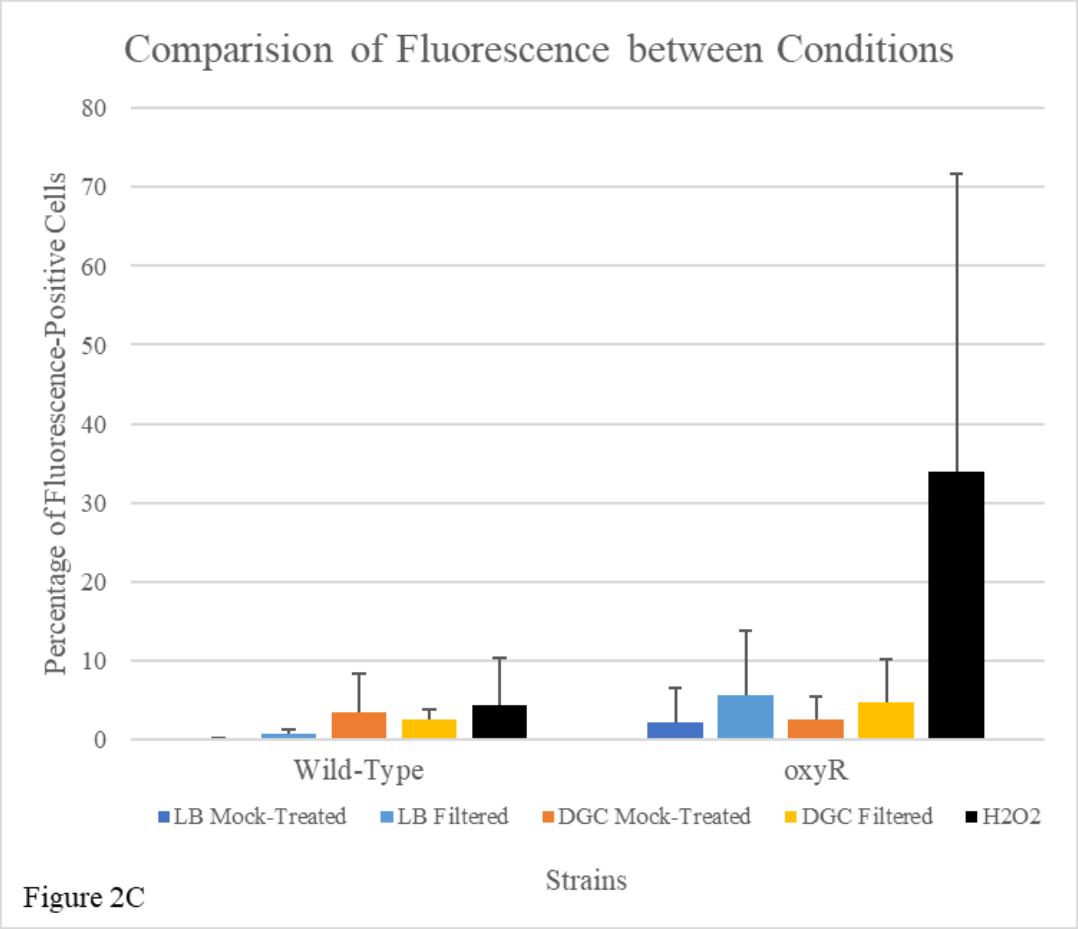

Figure 2: Filtering cells grown in complex medium does not significantly induce oxidative stress. Cells were cultured in either rich or defined growth medium to mid-log, filtered, and resuspended in fresh medium. Aliquots were removed, stained with H_2DCFDA and viewed as for Fig. 1. Representative images for (A) wild-type and (B) *oxyR* cells are shown, along with their respective hydrogen peroxide-treated controls for H_2DCFDA staining. (C) The percentage of fluorescent-positive cells for each growth condition is plotted for wild-type and *oxyR* strains. The bar graphs show an average of at least three independent experiments. Error bars represent plus one standard deviation.

Filtering cells grown in complex medium does not significantly induce oxidative stress

My previous results demonstrated the utility of H_2DCFDA in visualizing oxidative stress following hydrogen peroxide treatment, however it was unknown whether filtration following growth in complex medium induced a similar stress response. To determine this directly, cells were grown in either DGC (defined) or LB (complex) medium, filtered through a nitrocellulose

membrane, and resuspended in fresh growth medium. Treated cells were then stained with H_2DCFDA, and visualized using microscopy as described above. To assess the efficiency of H_2DCFDA staining I also visualized hydrogen peroxide-treated wild-type and *oxyR* cells in every experiment. If filtration causes a similar arrest in cellular replication as oxidative stress induced by reactive oxygen species, then filtered populations grown in LB would be expected to have an increased percentage of fluorescence-positive compared to mock-treated samples or those grown in DGC.

3.36% of wild-type cells grown in DGC and mock-treated were found to fluoresce. When treated with hydrogen peroxide, 4.27% of wild-type cells were found to fluoresce (Figure 2A). 2.5% of cells deficient in OxyR, grown in DGC, and mock-treated were found to fluoresce. When treated with hydrogen peroxide, 33.94% of *oxyR* cells were found to fluoresce (Figure 2B). This is consistent with a role of *oxyR* in responding to oxidative challenge, and demonstrates the efficiency of H_2DCFDA staining in detecting hydrogen peroxide-induced oxidative stress.

It has been previously shown that wild-type cells grown in defined medium and filtered do not experience the cellular replication inhibition that is seen in filtered, LB-grown cells. This suggests that DGC medium does not trigger the same cellular response as filtration of LB-grown cultures (Wang, Hackert, Courcelle, unpublished data). If filtration after LB, but not DGC, growth induces oxidative stress, then I predicted that both mock-treated and filtered wild-type cells grown in DGC should have similar H_2DCFDA fluorescence levels. 2.44% of wild-type cells grown in DGC and filtered were found to fluoresce, compared to 3.36% H_2DCFDA-positive fluorescence seen in mock-treated, wild-type cells (Figure 2A). This result is consistent with our hypothesis that filtration of wild-type cells grown in DGC does not cause oxidative

stress. When I examined *oxyR* cells grown in DGC and filtered, 4.64% of cells were found to fluoresce, compared to 2.5% H_2DCFDA-positive fluorescence seen in mock-treated *oxyR* cells, similar to what I observed in wild-type cells (Figure 2B).

I predicted that cells grown in LB and mock-treated would have a similar level of fluorescence to that seen in mock-treated cells grown in DGC medium. I also hypothesized that cells grown in LB and filtered would have an increased level of fluorescence over mock-treated cells grown in either type of medium, due to oxidative stress caused by filtration. 0.11% of wild-type cells grown in LB and mock-treated were found to fluoresce. When filtered, 0.64% of wild-type cells were found to fluoresce, about a sixfold increase (Figure 2A). In *oxyR* cells grown in LB and mock-treated, 2.2% of cells were found to fluoresce. When filtered, 5.63% of *oxyR* cells grown in LB were found to fluoresce, about a two-and-a-half-fold increase over its mock-treated counterpart (Figure 2B). While the increase in H_2DCFDA fluorescence following filtration of LB-grown cultures over mock-treatment appears to support my hypothesis that filtration is causing oxidative stress, I observed wide variation in the percentage of fluorescence-positive cells between experimental populations, as shown by the error bars included in my graphs (Figure 2C). Therefore, it is unlikely that filtration is causing significant oxidative stress in LB-grown cells.

DISCUSSION

Filtration of *E.coli* grown in complex medium leads to a temporary arrest in cellular replication that is restored approximately 15 minutes after the culture is resuspended in fresh medium (Wang, Hackert, Courcelle, unpublished data). The filtration effect on replication is similar to what is observed after oxidative shock due to peroxide species (6, 18, 25, 26). In my thesis I addressed the question, does filtration of LB-grown cells induce oxidative stress? I filtered LB-grown cells and compared the percentage of cells staining positive for the ROS-responsive dye H_2DCFDA to those seen in mock-treated, LB-grown cells. I found that there was no difference in the percentage of fluorescent-positive cells in filtered versus mock-treated cultures grown in LB.

There are several possible reasons that H_2DCFDA did not induce fluorescence in the filtered cells. While I cannot rule out the possibility that modest oxidative stress is induced during filtration sufficient enough to inhibit replication, such levels may be below the threshold of detection when using H_2DCFDA as an indicator. The most likely interpretation of my results is that filtration simply does not induce oxidative stress inside cells. If this is the case, then the inhibition of replication could be caused by one of several other possible variables between the complex and defined media. These might include differences in divalent metal concentrations-such as iron and manganese in the cell, differences in the osmotic stress and dehydration induced by these media due to potassium and magnesium concentrations, or possibly other differences of potentially a rare or minor component found in the complex media that is absent in minimal, defined media.

Iron plays a vital role in the metabolism of many organisms. It acts as a nutrient, functioning as a cofactor for multiple enzymes involved in metabolic pathways (6, 7, 18, 25, 30,

31). It also acts as an electron acceptor, extending the viability of cells for cultivation in both aerobic and anaerobic conditions (7). However, excess or unbound iron poses a potential threat to a cell. When in contact with hydrogen peroxide, a natural byproduct of cellular metabolism, iron can generate reactive oxygen species such as hydroxyl radicals via the Fenton Reaction (6, 18, 25, 30, 31). This can damage the integrity of various lipids, proteins, and DNA (6, 18, 25, 30, 31). To prevent oxidative damage to iron-associated enzymes, manganese, which does not react with hydrogen peroxide, may be used in place of iron to restore the function of certain proteins (6, 18, 25, 31).

Levels of iron and manganese are regulated within a cell to allow for the optimal presence and function of metal-dependent enzymes (18, 25, 30). If *E.coli* has too much iron, it could be toxic to the cell. If *E.coli* are lacking such metals, it may not be able to activate certain metal-dependent enzymes crucial for the functionality of the cell (18, 25, 30, 31). DGC and LB have different concentrations of iron and manganese. DGC contains 117.04 µM manganese and 489.75 µM iron. LB contains 184.21 µM manganese and 4,326.98 µM iron (18). Filtration may deplete intracellular metal concentrations, and it's possible that cells grown in DGC are better adapted to the minimal levels of iron and manganese present. Therefore, filtration may not cause as great a shock to them as to cells grown in LB. Filtration may also have the same effect on LB as if the cell was being starved of nutrients. If this is the case then cells grown in DGC, because it lacks other nutrients in the same concentration as LB, may also be more adapted to filtration (20).

The act of filtration may also lead to osmotic stress in *E.coli*. High-salt or high-solute environments lead to osmotic stress and dehydration in bacteria such as *Bacillus subtilis* and *Escherichia coli* (12, 13, 34). It has been proposed that increases in cytoplasmic potassium is the

main regulatory signal that activates other osmotically controlled responses (12). In *B. subtilis*, one response to high salt stress is to transiently raise the level of potassium ions, resulting in bacteriostasis (34). It has been shown that with the influx of potassium follows an efflux of intracellular magnesium, and this depletion of magnesium is the rate-limiting factor in the resumption of growth. Without magnesium import to compensate, *B. subtilis* is not able to counter the effects of a high-salt or high-solute environment, and the resumption of growth does not occur (34). DGC contains 392.1 µM magnesium, whereas LB contains 81.87 µM magnesium (33). It may be that filtration affects DGC-grown cells less due to the higher levels of magnesium in the medium. Since LB medium contains lower levels of magnesium, when filtered, LB-grown cells may not be able to import enough of the ion to restore it to a biocompatible level. This may lead to sustained osmotic stress, and an arrest in replication (34).

Finally, H_2DCFDA may not be sensitive enough to detect low levels of oxidative stress. It is possible that a certain threshold of reactive oxygen species needs to be present to activate H_2DCFDA. If the dye was unable to be altered from a non-fluorescent chemical to a fluorescence one, then the level of fluorescence observed may be inaccurate to the amount of stress the cells were experiencing. It may also be that filtration decreases cell permeability. If this is the cause, then H_2DCFDA would not be able to enter the cell and detect ROS, resulting in low fluorescence levels.

Future studies examining osmotic stress, supplementation with different metals, or use of a different oxidative stress dye will be required to determine the effects of filtration on replication progression.

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