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Pretreatment with Sub-minimal Inhibitory Concentration of Kanamycin Increases RpoH Levels in *Escherichia coli* SC122

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Pretreatment of *Escherichia coli* with sub-MIC of 4 µg/ml kanamycin led to an increase in cell viability and survival upon exposure to temperatures greater than 45°C. The viability of sub-MIC kanamycin pre-treated wild type *E. coli* SC122 and K165 (Δ*rpoH*) upon transfer to 45°C was assessed using spectrophotometry and spread plating by cellular enumeration. Cellular levels of RpoH in sub-MIC treated *E. coli* SC122 cultures grown at 30°C were monitored using western blotting. Increase of RpoH levels was used as an indicator of a heat resistant phenotype. At 45°C, sub-MIC of kanamycin pre-treated *E. coli* SC122 showed a higher growth rate between 20 - 40 minutes compared to the untreated cultures. As expected, no significant changes in turbidity and a rapid drop in cell viability were observed for all treatments of the K165 strain in identical conditions. RpoH detection via western blotting in SC122 sub-MIC of kanamycin treated cultures grown at 30°C revealed an increase in band intensity, correlating to an increase in RpoH amounts over time. We reported the increase of RpoH levels in *E. coli* SC122 in response to sub-MIC of kanamycin.

When *Escherichia coli* is exposed to temperatures of 42°Cand above, a heat shock molecular cascade is induced. The expression of heat shock proteins DnaJ, DnaK and GrpE involved in proper folding and degradation of non-functional proteins is upregulated in order for the organism to tolerate higher temperatures (1).Heat shock proteins are regulated at the transcriptional level by RpoH, which is responsible for guiding RNA polymerase (RNAP) to the promoters of the heat shock response genes, conferring protection to cellular proteins from heat denaturation. In the absence of, or in the case of an aberrant RpoH response, the accumulation of non-functional proteins results in a temperature sensitive phenotype and rapid cell death on exposure to high temperatures (2).

RpoH is present at very low levels at 30°C (2). Under non-heat shock conditions RpoH activity is repressed in two ways. First, free RpoH is neutralized through direct interactions with DnaK and DnaJ chaperone proteins, which facilitate its degradation, by FtsH protease (3). Secondly, *rpoH* mRNA transcripts form extended secondary structures that are unable to be processed and translated by ribosomes (4). However, heat induced changes in the *rpoH* mRNA secondary structure allows for ribosomes to interact with the mRNA. In addition, at temperatures above 42^{o}C, interaction between RpoH and DnaK is weakened due to a conformational change in RpoH, and due to DnaK/DnaJ complex chelated by the abundance of heat denatured proteins (2). Hence, in temperatures above 42^{o}C, a 17-fold increase of RpoH, mediated at the transcriptional and translational level, can be observed (7).

Understanding how low levels of antibiotics affect the bacterial physiology has important implications in discovery of novel intrinsic mechanism for antibiotic resistance. Due to the highly conserved nature of the heat-shock response system, the following results suggest a potential universal strategy that bacteria use to deal with low levels of antibiotics that are prevalent in our environment (2, 6). Furthermore, insights into sub-MIC of antibiotic-induced response in *E. coli* could provide a better understanding of the intracellular signaling and potential crosstalk in bacterial stress response systems.

Sub-MIC of kanamycin has been demonstrated to render *E. coli* more tolerant to high temperature conditions (1). Until now, the mechanism through which the sub-MIC of kanamycin induces this effect had not been identified. It is known that kanamycin binds to the 16s rRNA in the 30s ribosomal subunit, causing the ribosome to misread the mRNA template, resulting in the accumulation of truncated and misfolded proteins (7). The purpose of this experiment is to determine if kanamycin is acting through the inherent heat shock system of *E. coli* using RpoH as an indicator of this cascade.

MATERIALS AND METHODS

Bacterial strains. *E. coli* SC122 and *E. coli* K165 (Coli Genetic Stock Collection, #6768 and#6769 respectively) were chosen as the organisms for the experiment (8). Both strains have identical genotype characteristics; *lacZ53*(AM), *phoA5*(AM), λ^{-}, *tyrT9* (ts, AS), *tyrT47*(OS), *trp-48*(AM), *relA1*, *rpsL150*(strR), *malT66*(Am,λ^{R}), *spoT1*, with the additional amber mutation localized to the *rpoH* gene (Δ*rpoH601*(Am)) in the K165 strain. Unless otherwise stated, all strains were cultured in Luria Broth (LB) media (1.0% w/v Tryptone, 0.5% w/v Yeast Extract, 1.0% w/v NaCl). Overnight cultures were incubated at 30°C, shaken at 95 Rumford a minimum of 12 hours and diluted as needed with sterile LB media before the start of the experiment.

MIC microplate assay for kanamycin. Overnight cultures of wild types SC122 and Δ*rpoH*K165 *E.coli* were diluted to 0.010OD$_{500}$. Wells for each diluted culture were set up using a standard 96 well flat bottom microplate (Cat. # 82.1581.001, Sarstedt). Stock kanamycin of 100 µg/ml(Cat.#11815-032, Life Technologies) in LB solution was added to wells to achieve final concentrations of 0.5, 1.0, 2.0, 4.0, 8.0, 12.0, 16.0, 20.0 and 24.0 µg/ml. Kanamycin in LB 0 µg/ml and 100 µg/ml were used for the positive and negative controls, respectively. In all cases, well volumes were 200 µl with an initial turbidity of 0.005OD$_{500}$. Plate was incubated at 30°C oven and read by a BioTek Epoch microplate spectrophotometer every 24hours with final reading taken at 60hours when positive control cultures reached turbidity

of 1.000 OD_{500}. MIC for each strain was deemed to be the concentration of kanamycin at which the turbidity did not deviate from the negative control. A sub-MIC of kanamycin in LB at 30 ^{o}C for each strain was then taken as half the MIC.

Culture preparation and pretreatment with sub-MIC of kanamycin. Overnight cultures of wild type SC122 and K165 *E. coli* were diluted to turbidity of 0.200 OD_{500}.Each was separated to 3 Erlenmeyer flasks, with a single one receiving sub-MIC kanamycin appropriate for the strain. Cultures were shaken at 195 rpm in a30°C water bath for 1.5 hours to allow for the pretreatment effect in the sub-MIC kanamycin sample. Growth was monitored via a Milton Ray Spectronic 20D spectrophotometer to ensure that all treatments reached a minimum turbidity of0.600 OD_{500}, at which point an additional LB culture from each strain received sub-MIC kanamycin addition. The late addition of sub-MIC of kanamycin served as a control for the direct effects, rather than pretreatment, of kanamycin on mediating heat resistance in bacterial cultures. Three treatments for each strain were then transferred to a shaker at 195 rpm in a 45°C water bath.

SC122 and K165 cell growth and viability at 45°C with and without pretreatment of sub-MIC of kanamycin. Culture turbidity (OD_{500}) of all treatments was monitored using a spectrophotometer over the course of the experiment. Every 15 minutes, a sample from each treatment was diluted appropriately for spread plating. All liquid cultures were monitored and sampled for seven individual time points approximately 15 minutes apart. All LB plates were incubated at 30°C and counted after 36 hours of growth.

SC122 growth and protein isolation. Overnight SC122 culture was diluted to turbidity of 0.200 OD_{500}ina fresh 300ml LB medium with added 4 µg/ml of kanamycin (sub-MIC for SC122). Culture was shaken at 195 rpm in 30°C water bath. Starting immediately after the initial dilution, 30ml of SC122 culture was extracted in a 50 ml centrifuge tube every 15 minutes and placed on ice. Samples were centrifuged at 7500 x g for 10 minutes in 4°C. The supernatant was removed and pellets were washed with 1 ml of 10mM Tris, pH 8.0 and re-centrifuged at the previous setting. The cell pellets were re-suspended in 1ml of 10mM Tris, pH 8.0 and lysed using a MP Bio Fast Prep-24 instrument with 0.1mm glass beads and a single large non-plating, glass bead. The FastPrep was performed with 3 cycles of 20 seconds at 4 m/s, followed by a 20 second pause after each cycle. Lysates were centrifuged at 14,000 x g for 5 minutes at 4°C. Supernatants were then transferred to fresh microcentrifuge tubes and stored at -20°C until further use.

Bradford protein microassay. Bradford microassay was performed on all protein samples as well as bovine serum albumin (BSA) (Cat. #B90015, Fermentas) standards as described in (9).Protein dye reagent concentrate (Cat. # 500-0006, Bio-Rad) was diluted 1/3 with dH_2O prior to use. Samples were read on a Thermo Scientific Evolution 60S spectrophotometer at 595 nm. BSA standards were used to construct a standard curve and extrapolate the protein concentrations of the bacterial lysates.

SDS-PAGE. Protein concentrations were standardized by the results from the previous Bradford assay. Standardized proteins were combined in one-to-one ratio with2x SDS sample buffer (50 mM Tris pH 6.8, 100 mM DTT, 2% w/v SDS, 10% w/v glycerol, 0.1% w/v bromophenol blue). The samples were heated for 7 minutes at 95 ^{o}C. Denatured samples were loaded onto a 5% stacking gel above a 12% SDS-polyacrylamide gel submerged in 1x running buffer (25 mM Tris pH 6.8, 156 mM glycerine, 1% w/v SDS). Novex sharp protein standard (Cat. #LC5800, Life Technologies) and XP Western Blot protein standard (Cat. #LC5602, Life Technology) were added on to the either sides of the samples to aid in protein sizing and visualization. Samples ran vertically using the Bio-Rad Mini Protean 2 cell system at 60 V for 15 minutes followed by 100 V for 65 minutes.

Western blot. Proteins were blotted onto a nitrocellulose membrane (Cat. #162-0116, Bio-Rad) in a chilled transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol) and ran at 10 V in Bio-Rad Mini Protean 2 cell system overnight in a 4 ^{o}C room. After this, the membrane was submerged and washed for 5 minutes with TBS-Tween 20 (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% v/v Tween-20), followed by the incubation submerged in western blocking solution (Cat. #W0138, Sigma) for 1.5 hours. Membrane was then hybridized with mouse anti-RpoH IgG monoclonal antibody (Cat. #WP006, Neoclone). Membrane was incubated overnight at 4°C. After primary antibody incubation (≈16 hours), the membrane was rinsed 3 times for 5 minutes each with TBS-Tween 20 and then hybridized with rabbit anti-mouse IgG polyclonal antibody conjugated to alkaline phosphatase (Cat. #D0314, Dako), diluted to 1:500 in dH_20. The membrane was washed for 5 minutes with TBS-Tween 20, 3 times. BCIP/NBT liquid substrate system (Cat. #B6404, Sigma-Aldrich) was added directly onto the membrane and incubated at room temperature for 30 minutes in the dark. The membrane was then visualized directly and photographed using a digital camera.

RESULTS

MIC of kanamycin for *E. coli* SC122 was higher than that of *E. coli* K165. The calculated sub-MIC that was used for all subsequent kanamycin treatments was 4 µg/ml and 2 µg/ml for SC122 and K165, respectively. Therefore the *ΔrpoH*K165 strain was significantly more susceptible to kanamycin in LB than SC122.

Pretreatment of SC122with sub-MIC resulted in an increased turbidity. During pretreatment at 30°C, SC122 was observed to exhibit a faster growth rate, compared to K165**,** regardless of the presence of kanamycin. Following the transfer to 45°C, SC122 pre-treated with kanamycin showed an increase in turbidity between 20 and 40 minutes (Fig. 1. A). interestingly, the turbidity of pre-treated SC122 culture showed a rapid drop following 40 minutes of growth.

No overall turbidity difference was observed for K165 cultures between any treatments. The turbidity of pre-treated K165 did not deviate from its initial turbidity before transfer to a 45°C water bath (Fig. 1. B). In the late addition and the non-treated cultures, there was a slight increase in the turbidity in the initial 10 minutes after transfer; however, this was followed by a plateau that remained constant over the course of the experiment.

SC122 viability, following transfer to 45°C, differed based on the presence and the timing of kanamycin addition. The untreated SC122 showed constant cell viability throughout its incubation at45°C (Fig. 2). The late addition of kanamycin showed an initial plateau for the first 30minutes, followed by a sharp drop in cell viability to a second plateau at 38 minutes that remained stable until the end of the experiment. Viability in pre-treated SC122 culture showed an initial decline, followed by a rapid drop to the second plateau achieved by the late kanamycin addition.

K165 culture grown at 45°Cshowed a rapid decline in cell viability, regardless of treatment. The untreated sample decreased to less than 3000 CFU/ml by 20 minutes

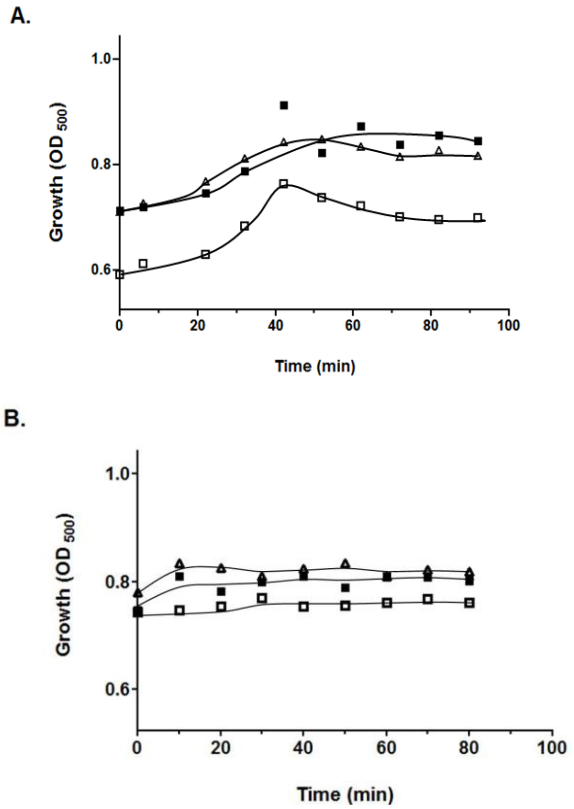

FIG 1 Growth of *E. coli* showing heat tolerance at 45°C where solid squares are untreated, open triangles are sub-MIC kanamycin treated at 30°C for 1 hour prior to 45°C exposure, and open squares are delayed addition of sub-MIC kanamycin at T = 0. (A) Wildtype SC122. (B) Mutant K165.

whereas, both kanamycin pre- and late-treated cultures showed no cell viability at 20 minutes. This difference was not significant; thereby it can be concluded that all samples showed a rapid decline by 20 minutes. The decimal reduction time for pre-treated culture was 4.0 minutes, whereas the late addition of kanamycin had 4.1 minutes. The non-treated culture had decimal reduction time for 21.0minutes.

RpoH presence in kanamycin-treated wild type *E. coli* SC122 culture peaked at 75 minutes post kanamycin addition. The band at approximately 32 kDa increased in intensity after 45 minutes of incubation at 30°C with sub-MIC of kanamycin (Fig. 3). In addition, the intensity of the protein decreased between 75 and 90 minutes (Fig. 3). Coincidentally, a smaller fragmented band appeared during this time transition. A similar decrease was observed between 45 and 60 minutes in the non-kanamycin treated samples at 45°C.Interestingly, SC122 overnight culture grown at 30°Ctreated with sub-MIC levels of kanamycin immediately prior to protein extraction showed a high intensity band at 32 kDa.

DISCUSSION

In *E. coli* SC122, sub-MIC of kanamycin increases RpoH levels, even in the absence of heat. The exact mechanism of this increase has not been shown, however, the effect of kanamycin may result from an accumulation of misfolded proteins. This accumulation would bind DnaK to the misfolded proteins, freeing the RpoH (2). Free RpoH protein could, then, bind to RNAP and promote binding to its promoter, leading to increased transcription of *rpoH*, *dnaK* and *dnaJ*. It is also possible that the additional DnaK chaperone proteins produced after kanamycin treatment could be rendered non-functional due to the effect of kanamycin on protein translation. Alternatively, the *rpoH* transcription may have been induced by the presence of sub-MIC of kanamycin since a transcriptional induction of *rpoH* in response to elevated temperatures has been found in other organisms (10). Kanamycin has also been shown to override the thermal activation mechanism in *E. coli* SC122.

A gradual increase in levels of detected RpoH protein was unexpectedly observed for 60 minutes after incubation in sub-MIC of kanamycin in 30°C. Previous studies revealed that in less than 3 minutes at elevated temperatures, a 17-fold increase in the concentration of RpoH was observed, followed by a rapid degradation and decline, confirming that the induction of heat shock proteins, along with RpoH, is a rapid response (5). The difference in the time response in this experiment may be due to antibiotic effects on protein synthesis. The interference by kanamycin may have reduced the protein synthesis rate in *E. coli*, resulting in a slower production rate of the protein. In comparison, a different observation in RpoH response was seen in the immediate-treated culture; an immediate peak in RpoH protein was observed after the addition of kanamycin (Fig. 3). Thus, the effects of kanamycin may elicit an immediate response to increase RpoH, similar to a heat response. The cause of this observed gradual increase may be due to experimental procedures; as cultures were sampled and stored in ice until protein isolation, slow degradation of RpoH may have occurred. This does not, however, completely explain the stability of RpoH for over an hour in the culture. In a normal heat stress culture, a rapid reduction in RpoH can be observed after the first fifteen minutes, followed by a plateauing towards the initial pre-stressed condition; the

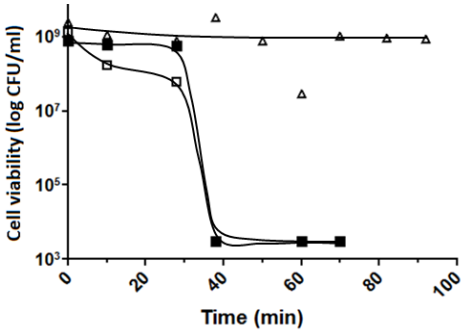

FIG 2 Survival of *E. coli* SC122 showing heat tolerance at 45°C where solid squares are untreated, open triangles are sub-MIC treated at 30°C for 1 hour prior to exposure to 45°C, and open squares are delayed addition of sub-MIC kanamycin at T = 0.

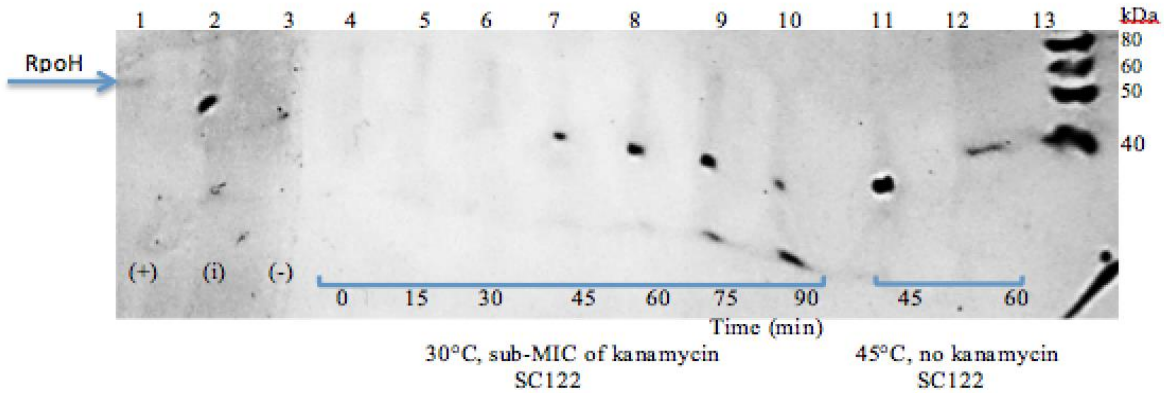

FIG 3 RpoH levels in *E. coli* SC122 lysates after corresponding time post-addition of sub-MIC kanamycin. Lane 1: (+) SC122 incubated at 45°C for two hours without sub-MIC kanamycin; lane 2: (i) SC122 incubated at 30°C with exposure to sub-MIC kanamycin immediately prior to protein isolation; lane 3: (-) K165 grown at 30°C without kanamycin; lane 4-10: SC122 simultaneously incubated at 30°C and sub-MIC kanamycin; lane 11-12: SC122 incubated at 45°C without kanamycin; land 13: XP Western blot protein standard.

transient half-life of RpoH protein at 45°C is only one minute (5). As regulatory proteins such as DnaJ and DnaK are either non-functional due to misfolding or are overwhelmed with other misfolded proteins due to combined effects of heat and kanamycin; the RpoH would be suspected to accumulate continuously. Interestingly, in between 75 and 90 minutes in 30°C culture, a small band below the RpoH is seen. This may be a fragmented part of the protein that has been degraded by the regulatory proteins.

The pretreated kanamycin culture showed negligible increases in heat tolerance at 45°C despite the increased RpoH response. However, a relatively faster growth rate was observed in the first twenty minutes, in comparison to the non- and late-treated cultures. This difference, however, was not significant enough to identify the trend as a faster adaptation in the pretreated culture. Due to a higher concentration of detected RpoH (Fig. 3), it was expected that similar behavior would be observed in heat shock proteins; thus, the pretreated culture was expected to have prolonged cell survival rate. Unexpectedly, a rapid decline in the cell viability after that initial twenty minutes was observed. Similarly, the decimal reductions for both strains in 45°C did not follow the expected observation correlating to the increase of RpoH.

This might be due to the increased potency of kanamycin at a higher temperature (11). As the cellular membrane becomes more permeable, allowing more kanamycin to enter into the cytoplasm, translation and DNA synthesis is irreversibly blocked. The antibiotic has also been known to cause irreversible damage to the cellular membrane (7). In addition to the detrimental effects of kanamycin, excessive amounts of RpoH can be deleterious and inhibits cell growth (4, 7). Thus, kanamycin-treated culture correlates to a lower cell viability and high decimal reduction time. Additionally, the relative rate of temperature change was different with the cell viability test and turbidity measurement. The media in the turbidity experiment underwent a suboptimal increase in temperature, whereas the culture used for spread plating was diluted and incubated in pre-warmed 45°C media. The difference between may have affected the adaptability of the culture. Another limitation for this experiment was that the kanamycin remained in the culture throughout the experiment. Additionally, the limitation in the western blot was that the RpoH may have been degraded over time, as the protein isolation samples were placed on ice in a 50 ml centrifuge tube, potentially allowing for slow RpoH degradation at the center of the tube due to uneven cooling throughout the sample. Thus, the gradual increase in the band intensity may not properly reflect the accumulation of RpoH in SC122. In determining the MIC for each strains, the MIC was determined at 30°C, which reflects the pretreatment of the cell cultures only, and did not reflect the physiology of the cells as well as the effect of kanamycin at 45°C. Another limitation in the cell viability experiment was that the loss of viability in all treatment levels was too rapid to be observed fully with the current technique, specifically between 25 and 38 minutes.

The observation from this study also showed a higher resistance to kanamycin by the *E. coli* SC122. The wild type SC122 had a two-fold higher MIC than the K165 Δ*rpoH* amber mutant. This increased tolerance against kanamycin may be due to the RpoH response and its adaptability against misfolded proteins.

Overall, sub-MIC of kanamycin was able to mimic a heat stress response by enhancing the accumulation of RpoH, but this did not correlate to higher heat tolerance. This kanamycin-dependent induction of RpoH, though, provided additional insight into the macromolecular mechanism of the heat shock cascade and its activation. Additionally, this thermal stress might results in an antibiotic resistance, similarly to previously observed resistance to rifampicin in *E. coli* from heat shock (12).

FUTURE DIRECTIONS

Given that the above findings do not specify the mechanism by which RpoH levels increase in *E. coli*, future studies can investigate mechanisms of upregulation. Quantitative real time PCR can be used to study increases in expression of heat shock genes when exposed to sub-MIC of kanamycin. This would indicate that kanamycin is having an effect on the expression system associated with the heat shock molecular cascade.

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