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Effect of *relA* Activation on UV and Antibiotic Sensitivity

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The stringent response in starvation conditions is a well documented activation of the RelA protein, which synthesizes guanosine 3', 5' bisphosphate (ppGpp). Aside from inducing global changes in gene expression, increased cellular ppGpp levels have previously been found to confer resistance to penicillin. We used *Escherichia coli* strains with *relA*$^+$ (NF314As19); *relA*$^+$ *valS*^{ts} (NF536As19); and *relA*$^-$ *valS*^{ts} (NF537As19) geneotypes to determine if RelA activation conferred resistance to other antibiotics besides the Penicillins, as well as to ultraviolet radiation. It was experimentally found that partial activation of RelA was achieved at 36°C in *relA*$^+$ *valS*^{ts} strains. While this strain was significantly more sensitive than the other two strains at 40°C to the five tested antibiotics (Ampicillin, Chloramphenicol, Ciprofloxacin, Kanamycin and Vancomycin), the *relA*$^-$ *valS*^{ts} strain was more sensitive than the *relA*$^+$ strain at 40°C to three of the antibiotics (Ampicillin, Chloramphenicol and Ciprofloxacin). *relA*$^+$ bacteria were the least sensitive to UV radiation and both temperature sensitive strains were very strongly affected by UV radiation.

The *relA* gene locus is one of many loci in *Escherichia coli* that promote cell survival amidst various environmental stressors and may be implicated in the tolerance of the bacterium to many bacteriocidal antibiotics. Its activation is noticeable during times of nutritional stress when the bacterium initiates a stringent response (11). Wendrich *et al.* (19) inferred that the stringent response serves two purposes, one of which is the down regulation of transcription of genes involved in the translation machinery (such as tRNA and rRNA), and the other is the up regulation of genes encoding metabolic enzymes (for example, those involved in amino acid biosynthesis, 11).

A recent model of the mechanism of action of RelA (a product of the *relA* gene) proposed by Wendrich *et al.* (19) states that during amino acid deprivation, there is a significant increase in the concentration of uncharged or deacylated tRNA making up 80 % of the total tRNA. These deacylated tRNA molecules bind to the empty ribosomal A site blocking the ribosome, albeit with low affinity. This blockage creates a protruding 3' extension of the mRNA which stimulates RelA binding to the ribosome, where it produces guanosine 5'-triphosphate-3'-diphosphate (pppGpp) and guanosine 3',5'-bisphosphate (ppGpp) using ATP and GTP. These final nucleotide products are actively involved in the stringent response (19).

Various experiments have shown the effects of ppGpp on bacteria. Specifically, it is involved in the control of transcription thereby affecting gene expression. It has been suggested that ppGpp plays a role in DNA replication, recombination and repair (1). In addition, ppGpp is also an activator of sigma S (6), which causes expression of genes responsible for entry into stationary phase or to combat various stresses (9). Research has also implicated that ppGpp inhibits peptidoglycan synthesis by inhibiting a major *E. coli* autolysin, soluble lytic transglycosylase, thereby causing a slower growth rate and increasing resistance to antibiotics (11).

Resistance to antibiotics was the focus of this research. We investigated the effects of the following antibiotics, which vary in their modes of action: Ampicillin, Vancomycin, Chloremphenicol, Kanamycin, and Ciprofloxacin; as well as looking at the effects of ultraviolet light (200 nm to 350 nm) on the survival of three *E. coli* strains which contain varying levels of cellular ppGpp.

Ampicillin and Vancomycin are antibiotics that affect peptidoglycan synthesis. Ampicillin is a member of the penicillin family of antibiotics containing a β-lactam ring. It has bacteriolytic effects due to its binding and inactivation of Penicillin Binding Proteins (PBP) which are components of peptidoglycan synthesis (18) Vancomycin, however, mimics PBP and binds to cell wall peptide-sugar building blocks (15) Chloremphenicol and Kanamycin are bacteriostatic and bacteriocidal antibiotics, respectively, designed to inhibit bacterial protein biosynthesis. Chloremphenicol binds the bacterial ribosome by adopting a conformation that mimics that of a peptidyl adenylyl terminus of tRNA. It therefore blocks further translation of the mRNA and subsequently all protein synthesis (4) Kanamycin is an aminoglycoside that binds the 30S ribosomal subunit preventing it from joining the 50S ribosomal subunit and inhibiting protein synthesis (http://www.merck.com/pubs/mmanual/section13/chapter153/153c.htm). Finally, Ciprofloxacin is a quinolone antibiotic that inhibits bacterial DNA gyrase (20), which is responsible for controlling the topological properties of DNA by introducing as well as

removing supercoils in the DNA. Ultraviolet exposure has been linked to high mutation rates in chromosomal DNA and was used here to assess resistance of ppGpp producing cells to DNA damage.

We examined three different *E. coli* strains, namely, the wild type strain, NF314As19 (*relA*$^+$), NF536As19 (*relA*$^+$ *valS*^{ts}) and NF537As19 (*relA*$^-$ *valS*^{ts}) (5). Using these last two temperature sensitive mutants, we could control RelA production by amino acid starvation due to *valS* inactivation and thereby observe the effects of ppGpp production on resistance to the antibiotics and UV exposure.

We hypothesized that ppGpp production would confer resistance to Ampicillin and Vancomycin since inhibition of autolysins would hinder peptidoglycan synthesis from proceeding. Thus, these antibiotics would not have the opportunity to affect this process. However, ppGpp production would not confer resistance to Chloremphenicol because the latter has been shown to inhibit RelA dependent ppGpp synthesis since it binds to the ribosome altering its functional state (2). Kanamycin would also produce similar results as Chloramphenicol since it would inhibit the ribosomal translational apparatus from forming thereby acting upstream of RelA activation. Increased ppGpp levels have been shown to lower the rate of DNA replication initiation but not to inhibit it (2). Thus, cells producing more ppGpp may confer only partial or no resistance to Ciprofloxacin. A similar hypothesis can be made about UV radiation resistance.

MATERIALS AND METHODS

Bacterial Strains – All *Escherichia coli* strains used in this experiment were kindly provided by Dr. William Ramey of the Microbiology and Immunology Department University of British Columbia. The *E. coli* strain NF314As19 (*leu*$^-$ *valS*$^+$ *relA*$^+$), the control strain, was a leucine negative auxotroph that expressed a normal valyl tRNA synthase and ppGpp synthase. The two experimental *E. coli* strains, NF536As19 (*leu*$^-$ *valS*^{TS} *relA*$^+$) and NF537As19 (*leu*$^-$ *valS*^{TS} *relA*$^-$), contained ppGpp synthase or no ppGpp synthase, respectively. Both were leucine negative auxotrophs, and contained temperature sensitive valyl-tRNA synthases, which were fully functional at low temperatures and completely inactivated at high temperatures.

Preparation of Growth Media – Luria Broth (LB) media and M9 minimal media were all prepared as described in the Microbiology 421 WebCT website. M9 minimal media however was supplemented with 50 ug/ml of leucine and 1ug/ml of thiamine to fulfill the growth requirements of the auxotrophic strains. LB media did not need to be supplemented because of its high concentrations of yeast extract and casamino acids.

Growth Curve - From overnight cultures of *E. coli* strains grown at 30ºC, 1-in-20 dilutions were made of these strains in modified M9 media and grown in three water baths set at 32ºC, 36ºC, 43ºC (data not presented for 32ºC and 43ºC). Turbidity readings at OD_{460} were taken at various time points with the Ultrospec3000^{TM} spectrophotometer to generate growth curves.

Preparation of Bacterial Strains for Radio-Labeled Uracil Incorporation and OD_{460} – Overnight cultures of bacterial strains were grown at room temperature in modified M9 media. 1-in-20 dilutions of these overnight stocks were grown at 30ºC in 60 ml of the same media for 2 hr prior to uracil incorporation. After initial incubations, 300 ml of 5 ug/ml cold uracil was added to each of the three strains. 6 ml of each of the strains was removed, and to this, 5 ul of 2-^{14}C-uracil (2.9 MBq/ml, 2.2 GBq/mmole) was added. The 6 ml samples were then split into sterile test tubes and placed in 32°C, 36°C, and 43°C water baths for scintillation count. The remaining 54 ml of each of the three bacterial strains were split into 3 flasks and grown in 32ºC, 36ºC, and 43°C for turbidity OD_{460} readings taken with the Ultrospec3000^{TM} photospectrophotometer. Scintillation and turbidity samples were taken at the same time.

Scintillation counting and OD_{460} reading – At time points 5, 10, 20, 30, 45, 60 minutes after the addition of cultures to water baths, 1ml samples were removed from the cold uracil conditioned cultures for absorbance OD_{460} spectrophotometer (Ultrospec3000^{TM}). 100 ul of hot uracil conditioned cultures were added to separate filter disks and left to air-dry. They were then conditioned in 50 ml cold TCA twice. The filters were placed in a 98°C oven for 2 hr before being placed into scintillation vials containing 3 ml of scintillation fluid. The vials were read through the scintillator to obtain the scintillation counts.

Preparation of Kanamycin Antibiotic Disks – 10 mg of Kanamycin was diluted into 1 ml of water to generate a 10 mg/ml Kanamycin stock solution. 3 ul of this stock solution was added to filter paper disks (10 mm diameter) that have been pre-autoclaved.

Antibiotics Resistance Testing – LB plates were preheated at 30ºC, 36ºC, and 40ºC. *E. coli* strains were spread onto these preheated LB plates, from their stock solutions. The LB plates containing the bacterial confluent lawns were then further incubated for 30 minutes before the antibiotic disks were placed onto the agar. (30 ug Kanamycin, 30 ug Vancomysin, 30 ug Ciprofloxin, 10 ug Ampicillin, and 30 ug Chloramphenicol) The diameters of the zones of inhibition were taken after a 24-hour period of incubation in the correct temperatures.

UV Resistance Testing – Stock solutions of the bacterial strains were 1-in-10 serially diluted and spread onto preheated 36ºC LB plates. These diluted bacterial plates were further incubated at 36ºC for 30 minutes before exposure to UV for the duration of 30 seconds. After exposure, all confluent lawns were incubated at 36ºC for 24 hours before plate count.

RESULTS

Bacterial Growth Curves – The purpose of making growth curves for the different strains at different temperatures was to find the temperature at which RelA is partially activated.

It was initially assumed that at this temperature of partial activation only the *relA*$^+$ *valS*^{ts} strain would undergo a reduction in growth; however, because both the *relA*$^+$ *valS*^{ts} and the *relA*$^-$ *valS*^{ts} strains had a ValS^{ts} mutation, incubation at this temperature prevented normal levels of protein synthesis, and thus a decrease in the growth of both strains. The ability to express functional RelA allowed a further reduction in growth of the *relA*$^+$ *valS*^{ts} strain via the stringent response, complementing the direct effect of decreased protein synthesis. When the growth curves were constructed, the M9 media did not contain sufficient leucine to support growth, containing a concentration of only

0.2µg/ml rather than the required 50 µg/ml. Therefore the *relA*$^+$ strain growth peaked at an absorbance of 0.8 for 36°C (where it was growing the most efficiently) and at 0.3 for 32°C and 43°C (Fig. 1). This was lower than would be expected had there not been an amino acid deficiency.

Fig. 1 Growth Curves for the three *Escherichia coli* Strains at the Semipermissive 36°C

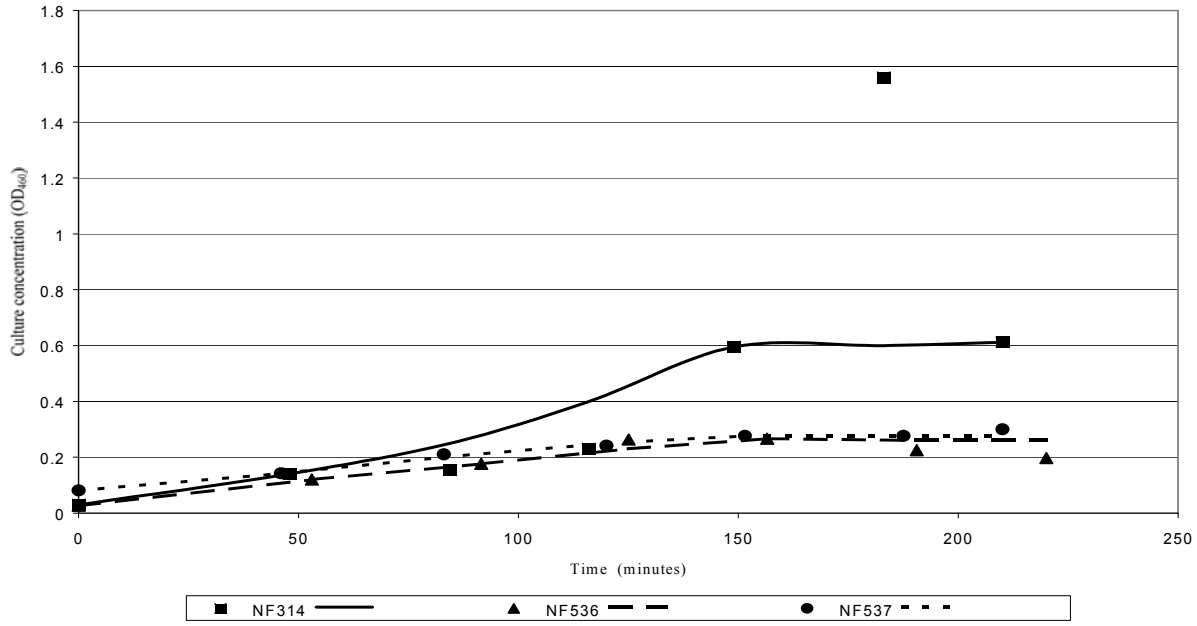
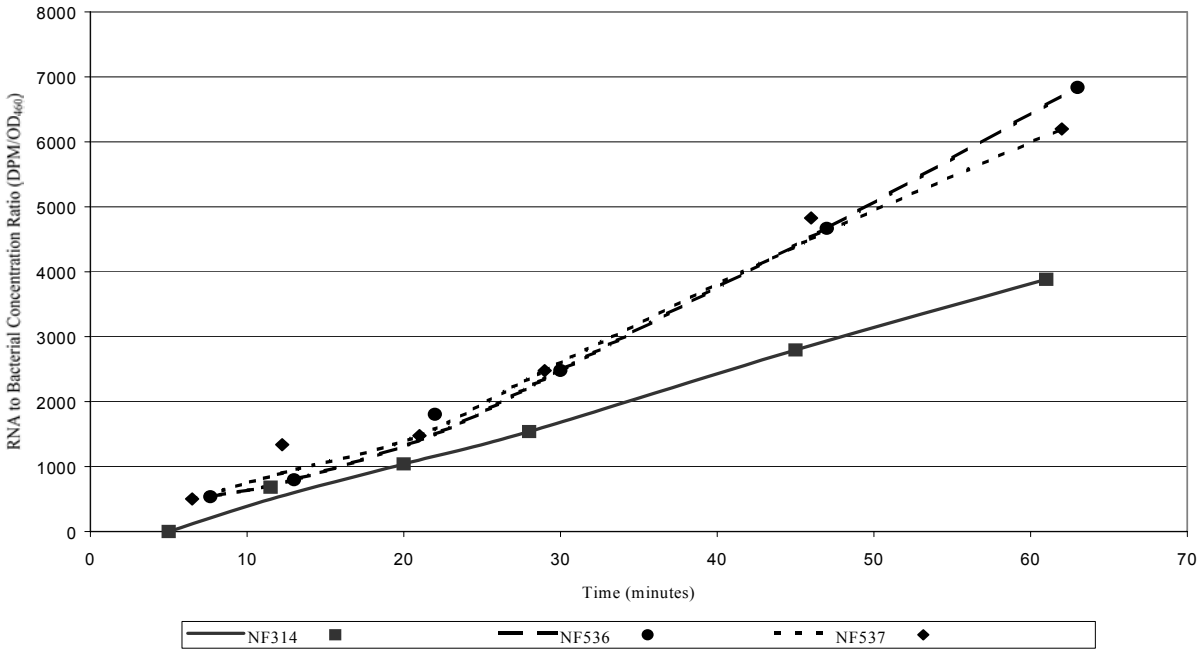
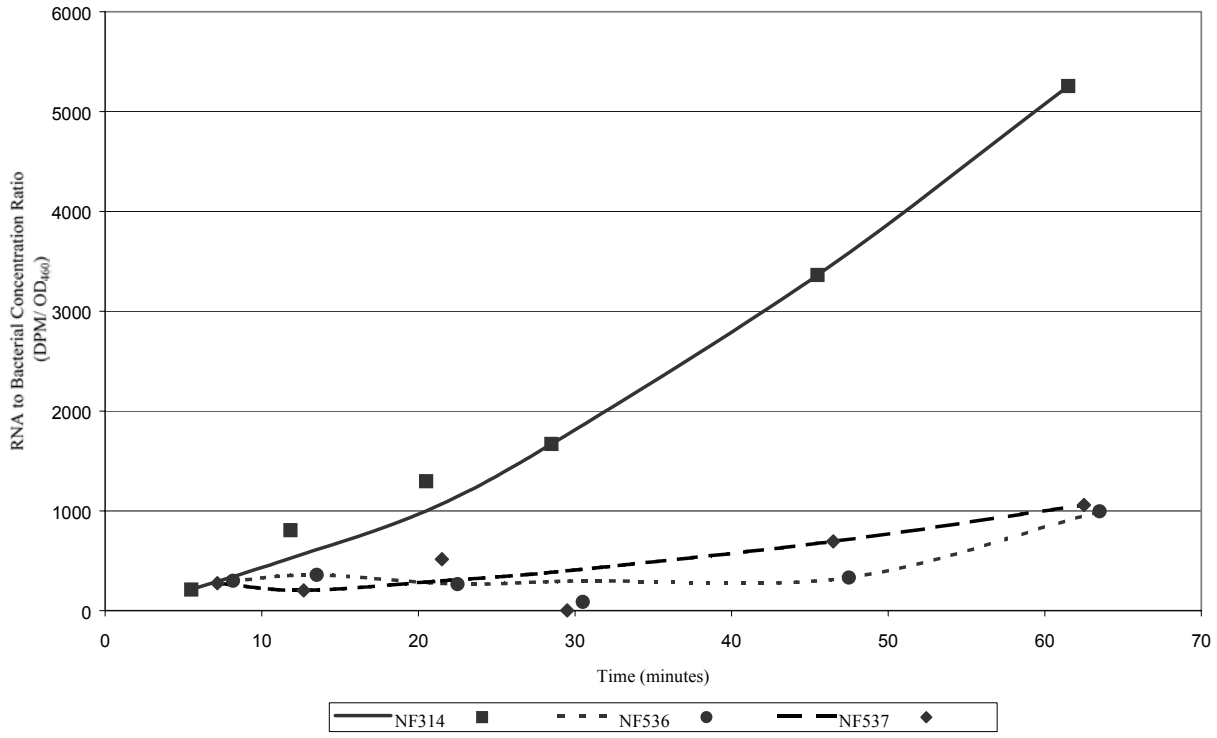


Fig. 2A Ratio of RNA to Turbidity at the Permissive 30°C: Labeled Uracil Incorporation and Bacterial Turbidity



Uracil Incorporation – Because ppGpp is known to inhibit RNA synthesis we hypothesized that at 36ºC the *relA*$^{+}$ *valS*^{ts} strain would have a lower RNA to protein ratio than the other two strains. RNA synthesis was measured by radiolabeled uracil incorporation at 30ºC, 36ºC and 40ºC. At 30ºC, when the temperature sensitive mutation in valS was not expected to have an effect, it was found that the *relA*$^{+}$ *valS*^{ts} and the *relA*$^{-}$ *valS*^{ts} strains had a slightly higher ratio of RNA synthesis to protein than did the *relA*$^{+}$ strain (Fig. 2A).

Fig. 2B Ratio of RNkA to Turbidity at 36°C: Labeled Uracil Incorporation and Bacterial Turbidity



Conversely, at 36ºC and 40ºC both the *relA*$^{+}$ *valS*^{ts} and the *relA*$^{-}$ *valS*^{ts} had lower ratios of RNA to protein compared to the *relA*$^{+}$ strain. Because a main effect of RelA activation and thus ppGpp synthesis is to inhibit RNA expression, our results indicate that this function is occurring in the *relA*$^{+}$ *valS*^{ts} strain, but in the case of the *relA*$^{-}$ *valS*^{ts} strain, there is some other mechanism of RNA synthesis regulation that is RelA-independent.

Antibiotic Disc Plates - According to our hypothesis, ppGpp should lend resistance to Ampicillin and Vancomycin but not to Kanamcyin, Chloramphenicol or Ciprofloxacin. We tested this hypothesis by growing cells in a lawn on LB agar plates for a disc diffusion assay. From the diameters of the zones of inhibition we inferred the level of sensitivity to the antibiotics. The major trends were that at 40°C, the *relA*$^{+}$ *valS*^{ts} was markedly more sensitive to all the antibiotics than the other two strains, with an 11.5 mm to 22 mm increase in diameter size when compared to the 30°C levels (Fig. 3). For Chloramphenicol, Ciprofloxacin and Kanamycin treatments, the *relA*$^{-}$ *valS*^{ts} strain showed more sensitivity than the *relA*$^{+}$ strain at 40°C with size differences ranging from 3.5 mm to 12.5 mm.

Ampicillin, Chloramphenicol, and Ciprofloxacin Resistance - At 30°C, all three strains are equally susceptible to the antibiotics Ampicillin, Ciprofloxacin, and Chloramphenicol (Figs 3A, 3B, 3C). With ciprofloxacin treatment the *relA*$^{+}$ strain was slightly more resistant to the antibiotic than the other two strains. At 36°C, there was a general trend ofincreased resistance to the three antibiotics. For Ampicillin and Ciprofloxacin, the negative and positive strains were more resistant than the control (20 mm vs. 25 mm for Ampicillin, and 25 mm vs. 31 mm for Ciprofloxacin). All the strains exhibited an equal increase in resistance to Chloramphenicol at 36°C (25 mm vs. 30 mm at 30°C). At 40°C, the *relA*$^{+}$ *valS*^{ts} strain became dramatically more sensitive to all three antibiotics, showing a

Fig. 2C Ratio of RNA to Turbidity at 40°C: Labeled Uracil Incorporation and Bacterial Turbidity

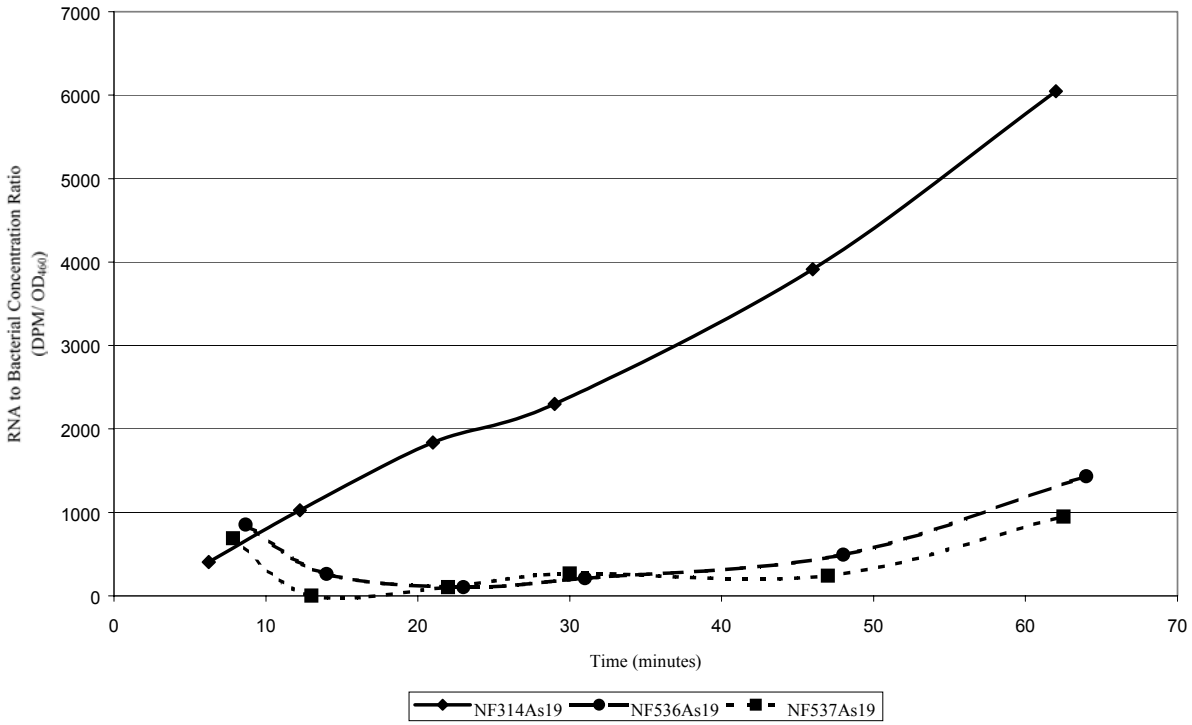


Fig. 3A Effects of Ampicillin on Bacterial Strains Growing in Different Temperatures

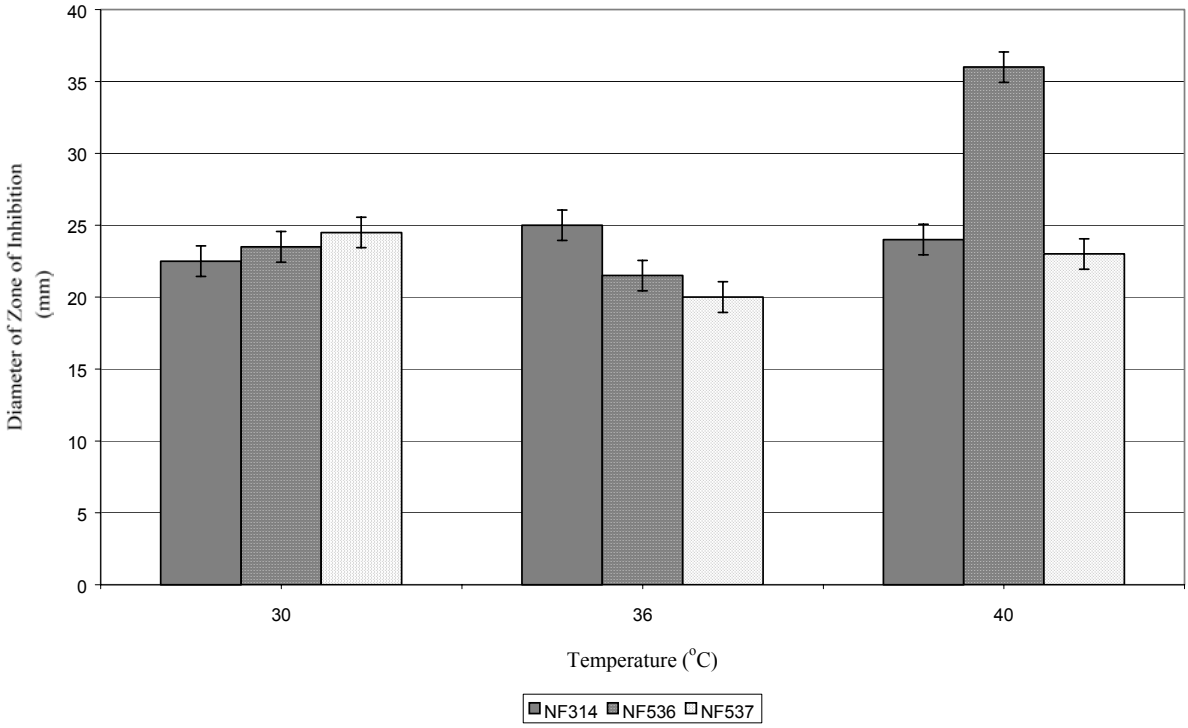


Fig. 3B Effects of Chloramphenicol on Bacterial Growth in Different Temperatures

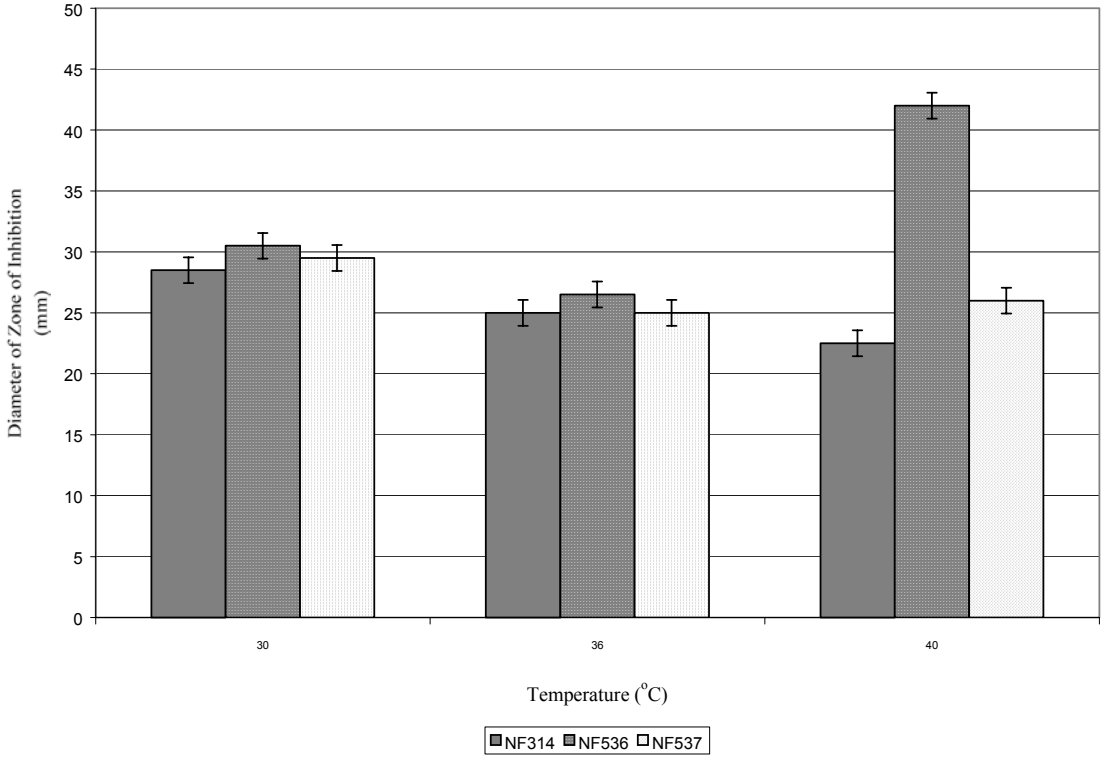


Fig. 3C Effects of Ciprofloxacin on Bacterial Growth in Different Temperatures

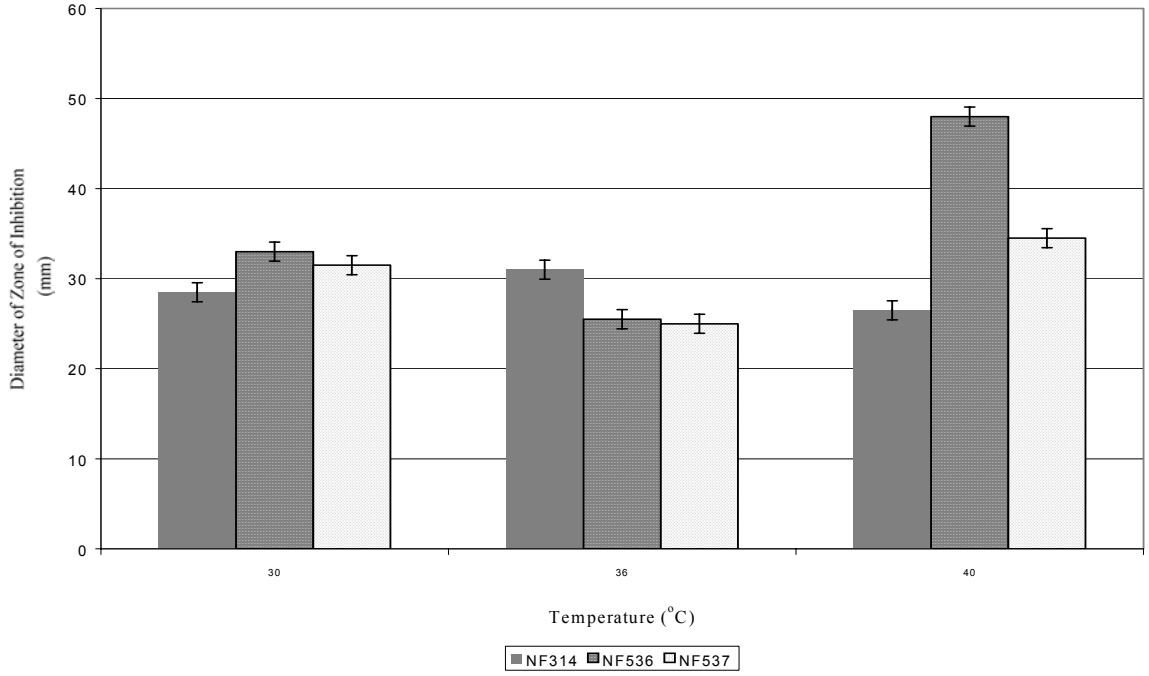


Fig. 3D Effects of Kanamycin on Bacterial Growth in Different Temperatures

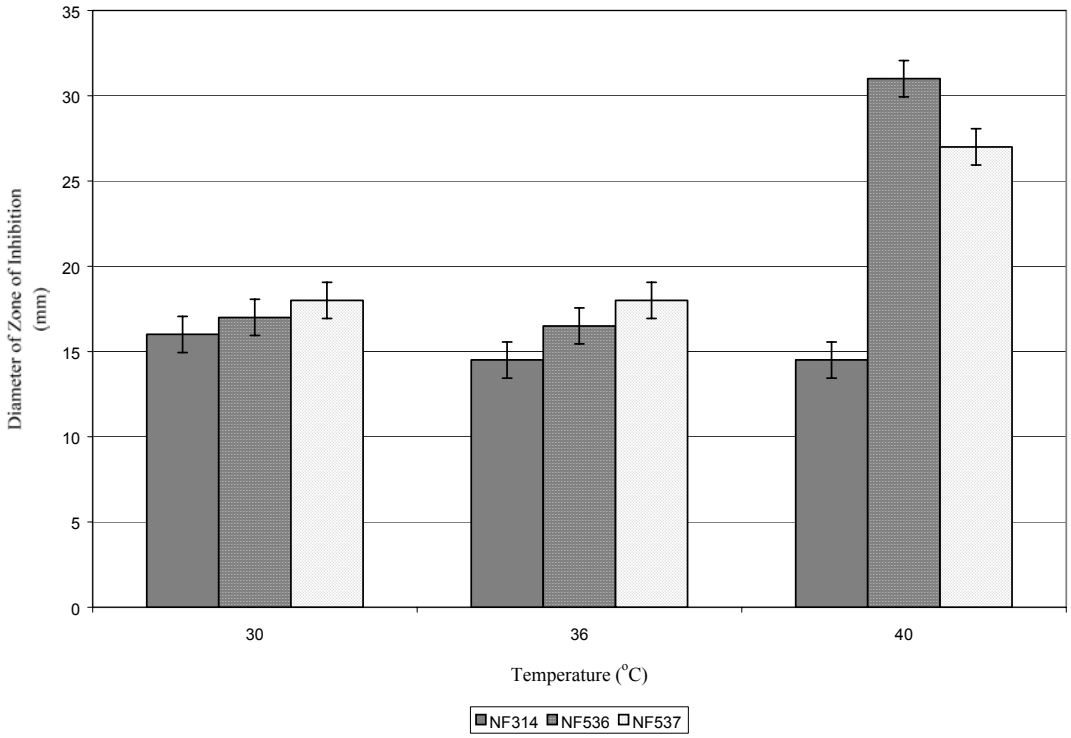
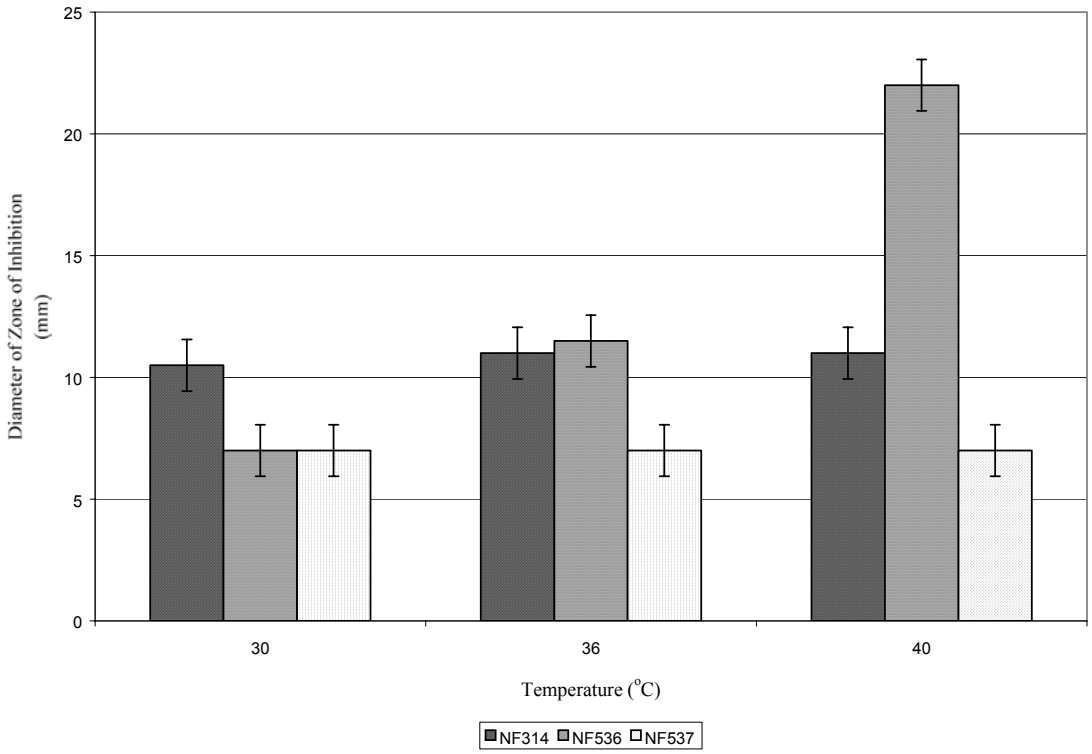
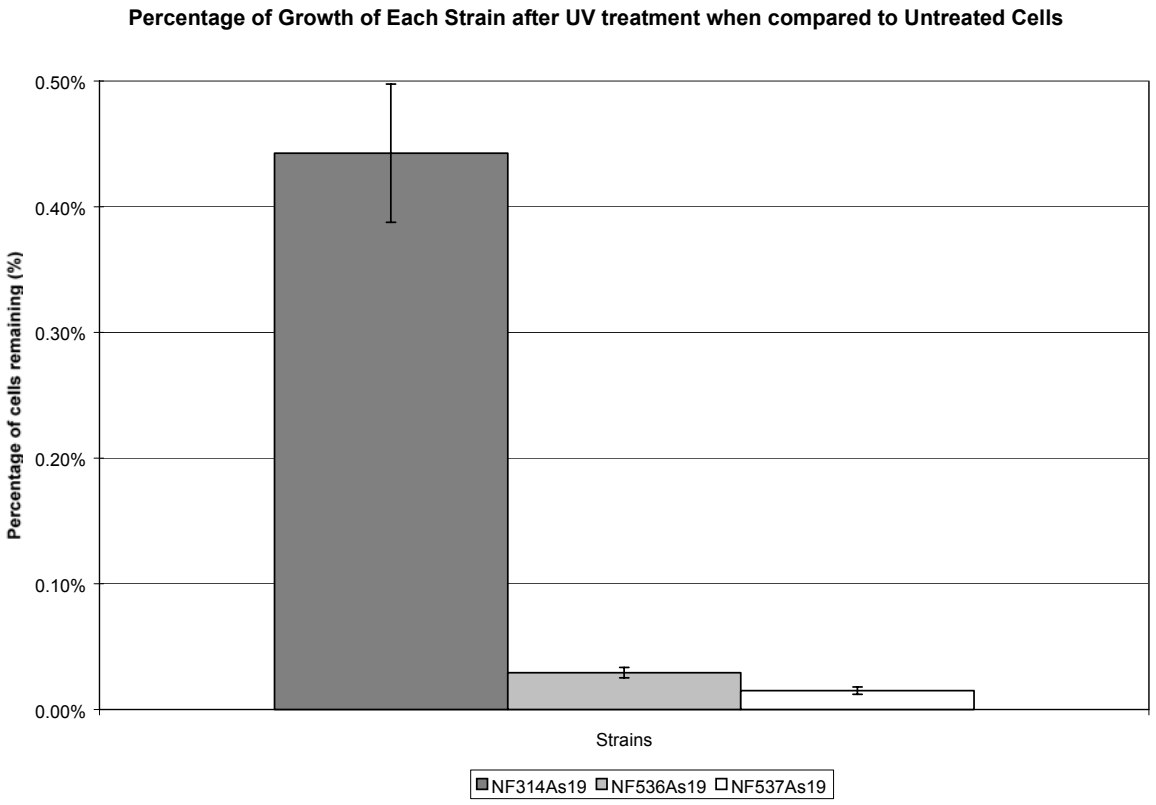


Fig. 3E Effects of Vancomycin on Bacterial Growth in Different Temperatures





10 mm increase in diameter for Ampicillin and Chloramphenicol and a 20 mm increase for Ciprofloxacin. For Chloramphenicol and Ciprofloxacin the *relA*$^-$ *valS*^{ts} strain also increased slightly in sensitivity.

Kanamycin Resistance – At 30°C, the zones of inhibition for the *relA*$^+$, *relA*$^+$ *valS*^{ts} and the *relA*$^-$ *valS*^{ts} strains showed similar results at 16 mm, 17 mm, and 18 mm respectively. At 36°C, all three strains also showed similar results. The *relA*$^+$ strain showed no change in its zone of inhibition at all three temperatures. There was a significant effect, however, on resistance of the *relA*$^+$ *valS*^{ts} and the *relA*$^-$ *valS*^{ts} strains at 40°C. The *relA*$^+$ *valS*^{ts} strain showed an increase in susceptibility to Kanamycin by a factor of approximately two, compared to the other two temperatures. The *relA*$^-$ *valS*^{ts} was similarly affected by the increase in temperature, and resulted in an increase of susceptibility by 1.5 times (Fig. 3d).

Vancomycin Resistance – (Fig. 3e) The *relA*$^+$ strain showed similar susceptibility results at all three temperatures (10.5 mm at 30°C, 11 mm at 36°C, and 11 mm at 40°C). As the temperature increased, however, the *relA*$^+$ *valS*^{ts} and the *relA*$^-$ *valS*^{ts} strains showed drastically different results from each other and from the control strain. The positive strain, *relA*$^+$ *valS*^{ts}, showed an increase in susceptibility from being completely resistant at 30°C (7 mm, diameter of the disk), to 11.5 mm at 36°C, to 22 mm at 40°C. With the change in temperature, the negative strain, *relA*$^-$ *valS*^{ts}, remained resistant to Vancomycin. For all three temperatures, the zones of inhibition were 7 mm (diameter of the antibiotic disk).

UV Resistance – The three *E. coli* strains grown in 32°C, 36°C, and 43°C after the exposure to 30 seconds of UV showed a drastic reduction of bacterial growth. Specifically, when the *relA*$^+$ strain was exposed to UV, a survival rate of only 0.44 % was shown as compared to non-UV exposed cells. The *relA*$^+$ *valS*^{ts} strain had only a 0.03 % survival rate, which is 15 fold lower then that of the control. The *relA*$^-$ *valS*^{ts} had an even lower survival rate of only 0.015 %. This is approximately 30 fold lower then the control strain, *relA*$^+$, and half that of the survival rate of the positive strain, *relA*$^+$ *valS*^{ts}. (Fig. 4).

DISCUSSION

The primary purpose of measuring radioactive uracil incorporation into cellular RNA was to indirectly measure the production of ppGpp. Since an increase in ppGpp concentration is inversely related to total RNA content, we hypothesized that at 30°C, all the strains would have a similar ratio of RNA to protein (as measured by bacterial concentration) (referred to as "ratio" from here on), which would steadily increase through time as more radioactive uracil would incorporate into the RNA. At 36°C, the *relA*$^+$ and the *relA*$^+$ *valS*^{ts} strains would continue the same trend as at 30°C with the latter strain having a slightly higher ratio than the former strain due to reduced protein production by partially inactivated ValS. The *relA*$^+$ *valS*^{ts} strain, however, would show reduced uracil incorporation through time into the RNA compared to the first two strains. This result would be due to its partial activation of RelA at 36°C (caused by the temperature dependent *valS*) increasing intracellular ppGpp levels. At 40°C, the *relA*$^+$ and the *relA*$^-$ *valS*^{ts} strains would show a lower rate of increase in the ratio compared to 30°C and 36°C due to effects of temperature on physiological condition. We also expected that at 40°C, the *relA*$^+$ *valS*^{ts} strain would show a severely depressed rate of increase in the ratio due to complete activation of RelA (by complete inactivation of ValS).

Notwithstanding, the results we obtained were surprisingly different. The curves plotted using the ratios obtained showed remarkably different trends at each temperature examined. At all temperatures, the curves for the *relA*$^+$ *valS*^{ts} and the *relA*$^-$ *valS*^{ts} were very similar. At 30°C, *relA*$^+$ strain showed a more gradual increase in the ratio over time compared to the other two strains. This may be attributable to a different optimal temperature for growth of this strain. Since the *relA*$^+$ *valS*^{ts} and the *relA*$^-$ *valS*^{ts} strains had a temperature sensitive mutation in *valS*, their optimal temperature might have been 30°C. However, the *relA*$^+$ strain, which had a wildtype *valS* gene, may have had a higher optimal temperature between 36°C and 40°C. The curves generated by this strain at those temperatures had a higher slope than at 30°C indicating increased RNA synthesis at higher temperatures than at lower ones. The curves generated by the *relA*$^+$ *valS*^{ts} and the *relA*$^-$ *valS*^{ts} strains at 36°C and 40°C were drastically lower than at 30°C for both strains. This effect was expected for the *relA*$^+$ *valS*^{ts} strain due to its increased RelA activation with rising temperature. For *relA*$^-$ *valS*^{ts}, however, the same argument cannot be made since it has an inactivated *relA* gene. We suggest that the decreased slope of the curves for this strain at both temperatures is due to the temperature sensitive mutation in *valS*. Somehow, this mutation may have reduce the rate of RNA synthesis of this strain at higher temperatures while also reducing its protein concentration effectively shifting its ratio curve lower than that at 30°C.

In order for bacteria to be able to withstand changes in temperature they must be able to alter the fatty acid composition of their membranes. At lower temperatures unsaturated fatty acids are favored over saturated ones so that a more fluid membrane is created. The opposite is true for higher temperatures where longer and more saturated fatty acids help to stabilize the membrane (13). In fact, at 30°C *Escherichia coli* has a ratio of unsaturated to saturated fatty acids of 1.6, and this ratio drops to 0.36 at 40°C (12).

ppGpp inhibits the synthesis of glycerol-3-phosphate acyltransferase (8) which is the enzyme responsible for adding fatty acyl chains to the glycerol-3-phosphate backbone of the phospholipids (13). Therefore at 40°C, when our three bacterial strains required alterations to their membrane (due to a change in temperature from 30°C to 40°C) only the *relA*$^+$ *valS*^{ts} strain was unable to make the changes efficiently. When the stress of membrane instability was combined with the stress of antibiotic inhibition the cells became significantly more sensitive to the effects of the antibiotics and underwent lysis.

The Ciprofloxacin, Chloramphenicol and Kanamycin patterns of the *relA*$^-$ *valS*^{ts} strain sensitivity at 40°C can be explained by the fact that the bacteria had had 30 minutes of incubation at 40°C prior to antibiotic disc addition to the plates; incubation at 40°C would cause a reduction in protein synthesis of ValS^{ts} strains. Upon disc addition, the *relA*$^-$ *valS*^{ts} strain already had a deficiency in proteins that the *relA*$^+$ strain did not. A shortage of necessary proteins would inhibit the growth of any organism under any conditions, explaining why the *relA*$^+$ strain was more resistant than the *relA*$^-$ *valS*^{ts} strain at 40°C. However, the latter strain was able to synthesize new phospholipids at 40°C and so was not as sensitive as the *relA*$^+$ *valS*^{ts} strain even though it had decreased protein synthesis.

We never saw examples where only the *relA*$^+$ *valS*^{ts} strain was more resistant to an antibiotic at 36°C. When this strain was more resistant, the *relA*$^-$ *valS*^{ts} strain was equally more tolerant suggesting that it was lack of protein synthesis and not ppGpp production that was responsible for this observation. Perhaps if ppGpp synthesis had occurred for 90 minutes before antibiotic addition, as was found to be necessary by Kusser *et al.* (10) we might have seen the *relA*$^+$ *valS*^{ts} strain having more tolerance to the antibiotics at 36°C than the *relA*$^-$ *valS*^{ts} strain.

Exposure to ultraviolet (UV) radiation has been known to cause two predominant lesions, namely the cyclobutane pyrimidine dimer (CPD) and the 6,4- pyrimidine photoproduct (6-4PP). These lesions are typically repaired by the nucleotide excision repair (NER) mechanism, which is best understood in *E. coli*. (3). Previous studies have shown

that illuminating growing *E. coli* cells with near-UV light induces ppGpp synthesis. At wavelengths shorter than 350 nm, it is speculated that a residue (5-methylaminomethyl-2-thiouracil) in the first position of the anticodon loop of tRNA Glu, tRNA Lys and one tRNA Gln isoacceptor is highly photoreactive and is modified due to UV light. The change alters these tRNAs such that they become poor substrates for their acylation enzyme (17). This process increases the concentration of deacylated tRNA molecules, which when bound to the ribosome recruit RelA and activate ppGpp production (14, 17, 19). Furthermore, Gong *et al.* (7) suggested that appropriate ppGpp levels activated RpoS (σ^S) expression, which in turn caused upregulation of RpoS-regulated stress-defending genes, like *dps* that produces Dps, a protein protecting DNA from oxidative damage (16).

The results obtained for treatment of the three strains of *E. coli* with UV radiation for 30 seconds showed that the *relA*$^+$ strain demonstrated the greatest resistance against UV-induced cell death (Fig. 4). This trend was not one that was hypothesized since we expected more ppGpp production in the *relA*$^+$ *valS*^{ts} strain than in the *relA*$^+$, and none in the *relA*$^-$ *valS*^{ts} strain at 36°C. Thus, we anticipated more or at least similar resistance in the *relA*$^+$ *valS*^{ts} strain compared to the *relA*$^+$ strain and clearly more resistance in the *relA*$^+$ *valS*^{ts} strain compared to the *relA*$^-$ *valS*^{ts} strain. The latter conjecture held true and can be attributed to the lack of ppGpp in the *relA*$^-$ *valS*^{ts} strain, which explains the 2-fold increase in percent viability in the *relA*$^+$ *valS*^{ts} strain compared to the *relA*$^-$ *valS*^{ts} strain. However, the former anticipation was contradictory to our results. This effect can be explained by the fact that ppGpp production is also increased in the *relA*$^+$ strain when exposed to UV radiation (typically below 350 nm), which stimulates amino acid starvation (17). Added to this phenomenon, the valS enzyme in the *relA*$^+$ strain is fully functional compared to the *relA*$^+$ *valS*^{ts} and the *relA*$^-$ *valS*^{ts} strains which contain a partially active form of the enzyme at 36°C. Therefore, the latter two strains are handicapped in producing sufficient amounts of protein to combat UV-related damage which explains the 30-fold and 15-fold increase in percent viability in the *relA*$^+$ strain compared to the *relA*$^+$ *valS*^{ts} and the *relA*$^-$ *valS*^{ts} strains, respectively.

FUTURE EXPERIMENTS

To test the hypothesis of membrane instability in the *relA*$^+$ *valS*^{ts} strain at 40°C we would look at phospholipids content and lysis of the three strains at the three different temperatures. We would expect the *relA*$^+$ *valS*^{ts} strain to have more of the saturated, longer chained fatty acids than the other two strains (13).

Examination of cell lysis would be done by growing the strains in liquid medium at the three different temperatures in the presence of the different antibiotics. We would expect the *relA*$^+$ *valS*^{ts} strain to lyse at 40°C and not the other two strains. It would also be beneficial to look at phospholipid content under these conditions.

To confirm that insufficient ppGpp levels after 30 min activation of RelA were responsible for the lack of resistance to the antibiotics at 36°C we would simply grow the cells for longer periods of time at the appropriate temperatures before the addition of the antibiotics to the plates.

Future researchers that investigate the effect of ppGpp on cellular function should express the *relA* gene on a plasmid in *relA*$^-$ cells, instead of attempting to induce *relA* by amino acid deprivation. Plasmid expression of *relA* at levels equivalent to that of the stringent response would allow faster and more controlled induction of *relA*, and would not require experimentation at different temperatures. Also, the varying effects of amino acid deprivation and that of *relA* would not be confused.

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