otsA single gene knock-out in *Escherichia coli* increases cell sensitivity to outer membrane destabilization by SDS and EDTA treatment

Albert Chang, Yu Gu, Kenny Hui, Chris Xi

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

SUMMARY Trehalose is a disaccharide consisting of two glucose molecules and is produced by various organisms, including *Escherichia coli* via the *otsBA* operon. It is proposed to be a stress protectant for cells under abiotic stress by stabilizing proteins and membranes. Here, we aim to elucidate if *E. coli* that are unable to synthesize trehalose due to an *otsA* gene deletion have increased susceptibility to abiotic stress that destabilizes the outer membrane by assessing cell viability. The surviving cell percentage of wild type MG1655 cells and mutant JW5312-3 cells lacking *otsA* were assessed after sodium dodecyl sulfate (SDS) and increasing concentrations of ethylenediaminetetraacetic acid (EDTA) treatment in culture media. Growth curve analysis and a low temperature viability assay as abiotic stress control were also performed. JW5312-3 cells were more susceptible at a lower EDTA concentration and to low temperature stress than MG1655, both indicated by lower surviving cell percentages. However, JW5312-3 were found to have higher growth yield than MG1655, though they have the same growth rate. Our findings suggest that trehalose' protective role in *E. coli* under abiotic stress may partly due to its outer membrane stabilizing effect and that deletion of one of the two genes in the *otsBA* operon only decreases cell viability and is not lethal to *E. coli*.

INTRODUCTION

Trehalose is a disaccharide consisting of two α-glucose monomers linked by a 1-1 glycosidic bond. It is upregulated in response to stress in *Escherichia coli* and has been shown to act as a stress protectant in various organisms including yeast, plants, insects and bacteria (1). These organisms contain a variety of evolutionary conserved pathways to synthesize trehalose in response to stressors including cold shock, heat shock, desiccation, high osmolarity, and oxygen radicals (2-7). While there exist multiple pathways for trehalose production (such as through the *treYZ* operon or TS pathway), the *otsBA* operon is the only method present in *E. coli* (8). The *otsBA* operon in *E. coli* is upregulated by RpoS, a stress activated transcription factor (9). It encodes for two proteins that are transcribed when the cell is under abiotic stress: OtsA is a trehalose synthase that converts glucose-6-phosphate into trehalose-6-phosphate and OtsB is a trehalose-6-phosphate phosphatase that converts trehalose-6-phosphate into trehalose. Trehalose has been proposed to be exported to the periplasm through stretch-activated protein channels that are activated upon membrane stretching inducing the export of various solutes and ions including trehalose (10). Once in the periplasm, trehalose may be cleaved into glucose by the enzyme trehalase (11). The subsequent localization of trehalose is currently unknown; it may be exported extracellularly, bind to the outer membrane (OM) or bind to the inner membrane (IM).

Trehalose has been demonstrated to play a protective role in *E. coli* under cold shock treatment. A proposed mechanism of protection is through outer membrane stabilization (3). *In vitro* experiments involving the use of trehalose for stabilization of proteins pre- and post- lyophilization have suggested that it can form hydrogen bonds with biological macromolecules to stabilize them (12). Furthermore, trehalose has conformational flexibility, which allows it to form stronger hydrogen bonds with irregular surfaces of macromolecules (13). Another experiment found that trehalose is also able to bind to metal ions preventing their precipitation and may play a role in stabilizing the lipopolysaccharide (LPS) in the outer membrane of *E. coli* (14).

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Address correspondence to:
https://jemi.microbiology.ubc.ca/

To investigate the validity of these hypotheses, we sought to determine if *otsA* plays a role in outer membrane stability. Δ*otsA E. coli* JW5312-3 and wild type MG1655 were subject to a sodium dodecyl sulfate (SDS) - ethylenediaminetetraacetic acid (EDTA) growth assay (15). This assay was previously developed (15) and was shown to be suitable to assess outer membrane stability in *E.* coli. SDS is an anionic detergent that can disrupt biological membranes and EDTA is a chelating agent that can sequester calcium ions associated with LPS. Together they can destabilize the outer membrane of *E. coli* resulting in cell lysis. Given the proposed membrane stabilizing role that trehalose plays in *E. coli*, we hypothesize that JW5312-3, deficient in *otsA*, a gene associated with trehalose synthesis, would be more susceptible to SDS-EDTA treatment compared to MG1655 cells.

We confirmed single gene deletion of *otsA* and replacement by a kanamycin resistance cassette in JW5312-3 and assessed the growth patterns of the two strains by comparing their growth rates and yields. We replicated the low temperature viability assay done by Kandror *et al.* (3) as an abiotic stress control and completed the SDS-EDTA growth assay comparing the growth yield between JW5312-3 and MG1655

METHODS AND MATERIALS

Bacterial strains. The two *E. coli* strains used are shown in Table 1. MG1655 were grown in Luria Bertani (LB) Lennox medium while JW5312-3 were grown in LB medium supplemented with 30 µg/mL kanamycin. MG1655 were streaked onto LB agar and JW5312-3 were streaked onto LB-kanamycin agar. Streaked plates were stored at 4°C.

Preparation of kanamycin stock solution. Kanamycin (30 µg/mL) stock solution was prepared by dissolving kanamycin sulfate (Lifelabs) in distilled water before filter sterilizing it through a 0.22 µm filter. Stock solutions were aliquoted into three separate 10 mL falcon tubes and stored at -20°C.

Genotypic confirmation by PCR and gel electrophoresis. The primer designs used to confirm whether *otsA* was present or absent in both of our strains are provided in Table 2. Genomic DNA was isolated from 1 mL overnight cultures of *E. coli* MG1655 and JW5312-3 with PureLink™ Genomic DNA Mini Kit (Invitrogen) as per the manufacturer's instructions. A NanoDrop3000 spectrophotometer was used to quantify the concentration and purity of the isolated genomic DNA. 50 µL PCR reactions were performed using 5 µL 10x PCR Buffer, 2 µL 50 mM $MgCl_2$, 0.1 µL 100 mM dATP, 0.1 µL 100 mM dTTP, 0.1 µL 100 mM dCTP, 0.1 µL 100 mM dGTP (Invitrogen), 0.5 µL of 100 µM forward primer (IDT) 0.5 µL of 100 µM reverse primer, 0.4 µl of Platinum™ Taq DNA Polymerase (Invitrogen), 2 µl of DNA template, and 39.8 µL of sterile water. Positive controls were performed using pUC19 template DNA and their respective primers (MBI Fermentas), while sterile water replaced template DNA in the negative controls. The T100 thermocycler (Bio-Rad Laboratories) conditions were programmed for an initial denaturation at 95°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 90 seconds, with a final extension at 72°C for 5 minutes. The PCR products were loaded onto 1% agarose gel stained with 1x SYBR® Safe DNA (Invitrogen) gel stain and subjected to electrophoresis in 1x TBE at 130V for 60 minutes. Amplicons were visualized and imaged under UV light using the ChemiDoc™ MP Imaging System (Bio-rad Laboratories).

Table 1. *E. coli* strains used in the study.

Strain	Mutation	Source
MG1655	WT (none)	Obtained from the Microbiology and Immunology Department at the University of British Columbia
JW5312-3	Δ*otsA747::kan*	Keio strain collection

Table 2. Primer Design.

Gene	Melting Temperature	Primer Sequence (5' – 3')
otsA	60.11°C	Forward: TGCCTACGGTGAGTTAAGCG
	59.75°C	Reverse: GATGTCTGGAGCTGGCTTGA

Growth Curve. Overnight cultures of *E. coli* MG1655 and JW5312-3 were diluted 1:200 in 2 mL LB and LB-kanamycin media respectively. LB and LB-kanamycin media without cells were used as controls. All samples were incubated in a 96-well plate on a shaking platform at 37°C for 16 hours. OD_{600} values were measured at 10-minute intervals using the Synergy™ H1 Hybrid Multi-Mode Microplate Reader (Biotek).

Low temperature cell viability assay. The method for the cold shock viability assay was adapted from the same experiment performed by Kandror *et al* (3). Overnight cultures of *E. coli* MG1655 and JW5312-3 were diluted 1:200 and incubated at 37°C for 3 hours to reach mid-logarithmic growth phase. Mid-logarithmic growth phase cells were serially diluted to concentrations 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} before plating. 50 µL of each dilution and undiluted cultures were plated on LB agar media if it was MG1655 and LB-kanamycin agar media if it was JW5312-3. The plated cultures were exposed to low temperature (4°C) stress for 0, 3, and 9 days before and transferred to a 37°C incubator. The cultures were incubated overnight for 16 hours before the colonies were counted.

96-well OD_{600}-based assay to determine susceptibility to SDS-EDTA treatment. Overnight cultures of MG1655 and JW5312-3 were diluted 1:200 and incubated at 37°C for 3 hours to reach mid-logarithmic growth phase. 10^6 of each strain was inoculated in triplicate in a 96-well plate with LB and supplemented SDS to a final concentration of 0.01%. JW5312-3 cultures were inoculated in LB-kanamycin in place of LB. Increasing concentrations of EDTA was added to each triplicate to achieve concentrations: 0 mM, 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, 0.7 mM, 0.8 mM, 0.9 mM, 1.0 mM, and 1.5 mM. Triplicates of LB and LB-kanamycin media with and without SDS were used as negative controls. The plates were grown overnight for 16 hours at 37°C. Growth was detected visually and endpoint OD_{600} readings were measured using the Epoch™ Microplate Spectrophotometer (Biotek). Cell viability was also tested with the EDTA concentration held at 0.1 mM and SDS supplemented to attain final concentrations of 0.01% to 0.10%, in increments of 0.01%.

RESULTS

Verification of *otsA* single gene deletion in JW5312-3 strain. To confirm that the JW5312-3 strain has the *otsA* gene deleted and replaced by the kanamycin cassette, PCR amplification was performed on isolated genomic DNA of both JW5312-3 and MG1655 cells. Primers were designed to amplify the 1.4 kb region containing *otsA*. As seen in Figure 1, MG1655 show PCR amplicons of *otsA* and JW5312-3 cells show PCR amplicons of smaller size than that of MG1655. In Figure 1, we see that the mutant amplicons are shorter/truncated when compared to wild type MG1655, indicating a deletion etc….. which is 0.6 kb smaller than *otsA*. This difference in amplicon size is confirmed by lower bands of JW5312-3 PCR amplicons compared to those of the MG1655. No DNA template was added to the reaction for the negative control and pUC19 was used for the positive control. The expected 150 bp product of pUC19 is see on the last two lanes of Figure 1.

JW5312-3 has higher growth yield but the same growth rate as MG1655. To assess cell viability and cell survival in our experimental groups, we generated growth curves for JW5312-3 and MG1655 to evaluate their growth patterns. From OD_{600} analysis (Figure 2A), our results show that JW5312-3 plateau at stationary phase at a cell concentration of approximately $2x10^9$ cells/mL, which is much higher than the MG1655 cells ($8x10^8$

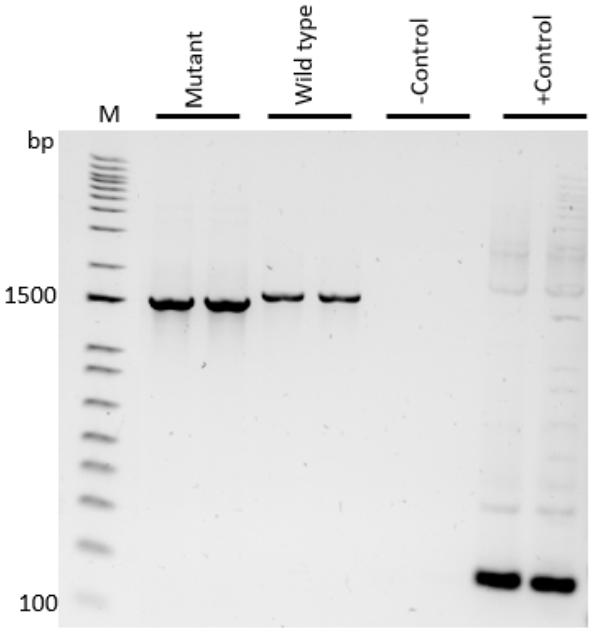

FIG. 1 Confirmation of *otsA* single gene deletion in *E. coli* strain JW5312-3 by PCR analysis. PCR amplification was performed on *E. coli* strains MG1655 ('Wild type') and JW5312-3 ('Mutant') using primers flanking *otsA*. PCR products were visualized by gel electrophoresis using 1% agarose gel. No DNA template was used for the negative control ('-Control') and pUC19 was used for the positive control ('+Control').

cells/mL). The logarithmic transformed values of cell number in Figure 2B indicates that both cultures increased in cell number at comparable rates with no significant differences and begin to plateau into stationary phase around three hours of incubation. Due to time constraint, the growth curve results were not replicated.

JW5312-3 mutant lacking *otsA* provides less protection against low temperature stress. A low temperature cell viability assay was performed as an abiotic stress control for MG1655 and JW5312-3 cells. By comparing the number of surviving colonies after 3 and 9 days of exposure at 4°C to colony numbers when unexposed to cold temperature, we observe that cold temperature stress decreases cell viability for cells of both strains (Fig 3). However, this decrease is much more prominent in JW5312-3. After 3 days at 4°C, 64% of MG1655 cells survived where was only 9% of the JW5312-3 cells did. After 9 days, surviving cell percentage decreased to 19% for MG1655 cells and to 3% for JW5312-3 cells. The difference in surviving cell percentage between both strains is largest after cold temperature exposure for 3 days, where the surviving cell percentage of MG1655 is more than 6 times that of JW5312-3.

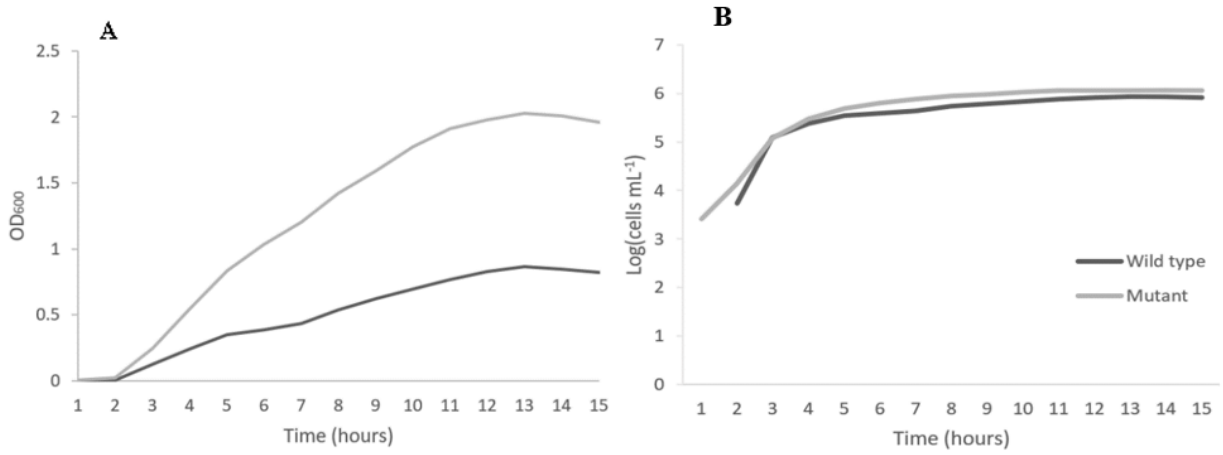

FIG. 2 Growth yield curves (A) and growth rate curves (B) of *E. coli* strains MG1655 and JW5312-3. 10^8 mid-logarithmic growth phase MG1655 ('Wild type') or JW5312-3 ('Mutant') cells were seeded in LB or LB supplemented with 30µg/mL kanamycin, respectively, and incubated overnight at 37°C. Absorbance was measured at 600nm at 10 minutes intervals for 16 hours. OD_{600} values were averaged between triplicates of the same experiment and plotted against time (A) and converted to cell mL^{-1} for 1 OD_{600} = 10^9 cells mL^{-1}, and the log plotted against time (B).

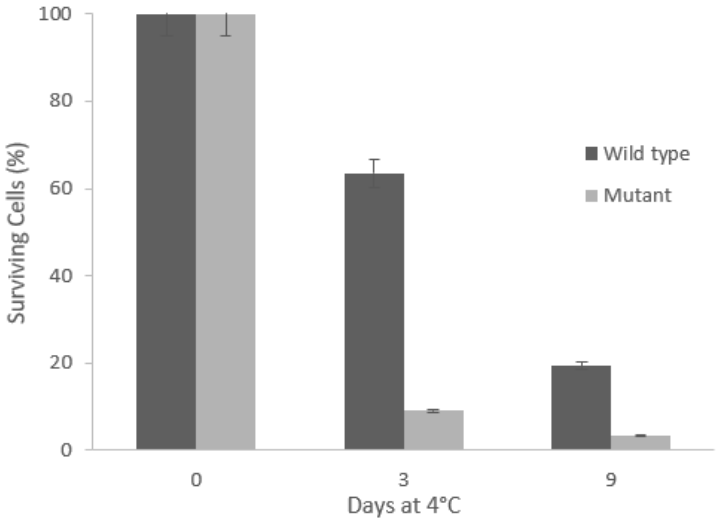

FIG. 3 Surviving cell percentage of *E. coli* strains MG1655 and JW5312-3 after cold shock at 4°C for 0, 3, and 9 days. Surviving cell percentage was calculated by normalizing the colony number at day 3 and day 9 to colony number at day 0 for both MG1655 ('Wild type') and JW5312-3 ('Mutant') strains. Five replicates by dilution from 10^{-4} to 10^{-8} at day 0 and four replicates by dilution from 10^{-4} to 10^{-7} at day 3 and 9 were included. Error bars indicate standard error.

JW5312-3 is more susceptible to lower EDTA concentration than MG1655. To investigate the effect of *otsA* deletion in *E. coli* on outer membrane stability, as SDS-EDTA cell viability assay for MG1655 and JW5312-3 cells was performed. SDS concentration was kept constant at 0.01% with increasing EDTA concentrations. From direct OD_{600} values analysis on Figure 4A, MG1655 had a higher initial OD_{600} value than JW5312-3 by 0.2 at 0 mM EDTA. The decrease in OD_{600} values for MG1655 under increasing EDTA concentrations was steeper than that of JW5312-3. Based on errors bars indicating standard error, no significant differences were observed between the two strains above 0.6 mM EDTA where both strains' OD_{600} values plateaued around 0.23. By comparing the relative OD_{600} values as a percentage of the initial OD_{600} values at 0 mM EDTA, the same trend was observed (Figure 4B).

JW5312-3 and MG1655 are equally susceptible at SDS concentrations higher than 0.02%. To investigate if SDS had similar effects on JW5312-3 and MG1655 as EDTA, the SDS-EDTA cell viability assay was repeated with a constant (0.1 mM) EDTA concentration and increasing SDS concentrations. The drop in OD_{600} value from 0% to 0.01% SDS was much greater for MG1655 (1.0 to 0.39) when compared to JW5312-3 (0.56 to 0.52) as seen

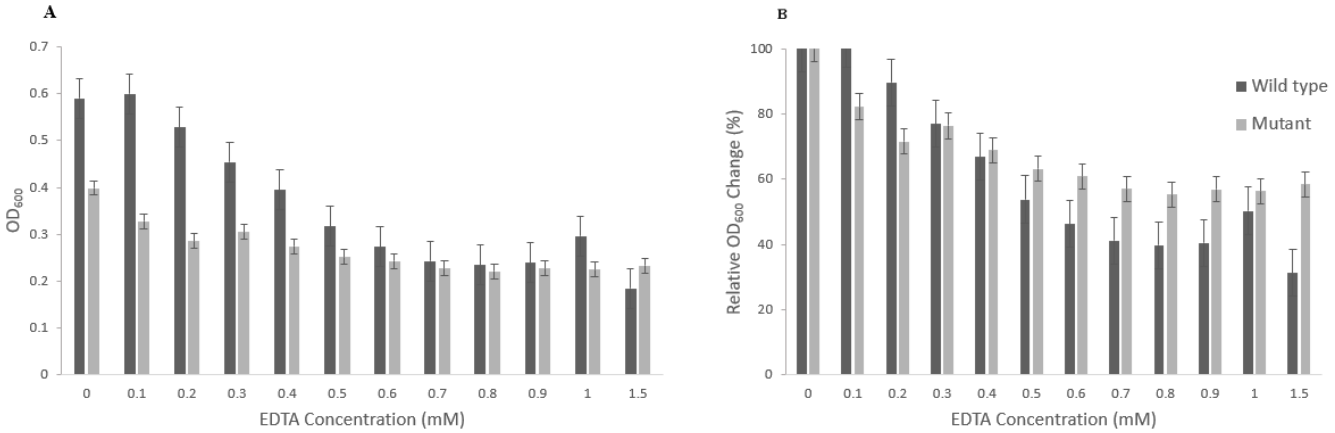

FIG. 4 *E. coli* strains MG1655 and JW5312-3 culture OD_{600} values (A) and relative OD_{600} change after SDS and increasing EDTA concentration treatment. 10^{6} mid-logarithmic growth phase MG1655 ('Wild type') or JW5312-3 ('Mutant') cells were seeded in LB or LB with 30μg/mL kanamycin, respectively, with 0.01% SDS and incubated overnight at 37°C. EDTA concentrations ranged from 0mM to 1.0mM at 0.1mM increment and at 1.5mM. An initial absorbance and an endpoint absorbance were measured at 600nm. OD_{600} values were averaged between triplicates and plotted against increasing EDTA concentration (A). Relative OD_{600} change as percentages were calculated by normalizing to OD_{600} value at 0mM EDTA as 100% (B). The experiment was duplicated, and the same trend was observed. Error bars indicate standard error.

on Figure 5A. There was no difference in OD_{600} values of both strains above 0.02% SDS. Accordingly, the relative OD_{600} (Figure 5B) decreased 62% for MG1655 and only 7% for JW5312-3 from 0% to 0.01% SDS. At 0.1% SDS, the OD_{600} value for JW5312-3 was 25% of the initial reading whereas 13% for MG1655.

DISCUSSION

The aim of these experiments was to determine if *otsA* affected cell viability in *E. coli*. This was done by incubating both wild type MG1655 and Δ*otsA* mutant JW5312-3 *E. coli* cells in a 96-well plate with constant (0.01%) SDS concentration while increasing the EDTA concentrations. This experiment was then repeated with the EDTA concentration held constant (0.1 mM) and instead increasing concentrations of SDS.

In the growth curve between the two strains was first conducted, the two strains had similar growth rates and reached stationary phase 3 hours into incubation. This contrasts the growth curves from literature for MG1655, where it typically reaches stationary phase roughly 6 hours into the incubation (13). An insufficient amount of media may have resulted in a build-up of waste products leading to an earlier stationary phase for our growth curve. As a result, the data past 3 hours may not be reflective of the actual growth rate of these two organisms. However, while the growth rates are similar, the growth yields are different. The growth yield of the mutant strain is much higher than wild type with endpoint OD_{600} values of roughly 1.75 and 0.75 respectively. Looking at Figure 2A, the difference in growth yield becomes noticeable 3 hours into the incubation when both strains reach stationary phase and this difference continues to increase as the incubation proceeds. Without abiotic stress in the growth medium that disrupts the OM of the cell and requiring the stabilization role of trehalose, the ability to synthesize trehalose does not aid in cell growth and replicating this gene segment may impair cell growth. Previous research has suggested that the replication of unnecessary genes may waster cellular resources and energy (14). Thus, deletion of *otsA* and its replacement by the kanamycin resistance cassette, which is smaller in size, may allow JW5312-3 cells to reach a higher cell numbers due to less gene transcription and transcription. Our results suggest that the ability to synthesize trehalose does not provide an advantage to *E. coli* growing in non-selective conditions that do not require protection from trehalose itself. However, we cannot conclude that they are indeed producing trehalose as measurements of trehalose in the non-selective media were not assessed in this study. Additionally, kanamycin resistance may give

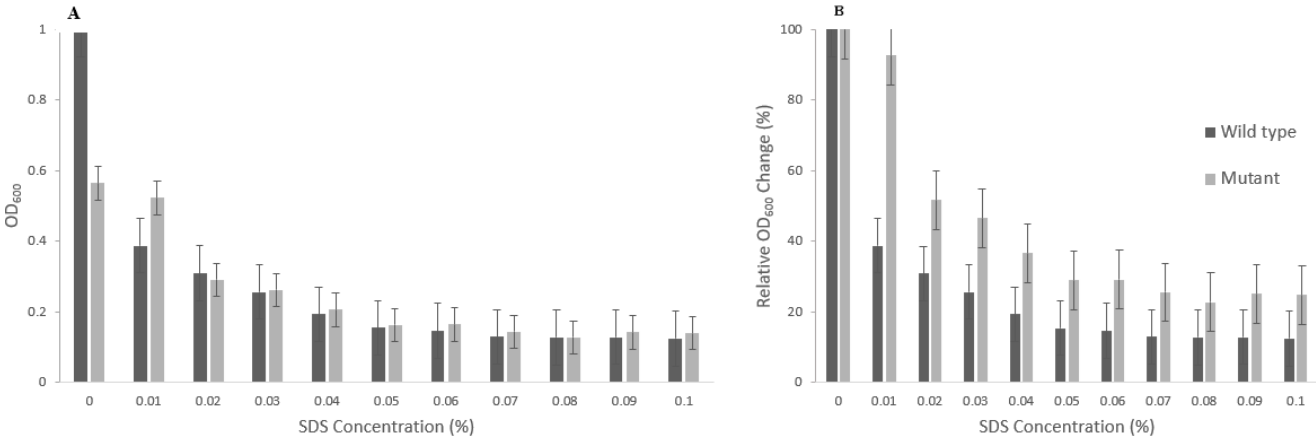

FIG. 4 *E. coli strains MG1655 and JW5312-3 culture OD_{600} values (A) and relative OD_{600} change after EDTA and increasing SDS concentration treatment. 10^6 mid-logarithmic growth phase MG1655 ('Wild type') or JW5312-3 ('Mutant') cells were seeded in LB or LB with 30μg/mL kanamycin, respectively, with 0.1mM EDTA and incubated overnight at 37°C. SDS concentrations ranged from 0% to 0.1% at 0.01% increment. An initial absorbance and an endpoint absorbance were measured at 600nm. OD_{600} values were averaged between triplicates of the same experiment and plotted against increasing SDS concentration (A). Relative OD_{600} change as percentages were calculated by normalizing to OD_{600} value at 0% SDS as 100% (B). Error bars indicate standard error.*

JW5312-3 cells growth advantage in kanamycin selective media whereas MG1655 may have been under strain competition if the media was contaminated by another strain that also grows in non-selective LB media.

Two variations of the SDS-EDTA assay were conducted. The first experiment kept the SDS constant at 0.01% while increasing the EDTA concentration from 0.1 mM to 1.5 mM. We see from Figure 4A that JW5312-3 reaches an OD_{600} of 0.3 at a concentration of 0.2 mM EDTA whereas the OD_{600} for MG1655 reaches 0.3 at a much higher concentration of 0.7 mM. Based on this data, it JW5312-3 is much more susceptible to EDTA treatment compared to MG1655. This may be explained by MG1655 synthesizing trehalose providing some form of stabilization for the outer membrane through hydrogen bonding or protection against chelating agents such as EDTA. However, at concentrations of EDTA higher than 0.7 mM, the stabilizing effects of trehalose seem to decrease. This may indicate that trehalose is not effective beyond a certain threshold of stressors, or at least in the amount that the cells are able to synthesize under these conditions. Another possibility is that at 0.7 mM EDTA, all the metal ions have been chelated and thus increasing EDTA concentrations beyond 0.4 mM will have no observable effect on the OD_{600} values. A third possibility may be that as the concentration of EDTA increases, more of the available bacterial cations are redirected to the outer membrane to stabilize LPS (18). Such an event would directly affect any cellular processes, which require metal ions, such as for ribosomal activity (19).

In the second experiment seen in Figure 5, contrary to what was predicted in the hypothesis, the OD_{600} of the MG1655 cells decreased to a greater extent than the JW5312-3 cells when compared to their untreated counterparts. Comparing only the change in OD_{600} it may appear that trehalose synthesis had little or possibly a negative impact on cell survival in the presence of SDS, although it must be taken into account that across all experimental replicates, the wild type cells have always had a higher untreated OD_{600} reading. Looking strictly at the OD_{600} values will reveal that both strains have similar optical densities from 0.02% SDS onwards. Unlike in Figure 4, the OD_{600} values continue decreasing as the SDS concentration increases, which is expected as SDS will continue partitioning into the outer membrane until the membrane lyses and is not restricted by the availability of substrate as in the case with EDTA.

MG1655 consistently had a higher time zero OD_{600} reading compared to JW5312-3 despite the same number of cells being seeded pre-incubation, which may be due to the effect of trehalose protecting MG1655 at time zero. These experiments had either 0.01% SDS or 0.1 mM EDTA and JW5312-3 had a lower OD_{600} in the presence of these reagents. This made it difficult to compare the OD_{600} values directly so the relative OD_{600} values were graphed as well (Figure 4B and 5B) and similar trends were seen. Another interesting observation is that for both SDS-EDTA assays, the negative controls showed opposite results compared to the growth curve despite identical growth conditions. In the growth curve, JW5312-3 had a higher growth yield compared to MG1655, but the opposite was true for our negative controls during our SDS-EDTA assay.

Conclusions We examined the role of *otsA* in outer membrane stability through an SDS-EDTA cell viability assay. Previous research has shown that the biosynthesis of trehalose increases cell viability in response to environmental stress but the localization of trehalose during these stresses is unknown. Here, we confirmed that trehalose plays an outer membrane protective role in *E. coli*. In agreement with our hypothesis, we demonstrate that JW5312-3 is more susceptible to increasing EDTA concentrations compared to MG1655. However, when testing the effect of increasing SDS concentrations, our results do not provide sufficient evidence that *otsA* aids in outer membrane stability.

Future Directions To further investigate the biosynthetic regulation of trehalose in *E. coli*, trehalose concentration in the supernatant of cells exposed to various abiotic stresses should be quantified. This can indicate if trehalose is synthesized in larger quantities when exposed to harsher or longer abiotic stressors. This could also shed light on the upregulation of different genes involved in trehalose synthesis pathways. Additionally, quantifying trehalose in the supernatant can confirm that cells do export and secrete trehalose extracellularly. To test if upregulation of trehalose can increase cell resistance to abiotic

stress, *otsBA* can be cloned onto a constitutively expressed vector and transformed into MG1655 cell prior to challenging them to abiotic stress. If these transformants show higher survivability than MG1655, we can deduce that overexpression of trehalose can make cells less susceptible to abiotic stress (16, 17). Previous research has also shown that during increased osmotic stress, glycine-betaine replaces the function of trehalose as a stress protectant (20). A strain lacking the gene required for glycine-betaine synthesis should be used in future experiments to ensure that it does not confer any cellular stability and impact the results (20). Finally, analysis of the growth curves between JW5312-3 and MG1655 should be looked into as our experiment showed conflicting results. Repeating the growth curve experiments may resolve these issues, as well as avoiding the use of the outer wells in the 96-well plates, which have a tendency to fluctuate in temperature and are more prone to evaporation (21).

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CONTRIBUTIONS

Brainstorming, planning, and execution of experiments as well as the writing and editing of the draft paper were a result of the entire groups shared efforts.

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