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Disruption of trehalose production in *Escherichia coli* K-12 does not confer sensitivity to SDS-EDTA-induced outer membrane stress

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SUMMARY Trehalose, a glucose disaccharide, has been reported to play a role in stabilizing the cell envelope of *Escherichia coli* under numerous forms of abiotic stress. The exact manner by which trehalose contributes to this stability remains under investigation, though a recent paper suggested that it stabilizes the outer membrane component of the cell envelope in response to a variety of environmental stressors, including the presence of SDS and EDTA. We sought to validate these findings with a more robust approach to the SDS-EDTA assay. A key enzyme in the biosynthetic pathway of trehalose is OtsA, a trehalose-6-phosphate synthase, as *otsA* mutants are unable to synthesize trehalose. We hypothesized that *otsA* deletion mutants would be hindered in their ability to stabilize the outer membrane, increasing their susceptibility to SDS-EDTA-induced stress. To test this hypothesis, we performed basal growth rate analyses and SDS-EDTA assays. We report that mutants with a disrupted trehalose biosynthesis pathway were not more sensitive to either form of membrane stress (SDS or EDTA) relative to the wild type. These findings suggest that trehalose may not promote outer membrane stability of *E. coli* during SDS-EDTA stress.

INTRODUCTION

Trehalose is a non-reducing glucose disaccharide with an α-1,1 linkage, originally described as an important storage component involved in glucose metabolism (1). More recently, trehalose has been implicated in several different roles in the various organisms that are capable of synthesizing it, from signaling to oxidation protection. However, its exact role in different species is diverse and is still under investigation (2). Its inert properties allow for high levels of accumulation without negatively impacting biochemical processes (3). Trehalose also has several important clinical implications. For example, repression of trehalose biosynthesis has been associated with decreased virulence in *Salmonella enterica* (4). Additionally, inhibition of trehalose biosynthesis pathways in *Mycobacterium tuberculosis* results in reduced virulence and improved survival in mouse infection models (4).

Despite its clinical significance, much remains to be known about how trehalose contributes to bacterial physiology. Overall, trehalose has been suggested to be important in growth and survival, as bacteria unable to synthesize trehalose have displayed decreased cell growth during osmotic stress, and trehalose overproduction has been found to increase the cell density of *Escherichia coli* cultures (3). This protective effect is believed to occur through action at the cell envelope, as trehalose is typically associated with bacterial membrane structures (2). In *E. coli* specifically, trehalose has been shown to contribute to cell envelope stability in the presence of certain stressors such as changes in osmolarity (3) and temperature

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(5). Trehalose has been proposed to contribute to this cell envelope stability through stabilization of the outer membrane (OM), via its ability to form flexible hydrogen bonds (6) as well as binding metal ions and stabilizing lipopolysaccharide (7). However, it remains unclear whether trehalose exerts its protective effects on *E. coli* by stabilizing the OM or another component of the cell envelope.

SDS-EDTA assays can be used in order to test outer membrane stability in *E. coli*. Sodium dodecyl sulfate (SDS) is a detergent known to disrupt the bacterial cell envelope (8). The OM of *E. coli*, along with other components of the bacterial envelope, has been found to play a crucial role in resistance to SDS treatment, specifically as a selective barrier (8). Therefore, the OM is likely the initial line of defence against this detergent. Ethylenediaminetetraacetic acid (EDTA), a chelator of Mg^{2+} and Ca^{2+} ions that are important for OM stability, permeabilizes the OM (9). Due to the disruptive properties of these two compounds, previous studies have used SDS-EDTA assays to investigate the role of specific outer membrane components in maintaining OM asymmetry and thus stability (10).

To investigate the role of trehalose in biological processes like OM stabilization, bacteria with mutations in trehalose biosynthesis pathways can be used. Although there are a number of metabolic pathways for trehalose biosynthesis in bacteria, *E. coli* only synthesizes trehalose through OtsA and OtsB, which are encoded in the *otsBA* operon (2, 3). OtsA, or trehalose-6-phosphate synthase, is a glycosyl transferase responsible for catalyzing the transfer of glucose from UDP-glucose to glucose-6-phosphate. OtsB, or trehalose-6-phosphate phosphatase, is a phosphatase that completes the synthesis reaction of trehalose (11). This pathway is upregulated when *E. coli* is under abiotic stress, including osmotic shock, desiccation, extreme temperatures, as well as entry into the stationary phase (2, 3, 12). Both enzymes are required for trehalose synthesis, and previous research comparing *otsA* and *otsB* deletion mutants found that both mutants equally reduced cell envelope stabilization and lowered trehalose levels under osmotic stress (12). As such, most studies investigating the role of trehalose use a single *otsA* or *otsB* knockout mutant.

In our study, we used an *otsA* mutant to assess the effect of trehalose on OM stability. Specifically, we investigated whether OtsA has a protective effect on *E. coli* in response to SDS-EDTA-induced outer membrane stress. One recent study had investigated this and found that mutants lacking *otsA* were more sensitive to concentrations of EDTA of 0.6 mM or less and equally as sensitive as the wild-type (WT) to concentrations of SDS greater than 0.02% (13). However, this study had four major flaws. First, an MG1655 strain was used as the WT strain, though the *otsA* mutant was derived from the BW25113 strain. Second, optical density readings were not conducted throughout growth in SDS-EDTA, and were instead only collected following the 16-hour endpoint. As such, the data lacks information about differences in the logarithmic growth stages of the two strains. Third, the base media used in the assay differed between the WT and the *otsA* trials: LB broth was used for growth of the WT strain, but LB broth supplemented with kanamycin was used for the *otsA* mutant. This may have differentially affected growth and introduced a confounding variable. Lastly, results from only one biological replicate were reported. We attempted to validate the findings from this study using a more rigorous experimental design to test the hypothesis that *otsA* mutants would be hindered in their ability to stabilize the OM and thus be more sensitive to SDS-EDTA stress relative to WT. Our results showed that under our experimental conditions, *otsA* mutants were not more sensitive to SDS-EDTA-induced stress than WT *E. coli* and, under some conditions, the mutants may have been more resistant to this stress. In summary, we challenge the previous findings that trehalose contributes to OM stability during SDS-EDTA induced stress, and highlight the need to further investigate the mechanism by which trehalose contributes to cell envelope stability under abiotic stress.

METHODS AND MATERIALS

Strains and media preparation. *E. coli* JW5312-3 (*otsA* mutant*)* and BW25113 (WT parent strain) were ordered from the Keio collection (*E. coli* Genetic Stock Center, Yale University). BW25113 was streaked out on Luria Bertani (LB) agar (1% tryptone, 0.5% yeast extract, 1%

TABLE. 1 Primer sequences used in PCR to confirm genotype

Gene	Direction	Sequence (5' to 3')	Source
otsA	Forward	TGCCTACGGTGAGTTAAGCG	Chang *et al.* 2019. JEMI
	Reverse	GATGTCTGGAGCTGGCTTGA	

NaCl, 1.5% agar in dH_2O) and JW5312-3 was streaked out on LB agar supplemented with kanamycin (30 µg/mL). Cultures were inverted and grown at 37℃ overnight and subsequently stored at 4℃ for downstream use.

PCR amplification of *otsA* and gel electrophoresis. Protocol adapted from Chang *et al*. (13). Isolated colonies of BW25113 and JW5312-3 were used to inoculate 5 mL of LB broth (1% tryptone, 0.5% yeast extract, 1% NaCl, in dH_2O) and LB broth + kanamycin (30 µg/mL) respectively, and were grown at 37℃ overnight while shaking at 150 rpm. Genomic DNA (gDNA) was extracted from the cultures with PureLink™ Genomic DNA Mini Kit (Invitrogen) following the protocol specified by the manufacturer. Yield and purity were determined by evaluating absorbance at 260, 280 and 230 nm with a Nanodrop 3000 (Thermofisher).

PCR reactions were carried out by combining 5 µL 10X PCR buffer (Invitrogen), 2 µL 50 mM $MgCl_2$, 0.1 µL 100 mM dATP, 0.1 µL 100 µM dTTP, 0.1 µL 100 mM dCTP, 0.1 µL 100 mM dGTP, 0.5 µL 100 µM forward primer, 0.5 µL 100 µM reverse primer, 0.4 µL Platinum™ Taq DNA Polymerase (Invitrogen), 39.8 µL PCR water and 2 µL of gDNA template. Primer sequences can be found in Table 1. Negative controls were prepared by replacing gDNA with PCR water. Using the Bio-Rad T100 thermocycler, PCR reactions underwent initial denaturation at 95°C for 2 mins followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 90 sec, then 72°C for 5 mins and infinite hold at 4°C. 10 µL of PCR products were loaded onto a 1% agarose gel with 1X SYBR Safe stain (Invitrogen) at 10,000X dilution and electrophoresis was carried out in 1X TBE buffer (5.4% Tris base, 2.75% boric acid, 2% EDTA diluted 5X in dH_2O) at 130 V for 60 mins. The gel was then visualized with the Bio-rad ChemiDoc™ MP Imaging System.

Amplicon sequencing. PCR products were purified with PureLink™ PCR Purification Kit (Invitrogen) according to the manufacturer's protocol and the Nanodrop 3000 (Thermofisher) was used to determine yield and purity. Samples were prepared for Sanger sequencing according to Genewiz criteria: 10 µL of PCR products were combined with 5 µL 5 mM *otsA* forward primer and sent to Genewiz for sequencing.

Growth curve. Overnight cultures were prepared by inoculating 5 mL of LB broth and LB + kanamycin broth with isolated colonies of BW25113 and JW5312-3, respectively, and grown at 37℃ overnight with shaking at 150 rpm. Samples of both overnight cultures were added to LB broth to reach an OD_{600} of 0.005 in a total volume of 200 µL per well. These cultures were grown in triplicate in a flat bottom 96-well plate alongside duplicate LB blank controls in the Microplate Reader (Biotek), which was set to linear shaking at 37℃ for 18 hours and measured OD_{600} at 15-minute intervals.

SDS-EDTA assay. Protocol adapted from Chang *et al*. (13). Overnight cultures were prepared by inoculating 5 mL of LB broth and LB + kanamycin broth with isolated colonies of BW25113 and JW5312-3 respectively and were grown at 37℃ overnight with shaking at 150 rpm. A 96-well flat bottom plate was prepared with 200 µL of LB media with 0.1 mM EDTA and varying concentrations of SDS (0.01%, 0.02%, 0.03%, 0.04%, 0.06%, 0.08%, 0.1%) to determine SDS sensitivity, or 0.01% SDS and varying concentrations of EDTA (0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, 0.9 mM, 1.2 mM) to determine EDTA sensitivity. In triplicate, overnight cultures of BW25113 and JW5312-3 were diluted in the

wells of varying SDS and EDTA concentrations in order to reach a starting OD_{600} of 0.005. Blank controls were included in duplicate for every SDS-EDTA condition. The plate was inserted into the Microplate Reader (Biotek) which was set to linear shaking at 37℃ for 18 hours with OD_{600} measured at 15-minute intervals. Each plate also included LB controls in triplicate inoculated with the same overnight cultures used in the SDS-EDTA wells. These were used to establish trial- and strain-specific baseline growth curves, which were then used to normalize the results of the SDS-EDTA assay to obtain fold change values of growth.

RESULTS

Confirmed *otsA* deletion in JW5312-3. In order to confidently investigate the role of trehalose on outer membrane stability, we first sought to confirm the genotypes of our strains. PCR amplification of *otsA* in gDNA of both strains was performed and analyzed by gel electrophoresis. The JW5312-3 amplicon displayed a slightly lower molecular weight than BW25113, around 1400bp and 1500bp respectively, suggesting that it had been disrupted (Supplemental Fig. 1). The Keio collection is developed through replacing the gene of interest with a kanamycin resistance cassette in order to inactivate it (14). To confirm that this was the nature of gene disruption in JW5312-3, we sent the amplicons for Sanger sequencing following PCR clean up. Results indicated that the JW5312-3 amplicon did indeed have sequence alignment with a kanamycin resistance cassette. Together, these data confirm that *otsA* in JW5312-3 is disrupted, providing confidence in the analysis of this strain to study the impact of trehalose on outer membrane stability. As such, JW5312-3 will hereafter be referred to as Δ*otsA* or *otsA* mutant, and BW25113 will be referred to as wild type (WT).

WT and *otsA* mutants had slightly different baseline growth dynamics. To assess baseline growth of the two strains, growth curves were constructed (Fig. 1). Although the two strains shared similar growth rates over the logarithmic stage of growth, they diverged in the stationary phase: the WT strain achieved a significantly higher average OD_{600} than the *otsA* mutant strain at 17-18 hours post-inoculation. When the percent difference was calculated separately for each biological replicate before comparison, the difference between the two strains reached statistical significance earlier in the assay, beginning at 12.25 hours (Supplemental Fig. 2). Together, these results indicate that the *otsA* mutants reach a significantly reduced OD_{600} compared to the WT during the stationary phase of growth.

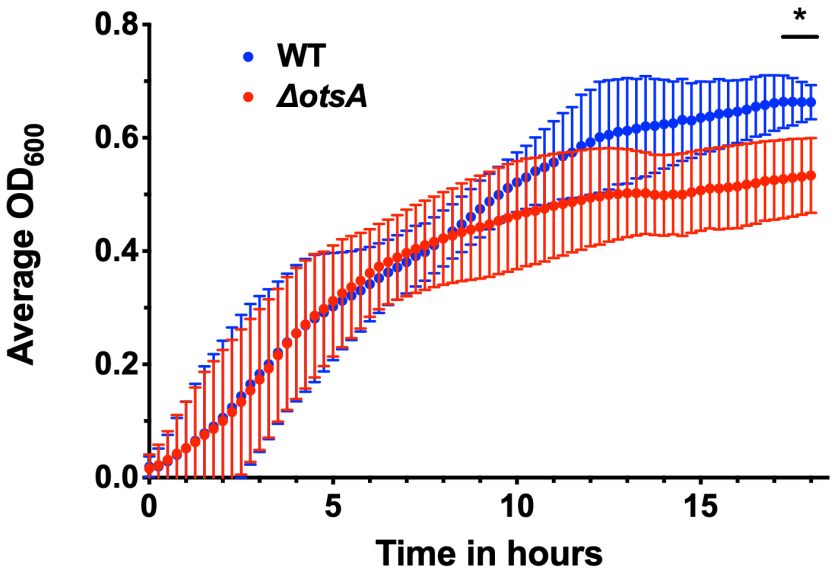

FIG. 1 WT and *otsA* mutant *E. coli* have slightly different baseline growth dynamics. Growth curves of WT (blue) and *otsA* mutant (red) over 18 hours in LB broth. Each point represents the average OD_{600} of three biological replicates, each with three technical replicates. Statistical analyses were performed with two-tailed unpaired parametric T tests. *, $P<0.05$. Error bars indicate standard deviation between biological replicates.

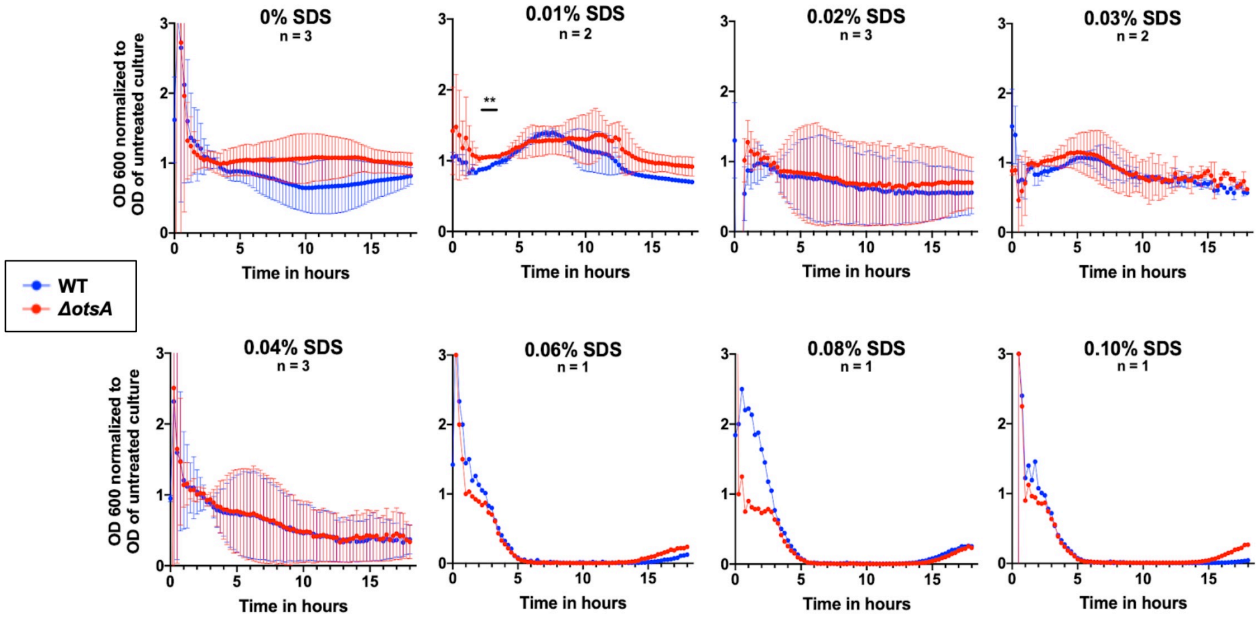

FIG. 2 *otsA* mutants are not more sensitive to SDS. Normalized SDS-EDTA assay results for varying concentrations of SDS, with WT (blue) and *otsA* knockout mutant (red) over 18 hours in LB broth supplemented with 0.1 mM EDTA. To normalize results to the untreated culture, the OD_{600} values from SDS-EDTA assay were divided by the OD_{600} values of the baseline (LB) growth curve corresponding to the correct genotype and timepoint (Fig. 1). Each point represents an average of the normalized OD_{600} values from the indicated number (n) of biological replicates, each with three technical replicates. Statistical analysis was performed with two-tailed unpaired parametric T tests. *, P<0.05; **, P<0.005. Error bars indicate standard deviation between biological replicates.

***otsA* mutants were not more sensitive to SDS-induced stress than the WT.** To investigate the role of trehalose in protection against SDS-EDTA stress, the WT and *otsA* mutant strains were first tested against varying concentrations of SDS with a constant low concentration of EDTA (0.1 mM). Results are shown normalized as fold change values of growth of the same strain at the baseline growth curve. Values below 1.0 indicate that SDS-EDTA treatment reduced the OD_{600} to levels below those observed in the untreated culture, which we interpret as the bacteria being hindered in their ability to stabilize the outer membrane (Fig. 2). Overall, increasing the concentration of SDS caused the normalized values to decrease, with little to no effect on OD_{600} at 0% and 0.01% SDS and an almost complete abolishment of growth at 0.06% and above. However, there was very little difference between the WT and *otsA* mutant strains within each condition. Both strains were equally susceptible to SDS-EDTA-induced stress across a range of SDS concentrations. The one exception to this observation was that at 0.01% SDS, between 2.25-3.25 hours, the *otsA* mutant achieved a significantly higher normalized OD_{600} as compared to the WT. Although the starting normalized OD varied between the two strains from 0-1 hours, these differences were not statistically significant and are likely explained by differing levels of condensation in the plate reader. Together, these results demonstrate that there was minimal difference in susceptibility to SDS-EDTA-induced OM stress between the *otsA* mutant and the WT strains, and indicate that the *otsA* mutant may have displayed slightly increased resistance to this stress at very specific conditions and times.

***otsA* mutants were not more sensitive to EDTA-induced stress than the WT.** To further investigate the differences in susceptibility to SDS-EDTA-induced OM stress between the *otsA* mutant and the WT, the two strains were also tested against varying concentrations of EDTA with a constant low concentration of SDS (0.01%). Across 0 mM, 0.1 mM, 0.2 mM, 0.4 mM, and 0.5 mM EDTA, the *otsA* mutant showed greater normalized OD_{600} values compared to the WT strain (Fig. 3). These differences emerged late in the assay, at 14 hours post-inoculation or later. This trend was present, although not statistically significant, in the

rest of the concentrations tested. These results indicate that the *otsA* mutant was not more sensitive to EDTA than the WT. In fact, at some EDTA concentrations and time points, the *otsA* mutant may have been less sensitive to SDS-EDTA-induced stress compared to the WT.

DISCUSSION

Numerous studies have demonstrated that trehalose provides protection against various abiotic stressors in *E. coli,* acting on an undetermined component of the cell envelope (2-4). A recent study by Chang *et al.* (13) determined that the knockout of *otsA* negatively impacted *E. coli* cell viability during SDS-EDTA-induced OM stress, which they suggested indicates that trehalose may act on the outer membrane specifically. We sought to validate these results with a more rigorous protocol using the correct parent WT strain, consistent growth media, OD_{600} readings at frequent intervals throughout the assay, and three biological replicates. Overall, the aim of our study was to investigate the role of trehalose in stabilizing the cell envelope of *E. coli* under SDS-EDTA-induced OM stress. Broadly, we demonstrate that under our experimental conditions, the *otsA* mutant did not present higher susceptibility to SDS-EDTA-induced stress than the WT. This suggests that trehalose may not promote OM stability in the context of this form of abiotic stress, as will be discussed below.

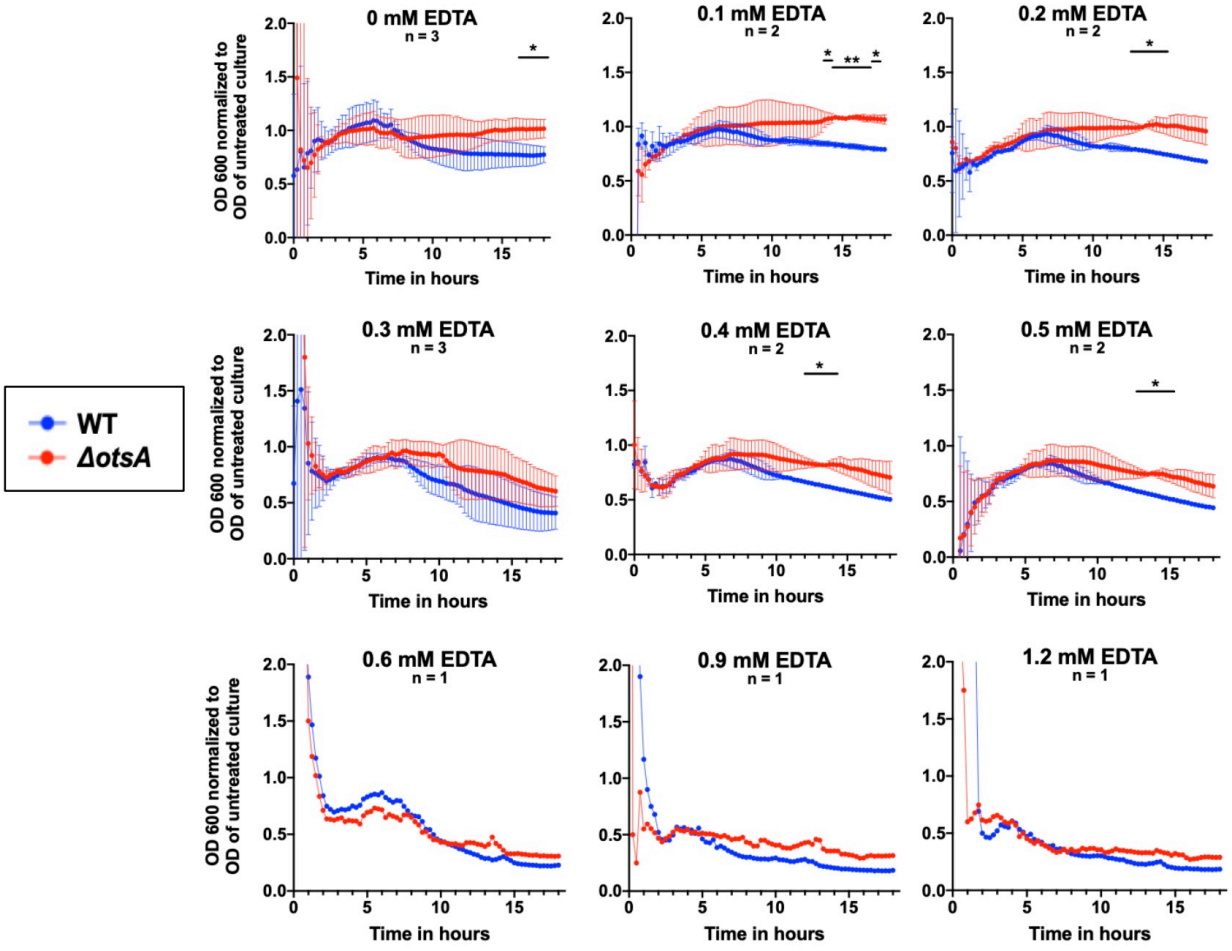

FIG. 3 *otsA* mutants are not more sensitive to EDTA. Normalized SDS-EDTA assay results for varying concentrations of EDTA, with WT (blue) and *otsA* knockout mutant (red) over 18 hours in LB broth supplemented with 0.01% SDS. To normalize results to the untreated culture, the OD_{600} values from SDS-EDTA assay were divided by the OD_{600} values of the baseline (LB) growth curve corresponding to the correct genotype and timepoint (Fig. 1). Each point represents an average of the normalized OD_{600} values from the indicated number (n) of biological replicates, each with three technical replicates. Statistical analysis was performed with two-tailed unpaired parametric T tests. *, $P<0.05$; **, $P<0.005$. Error bars indicate standard deviation between biological replicates.

We first demonstrated that the WT and *otsA* strains have slightly different growth dynamics. Our growth curve analysis indicated that the WT reached a significantly greater OD_{600} compared to the *otsA* mutant during the stationary phase. This is in contrast to results reported by Chang *et al.* (13), in which the *otsA* mutant attained a higher OD_{600} compared to the *E. coli* MG1655. However, MG1655 was incorrectly used as the parent strain instead of the correct parent strain (BW25113). There are several differences between the two strains, as BW25113 has certain genes deleted, replaced, or mutated compared to MG1655 (15). Since our assay used the correct parent strain, we were able to minimize the variation that arises from the different genetic backgrounds of the WT and mutant strains. Interestingly, a previous study investigating trehalose expression in yeast found that trehalose accumulated in cells during the transition between the exponential and stationary phases, and then was degraded during stationary phase (16). The authors suggested that trehalose could be consumed to provide carbon and energy to the cells, though it should be noted that the growth media was supplemented with glucose to support this process. Like yeast, *E. coli* are able to use trehalose as their sole carbon source (17). Thus, in our experiment, trehalose had the potential to act as a carbon and energy source for the WT cells under the baseline growth conditions. However, the carbon-limited nature of LB media brings into question the likelihood of this possibility. Consistent with this characteristic of LB media, the change in growth appeared at stationary phase; as the WT has an extra source of carbon (cell-produced trehalose), it would exhaust its carbon source later, and thus plateau at a higher OD_{600} (18). In contrast, the *otsA* mutant would not have been able to produce trehalose, which could limit its access to alternate sources of carbon and energy and result in a lower OD_{600}. As a result, our growth rate analyses suggested that cell-produced trehalose may also have been consumed as an energy and/or carbon source in *E. coli*. However, we cannot definitively conclude this, as trehalose concentrations were not directly measured during the assay.

These growth rate analyses allowed us to normalize our SDS-EDTA assays to the baseline growth rates of each strain. Through these SDS-EDTA assays, we demonstrated that the *otsA* mutants did not have increased susceptibilities to either SDS- or EDTA-induced stress, and in fact may have been more resistant to this stress compared to the WT. This is in contrast to our original hypothesis, as well as the previous study by Chang *et al.,* which found that the *otsA* mutant had increased susceptibility to certain EDTA concentrations (13). One possible explanation for the difference in results between our studies is that Chang *et al*. performed their assay with the *otsA* mutant grown in LB broth supplemented with kanamycin, which is a potential confounding factor. Upregulation of antibiotic resistance mechanisms in the presence of antibiotics is often energetically costly and can lead to reduced growth (19). This fitness cost has also been shown to increase under stressful growth conditions (20). As such, it is possible that the susceptibility of the *otsA* mutant to stress reported by Chang *et al.* was inflated due to reduced growth under antibiotic pressure, especially when coupled with SDS-EDTA-induced stress. To avoid this effect, we grew both strains in LB during our assay. Chang *et al.* only reported one biological replicate, which did not allow them to perform any statistical analyses of their results. As such, it is difficult to know whether or not the trends they observed were significant. In contrast, we performed three biological replicates for many of the concentrations we tested, which allowed us to draw conclusions regarding which of our results were statistically significant. These two reasons - the use of a consistent base medium without antibiotics and the use of multiple biological replicates with statistical analysis - gave us confidence in our results where they diverge from those of Chang *et al.* (13).

It was also surprising that the *otsA* mutant was less sensitive to SDS-EDTA stress compared to the WT in certain concentrations of EDTA. A previous study on protein costs in *E. coli* suggested that expression of unneeded genes can negatively impact cellular fitness and result in slower growth rates (21). Specifically, the authors found that cells had the highest fitness costs from producing unnecessary proteins when grown exponentially after a nutritional upshift from an overnight culture; our SDS-EDTA assays were conducted with a similar shift from an overnight culture. *E. coli* is able to utilize other compounds besides trehalose to adapt to environmental stress, including glycine betaine (3). Glycine betaine has been shown to be the preferred osmoprotectant molecule over trehalose, and may have been

synthesized from the choline that was present in the LB growth medium used in the SDS-EDTA assays (22, 23). Synthesis of trehalose may be considered unnecessary under high concentrations of SDS and EDTA, which may simulate potential osmotic stress-like conditions, if the glycine betaine pathway was indeed active. As a result, if the WT cells were continuing to produce even a low amount of trehalose in addition to glycine betaine, they may be at an energetic disadvantage compared to the *otsA* mutant that would solely be relying on glycine betaine. Although it is possible that the *otsA* mutant was also producing unnecessary protein due to its kanamycin resistance cassette, this is unlikely to have as large of an effect as the production of OtsA. Additionally, the OtsA protein is larger than the Kanamycin resistance protein (54 kDa and 31 kDa respectively (24)) and its expression may come at a higher energetic cost than production of *kanR* because OtsA uses UDP-conjugated glucose in the process of trehalose production. Thus, our results may have indicated that trehalose synthesis was unnecessary under EDTA stress and placed the WT at a growth disadvantage. Additionally, although our experimental model was specific for the outer membrane, there may have been unexpected non-specific effects on the cells due to the metal-chelation ability of EDTA (9).

The study by Chang *et al.* was unable to comment on the temporal patterns of their EDTA or SDS results due to a lack of continuous OD_{600} measurements. In contrast, our experiment involved continuously recording the OD_{600} measurements (at 15-minute intervals), allowing for greater insight into when various patterns arise. As a result, we were able to observe that the decreased susceptibility of *otsA* mutants to SDS-EDTA-induced stress emerged late in the assay, around 14 hours post-inoculation or later, well into the stationary phase. However, it is unclear why this pattern emerged late in the assay. One possible explanation is that the WT may have lost its growth advantage. Above, we tentatively suggested that the WT may be using trehalose as an alternative carbon or energy source at stationary phase in the baseline growth curve. This ability could give the WT a growth advantage, resulting in the observed higher OD_{600} as compared to the *otsA* mutant. However, if the WT was using trehalose to stabilize its OM in response to SDS-EDTA-induced stress, it may have a reduced ability to use trehalose as a carbon source. This theory is supported in the literature, as *E. coli* has been shown to differentially utilize trehalose depending on the stressors present in the environment (17). Horlacher and Boos found that low osmolarity promotes the catabolism of trehalose to be used as a carbon source, while high osmolarity promotes the production of trehalose for use as an osmoprotectant (17). Similarly, in our study, the WT may be catabolizing trehalose in the baseline growth curve and not under SDS-EDTA-induced stress, as it is used as an osmoprotectant instead. If this is correct, it would explain why the WT had lower normalized OD_{600} values– we normalized the OD_{600} values of the WT from the SDS-EDTA assay by using the growth curve where they had this growth advantage from trehalose catabolism. As such, the normalized OD_{600} values for the WT may have been lower than those of the *otsA* mutants because they were being compared to a higher stationary phase OD_{600} value, rather than being comparatively more susceptible to SDS-EDTA-induced stress. Important to note is that in the study by Horlacher and Boos, the minimal media used contained trehalose as the sole carbon source (17) and therefore it remains unclear whether cell-produced trehalose can be used as a growth supporting carbon source.

Study limitations. One limitation of the SDS-EDTA assay included the range of concentrations that were tested, which may not have been optimized for our specific strains. As demonstrated at the higher concentrations, SDS-EDTA-induced OM stress significantly inhibited the growth of both the mutant and WT *E. coli*, with no differences between the two strains that could be observed. Importantly, trehalose was not directly quantified in this study so we cannot definitively attribute these results to its presence in the OM. There exists the possibility that trehalose was not synthesized upon SDS-EDTA-induced OM stress, resulting in the lack of the protective effect that we expected to see in the WT. In addition to trehalose quantification, pre-treatment of the cells in a documented trehalose-inducing abiotic stressor such as low temperature shock (4) could be performed prior to the SDS-EDTA assay to ensure its presence during the target assay. However, it is worth noting that this treatment could

result in a wide range of gene expression changes and other methods such as an *otsA* overexpression plasmid could also be used for more gene specificity.

These results highlight the complexity of potential functions of trehalose in different abiotic stress conditions, as well as its possible associations with other osmoprotectants. Trehalose has also been implicated in several pathogenic mechanisms in a variety of organisms, representing its role in an emerging field of clinical research (4). Thus, better elucidation of its physiological functions in bacteria may offer greater insight into its roles in host infections and/or immune evasion (4). Clearer characterization of the relationship between the trehalose biosynthetic pathway and other compensatory mechanisms, as well as cellular localization, is needed. This insight may also allow for the development of more targeted antimicrobial therapies, a significant contribution in the broader context of antimicrobial drug resistance.

Conclusions Our study investigated the role of OtsA (an essential enzyme in the biosynthesis of trehalose) in protecting *E. coli* K-12 against SDS-EDTA-induced OM stress. Initially, we hypothesized that the *otsA* mutant would have increased susceptibility to SDS-EDTA-induced membrane stress due to a loss of trehalose synthesis and thus decreased stabilization of the outer membrane. Contrary to this hypothesis and the previous findings that we sought to validate (13), our data showed that trehalose did not provide a protective effect against SDS-EDTA-induced outer membrane stress, as our wildtype did not show a consistently higher normalized OD_{600} reading than our mutant strain in any SDS or EDTA concentration tested. We take this to suggest that trehalose disruption does not promote outer membrane sensitivity under SDS-EDTA-induced stress. As such, the mechanism by which trehalose protects *E. coli* against abiotic stressors remains unclear and is a promising area of future research.

Future Directions There are numerous approaches that could be taken in order to more thoroughly investigate the protective role of trehalose in the *E. coli* cell envelope. Although we determined that *otsA* was disrupted in the mutant strain, we did not directly test for the presence or absence of trehalose. Future studies should look to quantify the amount of trehalose in cells to further confirm its role and regulation in *E. coli* under abiotic stress. One possible approach to this would be via a trehalase assay, in which trehalose present in the lysates from cells of interest is converted to glucose and then quantified with commercial assay kits (25). Additionally, the amount of *otsA* mRNA could be measured by RT-qPCR at various time points during the SDS-EDTA assay. This could provide more information about whether the bacteria are upregulating trehalose biosynthesis genes, and if so, when and under what conditions. OtsA could also be overexpressed using a plasmid, which may add further specificity with respect to the induction of trehalose production. Another method to increase confidence in the *otsA* mutant phenotype could involve determining if the viability of the *otsA* mutant decreases during a cold shock, as *otsA* mutants have been found to have decreased survival at 4°C (5); however, a cold shock may potentially induce other undesirable gene expression changes, which must be carefully considered. Together, these approaches could provide further confidence that the WT contains trehalose and that the *otsA* mutant does not. Without that information, we cannot definitively attribute any protective or non-protective effects to the presence of trehalose in the cells under abiotic stress.

We suggested that one explanation for the lower normalized OD_{600} observed in the WT strain, compared to the *otsA* mutant under certain conditions during the SDS-EDTA assay, was that the WT could have lost the growth advantage that it previously had in the untreated baseline growth curve. Specifically, we proposed that the WT was catabolizing trehalose during the baseline growth curve and not under SDS-EDTA-induced stress. qPCR of genes involved in the catabolic pathway of trehalose (e.g. *treB, treC,* or *treR*) in the WT grown in LB broth supplemented with or without various concentrations of SDS and EDTA could add confidence to this explanation (17).

A comprehensive investigation of the protective effects of trehalose should utilize a wider variety of abiotic stressors in future studies. Trehalose has been reported to be protective against osmotic stress (3), temperature shock (4), and desiccation (2). Assays utilizing these

forms of abiotic stress could be used to confirm the phenotype of the *otsA* mutant, or as pretreatments prior to an SDS-EDTA assay if the outer membrane is the focus of investigation. Again, the quantification of trehalose and/or the verification of its presence is crucial, as this would further validate the attribution of any effects to the loss of trehalose synthesis in the *otsA* mutant. Furthermore, we focus largely on the OM of *E. coli*, though there are other cell envelope structures through which trehalose may be providing support under abiotic stressors. Future studies should also aim to elucidate which structural component of *E. coli* that trehalose is able to act on, in order to support structural integrity under abiotic stress (e.g. using treatments that target the peptidoglycan component of the cell envelope).

Alternate pathways should also be investigated to determine if a lack of trehalose in the *otsA* mutant can be compensated by another molecule. Glycine betaine is a well-known osmoprotectant that is also synthesized by *E. coli* via the BetBA pathway during abiotic stress (26). A strain containing a double knockout of the *otsBA* and *betBA* operons could account for both biosynthetic pathways and provide further insight into their osmoprotective roles. Since glycine betaine is synthesized from its precursor, choline, an alternative method may be to grow the cells in media without choline (e.g. M9 minimal media). Finally, it might be interesting to investigate the effect of trehalose in antibiotic resistance, as antibiotic treatment is a form of abiotic stress that has yet to be investigated in the context of trehalose cell envelope stabilization. This would have implications for potential mechanisms of drug resistance in *E. coli* and could be explored as a target for antimicrobial therapy.

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