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The Effect of Sequential High Temperature and Hyperosmotic Shock on Rpos Expression and Cell Viability in *Escherichia coli* B23

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σ^s, commonly known as RpoS, is a subunit of bacterial RNA polymerase and a general stress regulator which is up-regulated when bacteria are subjected to sub-optimal environmental conditions such as high temperature and high osmolarity. The objective of this study was to investigate the ability of *Escherichia coli* B23 cells to cross-protect against high osmolarity by first subjecting the cells to high temperature shock then exposing the cells to hyperosmolarity. Cell viability was then measured to estimate overall cell survival. The cells that were dual-treated had a 1.5-fold reduction in cell viability as compared to the cells subjected to only hyperosmolarity. RpoS expression in the different treatments was examined by Western immunoblotting with a monoclonal RpoS-specific antibody. The heat shocked and control cells had approximately equal amounts of RpoS expression. The dual-treated cells had the highest level of RpoS expression, whereas the cells subjected to just hyperosmolarity had the lowest. No cross-protection was observed as cell viability in dual-treated cells was unexpectedly low compared to cells subjected to just hyperosmolarity; a possible explanation is that increased RpoS expression could have increased production of genes responsible for cell death, therefore, sacrificing part of the population to increase nutrients available and to better the chance of survival for the rest of the population. Future experiments including improvements of the experimental procedure, alterations in the order and number of stress conditions and using RpoS$^-$ mutant strains may be helpful to better understand the mechanism of RpoS in response to sequential stresses.

Escherichia coli, a gram negative enteric bacterium, is constantly exposed to adverse environments, such as UV radiation, temperature or osmotic shock, oxidative stress and nutrient deprivation, forcing it to adapt in order to survive. Fortunately for *E. coli,* they have developed a mechanism to partially counteract these stresses through the general stress response regulator, σ^s, which allows the up-regulation of genes that help the cell survive these conditions (7).

σ^s (or RpoS) is a 38 kDa sigma subunit of RNA polymerase that was originally found to be up-regulated as *E. coli* entered stationary phase, which itself is considered a form of stress (5, 11). It was later discovered that RpoS was also up-regulated when *E. coli* was subjected to other stress conditions including limiting nutrient concentrations, temperature and osmotic shock, and high population density (6). Regardless, the current understanding of RpoS is that it transcriptionally activates a subset of genes by directing the RNA polymerase to alternate promoters, resulting in the expression of new genes. These new genes are generally involved with stress resistance, cell morphology, metabolism, and virulence to help the cell adapt to the stress conditions and then to promote cell death if unable to adapt (6). Because the stress response system results from a variety of external environmental cues to cause dramatic changes within the cell, tight regulation of RpoS is essential to the cell survival. Therefore, regulation of RpoS levels occurs at virtually every step, including input at transcription, translation, and proteolysis (7).

Because RpoS is believed to direct the RNA polymerase to alternative promoters (7), theoretically, one single stress condition can lead to increased protection against all other stresses, since the same subset of genes should be activated as part of the response (6). Indeed, even before the characterization of RpoS, it was noted that *E. coli* at stationary-phase showed increased resistance to high osmolarity and high temperature conditions (9, 10). A more recent example involved *E. coli* O157:H7 (a pathogenic strain causing human foodborne illness), where it was found that alkaline cleaners increased the bacterial resistance to heat and sanitizers (14). It is hypothesized that this cross-protective mechanism may provide cells with an ability to survive against numerous stresses not yet encountered after being subjected to an initial stress (7).

The objective of this study was to test the potential ability of heat shocking *Escherichia coli* B23 to cross-protect against high osmolarity. Although previous studies have demonstrated increased bacterial resistance to heat upon prior exposure to alkaline cleaners, our work tests the

effects of consecutive heat and hyperosmolarity stersses not only on cell survival but also RpoS expression levels. We hypothesize that heat shocking *Escherichia coli* B23 cells will result in cross-protection, leading to increased cell viability and RpoS expression relative to control samples. Initially, we subjected logarithmic-phase wild-type strain *E. coli* B23 to heat stress, and immediately after, to high osmolarity stress. The effects of the stresses were monitored through cell viability and RpoS expression assays.

MATERIALS AND METHODS

Bacterial culture. *Escherichia coli* K12 B23 was provided by Dr. William Ramey (Department of Microbiology & Immunology, University of British Columbia).

Growth conditions and induction of stress. Cells were seeded from stationary overnight culture at a dilution of 1/20 in Luria-Bertrani (LB) media (0.5% yeast extract, 1% tryptone, 0.5% NaCl, 0.2% glucose). Re-seeded cultures were grown at 37 °C with agitation (125 rpm) and aeration. At 45 min (OD_{660} ~0.5), samples selected for heat treatment were transferred to growth at 45 °C for 15 min. At 60 min, samples selected for hyperosmolarity treatment were supplemented with NaCl to increase the total salt concentration to 0.8 M and were grown for a further 15 min at 37 °C.

Viability assay. At 75 min of growth, samples were diluted (10^{-6}, 10^{-7} and 10^{-8}), and 1 mL of each diluted sample was plated on LB media with 1.5% agar and grown at 37 °C until the appearance of distinct colonies was observed (~24 h).

Cell lysis. At 75 min of growth, 25 mL of each sample was centrifuged at 7,500 x g in a J2-21 centrifuge (Beckman) at room temperature for 10 min. Cells were washed with 10 mL Tris buffer (10 mM, pH 8) and re-centrifuged before resuspending cell pellets in either 2 mL (Trial 1) or 1 mL (Trial 2) Tris buffer for sonication. Lysis was then performed by pulse sonification with a Microson Ultrasonic Cell Disruptor (Misonix, Farmingdale, NY) for 5 min. Lysates were microfuged at 14,000 x g in a 5415 D centrifuge (Eppendorf, Germany) at 4 °C and stored at -20 °C for further use.

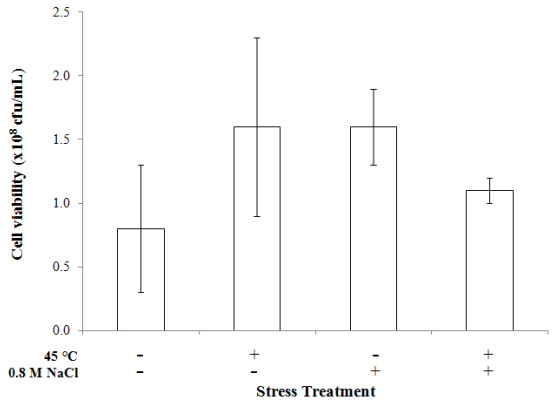

FIG. 1. Viability assay for cross-protection from hyperosmolarity by high temperature in *E. coli* B23. Cell viability was measured by cell plating and is expressed in viable cell concentration units (cfu/mL). Uncertainty was expressed as the magnitude of mean absolute deviation from three replicates per each dilution plated.

Total protein assay. Lysates were analyzed for protein concentration by the Bradford assay as previously described (2). Bradford reagent was purchased from Bio-Rad Laboratories, and chicken egg albumin (Sigma-Aldrich) was used as a protein standard.

SDS-PAGE. *E. coli* K12 B23 lysates were combined in equal amounts with a 2x sample buffer (50 mM Tris pH 6.8, 100 mM dithiothreitol, 2% sodium dodecyl sulphate [SDS] (Sigma-Aldrich), 0.1% bromophenol blue, 10% glycerol). Samples were then heated to 90 °C for 3 min and microfuged at 14,000 x g at room temperature for 10 s. Protein content was normalized by altering volume of sample loaded as judged by the Bradford protein assay. Samples were loaded onto a 12% SDS-polyacrylamide gel (Bio-Rad) and run vertically in running buffer (25 mM Tris base pH 6.8, 192 mM glycine, 1% SDS) at 180 V in a Bio-Rad PAGE apparatus for 45 min. Duplicate gels were made and run; one was used for Western blotting and immunodetection, while the other was stained with PageBlue™ Staining Solution (Fermentas Life Sciences, cat. # R0579) for 2 h to confirm protein normalization in parallel treatment wells.

Western blotting and immunodetection of RpoS. Following SDS-PAGE, blotting onto a Hybond™-P membrane (Amersham Biosciences, cat. # RPN2020F) was performed in transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol) at 100 V in a chilled Bio-Rad electroblotting apparatus. Following blotting, the membrane was treated with Western blocking reagent (Roche, cat. # 11 921 673 001) for 10 min. Following two successive washes with TBS-Tween 20 (50mM Tris, 150 mM NaCl, 0.2% KCl, 0.5% Tween 20, pH 7.5), hybridization with a primary mouse anti-RpoS antibody (Neoclone Biotechnology, cat. # W0009) diluted 1/1000 in Western blocking reagent was performed for 1 h at room temperature on a shaking apparatus. Following three additional washes with TBS-Tween 20, hybridization with a secondary alkaline peroxidise-linked goat anti-mouse Ig (Chemicon International, cat. # AP124A) diluted 1/3000 in Western blocking reagent was performed for 1 h at room temperature on a shaking apparatus. The membrane was washed three more times with TBS-Tween 20 and twice with substrate buffer (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) before the addition of BCIP/NBT substrate (Sigma-Aldrich cat. # B3804) for colorogenic detection of RpoS.

RESULTS

Effects of heat and NaCl exposure, individually and sequentially, on cell viability. *E.coli* B23 cells were grown to early logarithmic growth phase prior to exposure to 45 °C and subsequent 0.8 M NaCl (dual-treated cells) before plating on LB media. As shown in Figure 1, cell viability in the control (no heat shock or hyperosmolarity) appeared to be lower than cell viability in all stress treatments but showed high deviation. Cell viability was roughly equal in individually-stressed treatments (45 °C alone or 0.8 M NaCl alone), while the dual-treated cells showed a 1.5-fold lower viability than either heat or hyperosmolarity treated samples. The results for dual-treated cells showed the least deviation, and the 1.5-fold reduction in viability in this treatment compared to the viability of cells treated with 0.8 M NaCl alone was significant. The greatest variability in viability was observed for the control sample and the sample exposed to 45 °C alone.

Normalization of protein levels from *E. coli* B23 lysates in SDS-PAGE. Figure 2 shows the results of a SDS-PAGE gel stained with Coomassie Blue G-250 dye. Lanes 1-4 are from Trial 1 and Lanes 6-9 are from Trial 2. The amount of protein loaded in each trial was roughly equal for all treatments, as shown by similar intensities of bands in Lanes 1 through 4 and Lanes 6 through 9. Lane 5 represents protein standards. Individual bands could not be discerned due to poor resolution of staining.

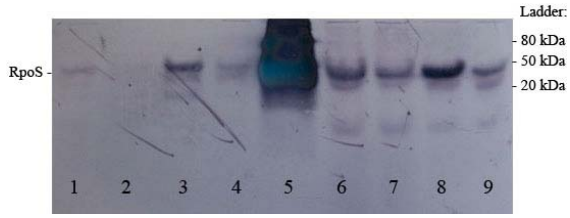

FIG. 3. Detection of RpoS via Western immunoblotting of *E. coli* B23 cell lysates resolved with 12% SDS-PAGE. Trial 1: 1-control, 2-0.8 M NaCl, 3-45 °C + 0.8 M NaCl, 4-45 °C; Ladder: 5-MagicMark™ XP Standard (Invitrogen); Trial 2: 6-control, 7-0.8 M NaCl, 8-45 °C + 0.8 M NaCl, 9-45 °C.

Effects of heat and NaCl exposure, individually and in sequence, on RpoS expression. Figure 3 shows the results of RpoS detection by Western immunoblotting. Although protein standards in lane 5 were poorly resolved, the three ladder bands could be identified and their sizes were labeled. The band consistently appearing in all lanes at approximately 38 kDa was putatively identified as RpoS. In both trials, an identical trend of RpoS expression was observed. Sequential treatment of heat and hyperosmolarity (45 °C + 0.8 M NaCl, Lanes 3 & 8) resulted in the highest RpoS induction. The sample exposed to 0.8 M NaCl alone (Lanes 2 & 7) showed the lowest RpoS expression level, while the levels between control (Lanes 1 & 6) and the sample exposed to heat shock alone (Lanes 4 & 9) were approximately the same. Lower molecular weight bands of putative RpoS protein were observed in all lanes (~35 kDa), and bands representing fragments of the size less than 20 kDa were also detected in Trial 2.

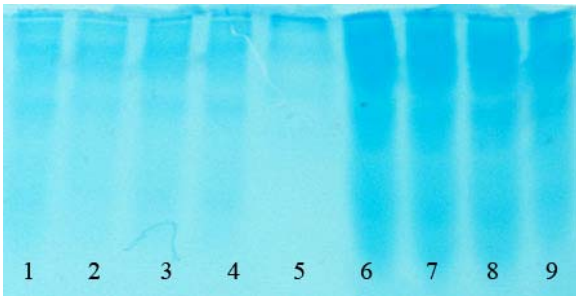

FIG. 2. Total protein visualization in *E. coli* B23 cell lysates resolved with 12% SDS-PAGE and stained with Coomassie Blue G-250 dye (PageBlue™ Protein Staining Solution) for 2 h. Trial 1: 1-control, 2-0.8 M NaCl, 3-45 °C + 0.8 M NaCl, 4-45 °C; Ladder: 5-MagicMark™ XP Standard (Invitrogen); Trial 2: 6-control, 7-0.8 M NaCl, 8-45 °C + 0.8 M NaCl, 9-45 °C.

DISCUSSION

Prior to the immunodetection of RpoS, we confirmed the normalization of protein levels in parallel treatments so that any difference in Figure 3 would be contributed to differential expression of RpoS rather than the difference due to the loading of protein by measuring approximate protein concentration in the samples using the Bradford Assay. The concentrations were used to load 13 µg of protein per lane in Trial 1, and 64 µg of protein per lane in Trial 2 (hence the difference in band intensity between the two trials). An increased amount of protein was loaded in Trial 2 because we were afraid that the lack of RpoS detection observed in a Trial 1 Western immunoblot (data not shown) may have been due to low amounts of protein loaded. No band sizes were marked on the gel in Figure 2 due to poor resolution of protein standards, and individual bands could not be discerned on the gel due to poor staining. The protein concentration was deliberately increased in Trial 2 by sonicating cells in smaller volumes.

Contrary to our original hypothesis, we did not observe any visible cross-protection between cells that were subjected to both heat shock and increased osmolarity when compared to cells that were subjected to just heat or just osmolarity shock (which were our controls). While these cells did appear to exhibit an increase in the level of expression of RpoS (Fig. 3), there was an unexpected decrease in the cell viability (Fig. 1), indicating a lack of cross-protection between the two environmental stresses to which these cells had been exposed.

The expression level of RpoS in each of the different treatments was compared using a Western immunoblot followed by visualization with a monoclonal RpoS-specific antibody detection system. Coomassie Blue staining of our SDS-PAGE gel showed an equal intensity of staining for all four treatments within a given trial, confirming that approximately the same amount of total protein was

loaded into each lane and that the differences in RpoS level (represented by the intensity of the band on our Western blot membrane) could be attributed to the different conditions present in each treatment and not to loading a different amount of total protein into each lane. The intensity of the band observed in the control sample (that was not treated with either heat shock or increased osmolarity) was assumed to be representative of the basal expression of the RpoS.

While high-temperature stress has previously been shown to increase the half-life of RpoS by decreasing its proteolytic degradation (7), our high-temperature treatment exhibited roughly the same level of RpoS as the control sample. This observation may have been due to the fact that our cells incubated in the 37 °C shaking water bath for approximately fifteen minutes following our fifteen-minute high-temperature treatment (at 45 °C) prior to cell lysis and protein harvesting. This time might have been sufficient for the RpoS level to return to a basal level and would explain why these two treatments had similar band intensities and similar RpoS expression levels.

It has been previously speculated that hyperosmolarity was involved in the regulation of RpoS by inducing translation of the RpoS mRNA and also by decreasing degradation of the RpoS protein (7). These regulatory changes should have resulted in an increase in RpoS protein level in the treatment subjected to osmolarity shock, however, we observed a decrease in RpoS level as compared to the control sample (Fig. 3, Lane 7 vs. 6). It should be noted that because RpoS mRNA remains at a constant level regardless of the presence of environmental stresses (7), we have concluded that transcriptional regulation by the high salt concentrations contributed only minimally in this case. It has also been previously demonstrated that a rapid increase in RpoS translation should occur in the presence of environmental stresses (7); therefore, the translational regulation by the high salt concentrations can also be eliminated as a possible explanation for our results. During translation, proteins undergo some form of post-translational folding. Therefore, we theorize that the high salt concentrations had an effect on the proper 3-dimensional folding of the RpoS protein (3). This misfolding may have resulted in the segregation of the amino acid residues required for interaction with RpoS-specific antibody, thus making the interaction between RpoS and the antibody less probable and causing us to see a decrease in the level of RpoS in this treatment.

The expression level of RpoS was observed to be the highest in the cells that had been sequentially exposed to both heat shock and hyperosmolarity; however, this increase in expression level cannot be directly attributed to any one of regulation of transcription, regulation of translation or protein degradation of the RpoS without further experimentation. It has been previously shown that heat shock causes a decrease in the amount of RpoS protein degradation (7); however, the lack of an appropriate heat control sample (cultivated immediately following heat treatment) made us fail to have a direct comparison between the amount of RpoS expressed due to a dual-treatment as opposed to the amount expressed due to heat treatment alone. However, the increase in expression level of RpoS in our dual-treated sample can still be at least partially attributed to the heat treatment due to the fact that we observed a decrease in expression level of RpoS in the sample exposed to increased osmolarity alone and would, therefore, expect a similar result in the dual-treated sample, if the heat-treatment had no effect on the observable level of RpoS.

Taking into account that the above-mentioned assumptions are correct, we hypothesize that the increase in RpoS protein expression in the dual-treated sample was due to an additive effect caused by both of the environmental stresses to which the sample was exposed. Firstly, the heat shock to which the cells were exposed probably resulted in both an increase in the amount of RpoS being produced in the cell as well as a decrease in protein degradation, therefore, causing an increase in the half-life of the RpoS within the cell (7). The hyperosmolarity conditions to which the cells were subsequently exposed probably caused an increase in the rate of translation of the RpoS mRNA present within the cell, thus, further increasing the amount of RpoS protein within the cell. Unlike the treatment subjected to hyperosmolarity shock alone, we hypothesize that the RpoS being produced in the dual-treated sample was not as likely to undergo misfolding as a result of high salt concentrations (7) due to the already high relative concentration of RpoS present within the cell after heat treatment. The presence of these properly-folded RpoS generated during the heat treatment may have helped to prevent the aggregation of any new RpoS synthesized (4). In addition, the preceding heat shock treatment resulted in the increased presence of heat shock proteins, chaperone proteins designed to aid in the establishment of proper 3-dimensional protein conformation. The increased presence of these proteins may also play a key role in facilitating proper RpoS protein folding in this treatment (8).

Finally, we are observing bands in the lower molecular weight region (less than 35 kDa) of the gel in Lanes 6-9, Trial 2 (Fig. 3). Since the protein within these bands reacted with RpoS specific antibody, it can be hypothesized that these bands represent degraded RpoS that has retained the ability to interact

with RpoS-specific antibody. The RpoS might have been denatured in the process of cell lysis due to the heat generated during sonication. Another possibility is that since cell lysates were stored at -20 °C, crystal formation could have damaged RpoS. It has been previously shown that proteins tend to absorb on the ice crystals and suffer a loss of conformational stability (12). This loss of stability has been shown to make the protein more prone to irreversible degradation (12). Finally, although lysates were frozen overnight before being run on SDS-PAGE gel, the action of proteases at low temperatures cannot be disregarded. RpoS requires a specific protease ClpXP that cuts at a specific stretch of amino acid residues (13). This site-specific degradation would produce peptides of specific size, resulting in the formation of distinct bands rather than a smear. The exact sizes of the RpoS fragments that result from ClpXP cleavage could not be tracked in literature. Thus, a possible explanation for the recurring low molecular weight band of the same size in Lanes 6-9 is ClpXP protease specific degradation. This band might represent an RpoS peptide that has retained the epitope required for antibody binding. An equally possible explanation is a low level of cross-reaction to a different protein. This would mean these unexpected bands represent proteins other than our desired RpoS protein. The difference between these two explanations is important because the former means that the degradation of a sample may be the causation of the smaller bands, whereas the second explanation means that proteins other than RpoS reacted with our antibody.

It has previously been shown that cells exposed to one environmental stress are protected against successive environmental stresses through the induction of a general stress response involving the up-regulation of RpoS protein (7, 15). However, the results obtained were from different environmental stresses. In addition, from our viability assay, the cell survival decrease observed disagrees with the current understanding of the cross-protection mechanism involving the general stress response and the expression of RpoS.

While the average viability value for the control sample appears to be the lowest, the high error value associated with it reveals that the actual viability in this sample fits into a larger range of values that overlaps with the ranges of values obtained from the other treatments. Based on this observation, we concluded that there was not a significant enough change in viability between the different treatments in order to reliably conclude whether or not cross-protection between the two environmental stresses actually occurred. However, assuming that the viability assay simply did not accentuate the differences between the different treatments and that the general trends that were observed are actually reliable, there are a few possible explanations that account for the results that we obtained.

One possible explanation for the 1.5-fold decrease in viability observed in the dual-treated sample is that the dual environmental stress imposed on these cells may have caused enough of a compromise to their overall physiology in order to cause them to die at a increased rate when exposed to the hot spreaders used in cell plating, thus, yielding a lower observed viability value than cells that were only exposed to a single environmental stress. However, this type of error is more likely to be due to the differences in residual heat than differences in cell physiology.

Another possible explanation for our viability assay results relates to the up-regulation of the genes responsible for programmed cell death. RpoS has previously been shown to increase the expression of genes involved in apoptosis during stationary phase in an attempt to eliminate a portion of the bacterial population, thereby increasing the amount of nutrients available for the remaining cells and increasing the overall chance of survival of the bacterial population (1). The transcription of the entericidin gene locus, which is believed to be directly involved in programmed cell death, is normally up-regulated by the RpoS and down-regulated by the EnzV/OmpR (1). However, high salt concentrations were found to have an inhibitory effect on the EnzV/OmpR, thereby preventing them from down-regulating the transcription of the entericidin gene locus (1). Therefore, it is believed that the alteration of the regulations of this particular locus of a moribund cell, results in the programmed cell death, resulting in the sacrifice of the cell to help benefit the population (1). Although these explanations can partially explain our results, they are only theoretical models proposed for the effect of RpoS on cell viability. To understand the mechanisms involved, further experimentation is necessary.

FUTURE EXPERIMENTS

In this experiment, one of the main issues was our temperature control. Since there was a 15 minute period at which the cells were at 37 ^{o}C after the heat shock treatment at 45 ^{o}C, we could not directly compare the levels of RpoS expression between the temperature control and the dual-treated sample; an improvement can be made by lysing the cells immediately after heat shock instead of returning the sample to 37 °C after growth at 45 °C. A second problem with our experiment was the great deal of

inconsistency in the colony counts between replicate plates. Because only triplicates were performed, it was difficult to determine which numbers were outliers and which ones were statistically relevant. Therefore, another important modification would be to increase the number of replicates in the viability assay in order to increase the reliability of the values obtained from it.

Since RpoS is a general stress response regulator, it would be important to investigate whether the order in which cells are exposed to environmental stresses has any effect on RpoS expression and cell viability. Thus, an experiment should be done to look at the level of RpoS expression in cells first exposed to high osmolarity and then to heat shock in order to assess the role of the order of environmental stresses on the level of RpoS expression. Simultaneously, it would be useful to measure the amount of RpoS quantitatively, instead of simply looking at RpoS expression levels comparatively, and to expose the cells to more than just two environmental stresses in the hope of determining the "stress saturation" level of RpoS (the maximum amount of RpoS that is expressed before cell death occurs). A more complex future experiment would involve studying stress response in an $RpoS^-$ mutant strain. By using the same treatments as in our original experiment, we can compare viability results between an $RpoS^-$ mutant strain and wild type *E. coli* B23 as an alternative method of assaying for possible cross-protection. If the viability results for the $RpoS^-$ mutant strain were approximately the same between the mutant controls and the dual-treated mutant, then we can assume that $RpoS^-$ may be involved in altering the viability of the cells when exposed to stress. A final experiment would be to repeat our experiment for mutants in the original strains of *E. coli,* and see if these mutants have a fitness advantage. If they do, then there might be an evolutionary significance attached to RpoS expression for these strains of bacteria, and may also be worth investigating further.

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