Journal of Experimental Microbiology and Immunology (JEMI) Vol. 18: 24 – 29

RpoS May Not Play a Central Role in Coordinating Heat Shock Stress Response and Antibiotic Tolerance

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The stationary phase sigma factor, RpoS, is a mediator of the general stress response in *Escherichia coli* exposed to environmental stress. The general stress response confers protection to other stresses including increased tolerance to tetracycline. Although RpoS is generally up-regulated under stress conditions, the regulatory RNA *oxy*S, up-regulated in the presence of oxidative stress, negatively regulates *rpo*S expression. This study addressed the regulatory relationship between RpoS and *oxy*S, and the resulting impact on tetracycline resistance. A culture subjected to heat shock stress was subsequently exposed to oxidative stress using a wild type *E. coli* and an isogenic derivative bearing a deletion of *rpo*S. Contrary to expectations, RpoS expression decreased following heat shock treatment and increase in tetracycline tolerance was not observed. PCR amplification of the *oxy*S RNA converted to cDNA showed a subtle increase in *oxy*S expression following oxidative stress. The impact of oxidative stress and the *oxy*S RNA on RpoS expression remains unclear. Thus, our data suggests that RpoS may not play a central role in coordinating the heat shock stress response and tolerance to tetracycline.

The ability of bacteria to rapidly adapt to environmental conditions is advantageous for their survival. In *Escherichia coli*, rapid adaptation can be partially attributed to the alternate sigma factor RpoS, which accumulates under stress conditions replacing the normal, or vegetative, sigma factor RpoD (1). Nutrient deprivation, as well as changes in osmolarity, pH, temperature, or exposure to UV radiation are all conditions that result in the up-regulation of RpoS (2). As a master regulator of the general stress response, RpoS induction by a single stress triggers the expression of genes that confer tolerance to many different types of stress (1). This is referred to as cross-protection.

One example of cross-protection augmented by RpoS accumulation is antibiotic tolerance. Antibiotic tolerance is a medically relevant bacterial phenotype that has been shown to increase during a heat shock-dependent general stress response (3). Acquired antibiotic tolerance during a stress response occurs when the cell up-regulates efflux pumps that actively pump antibiotics out of the cell. Efflux pumps can be specific for a particular antibiotic, such as the tetracycline efflux pump encoded by the *tetA* gene (4). Alternatively, and commonly the case during a stress response, the presence of multidrug resistance pumps act to nonspecifically pump various antibiotics and other small molecules out of the cell (5). RpoS has been implicated in regulating the expression of the *acrAB* multidrug resistance pump conferring decreased antibiotic susceptibility (6).

Repression of RpoS can occur through the action of the regulatory RNA *oxy*S (7). *oxy*S prevents translation of RpoS by sequestering the RNA binding protein Hfq, which is required for RpoS translation (8). Expression of *oxy*S RNA is activated by the transcriptional regulator OxyR during oxidative stress (8). *oxy*S regulates the expression of over 40 genes, and by doing so induces various cellular processes that protect against reactive oxygen species such as hydrogen peroxide (9, 10). Therefore, *oxy*S represses RpoS translation in the presence of hydrogen peroxide. In turn, reduced RpoS expression may diminish the general stress response, which may include antibiotic tolerance.

In this study, it was determined whether *oxy*S-dependent inhibition of antibiotic tolerance is conferred in *E. coli* that have induced a general stress response. To test this hypothesis, we subjected *E. coli* cells to heat shock, followed by hydrogen peroxide-induced oxidative stress. Antibiotic tolerance to tetracycline was measured before and after heat shock and oxidative stress to detect any changes that may be conferred by varying RpoS and *oxy*S levels. By these means, we aimed to better understand the regulation of antibiotic tolerance by opposing RpoS and *oxy*S activity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study were an *rpoS*⁻ mutant *E.coli* strain JW5437-1 (F-, Δ(*araD-araB)*567, Δ*lacZ4787*(::rrnB-3), λ⁻, Δ*rpoS746::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514)* and *E.coli* wild type strain ZK126 (F-, Δ(*argF-lac)169*, λ⁻, *IN(rrnD-rrnE)1*, *rph-1*, *tnaA5)*. These strains were obtained through the Coli Genetic Stock Center at Yale University (CGSC). Bacteria were grown in Luria Broth (LB) (1.0% w/v Bacto tryptone, 0.5% w/v Bacto yeast, 1.0% w/v NaCl, pH 7.0) at 37°C on a water bath shaking at 200 rpm.

Heat shock and oxidative stress induction conditions. After the overnight growth, the cultures were diluted to an OD_{660} of 0.3 with LB media. They were then grown at 37°C in a shaking water bath to an OD_{660} of 0.5. At this timepoint (0min), samples were taken from each culture to represent non-stressed control samples. The cultures were then separated into two flasks, one was heat shocked at 42°C for 30 minutes and the other remained at 37°C. After 30 minutes, samples for the assay were taken from the heat shocked culture for the heat shocked only control. After taking the samples, the heat shocked culture was split into three different flasks: the first flask was exposed to hydrogen peroxide at a final concentration of 50 uM, the second flask had a final concentration of 100 uM of hydrogen peroxide, and the third flask had no hydrogen peroxide

and continued to grow in the 37°C water for 15 minutes. The non-heat shocked culture was also split into 2 flasks at 30 minutes: the first flask was exposed to a final concentration of 50 uM hydrogen peroxide while the second flask had a final concentration of 100 uM of hydrogen peroxide. After 15 minutes the samples for the assays were collected representing samples with or without heat shock at varying concentrations of hydrogen peroxide depending on the stress condition it received.

Minimum inhibitory concentration of tetracycline. The minimal inhibitory concentration of tetracycline for each strain and test condition was determined using a broth microdilution assay. The antibiotic concentrations used were 0, 0.5, 1, 2, 4, 8, 16, 32 ug/ml in 100ul of distilled water. These concentrations were obtained by making a 5 mg/ml tetracycline stock in 70% ethanol and then diluting the stock to 32 ug/ml with distilled water. A 2 fold dilution series was then used to obtain the desired final concentrations. The 1 ml of sample for each stress condition was diluted with LB media to stop the hydrogen peroxide reaction and then standardized to an OD_{660} of 0.3. It was then diluted to 1/150 with LB media to give a final OD_{660} of 0.002. 100 ul of this diluted culture was added to each well already containing 100ul of antibiotic, subsequently diluting the antibiotic and the culture by a half. The 96-well plate was then incubated at 37°C for 20 hours and visualized by eye to determine the lowest concentration of tetracycline that inhibited growth.

SDS-PAGE. $5x10^8$ cells were re-suspended in 1 × SDS sample buffer (0.25 M Tris pH 6.8, 20% glycerol, 4% SDS, 10% β-Me, 0.1% bromophenol blue) and boiled for 5 minutes at 95 °C. Samples were then loaded onto a 5% acrylamide stacking gel above a 7.5% acrylamide gel. 15 ul of Novex Sharp Pre-stained Protein Standard (Life Technologies) was loaded for protein sizing. The gel was submerged in 1x electrophoresis buffer (0.1 M Tris-base, 0.38M Glycine, 0.1% SDS) and samples ran at 100 V for 70 minutes.

Western blot assay for RpoS. Proteins were blotted from the SDS-PAGE gel onto a nitrocellulose membrane at 100 V in transfer buffer (25 mM Tris-base, 150 mM glycine, 20% methanol). The blotting tank was placed on ice during transfer for 1 hour. Following transfer, the membrane was washed with TBST buffer (0.1 M Tris-base, 0.15 M NaCl, 0.1% Tween 20) and incubated overnight at 4 °C in blocking buffer (5% nonfat dry milk in TBST). Membrane was then hybridized with primary mouse IgG1 monoclonal antibody to RpoS (NeoClone) diluted 1:1000 in TBST and 0.1% blocking buffer for 1 hour at room temperature. After washing in TBST, the membrane was incubated with goat anti-mouse secondary antibody conjugated to alkaline phosphatase diluted 1:5000 in TBST and 0.1% blocking buffer for 1 hour at room temperature. The membrane was then washed with TBST buffer then incubated for 15 minutes with BCIP/NBT reagents (Invitrogen) for protein detection.

Total RNA extraction and cDNA conversion. Total RNA was isolated using a modified protocol from Qiagen's RNeasy mini kit. Sample culture cells were lysed in lysozyme solution (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1 mg lysozyme) followed by the addition of Trizol and chloroform. Lysates were mixed well and then centrifuged to separate the nucleic acid phase. 70% ethanol was added to this phase, mixed, and transferred into an RNeasy Mini spin column. The column was centrifuged to rid the flow through, followed by 1 wash with Buffer RW1, and 2 washes with Buffer RPE. The column was dried via centrifugation, incubated with RNase-free water and centrifuged to elute the RNA. Isolated RNA was converted to cDNA using SuperScript II Reverse Transcriptase kit (Invitrogen) following the manufacturer's instructions. Analysis of total nucleic acid was performed on the cDNA using the NanoDrop 2000c Spectrophotometer (Thermo Scientific) to determine the volume needed for final cDNA samples of 100 ng to undergo PCR.

PCR amplification of *oxyS* cDNA and agarose gel electrophoresis of product. Primer sets for the *oxy*S target gene and the *cys*G housekeeping gene were designed using the NCBI/Primer-BLAST online primer designing tool. Primer sequences are as follow: *oxyS* (Forward: 5'-CCTGGAGATCCGCAAAAGTTC-3'), (Reverse: 5'-GCGGCACCTCTTTTAACCCT-3'); *cysG* (Forward: 5'-ACGACCGTCTGGTTTCTGAC-3'), (Reverse: 5'-GGAACCACCGAGAACGGAAT-3'). The PCR cycle consisted of initialization for 5 minutes at 95°C, denaturation for 30 seconds at 95°C, annealing for 40 seconds at 61°C, elongation for 30 seconds at 68°C, 30 cycles of the denaturation to elongation steps and final elongation for 4 minutes at 72°C. 12 ul of each PCR product was loaded on a 1.5% agarose gel with 2 ul of 6x DNA loading dye from Thermo Scientific. Thermo Scientific Gene Ruler 100 bp DNA ladder was loaded to evaluate size of observed bands. Un-amplified RNA, un-amplified cDNA and PCR amplified RNA from non-treated samples were loaded on a separate 1.5% agarose gel as controls. The gels were placed in 1x TAE running buffer (1 mM EDTA, 40 mM Tris-acetate, pH 8.5) at 100 V for 80 minutes. The gels were then stained in 0.5% ethidium bromide for 30 minutes before visualization using ultraviolet light and the MultiImage light cabinet (Alpha Innotech).

Spot density analysis of *oxyS* RT-PCR and RpoS western blot data. Integrated spot density values were taken with an analysis tool called Spot Denso, on alphaimger, for each band of the *oxy*S and *cys*G amplicons. Spot density values were also taken for the predicted RpoS bands of the western blot. The integrated density of each band was measured by outlining it through the program's "measure" command, while avoiding inclusion of background density. Density values for the *oxy*S amplicons were normalized to the values for *cys*G amplicons by dividing the *oxy*S value by the *cys*G value for the corresponding lane.

RESULTS

***oxy*S RNA expression increases with oxidative stress.** Wild type *E. coli*, strain ZK126, and an *rpo*S$^-$ strain, JW5437-1, were heat shocked at 42°C for 30 minutes to induce RpoS expression and the general stress response. Following heat shock, the *E. coli* cells were exposed to either 50 uM or 100 uM of hydrogen peroxide for 15 minutes. This oxidative stress aimed to promote *oxy*S expression in order to inhibit RpoS activity. A sample of each strain was also grown without any oxidative stress to see if *oxy*S levels varied over time after heat shock only. Additionally some samples received no heat shock to determine if *oxy*S levels are affected at all by the heat shock stress. Samples were taken from the culture before and after both heat shock and oxidative stress to measure *oxy*S levels. To do this, total RNA was extracted from the cells. cDNA was synthesized and subjected to PCR. To ensure there was no DNA contamination, total RNA from cells was also subjected to PCR. The resulting products were run on an agarose gel. Both the *oxy*S product (95 bp) and the reference *cys*G product (244 bp) were visible (Fig 1). Additionally we saw no bands present from the PCR of total RNA ensuring the bands observed were not due to

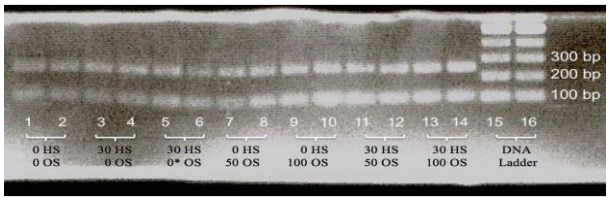

FIG 1 Agarose gel electrophoresis of cDNA PCR products. Odd-numbered lanes were WT samples, even-numbered lanes were *rpo*S⁻ samples. Labels represent differential treatments of the two strains, shown in the gel as pairs.
*HS= heat shock
**OS= oxidative stress in μM

DNA contamination (data not shown). Spot densitometry analysis of each band showed similar expression levels of *oxy*S between each condition (Table 1). However, samples that received similar heat shock treatment but varying oxidative stress, showed a slight increase in *oxy*S expression with increasing oxidative stress that was similar in magnitude as that observed after heat shock.

TABLE 1 *E. coli* wild type and *rpo*S⁻ mutant strain relative expression of *oxy*S small regulating RNA normalized to *cys*G expression derived based on pixel count comparisons.

Treatment		*oxy*S expression	
Heat Shock at 42°C (mins)	Oxidative Shock (uM)	WT	*rpo*S⁻
0	0	0.98	0.99
30	0	1.00	1.00
30	0*	0.97	0.96
0	50	0.98	0.97
0	100	1.00	1.00
30	50	1.01	0.98
30	100	0.98	0.99

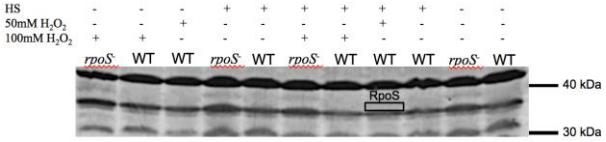

FIG 2 Total lysate Western blot for RpoS protein in *E. coli* wild type and *rpo*S⁻ mutant strains after exposure various stresses.
*HS= heat shock
**OS= oxidative stress in μM

RpoS protein expression may decreases after heat shock and oxidative stress. To confirm that the *rpo*S⁻ strain did not express RpoS protein, a western blot was performed on each sample total lysate (Fig. 2). The *rpo*S⁻ strain had lower band intensity at 38kDa, the size of RpoS protein, compared to the wild type *E. coli*. Spot densitometry analysis gave a lower pixel count for the theoretical location of RpoS in the *rpo*S⁻ strain compared to wild type counterparts (Table 2). This confirmed the absence of RpoS in the mutants, and pixel counts in this position are attributable to background. Additionally, spot densitometry was used to measure the relative difference in RpoS expression between wild type samples subjected to different conditions. RpoS expression following each treatment was compared to RpoS expression levels prior to treatment. After heat shock, there was a decrease in RpoS expression followed by a slight increase after subsequent oxidative stress, but not to the original level observed before treatment. Cells that were heat shock only and allowed 15 minutes to recover also showed a decrease in RpoS expression, but to a lesser extent than the cells measured for expression levels directly after heat shock.

TABLE 2 Summary of average pixel counts of RpoS band denoting RpoS expression in *E. coli* wild type and *rpo*S⁻ mutant strains after various stresses.

	WT	*rpo*S⁻
Treatment	Pixel Count $x10^{-4}$	Pixel Count $x10^{-4}$
No treatment	2.60	1.91
HS only	1.98	1.76
HS + 100mM H_2O_2	1.66	1.47
No HS + 100mM H_2O_2	2.05	1.72

Antibiotic tolerance to tetracycline changes in *rpo*S⁻ but not wildtype *E. coli* after heat shock and oxidative stress. After differential treatment of the wild type and *rpo*S⁻ mutant *E.coli* strains, the samples were subjected to serial dilutions of tetracycline. The MIC of the samples is shown in Table 3. There was no growth detected for the negative control containing only fresh LB media, while there was an MIC detected for all the samples suggesting that the antibiotic was effective at inhibiting growth. The MIC for the wild type strain did not vary (2 ug/ml), whereas the MIC for the mutant *rpo*S⁻ varied between 2 ug/ml and 4 ug/ml. This variation observed in the mutant strain appeared to correlate with the presence or absence of stress treatment. The uncertainty of the MIC assay is ±1 well, and since all of our samples were only 1 well apart from each other this means that they are all within the range of uncertainty for each other.

TABLE 3 Summary of the tetracycline minimum inhibitory concentrations of *E. coli* wild type and *rpo*S⁻ mutant strains after being subjected to different stresses.

Treatment		MIC (ug/ml)	
Heat Shock at 42°C (mins)	Oxidative Shock (uM)	WT	*rpo*S⁻
0	0	2	4
30	0	2	4
30	0*	2	2
0	50	2	2
0	100	2	2
30	50	2	4
30	100	2	4

DISCUSSION

In this experiment, we aimed to assess whether the specific combination of the general stress regulator RpoS and the small regulatory RNA *oxyS* can cause measurable fluctuations in *E. coli* tetracycline tolerance. We approached the problem through putting a wild type strain as well as an *rpo*S⁻ strain of *E. coli* through a series of stress signals. First, heat shock was applied in attempt to up-regulate RpoS, which we expected to increase tetracycline tolerance. Next, we applied oxidative shock through addition of hydrogen peroxide

to up-regulate *oxyS*, which we expected would inhibit intracellular RpoS. The main assessment tools we used were western blotting and PCR. However, we encountered technical challenges with both. Despite the technical challenges that may impede our ability to draw definite biological conclusions, it is still worthwhile to closely examine the data for any possible trends.

In the *E. coli* wild type strain, RpoS expression did not significantly change with heat shock (Table 2). This contrasts previous JEMI publications (12), which show increased RpoS expression after heat shock. There are a few possible explanations for this observation. Firstly, RpoS could have been significantly up-regulated prior to the beginning of heat shock, by the overnight culture growth conditions. This would mean that the RpoS levels before heat shock may have been higher prior to heat shock, which may explain the absence of a further increase in RpoS expression. Since there are no obvious reasons that the overnight growth should have induced RpoS activity, however, we suspect it is possible that the 30-minute heat shock period at 42^{o}C may not have been potent enough to up-regulate RpoS expression.

As seen in Table 1, *oxyS* levels in the wild type strain remained relatively consistent after heat shock at baseline level of expression, as expected. Immediately after heat shock, we observed a slight decrease in *oxyS* expression, but it was returned to pretreatment levels as the cells recovered over time. This suggests a model in which *oxyS* down-regulation occurs during heat shock. With subsequent oxidative stress, we saw a slight increase in *oxyS* RNA expression and further decrease in RpoS. This increase in *oxy*S was small and while we confirmed that the levels of hydrogen peroxide applied to the cells were non-lethal, we cannot conclude that the concentration of hydrogen peroxide was high enough to induce a measurable up-regulation of *oxyS*. Further, we did not observe the expected increase in antibiotic susceptibility in the wild type strain with any of the treatments, which is consistent with the decreasing pattern of RpoS described earlier (Table 2). With lower levels of RpoS, a general stress regulator, a constant or decreasing antibiotic tolerance is plausible because we expected RpoS to be a factor in up-regulation of tetracycline tolerance.

Next we analyzed data for the JW5437-1 *rpo*S^{-} *E. coli* strain in a similar manner. A Western blot of RpoS (Fig. 2) consistently showed lower pixel counts compared to the wild type. As such, it is reasonable to assume that the *rpo*S^{-} strain was indeed lacking RpoS, and the faint banding observed in the mutant lanes is background from non-specific antibody binding. *oxyS* expression in the *rpo*S^{-} strain was observed to increase slightly with heat shock (Table 1). Antibiotic tolerance was unchanged before and after heat shock, although given time to recover post-heat shock there was a decrease in tolerance (Table 3). These results suggest an RpoS-independent pathway that maintains antibiotic tolerance in response to heat stress. One possible pathway involves the specific heat shock-regulating sigma factor RpoH (13). With subsequent oxidative stress of the *rpo*S^{-} strain, *oxyS* expression increased, consistent with the wild type strain as well as descriptions in previous literature (8, 9, 10). Antibiotic tolerance increased again with oxidative stress back to pretreatment levels of tetracycline tolerance, but cells without heat shock remained at a lower tolerance. Again, increased tolerance under oxidative stress in the absence of RpoS suggests an alternate compensatory pathway.

In reference to the measurements of *oxy*S RNA expression, there is indication that the *E. coli* housekeeping gene *cys*G, chosen with the intention of providing a constant level of expression throughout all treatment options, was variably expressed (Fig. 1). CysG is an enzyme involved in metabolism, which should only change its expression based on changes in the nutritional environment (14, 15). All cultures used in this experiment were grown on Luria broth prepared in one batch using the same recipe, so there is no reason to assume significant deviations in any of the cultures' nutrient levels. The *cys*G gene in *E. coli* chromosome is preceded by a *nir*B gene, for which expression is strictly regulated by oxygen repression, nitrite induction during anaerobic growth, and activation by the Fnr protein (14). In the aerobic growth condition employed in this experiment, however, *cys*G is transcribed constitutively using its own promoter and has no known preceding transcription terminators (14). As such, it is expected to exhibit constant levels of expression throughout both heat shock and oxidative stress. While both *cys*G and *oxyS* levels fluctuated, they did so maintaining a consistent ratio. Thus, the changes in *cys*G expression are assumed to be an issue in loading. With that said, we did not see significant changes in *oxy*S expression over the course of the experiment in either strain.

The apparent lack of measurable change in all of our assay results may fundamentally suggest that heat shock, the first step in our experiment, was not the best stressor in terms of inducing a measurable up-regulation of RpoS. Alternatively, previous JEMI publications have suggested that heat shock may result in excessive production of RpoS that actually reduced tetracycline tolerance (16). While this is possible, we cannot infer anything with regards to this from our results (Fig. 2), as there was too much background detection in the Western blot to see clear fluctuations in RpoS expression due to the treatments.

Although there was an observed trend for increasing *oxyS* RNA with oxidative stress, this was only seen with one biological replicate. Therefore, we cannot draw any conclusions to the significance of these changes. It is

possible that the changes observed are simply due to the natural variability in biological organisms, or variability of technical procedures. In this case, it would be reasonable to conclude there was no real difference in *oxyS* throughout the experiment. The lack of expected changes in antibiotic tolerance in the wild type strain, where we expected to see an increase in antibiotic tolerance with heat shock and decrease with oxidative stress, may actually be indicative that RpoS is not the principal determinant in producing a significant tetracycline tolerance in response to heat shock. There is also indication that RpoS is more important in cells recovering after prolonged heat shock (17). This suggests that a larger fluctuation in RpoS expression might have been observed if cultures were subjected to heat shock for periods longer than 30 minutes (17). Similarly, while there may be interactions between RpoS and *oxy*S in the cell, the specific combination likely does not play a prominent role in the determination of tetracycline susceptibility in *E. coli*.

FUTURE DIRECTIONS

In our experiment there was a significant amount of non-specific binding for the RpoS Western blot. In future experiments it may be useful to use AP-Rabbit Anti-Mouse IgG (H+L) Conjugate (ZyMax Grade) as a secondary antibody instead of the goat anti-mouse secondary antibody that we used. The rabbit anti-mouse secondary antibody has been able to show less non-specific binding, which would help in analyzing the western blot results (L. Yang, submitted for publication). Furthermore, the MIC assay as well as the measurements of *oxyS* RNA and RpoS protein should be repeated to increase confidence in the results and ensure its reproducibility and significance.

oxyS RNA levels did not significantly change between the different samples. This made it difficult to determine whether an increase in *oxyS* levels is sufficient in decreasing antibiotic susceptibility. In a future experiment it may be helpful to clone *oxy*S onto a plasmid under an inducible promoter. This would allow to control o*xyS* RNA expression and determine its role in repressing cross protection conferred by the general stress response.

Similarly, RpoS levels did not significantly change between the different samples. As suggested above, our induction of RpoS through heat stress may not have been long enough to significantly increase RpoS expression. In a future experiment it would be advised to either heat shock for longer periods of time. Since heat shock is not the primary stressor that RpoS responds to it would be advisable to also try different stressors to increase RpoS.

ACKNOWLEDGEMENTS

This study was generously supported by the Department of Microbiology and Immunology of the University of British Columbia. We would like to sincerely thank Dr. William Ramey, Dr. David Oliver and Andrew Cameron for their constant support, valuable guidance and endless patience. Our gratitude also goes to the employees of the department's media room for providing us with all the necessary material for our experiment.

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