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The Genes of *dps*, *relA*, and *spoT* are Each Important in the Resilience of *Escherichia coli* Exposed to UVA-Radiation during Stringency

Daniel Kuo, Jessie Yu, Nicole Lockyer, and Yu-Chin Peggy Lin
Department Microbiology & Immunology, University of British Columbia

Dps protein provides important protective effects on DNA against UVA irradiation. A previous study found that its presence during stringency increased survival of *Escherichia coli* upon exposure to UV, but in its absence, stringency still triggered an increase in survival. Since it was uncertain whether the observed protective effects triggered by stringency were due to Dps and other protein mechanisms or mostly to Dps alone, the aim of this study was to elucidate the relative importance of Dps. For the *E. coli* K12 strains BW23113 (parental *dps*), and JW0797-1 (*Δdps* mutant), valine was used to establish stringency while chloramphenicol (CAM) was used to simulate *ΔrelAΔspoT* double mutations, and inhibit protein synthesis. The experiments showed that even in the presence of Dps, the lack of stringency and protein synthesis lowered the resilience. Dps was most important for resilience against UVA, while protein synthesis and stringency were also significant and required to provide complete protection.

Ultraviolet (UV) radiation causes DNA mutations through the induction of oxidative stress (9). Previous studies (1, 10) have shown that the presence of Dps, a DNA-binding protein, improves *E. coli* resilience against UVC; although, it does not appear to be the only factor involved. While not particularly mutagenic, UVA is the predominant form of solar radiation that reaches the surface of the Earth, covering the wavelength range of 320-400 nm (12).

Escherichia coli species possess various adaptive mechanisms for persisting in unfavourable environments. One important mechanism involves the activation of the stringent response, which occurs in the event of nutrient starvation, energy shortage, or other environmental stresses (4). Under stringency, the key enzymes involved are RelA and SpoT, which catalyze the synthesis and accumulation of guanosine 5'-diphosphate, 3'-diphosphate and guanosine 5'-triphosphate, 3'-diphosphate, collectively referred to as (p)ppGpp (14). Specifically, RelA activates (p)ppGpp synthesis, while SpoT mediates its elevation by encoding both degradation and synthesis activities (14). (p)ppGpp contribute to the regulation of various affected cellular processes such as growth, adaptation, secondary metabolism, survival, persistence, cell division, and competence (14).

A downstream effect of (p)ppGpp synthesis is the production of Dps (2). Dps is abundant in stationary-phase *E. coli* and is required for persistence in this phase (13). However, it has also been discovered to be important in conferring protection against oxidative stresses and starvation (2,6). Islam *et al* (10) treated wild-type and *dps* mutant *E. coli* with valine to investigate the role of Dps in UV resistance, and found that even in the absence of Dps, stringency resulted in increased survival. Gallant *et al* (7) found the ribosome inhibitor chloramphenicol inhibited the synthesis of ppGpp and, thus, prevented the production of a stringent response. Chloramphenicol prevents the formation of proteins by trapping aminoacyl-tRNA at the ribosomes (16); thus, no stringent-response proteins were synthesized, including the key effector molecule (p)ppGpp.

This study used a chloramphenicol- and valine-treated *Δdps* strain, simulating a *ΔspoTΔrelAΔdps* relaxed triple mutant, and exposed it to UVA radiation. The goal was to assess the value of the role of *dps* during stringent and non-stringent, protein synthesis inhibited conditions with respect to *E. coli* resilience to UVA. Dps proved to be the most important for resilience against UVA; however, RelA and SpoT were required for a collaborative UVA defence system.

MATERIALS AND METHODS

Bacterial strains. *Escherichia coli* strains BW23113 (5) and JW0797-1 (3) were obtained from the University of British Columbia Department of Microbiology and Immunology MICB 421 culture collection and the Yale University Coli Genetic Stock Center, respectively. *E. coli* BW23113 is the isogenic parental strain to JW0797-1, a K12 *dps* mutant strain. Full genotypes are available at the Coli Genetic Stock Center.

Growth conditions and media preparation. The supplied *E. coli* strains were grown overnight in M-9 minimal salts media, prepared as described previously (15) with the following final concentrations per litre of water: 0.5 g NaCl, 7.0 g Na_2HPO_4, 3.0 g KH_2PO_4, 1.0 g NH_4Cl, 0.2 g $MgSO_4 \cdot 7H_2O$, and 0.2% glycerol. For plating purposes, Luria-Bertani (LB) plates were prepared by dissolving 10 g tryptone, 5 g yeast extract (Bacto, 7116730), 15 g of agar (Invitrogen, G08-33), and 10 g NaCl (Fisher Scientific, 102039). The pH was adjusted to 7.0 using 1 M NaOH.

Genotype verification. PCR amplification of the *dps* DNA fragment in the culture strains was done using whole cell PCR as described in a previous study (18). The amplification was done with a set of *dps* partial primers, dps-II-2zeta-12w (forward primer 5'ATGAAATGCTGGATGGCTTC3',reverse complement primer 5'GGTCAGCCAGTTCTTTCAGG3'), specific to the inner regions of the *dps* gene corresponding to a product size of 183 bp. Primers were designed using Primer3: WWW Primer Tool (University of Massachusetts Medical School, U.S.A). The PCR mix was prepared using 48% dH2O, 3.2 mM dNTP, 2.5 mM

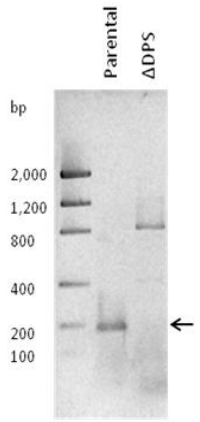

FIG 1 Genotyping of *E. coli* parental and Δ*dps*strains confirms the absence of the dps gene in the mutant. Genotyping was done using *dps*partial primer dps-II-team2zeta-12w corresponding to a product size of 183 bp (denoted by arrow). The presence of a non-specific band at ~800bp is likely an insertion or non-specific priming caused by the mutational changes.

$MgCl_2$, 0.05 U/µl Taq polymerase, 1X PCR buffer, and 4 µM dps-II-2zeta-12w primers. The PCR conditions were according to a 3-step heating cycle of 94°C denaturation, 42°C annealing, then 72°C elongation done to a total of 30 cycles, with an additional 10 minutes 72°C final elongation. The resulting PCR products were ran on 2% agarose gels in 1X TBE running buffer and stained in ethidium bromide bath at 0.5 µg/ml.

Bacterial growth curve. The supplied *E. coli* strains were streaked for purity onto LB plates and used to inoculate M-9 minimal salts media and grown overnight with aeration (150-200 rpm) at 37°C. The overnight cultures were adjusted with M-9 minimal salts media to achieve OD_{460} readings between 0.15 and 0.18 using a Spectronic 20D+ spectrophotometer. The cultures were split into four conditions: parental, parental plus chloramphenicol, *Δdps*, and *Δdps* plus chloramphenicol. Chloramphenicol (Sigma, 73H017426, 2 mg/ml stock) was added at a concentration of 30 µg/ml to inhibit protein synthesis. The cultures were subsequently incubated in a 37°C shaking water bath at 200 rpm, and the OD_{460} was measured periodically for 170 minutes. At 80 minutes, each culture was split again and 100 µg/ml of valine (Sigma, 80H0457, 100 mg/ml stock) was added. An inhibitory level of valine limits isoleucine synthesis, resulting in amino acid starvation which induces stringency (17)

Specific [^{14}C]-uracil incorporation assay. Overnight cultures were diluted with M-9 minimal salts media to achieve OD_{460}readings between 0.15 and 0.18 and incubated in a 37°C water bath shaking at 200 rpm for 50 minutes to reach log phase. For each culture, 1 µg/ml non-radioactive uracil (Sigma, 114H0272, 1 mg/ml stock) and 100 µg/ml valine were added to two flasks, along with 30 µg/ml chloramphenicol in one of the flasks. The remaining procedure for the [^{14}C]-uracil incorporation assay and concurrent growth assay was performed as described previously (15), over a period of 45 minutes. The labelled uracil incorporation assay was used to monitor RNA synthesis under all conditions, and, thus, confirm valine-induced stringency, as well as chloramphenicol-induced relaxation. Values for specific incorporation were obtained through dividing the activity in bequerels/ml by the turbidity of each corresponding time point.

Protein quantification Lowry assay. Done concurrently with the growth and incorporation assay described above, samples were taken from each of the non-radioactive cultures at the first and last time point. The samples were assayed at undiluted and ¼ diluted concentrations, and standard curve generated using chicken egg albumin (Sigma, 54H7070, 500 ug/ml stock). Reagents were prepared as described (11) using 2% sodium potassium tartrate, 1% hydrated copper sulfate, 5% sodium carbonate, and 1N yellow Folin-Ciocalteu reagent.

UVA-irradiation and survival assay. Overnight cultures were diluted with M-9 minimal salts media to obtain OD_{460} readings between 0.20 and 0.30 and incubated at 37°C with aeration at 150-200 rpm for 30 minutes to reach log phase. The cultures were read again at OD_{460} to ensure it did not exceed 0.30, and then serially diluted with M-9 minimal salts media to obtain 10^{-5} and 10^{-4}dilutions for the parent and *Δdps* strains, respectively. Valine (100 µg/ml) was added to each of the final dilution tubes, and chloramphenicol (30 µg/ml) was added to only one of the final dilution tubes for each culture. 3 ml of each culture condition was irradiated in 100 mm x 15 mm polystyrene petri dishes (Fisherbrand) 10 cm under two 20 W Blacklight bulbs (366 nm peak wavelength), similar to a previous study (8). To ensure a uniform UVA dosage of 15.9 mW/cm^2, each petri dish (lids removed) were magnetically stirred at 205 rpm. Dark controls of each sample were covered in aluminum foil to prevent exposure to UV radiation. 100 µl duplicate samples were removed from each condition and spread onto LB agar plates using 5 sterilized glass beads over 180 minutes; dark controls were only plated at 0 and 180 minutes, although duplicate 100 µl samples were removed at each time point. Plates were incubated at 37°C overnight and assessed for growth through the quantification of colonies. Plate counts were averaged and normalized as percent survival values using the number of colonies that survived on the respective control plates which were not exposed to UVA.

RESULTS

Genotyping verification. To verify that the Dps protein was completely absent from the mutant strain, the *dps* gene was amplified using a set of partial primers, dps-II-team2zeta-12w, with a product size of 183 bp as indicated by the arrow (Fig. 1**)**. The parental *E. coli* K12 strain (BW23113) and the *Δdps* strain (JW0797-1) showed the correct genotypic profiles for the deletion of *dps* in the JW0787 strain provided by the Yale University Coli Genetic Center. An unknown band of approximately 800 bps was also captured in the gel electrophoresis, likely due to non-specific priming or insertion in/near the *dps* region (Fig. 1). This may have been due to non-specific priming of a larger fragment due to changes during the mutational analysis or an insertion in the primed region. The darker region at the bottom around 50 bp was due to a loose gel fragment refracting the UV light. However, neither of the mentioned factors above should have affected the validity of the genotype. The non-specific fragment was significantly larger than the PCR product of 183 bp and the 504 bp *dps*, and therefore unlikely to signify ineffective deletion. Furthermore, in the scenario of a large insertion within *dps*, the resulting gene would be non-functional. Overall, the results were in line with expectations for the absence of the *dps* band in the *Δdps* strain.

Growth curve. The growth rates of the parental *E. coli* strain and the *Δdps* strain displayed similar characteristics (Fig. 2). Without valine or chloramphenicol, both cultures showed an increasing growth concentration that was consistent with log phase growth (Fig. 2). Valine addition immediately reduced the growth rate compared to the

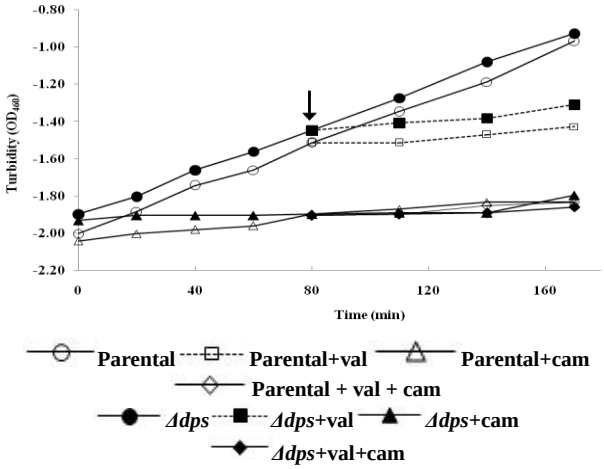

FIG 2 Effect of chloramphenicol and valine on growth of a parental and *Δdps* mutant strain of *E. coli* K12. Arrow indicates valine addition at 80 minutes.

untreated cells (Fig. 2). This cessation of growth that occurred in both *RelA* and *RelA*$^+$ cells was indicative of the expected isoleucine deprivation that subsequently resulted in the lack of protein synthesis, which was not dependent on stringency. Chloramphenicol, as expected of a bacteriostatic antibiotic inhibiting protein synthesis, significantly hindered the growth of both cultures (Fig. 2). A small increase in slope was observed for the parent strain, which may have been due to a concentration of chloramphenicol that was not fully inhibitory; however, it appeared sufficient in inhibiting the growth of the *Δdps* strain. Although the chloramphenicol relaxed the valine-induced inhibition of RNA synthesis, protein synthesis was still inhibited and little to no growth was observed in the chloramphenicol- and valine-treated strains.

Specific [^{14}C]-uracil incorporation assay. Specific incorporation for both the parental *E. coli* strain and the *Δdps* strain was severely diminished in the presence of valine, which indicated the successful stringent inhibition of RNA synthesis (Fig. 3). With the additional inclusion of chloramphenicol, the specific incorporation increased for both strains, indicating RNA synthesis was relaxed despite the presence of valine. These results showed that valine caused stringency that could be relaxed by adding chloramphenicol. Incorporation for the parental strain occurred faster than the mutant, which resulted in 2X more incorporation by 45 minutes (Fig. 3).

Protein quantification Lowry assay. Direct quantification of protein content confirmed the growth curve results. For all samples, there was minimal net protein synthesis (data not shown), which assured the inhibition of protein synthesis by CAM, and effective stringency induction by valine.

UVA-irradiation and survival assay. Ideally, a triple mutant for *ΔspoTΔrelAΔdps* would be compared to a *Δdps* strain; however, due to time limitations, the triple mutant was simulated via addition of chloramphenicol. The synthesis of RelA and SpoT enzymes were inhibited through concurrent additions of valine and chloramphenicol, limiting protein accumulation, and thereby simulating conditions comparable to the mutant. All conditions showed a clear trend of decaying percent survival over time except the parental strain grown in valine, which showed fluctuating percent survival (Fig. 4). Nevertheless, after 30 minutes of UVA exposure, the parent strain treated with valine showed better survival than all other treatments. Additionally, the Δ*dps* strain consistently had lower percentage of survival compared to its counterpart parental condition at each time point (Fig. 4). This suggested the Δ*dps* strain did not have the same capability as the parent to withstand UVA-irradiation under both the relaxed condition and stringent response. Furthermore, the addition of chloramphenicol, the relaxed state inducer, to each condition reduced percent survival faster than the same strain grown in valine alone for both the parent and Δ*dps* strains; percent survival diminished 4X faster when chloramphenicol was added in the Δ*dps* strain, providing evidence that the artificially induced *ΔspoTΔrelAΔdps* could incapacitate the ability of the cells to repair UVA damage. This is consistent with the expectation that protein synthesis inhibition contributed to survivability separately from *dps*.

DISCUSSION

Valid conclusions from the survival assay depended on successfully simulating the *ΔspoTΔrelA* mutants, and establishing stringency. Results congruent with expectations from the PCR, growth curve, Lowry, and incorporation assays confirmed that the use of chloramphenicol and valine simulated double mutants for the chosen strains, and resulted in relaxed or stringent conditions.

Several inferences could be made from the UVA irradiation and survival assay. Firstly, during stringent response, the decreased survival of the Δ*dps* strain suggests that it does not have the same capability as the parent to withstand UVA-irradiation; the presence of Dps was a significant contributor towards the resilience of UVA irradiation, reaffirming previous studies (10). Secondly, the decrease in percent survival of both strains after chloramphenicol treatment showed that the inhibition

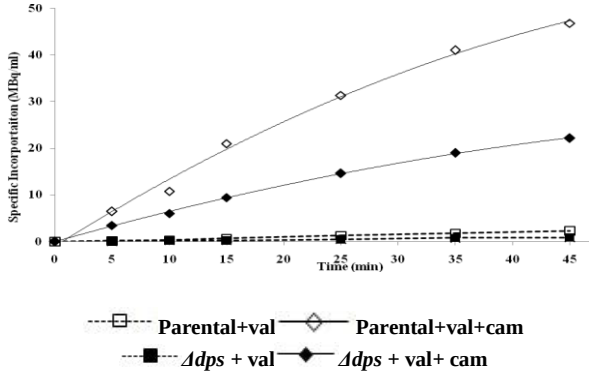

FIG 3 Effect of chloramphenicol on RNA accumulation in parental and *Δdps* mutant *E. coli* K12 strains under stringency.

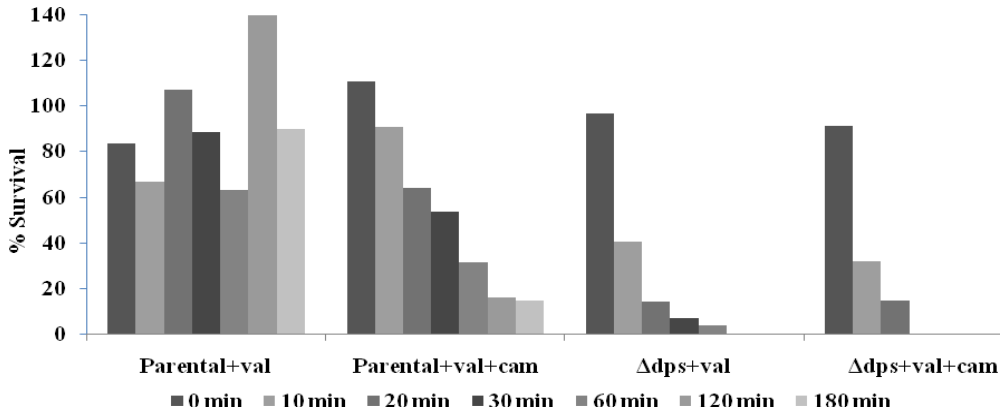

FIG 4 Survival of *E. coli* K12 parental and Δ*dps* strains under UVA exposure in stringent or relaxed condition. A UVA dose of 15.9 mW/cm^{2}was used.

of protein production correlates to resilience to UVA exposure in spite of the presence of Dps. This suggests key mechanisms involving protein synthesis exist and contribute to survival against UVA, separate from stringency. It also indicates that the artificially induced *ΔspoTΔrelAΔdps* could incapacitate the ability of the cells to repair UVA damage. This was a confirmation of an observation from a previous study in which *E. coli* cultures remained resilient against UVA irradiation even after Dps removal (10).

The causation of the relaxed state using chloramphenicol prevents stringency and causes protein synthesis inhibition, separating the two factors. Higher mortality during protein synthesis inhibition suggests that the production of certain proteins in response to radiation stress may be more important to cell survival than stringency. Similar trends being observed both in the presence and absence of *dps* suggest that the mechanism in effect works in addition to the UVA protection conferred by Dps. While the presence of Dps provides noticeably higher protection against irradiation, its protective effects do not fully compensate for the loss of protein synthesis, as indicated by the increased mortality of the parental strain with chloramphenicol addition. The non-UV-exposed controls retained high percent survival, eliminating the possibility that mortality occurred from valine induced amino acid starvation or chloramphenicol-induced protein synthesis inhibition. One possible limitation in the simulated mutant is that due to the complete protein inhibition effects of chloramphenicol, the cultures could not be grown in its presence. Thus, there may have been differences in residual protein synthesis that might have affected the results.

The study has shown evidence for a system of concerted defence against UVA stress in *E. coli*, which is not required for the organism's survival under optimal conditions. The synthesis of other significant proteins upstream of Dps such as RelA and SpoT cannot confer the same level of resilience against UVA as Dps does.

Nevertheless, they are still required to work in conjunction with Dps to provide complete defence in response to the UVA stress, even though protein synthesis is more important than stringent response in providing protection.

FUTURE DIRECTIONS

Built on the work of Islam *et al* (10), the use of a simulated *ΔspoTΔrelAΔdps* relaxed strain made by utilizing chloramphenicol suggests significant results, and further investigation following similar designs using a true *ΔspoTΔrelAΔdps* triple mutant is a logical follow-up. Following the knock-out method used by Baba *et al* (3), a *ΔspoTΔrelA* double mutant could be used to delete the *Dps* gene to generate the triple mutant. This would compensate for the weakness of the current study, where chloramphenicol treatment indiscriminately inhibits all protein synthesis at the translational level and could not be altered to target single proteins of interest such as RelA, or SpoT. In addition to the proposed design, the survival assay of the true *ΔspoTΔrelAΔdps* triple mutant could be compared to the chloramphenicol-induced *ΔspoTΔrelAΔdps* relaxed strain to assess reproducibility between the two models. Quantifying the amount of Dps, SpoT and RelA proteins present in the true triple mutant and the simulated mutant would also help determine their comparability, and the validity of the model.

The *E. coli* strains could be exposed to UVB and UVC radiation using the UV Stratalinker® Model 2400 to compare the effectiveness of Dps, RelA, and SpoT during stringency under various UV wavelengths. Further experiments could also test levels of UVA radiation that mimic the actual amount that reaches the Earth's surface to reflect how these resilience genes act in the current environment.

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