The Molecular Chaperone DnaJ is Involved in the Peroxide-induced Oxidative Stress Response in *Escherichia coli*

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The chaperone protein DnaJ and sigma (σ) factor σ32 (often called RpoH) play important roles in the heat-shock response in *Escherichia coli*. Previous studies have demonstrated increased heat tolerance accompanied by increased RpoH concentrations in *E. coli* treated with a sub-minimal inhibitory concentrations (MIC) of kanamycin. DnaJ, along with other chaperone proteins, forms a complex with RpoH to facilitate its degradation in the absence of stressful stimuli. In this study, we explored the relative importance both proteins in the oxidative stress response in the presence and absence of a sub-MIC of kanamycin. Phagocytes of the human immune system often use reactive oxygen species upon contact with foreign bacteria to facilitate killing and prevent potential infection. Tolerance of *E. coli* strain C600, dnaJ mutant strain MF634, and ΔrpoH strain K165 to peroxide-induced oxidative stress was assessed by (i) monitoring cell growth in broth cultures containing H₂O₂ and by (ii) measuring zones of H₂O₂ growth inhibition in a solid phase diffusion disc assay. In broth cultures, growth of the wild-type strain was less negatively impacted by H₂O₂ compared to the dnaJ and ΔrpoH mutants. In the disc diffusion assay, zones of inhibition for WT were significantly smaller than those of the dnaJ mutant. The presence of a sub-MIC of kanamycin did not produce smaller zones of inhibition for either WT or mutant strains in the disc diffusion assay. Western blot detection analysis showed that in the absence of a sub-MIC of kanamycin, RpoH concentrations were higher in the dnaJ mutant compared to the WT strain. Results of this study indicate that DnaJ is an important mediator of the oxidative stress and that a lack of a functional copy of *dnaJ* is sufficient to cause impairment of the oxidative stress response in *E. coli*.

In bacteria, a highly conserved “heat-shock” stress response is induced in response to stimuli such as heat, radiation, starvation, and oxidative agents (1). The heat-shock response has evolved to minimize the inhibitory effects caused by various stressors, including the improper folding of cellular proteins (2). In *E. coli*, the heat-shock response involves the induced expression of a set of genes that encode heat-shock proteins – many of which are molecular chaperones involved in assisting transit across membranes, targeted proteolysis, and polypeptide folding (3). Stress-induced transcription of heat-shock genes is controlled by binding of the sigma factor RpoH - encoded by *rpoH* - to RNA polymerase, allowing for specific binding of RNA polymerase to gene promoters associated with various heat-shock proteins crucial to the bacterium’s survival in stressful conditions (4).

The heat shock proteins DnaK, DnaJ, and GrpE are part of a system that is responsible for negative regulation of σ32 (5). In the absence of oxidative stress, these molecular chaperones maintain low intracellular concentrations of σ32 by sequestering free sigma factor, causing disassociation from RNA polymerase, and facilitating its degradation via FtsH proteases (5, 6). When protein misfolding occurs as a result of heat- or oxidation-induced denaturation, chaperone proteins DnaK and DnaJ preferentially bind to misfolded proteins and allow for σ32 to re-associate with RNA polymerase, resulting in increased transcription of heat-shock proteins (6).

Different cellular responses may be stimulated depending on the type of oxidative agent introduced and stress responses elicited by peroxides (H₂O₂) or superoxide radical ions (O₂⁻). These responses produce upregulation of different cellular proteins, referred to as the peroxide and superoxide stimulons, respectively (7, 8). Studies have demonstrated that *E. coli* dnaK deletion and null mutants are severely impaired in both thermostolerance and resistance against H₂O₂-induced stress (2, 9).

The molecular chaperone DnaJ is believed to be the primary regulator of RpoH degradation and has been reported to facilitate the effective binding of DnaK to form a DnaJ-DnaK-RpoH complex (5, 6). Little is known about DnaJ in terms of its importance in regulation of the oxidative stress response and study of a dnaJ null mutant of *E. coli* may yield insight into the mechanisms underpinning the bacterial oxidative stress response.

Kanamycin is a member of the aminoglycoside class of antibiotics that inhibits bacterial growth by interfering with the translocation of amino acids at the site of the bacterial ribosome during protein synthesis, resulting in an increased frequency of mistranslated and misfolded proteins (10). Via an unknown mechanism, *E. coli* pretreated with a sub-minimal inhibitory concentration (sub-MIC) of kanamycin exhibited increased thermotolerance accompanied by elevated cellular concentrations of RpoH (11). Aminoglycoside antibiotics such as kanamycin may be used to treat bacterial infections and greater understanding of the mechanism of how sub-minimal inhibitory levels of kanamycin modulate the *E. coli* stress response may prove valuable. Hydrogen peroxide (H₂O₂) also plays a critical role in the human innate immune response system, generating multiple products that exhibit bactericidal activity (12). Resistance to oxidative stress linked to exposure to a sub-MIC of kanamycin may have important implications in the pathogenesis of *E. coli*.

We hypothesize that a strain possessing a mutation in *dnaJ* will be more sensitive to oxidative stress compared to
a strain with an rpoH deletion. We also hypothesize that introduction of a sub-MIC of kanamycin will produce a protective effect against peroxide-induced oxidative stress as it has been shown to do for the heat shock response. This study examined the relative importance of DnaJ and RpoH in mediating the oxidative stress response, with an emphasis on the peroxide stimulus. Additionally, an investigation was conducted on whether co-incubation with a sub-minimal inhibitory concentration of kanamycin was sufficient to generate increased tolerance against oxidative stress and if cellular concentrations of RpoH were elevated in a dnaJ null mutant.

**MATERIALS AND METHODS**

**Bacterial strains.** *E. coli* strains C600 (WT), MF634 (dnaJ mutation, C600 derivative), SC122 (WT in respect to K165), and K165 (∆rpoH relative to SC122) were selected for use in experimentation (correspond to Coli Genetic Stock Collection #3004, #5828, #6768, and #6769 respectively). *E. coli* C600 is a strain derived from *E. coli* K12 and possesses the following genotype characteristics: thr-1, leuB6, fhuA21, cysH-1, lacY1, glnX44 (Am), rfbC1, glpR2805 (Am), and thiE1. MF634 is a mutant strain derived from the C600 strain exposed to an ultraviolet light dose, generating a null mutation in dnaJ that rendered the strain temperature sensitive (dnaJ259 (ts)) (13). *E. coli* SC122 possesses the genotype characteristics: lacZ53 (Am), phoA5 (Am), xylT49 (OS), tyrT91 (ts, AS), trp-48 (Am), relA1, rpsL150 (strR), madT66 (Am, λ), and spoT1. *E. coli* K165 is genetically identical its parent strain SC122 with the exception of an amber mutation mapped to the rpoH gene (∆rpoH601 (Am)) (14). All overnight cultures were incubated in Luria broth (LB) (1.0% w/v tryptone, 0.5% w/v yeast extract, 1.0% w/v NaCl) at 30°C in a shaking incubator at 175 rpm unless otherwise stated.

**Microplate MIC assay for kanamycin.** Overnight cultures of *E. coli* C600 and dnaJ mutant MF634 were normalized to 0.01OD<sub>600</sub> and added to wells of a standard 96-well microplate in volumes of 100 µl. Stock kanamycin of 100 µg/ml diluted in sterile LB was added to the diluted culture in wells to obtain final concentrations of 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 16.0, 20.0, 25.0, and 32.0 µg/ml. Positive and negative controls containing culture with no kanamycin added and 100 µg/ml kanamycin were included in the microplate. Sterile LB was also included to test for contamination. All test samples were performed in duplicate. The microplate was incubated at 30°C and results were recorded at 21 hours. The minimum inhibitory concentration was determined to be 12.0 µg/ml based on visual confirmation of the absence of turbidity in the wells containing concentrations of kanamycin equal to or higher than 12.0 µg/ml. Observations were performed again at 46 hours post-incubation and no development of turbidity was observed for previously clear wells. The sub-MIC of kanamycin for broth cultures was arbitrarily set at 6.0 µg/ml by halving the determined MIC (10). For spread plates, strains were unable to grow on plates containing 4.0 µg/ml kanamycin and a lower concentration of 2.0 µg/ml was used.

**Growth of broth cultures in the presence of hydrogen peroxide.** Overnight cultures of WT, dnaJ mutant, and ∆rpoH strains were diluted in Klett flasks of sterilized LB and equalized to approximately 0.01 OD<sub>600</sub>, with final volumes of 30 ml. The flasks were placed into a 30 °C shaking water bath at 120 rpm, with 17 µL of 35% H<sub>2</sub>O<sub>2</sub> (corresponding to 4.37 mM) introduced in each flask at 60 minutes after the start of incubation. The rate of shaking was increased to 195 rpm at 60 minutes to ensure even distribution of the added H<sub>2</sub>O<sub>2</sub>. The turbidity in Klett units (250 K.U = 1OD<sub>600-700</sub>) was read via a Klett-Summerser Photoelectric Colorimeter with a red filter at 0, 10, 20, 40, 60, 70, 80, 90, 100, 120, 160 and 200 minutes. The Klett-Summerser Colorimeter and its associated flasks were used in order to minimize the risk of contamination during readings across the multiple time points.

**Disc assay for peroxide sensitivity.** Two sets of sterile LB agar (LB broth with 12% w/v agarose, one set containing 2 µg/ml kanamycin) plates were prepared. Overnight cultures of *E. coli* C600, MF634, and K165 were diluted to approximately 0.25 OD<sub>600</sub> and 100 µl was spread on plates and allowed to set for 10 minutes. Sterile 7mm filter disks were placed onto quadrants of agar and 5 µl of varying concentrations of hydrogen peroxide (5%, 1%, 0.5%, 0.1%) were added to the filter disks. Plates were allowed to sit upright for 10 minutes at room temperature prior to incubation at 30°C. All plates were performed in triplicate. Zones of inhibition were measured at 21 hours after incubation. Statistical analysis was conducted using the 2-tailed student’s t-test assuming unequal variances. A significant level of α=0.05 was used for comparison of mean diameters of growth inhibition.

**Growth of C600 and MF634 and isolation of protein.** Overnight cultures of the *E. coli* strains C600 and MF634 were diluted to approximately 0.200 OD<sub>600</sub> in flasks containing 100 ml LB or 100 ml LB containing a sub-MIC of 6.0 µg/ml kanamycin. To generate a positive control for detection of RpoH, 50 ml of LB containing 4.0 µg/ml kanamycin was inoculated with *E. coli* SC122 and incubated at 30°C at 175 rpm for 120 minutes (11). For the negative control, a 50 ml culture of ∆rpoH601 strain K165 was incubated for the same duration and under the same conditions as SC122 prior to protein isolation. The flasks containing newly inoculated LB were placed in a 30°C shaking water bath at 195 rpm. Turbidity measurements (OD<sub>600</sub>) were taken at 0, 10, 20, 30, 40, 60, 90, 120, and 215 minutes. Samples were taken from each treatment at 0, 20, 40, 60, 90, 120, and 215 minutes, equalized according to OD<sub>600</sub> and centrifuged at 13,200 rpm in an Eppendorf 5415D bench top centrifuge. Following centrifugation, supernatant was removed and cell pellets were suspended in 50µl of sample buffer (0.125M Tris pH6.8, 10% v/v glycerol, 2% w/v SDS, 5% v/v β-mercaptoethanol, 0.02% w/v bromophenol blue). Samples were then heated on a VWR Scientific Heatblock II for 5 minutes at 95°C, briefly centrifuged, and transferred to sterile microcentrifuge tubes prior to storage at -20°C.

**SDS-PAGE.** Lysate samples standardized according to OD<sub>600</sub> readings were separated on a 12.5% SDS-polyacrylamide submerged in 1x running buffer (25 mM Tris pH 8.3, 192 mM glycerol, 0.1% w/v SDS) as according to the Laemmli protocol (15). Novex Sharp Protein Standard (Cat. #LC5800, Life Technologies) and XP Western Blot Protein Standard (Cat. #LC5602, Invitrogen/Life Technologies) were loaded in volumes of 4 µl alongside experimental samples for use in subsequent estimation of protein sizes and confirmation of successful Western blot transfer and probing. Samples were run using the Bio-Rad Mini Protean 2 cell system at 100V for 60 minutes.

**Western Blot.** Proteins were transferred onto nitrocellulose membranes (Cat. #162-0116, Bio-Rad) in a chilled transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol). The transfer was run at 100V for 60 minutes during which the box was cooled by placement in ice. The transferred membranes were submersed/washed with 1x TBS Tween-20 (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% w/v Tween-20) for 1 cycle of 15 minutes and 2 cycles of 5 minutes prior to incubation in blocking solution (5% w/v skim milk powder in TBS Tween-20 solution) for 1 hour. The mouse anti-c32 IgG monoclonal antibody (Cat. #WP006, Neoclon) was previously diluted 1:1000 using TBS Tween-20, and incubated with pieces of nitrocellulose membrane containing *E.coli* K165 (∆rpoH601) lysate immobilized using the Bio-Rad Bio-Dot SF Cell system. This step was performed in order to
FIG 1  Wild-type (◇), dnaJ mutant (▲), and ΔrpoH mutant (□) E. coli strains exhibiting recovery from peroxide-induced stress as indicated by growth during aerated incubation at 30°C. Addition of H₂O₂ at t=60 minutes to a final 4.37 mM concentration is indicated by an arrow.

minimize the non-specific binding capacity of the primary antibody. The hybridization step was conducted with further diluted 1:2500 primary antibody solution (mouse anti-σ32 IgG monoclonal antibody in TBS Tween-20 and 1% w/v skim milk powder) and incubated for approximately 20 hours at 4°C without shaking. The membrane was then allowed to shake for 1 hour before 3 wash cycles using TBS Tween-20 were performed. The secondary antibody AP-Rabbit Anti-Mouse IgG (H+L) Conjugate (ZyMax Grade) (Cat#81-6722, Invitrogen) was diluted 1:2500 in TBS Tween-20 and membranes were incubated in 10 ml of the diluted secondary antibody for 1 hour. The membrane was washed again with TBS Tween-20 for 3 cycles as previously described. BCIP/NBT Reagent Kit (N-6547, Life Technologies) was diluted to 1X in distilled H₂O and 10 ml of the diluted solution was applied directly onto each membrane, allowing for incubation with shaking at room temperature for 40 minutes. Membranes were photographed using the Multilmage Light Cabinet (Alpha Innotech) and relative band intensities were subsequently analyzed using the AlphaEase FC software (version 4.1.0, Alpha Innotech).

RESULTS

The minimum inhibitory concentrations of kanamycin for E. coli strains C600 and MF634 were equivalent. The MIC assay was performed in order to confirm that both strains did not possess resistance to kanamycin and to find a concentration of kanamycin that would not visibly inhibit growth. The wild-type and the dnaJ mutant strains were found to be sensitive to a kanamycin concentration of 12.0 µg/ml in the microplate assay and were unable to grow on LB agar containing a kanamycin concentration of 4.0 µg/ml. Additionally, the ΔrpoH strain was also unable to grow on LB agar containing a kanamycin concentration of 4.0 µg/ml. The data showed that a lower concentration of kanamycin – relative to the broth- proved to be inhibitive for growth on solid media.

E. coli dnaJ and ΔrpoH mutants show increased sensitivity to H₂O₂. In order to determine the relative importance of each gene in the oxidative stress response, the wild-type and two mutant strains were exposed to a low concentration of H₂O₂ after being permitted to grow for 60 minutes. We found that the WT, dnaJ mutant, and ΔrpoH mutant strains had similar rates of growth in the absence of H₂O₂. After addition of H₂O₂ at 60 minutes post-incubation a marked decrease in growth rate was observed for all three strains around 70 minutes (Fig 1). The wild-type strain appeared to recover by 80 minutes and continued to increase in turbidity. The growth of the ΔrpoH mutant strain decreased between 70 and 90 minutes and began increasing in turbidity after approximately 120 minutes. The dnaJ mutant strain exhibited a similar pattern of growth compared to the ΔrpoH mutant, undergoing a period of stalled growth followed by recovery after the 120 minute time point. These results indicated that strains possessing a non-functional dnaJ gene or a deletion of rpoH were more negatively impacted by H₂O₂ compared to the wild-type strain.

The dnaJ mutant displayed greater sensitivity to peroxide-induced stress than wild-type and ΔrpoH strains. This assay was performed as an alternative to the broth culture growth experiment to ascertain if a quantifiable difference existed between the two mutant strains. Zones of inhibition were not observed for any of the strains at 0.1% H₂O₂. Zones of inhibition of the WT
strain were significantly smaller than the dnaJ mutant when treated with 5% H₂O₂. While no significant differences were observed for treatments with 1% and 0.5% concentrations of H₂O₂, there is a visible difference for 1% H₂O₂ (Fig 2A). No significant differences between the WT and ΔrpoH strains were observed for the 5%, 1% or 0.5% H₂O₂ treatments. Based on this data, it was determined that only the dnaJ mutant was significantly impaired in its ability to resolve peroxide-induced oxidative stress. Additionally, the difference was more apparent at higher concentrations of H₂O₂.

Sub-MIC treatment with kanamycin did not impact the viability of WT, dnaJ mutant, and ΔrpoH mutant strains grown in the presence of H₂O₂. Between the control and kanamycin conditions, no significant differences were observed for any of the strains treated with 5%, 1%, and 0.5% H₂O₂. The only possible difference existed between the control and kanamycin conditions for MF634 treated with 5% H₂O₂ (Fig 2A).

Cellular concentrations of RpoH were elevated in the dnaJ mutant relative to the wild-type strain. Quantification of RpoH via Western blot detection was performed to better understand the role of DnaJ in the mechanism through which increased thermostolerance results for strains treated with a sub-MIC of kanamycin (11). Based on band intensity, it was found that samples from the dnaJ mutant strain contained a higher concentrations of RpoH compared to the wild-type (Fig 4). This difference was most apparent for samples taken at the 90-minute time point. Conversely, for samples taken from culture incubated in the presence of a sub-MIC of kanamycin, WT samples appeared to contain higher concentrations of RpoH. For dnaJ mutant-derived samples, the samples taken from the control and sub-MIC kanamycin treatment did not appear to vary significantly in terms of RpoH concentration.

DISCUSSION

The sigma factor RpoH is important for the transcription of multiple genes involved in the oxidative stress response system (3, 4, 5). In the absence of stressful stimuli, negative regulation of RpoH is performed through the action of the various molecular chaperones DnaJ, DnaK, and GrpE (6). These molecular chaperones are also referred to as “heat shock proteins” and are involved in reducing the negative effects on produced by exposure to high temperatures, reactive oxygen species, and starvation (6, 7). The importance of DnaJ in the oxidative stress response is relatively unknown and in this study, we sought to compare how a mutant possessing a non-functional copy of dnaJ would fare relative to a mutant with an rpoH deletion. Two assays in which H₂O₂ was introduced to broth cultures and bacterial culture spread on LB agar were performed and growth rates and zones of inhibition were used to gauge the relative sensitivities of the mutants (in addition to the WT strain) to peroxide-induced oxidative stress.

The slower recovery of the dnaJ and ΔrpoH strains from peroxide-induced stress relative to the wild-type strain implicated in DnaJ and RpoH in the E. coli oxidative stress response. It was previously demonstrated that E. coli rpoH mutants’ susceptibility to peroxide-induced oxidative stress was similar wild-type unless they also possessed mutations in genes encoding two superoxide dismutases (sodA and sodB).
FIG 3 Growth curve of cultures of WT (■) and dnaJ mutant (▲) from which protein samples were isolated for use in SDS-PAGE and Western blot analysis.

(7). However, data from another study using a different rpoH deletion mutant suggested that strains lacking the rpoH gene were more sensitive to peroxide-induced stress in exponentially growing cultures (16). Our data corroborated the observation that a lack of a functional rpoH gene resulted in an impaired ability to recover from peroxide-induced stress. However, it should be noted that the ΔrpoH strain K165 was not derived from the C600 strain, but from the parent strain - SC122 - which possesses different genotypic characteristics. The two parent strains may exhibit variation in terms of sensitivity to oxidative stress due to genetic differences. Greater sensitivity of the dnaJ mutant to peroxide-induced stress was inferred from results of the H₂O₂ diffusion disc assay, with the mutant strain producing zones of inhibition significantly greater in diameter than observed for the wild type at the 5% H₂O₂ level as well as notably larger zones at the 1% H₂O₂ level (Fig 2A.).

While the importance of DnaK has been investigated previously, the role of DnaJ in resistance against peroxide-induced oxidative stress has not been studied in detail (2, 9). The significant differences in inhibition zone diameters between the wild type and mutant strain indicated that DnaJ may play an important role in minimizing the effect of oxidative damage in E. coli. The lack of any significant difference in inhibition zone diameters between the control and kanamycin conditions for all three experimental strains indicated that the sub-MIC of kanamycin – at least at a concentration of 2 µg/ml – was insufficient to convey increased resistance against peroxide-induced oxidative stress. The similar inhibition zone diameters for the WT strain and the ΔrpoH strain contradicted the results depicted in Figure 1. This could be explained by the different natures of the two assays, with one measuring growth rate recovery and the other measuring tolerance for varying concentrations of H₂O₂. It is possible that with the disc assay, the protection against oxidative stress associated with RpoH was too low to be observed or measured accurately.

Protein carbonylation is a modification of amino acids that is indicative of oxidative stress (17). It has been suggested that DnaK and DnaJ overproduction reduces stasis-induced carbonylation by keeping carbonylated proteins in a soluble form that is susceptible to cellular proteases (17). If the dnaJ mutant was incapable of expressing functional DnaJ, the mutant strain may not have been able to degrade carbonylated and misfolded proteins created during a period of peroxide-induced oxidative stress. The WT and ΔrpoH strains, possessing functional copies of the dnaJ gene, would have been more capable of negating the inhibitory effects of H₂O₂.

We predicted that in the absence of functional DnaJ, negative regulation of RpoH via targeted degradation by FtsH proteases would be reduced (5, 6). Western blot detection of more RpoH for the dnaJ mutant
degradation and is found in higher cellular concentrations (4, 5). The increase in RpoH concentration in kanamycin-treated samples was observed for the wild-type strain but inconsistent for the dnaJ mutant. Based on our results, observed increases in RpoH concentrations associated with the presence of a sub-MIC of kanamycin can be primarily attributed to a disruption of post-translational regulation of RpoH.

We proposed a model in which the presence of a sub-MIC of kanamycin resulted in sequestration of DnaJ due to preferential binding of the chaperone protein to misfolded proteins produced by the action of the antibiotic. This mechanism would have prolonged the cellular half-life of RpoH by preventing targeted proteolysis by FtsH proteases, yielding greater cellular concentrations of RpoH. Our data shows, based on the absence of the kanamycin-associated increase in RpoH in dnaJ mutants, this model may be accurate. However, we cannot rule out kanamycin-associated transcriptional regulation without further experimentation.

Taken together, our data demonstrates the involvement of DnaJ in the peroxide-induced stress response in E. coli and suggests that a deficiency in RpoH may be sufficient to cause impairment of the same stress response. Consistent with our hypothesis, a lack of the heat shock protein DnaJ proved to be more detrimental to the survival of E. coli compared to absence of the sigma factor RpoH. A sub-MIC of kanamycin was shown to increase RpoH concentrations via disruption of DnaJ-mediated post-translational regulation of the sigma factor. However, the sub-MIC of kanamycin used was determined to be inadequate in conferring resistance against peroxide-induced oxidative stress.

**FUTURE DIRECTIONS**

Future studies may involve generating deletion mutants of dnaJ, dnaK, and rpoH from a single parent strain and assessing the relative importance of each gene in modulating the E. coli oxidative stress response. In this study, we compared the wild type C600 strain to the genotypically similar dnaJ null mutant (MF634) and a ΔrpoH mutant with an amber mutation (K165) derived from a different parent strain. Consequently, we were unable to eliminate individual strain differences as potential factors in observed resistances to oxidative stress. The use of deletion mutants derived from a single strain (e.g. using a homologous recombination system to replace target genes with antibiotic resistance markers) may facilitate the experimental process and provide data of greater significance. The experiments performed in this study could also be repeated with paraquat treatment replacing H$_2$O$_2$. Results from this experiment would demonstrate if DnaJ and RpoH are as crucial in the superoxide stimulon as in the peroxide stimulon.

**ACKNOWLEDGEMENTS**
We would like to thank the UBC Department of Microbiology and Immunology for providing us with various reagents and bacterial strains as well as the staff of the Wesbrook building for allowing the use of equipment and supplies. We would like to thank Dr. William Ramey, Dr. David Oliver, and Cedric Brimacombe for their invaluable input in regards to the design, refining, and troubleshooting of our project. We would also like to thank Andrew Cameron for guidance in execution of the Western transfer procedure.

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