Dps Protects Extra-chromosomal DNA in *Escherichia coli* Grown to Early-stationary Phase from Hydrogen Peroxide Damage

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DNA binding protein in starved cells (Dps) confers multiple modes of protection against various environmental stresses. This study investigated the protective role of Dps during oxidative stress to extra-chromosomal DNA by first transforming wildtype ZK126 and *dps* mutant ZK1146 *E. coli* cells with pKD46, a plasmid with temperature sensitive replicon, and then exposing the transformed cells to various concentrations of hydrogen peroxide. The frequency of the mutation of the temperature-sensitive gene to temperature insensitivity induced by hydrogen peroxide was determined by comparing the number of single colonies that arose at 30°C with the number of mutants that grew at 42°C. The mutation frequency to temperature insensitivity in the *dps* mutant strain was higher across all tested concentrations of hydrogen peroxide than the frequency in the wildtype strain, supporting the notion that Dps protects extra-chromosomal DNA. However, no clear correlation was observed between increasing hydrogen peroxide concentrations and increasing mutation frequency for either the wildtype or the *dps* mutant strains. This absence of trend may be due to the induction of other DNA repair mechanisms and should be further tested.

**Materials and Methods**

**Bacterial strains and plasmid.** The strains used in this study were *E. coli* ZK126 [W3110ΔlacU16ma2] (wild-type control), and *E. coli* ZK1146 [ZK126dps::cam] (*dps* mutant). The plasmid pKD46, in addition to conferring ampicillin resistance, was used as a temperature sensitive conditional plasmid (2). The bacterial strains and the plasmid were supplied from the MICB 421 culture and plasmid collections, Department of Microbiology and Immunology, the University of British Columbia.

**Plasmid isolation.** *E. coli* BW25141 (lacI* Q* rrrB214 ΔlacZ188 βphoBR580 hsdR514 ΔaraBAD153 ΔaraBAD153)
galU95 end-A32511 uidA (ΔMhuF+pir+ recA1) (2) stock culture containing the pKD46 plasmid was streaked onto an LB plate supplemented with ampicillin (Sigma, #A-9518). All ampicillin-supplemented LB media (LB+Amp) used in this study had final ampicillin concentrations of 100 µg/mL. The inoculated plates were grown overnight at 30°C to get single colonies. Five colonies were then inoculated into 10 mL LB+Amp and grown overnight at 30°C. The plasmid was then purified from the overnight cultures using the GeneJET plasmid miniprep kit (Fermentas, #K9505) and eluted in 50 µL sterile distilled water. The purity of the plasmid was determined by measuring its absorbance at 260 and 280 nm using the UV/Vis spectrophotometer (Beckman, DU530).

**Electro-competent cell preparation.** *E. coli* ZK126 and ZK1146 stock strains were streaked on Luria Bertani (LB) plates (10 g/L tryptone (BD Biosciences, #211705), 5 g/L yeast extract (BD Biosciences, #711673), 10 g/L NaCl (EMD Biosciences, #45259605), and 15 g/L agar (BD Biosciences, #1251318)) and grown overnight at 37°C to obtain single colonies for preparation of overnight cultures. Electro-competent cells of both strains grown in LB were prepared according to the protocol described by Sambrook and Russel (13). After the competent treatments, 40 µL of the electro-competent cells were used directly for transformation.

**Transformation.** Electroporation was carried out as previously described (13). Briefly, 40 µL of cells (concentration ~2×10^10 cells/mL) from each strain were transformed directly with 5 µL pKD46 plasmid using MicroPulsed Electroporator (Bio-Rad, #165-2100) in 0.2 cm MicroPulsar cuvettes (Bio-Rad, #165-2092). The cells were then diluted in 1 mL SOC medium (SOB medium + 20 mM (NH_4)glucose (Sigma, #G-50000)) and allowed to recover at 30°C for 1 hour on a shaking platform. After recovery, 100 µL of the suspension was plated onto SOB plates (20 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl, 2.5 mM KCl, 10 mM MgCl_2, and 15 g/L agar, pH 7.0) supplemented with 100 µg/mL ampicillin and incubated at 30°C overnight.

**Culturing methods.** A single colony of both strains that had been transformed with the pKD46 plasmid was streaked onto two LB plates supplemented with ampicillin. One plate was incubated at 30°C and the other at 42°C. No growth of transformed cells was expected at 42°C. This step was done to ensure that the transformed cells had the desired characteristics: temperature-sensitivity and ampicillin-resistance. Resistant colonies that grew at 30°C became our source of cells for further experiments.

**Determination of stationary phase.** Three colonies of transformed ZK126 and ZK1146 cells were inoculated into 3 mL of LB+Amp broth, and the same inoculants were streaked onto an LB+Amp agar plate. The liquid cultures were incubated at 30°C on a shaking platform overnight while the control plates were incubated at 42°C. The next day, one overnight culture of each strain that showed little or no growth at 42°C was inoculated into 200 mL LB media with ampicillin and incubated at 30°C in a shaking waterbath (260 rpm). After 3 hours of incubation, turbidity readings were taken at various time points over a period of 3 hours. Cells were deemed to be in the early-stationary phase when their OD_{660} readings were around 1.2. The turbidity was measured using a spectrophotometer Spectronic 20.

**Mutagenesis assay.** After the cells reached the stationary phase, 1 mL samples were taken and subjected to hydrogen peroxide treatments at final concentrations of 0.1 mM, 1 mM, and 2 mM for 10 minutes at 30°C on a shaking platform. A sample from each strain was also treated with dH_2O as a control. The cells were collected by centrifugation (Eppendorf, 5415C) for 1 minute at 9,300 x g. After a wash step with 1 mL of LB media with ampicillin, the cells were pelleted and resuspended in 1 mL LB+Amp broth. Samples were subsequently diluted and plated onto LB+Amp agar plates at final plated dilutions of 10^7 to 10^8 and incubated at 30°C overnight to determine the number of surviving cells. The same samples were also plated at final diluted dilutions of 10^2 to 10^5 and incubated at 42°C overnight to determine the number of heat-insensitive mutants. The mutation frequency was the concentration of viable cells calculated for plates incubated at 42°C divided by the concentration of viable cells calculated for the corresponding plates incubated at 30°C.

**RESULTS**

**Growth characteristics of *E. coli* ZK1126 and *E. coli* ZK1146 containing plasmid pKD46.** The growth curve was generated by measuring the turbidity readings of both wildtype *E. coli* ZK126 and *dps* mutant *E. coli* ZK1146 containing plasmid pKD46 that were grown in LB broth supplemented with ampicillin, over the course of five hours (Fig. 1). Both strains entered the exponential phase without a noticeable lag phase after re-inoculation from an overnight starter culture. In addition, similar growth rates were observed in both the wildtype and mutant strains thus, both strains reached their stationary phases at approximately equal time, which was about 320 minutes. Previous experiments have shown that these strains only took 150 minutes to reach stationary phase (10) when grown in LB+Amp broth at 37°C. However, these strains were grown at 30°C, for the purpose of retaining the pKD46 plasmid in the cells, and not at their optimal growth temperature hence, the time that these cells took to reach stationary phase was expected to increase significantly. Figure 1 shows the growth characteristics of both cell types, demonstrating that the cells were sampled at early-stationary phase as intended.

pKD46 plasmid DNA is protected by Dps upon hydrogen peroxide treatment during early-stationary phase. The pKD46 plasmids that were introduced into both the wildtype and *dps* mutant strains were susceptible to hydrogen peroxide damage even in low concentrations, such as 0.1 mM. All tested hydrogen peroxide treatments resulted in higher pKD46 plasmid mutation rates in *dps* mutant cells than wildtype cells, suggesting that wildtype cells demonstrated greater resistance to hydrogen peroxide damage than *dps* mutants (Fig. 2).

The mutation frequency of the plasmids was approximately two-fold higher in *dps* mutant strains compared to the wildtype strains when both strains were exposed to 0.1 mM and 2 mM hydrogen peroxide treatments. However, a 50-fold higher plasmid mutation frequency was found in *dps* mutant strains upon 1 mM hydrogen peroxide...
FIG. 1. Growth characteristics of *E. coli* ZK126 and *E. coli* ZK1146 containing plasmid pKD46. A 1-in-50 dilution of overnight wildtype (ZK126) and *dps* mutant (ZK1146) strains of *E. coli* containing plasmid pKD46 were grown aerobically in LB media at 30°C in a shaking water bath at 50 rpm until they reached the stationary phase. The arrow in the figure indicates the point at which the two bacterial strains were sampled for the mutagenesis assay.

FIG. 2. Effect of Dps protein on pKD46 plasmid protection upon treatment with different concentrations of hydrogen peroxide during stationary phase. For each hydrogen peroxide concentration and strain, two plates were plated and each plate was incubated in either the 30°C or 42°C incubator. Mutation frequency was calculated by taking the ratio of growth in 42°C and the growth in 30°C. The mutation frequency for each hydrogen peroxide treatment and strain was corrected by subtracting the mutation frequency of the appropriate controls of each strain (at 0 mM hydrogen peroxide).

treatments. As shown in figure 2, no apparent trend could be observed between hydrogen peroxide concentrations and frequency of plasmid mutations in either strain. In the wildtype strain, the plasmid mutation frequency decreased from 0.1 to 1 mM hydrogen peroxide concentrations by about 90%. The mutation frequency was increased in the 2 mM treatment compared to 1 mM treatment, yet it was still about 35% lower than the mutation frequency from the 0.1 mM treatment. For the *dps* mutant strain, the plasmid mutation frequency increased from 0.1 to 1 mM hydrogen peroxide concentrations by about 80% whereas, the plasmid mutation frequency decreased from 0.1 mM to 2 mM hydrogen peroxide concentration by about 40%. Occurrence of occasional spontaneous mutations, depicted by growth of a few colonies for strains grown at 42°C with 0 mM hydrogen peroxide treatment was taken into account in the calculation by subtraction.
DISCUSSION

The mutagenesis assay developed in this study was used to further investigate the protective roles of Dps by examining the hydrogen peroxide induced damage on extra-chromosomal DNA in the absence and presence of Dps. By introducing the pKD46 plasmid to the bacterial cells, damage on the heat-sensitive gene encoded on the plasmid would allow the cell to grow at 42°C, a temperature that is growth-inhibiting for cells carrying non-mutated plasmids (2). This protocol design has an advantage over conventional loss-of-function mutagenesis assays. Mutations on the heat-sensitive gene allow cells to grow at a non-permissive temperature in the presence of the selecting antibiotic, allowing for an assay that is based on positive selections. This design has significantly improved the sensitivity of the assay in terms of detecting the number of mutants and minimized the number of steps in the protocol. Since a plasmid is a non-chromosomal piece of DNA, we expected to see less stringent defense mechanisms exerted by the cell to protect it against mutations than those exerted on chromosomal DNA, making a plasmid susceptible to higher mutation frequency. The appreciable number of mutants that grew at 42°C suggests that this might be the case. However, we did not specifically measure the mutation frequency of an analogous function in the chromosome, so we could not compare the frequency.

The plasmid mutation frequency of dps mutants was low, indicating that this strain may also encode other genes that may protect the plasmid from oxidative damage. In fact, other studies have shown that the presence of several proteins such as catalases, alkyl hydroperoxide reductase, and glutathione reductase help protect chromosomal DNA against oxidative damage (4). The genes encoding these enzymes are upregulated by RpoS in the stationary phase (12). Since these enzymes act by neutralizing the surrounding reactive oxidative species, the production of these proteins could possibly protect plasmid DNA from oxidative damage as well. In addition to these enzymes, other studies have shown that the superoxide stress response and peroxide stress response systems that are induced by different oxidative stresses are also critical for the protection of E. coli against oxidative stresses (11). However, the noticeable difference in mutation frequency was found between wildtype and dps mutant strains, suggesting that Dps plays a major role in the protection of plasmid DNA against oxidative stress.

No obvious correlation was observed between the mutation frequency and hydrogen peroxide concentration. As the hydrogen peroxide concentration increased, the frequency of plasmid mutations for each strain was expected to increase. The erratic trends seen in figure 2 could be due to the presence of other DNA protection and repair mechanisms in the cell besides Dps. Some of these mechanisms could be regulated under the same system but others may be regulated independently from one another. Therefore, these different systems may require higher levels of hydrogen peroxide before being induced. Additionally, experimental errors, such as inaccurate dilutions due to pipetting or vortexing could contribute to the erratic trends. Despite the erratic variation in trends, the wild type strain clearly showed lower mutation frequency compared to the dps mutant strains under all conditions tested, suggesting that Dps plays a role in plasmid DNA protection against hydrogen peroxide damage during early-stationary phase.

FUTURE EXPERIMENTS

This mutagenesis protocol could be used to identify proteins in different bacterial strains that are capable of protecting extra-chromosomal DNA against different types of mutagens such as UV radiation or carcinogenic compounds. Knockouts of potential candidate genes could be assessed by performing the mutagenesis assay that was developed for this experiment. For example, previous studies have shown that topoisomerase I also plays a role in protecting E. coli from oxidative damage (14). To see if topoisomerase I and Dps work together, the experiment can be repeated on topA and dps double mutants and observe if there are any changes in the mutation rate. We should expect that if topoisomerase I and Dps work together to protect DNA, there should be a higher mutation rate, and therefore greater number of live cells, in these cells compared to the dps mutants.

Results from this study have shown that no obvious trend was observed between hydrogen peroxide concentration and mutation rates and this may be caused by the cumulative effects from the interplay of different DNA protection mechanisms in the cell. To further visualize the hydrogen peroxide induced damage as well as to eliminate the possibility of experimental errors, this experiment should be repeated but the number of mutagen treatments should be increased, adding 0.01 mM, 10 mM, 45 mM, 100 mM, and 1 M. The method can determine the role of Dps at varying
levels of mutagen exposure and to obtain a clear trend for varying concentrations. Moreover, varying the length of exposure, from minutes to days, can also be investigated to determine the effectiveness of Dps in protecting DNA from a prolonged mutagen exposure.

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REFERENCES


