

## The Effect of a $\beta$ -lactamase Inhibitor on Ampicillin Resistance Induced by Activation of the Stringent Response in *Escherichia coli*

ELAINE MA, ADRIENNE PELTONEN, GENNY STANCHEVA, and  
LUBOMIR VLCEK

*Department of Microbiology and Immunology, UBC*

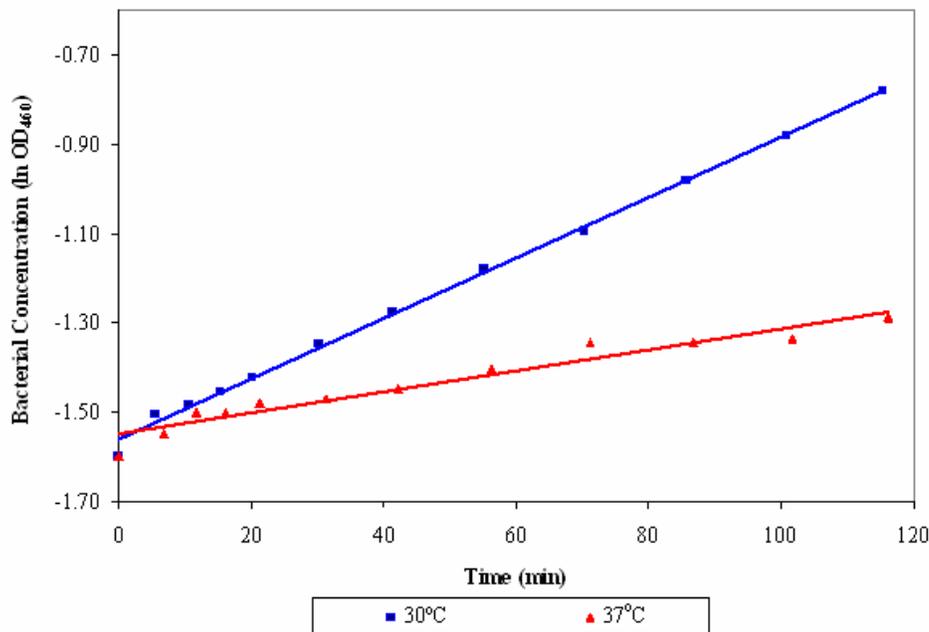
**Bacteria can enter a protective state known as the stringent response when faced with unfavourable or stressful environmental conditions. This state is characteristically associated with decreased rates of protein synthesis and the up-regulation of amino acid biosynthesis pathways but it affects a wide range of other physiological processes and responses in the cell. Namely, previous studies have shown that cells undergoing stringent response become partially resistant to  $\beta$ -lactam antibiotics, but the mechanism of this resistance has not been characterized. This study aimed to investigate whether the observed antibiotic resistance may in fact involve bacterial  $\beta$ -lactamase production, the most widespread mechanism contributing to  $\beta$ -lactam resistance. We report that one  $\beta$ -lactamase inhibitor, clavulanic acid, was not helpful in reversing ampicillin resistance under stringent response. However, since clavulanic acid is not active against all types of bacterial  $\beta$ -lactamases, we cannot conclude with certainty whether or not these enzymes play a role in stringent response-induced ampicillin resistance.**

Activation of the stringent response in bacteria plays an important role in survival and protection from adverse environmental conditions such as declining nutrient concentrations. The best understood trigger of this adaptive response is amino acid starvation, consistent with the observation that cells depend heavily on protein synthesis for growth. When an amino acid becomes depleted, its cognate uncharged tRNA accumulates, binds to the ribosomal A site and activates the ribosome-associated enzyme RelA to synthesize guanosine tetraphosphate ((p)ppGpp), the key nucleotide alarmone responsible for the pleiotropic cellular changes observed in cells undergoing stringent response (14). (p)ppGpp alters cellular gene expression by binding RNA polymerase and causing decreased transcription of genes involved in protein synthesis, such as the rRNA genes, and increased transcription of amino acid biosynthesis genes (2). These changes allow the bacteria to survive for the duration of the nutrient deficiency, albeit with a slower growth rate.

Although protein synthesis and amino acid synthesis genes are the best known targets of (p)ppGpp, this alarmone actually affects many other cellular functions and processes. It has been demonstrated that (p)ppGpp also inhibits peptidoglycan (PG) cell wall synthesis (5) and confers some resistance to  $\beta$ -lactam antibiotics (1,11). The observed  $\beta$ -lactam resistance has previously been attributed to the inhibition of PG synthesis by ppGpp, since penicillins can only lyse cells actively synthesizing PG. However, other studies have shown a link between activation of the stringent response and induction of  $\beta$ -lactamase genes responsible for rendering these antibiotics ineffective

by hydrolyzing their  $\beta$ -lactam rings (4, 8, 9). Furthermore, some types of  $\beta$ -lactamases have been found to play additional important roles in bacterial cells and may not be restricted to  $\beta$ -lactam resistant strains. For example, Henderson *et al.* (4) showed that  $\beta$ -lactam sensitive strains can express members of the  $\beta$ -lactamase family that are closely related to penicillin-binding proteins (PBPs), and like PBPs, are crucial for normal cell wall metabolism, independent of their role in antibiotic resistance. Therefore, the presence of  $\beta$ -lactamase genes in  $\beta$ -lactam sensitive bacterial strains cannot be automatically ruled out.

This study tested whether the partial ampicillin resistance previously observed (1) may have involved a stringent response-induced expression of  $\beta$ -lactamase or whether this was a  $\beta$ -lactamase-independent process indirectly caused by the inhibition of cell wall synthesis during stringent response. To test these alternate explanations, we used *E. coli* strain NF536As19 induced into stringent response at temperatures above 30°C due to inactivation of the valine amino acyl tRNA synthetase resulting in starvation for valine (1), and looked at how clavulanic acid, a  $\beta$ -lactamase inhibitor, affected the observed ampicillin resistance by monitoring culture turbidities. Clavulanic acid is a compound which interacts with some classes of bacterial  $\beta$ -lactamase enzymes, progressively inactivating them and restoring sensitivity to  $\beta$ -lactam antibiotics (6). This study demonstrated that clavulanic acid was not effective at reversing the observed ampicillin resistance under stringent response, indicating that the resistance mechanism did not involve expression of  $\beta$ -lactamase enzymes responsive to this inhibitor.



**FIG. 1** Growth characteristics of log-phase *Escherichia coli* NF536As19 grown in M9 minimal media with shaking at 30°C and 37°C. Overnight culture was diluted to a starting OD of 0.15, incubated at 30°C for one hour, and then split into two samples, one for each temperature, at time  $t=0$ .

## MATERIALS AND METHODS

**Bacterial Strains.** *E. coli* NF536As19 (*leu relA*<sup>+</sup> *valS*<sup>ts</sup>), DH5 $\alpha$  (wild-type control), and DH5 $\alpha$  containing plasmid pBR322 were obtained from the MICB 421 culture collection in the Department of Microbiology and Immunology, University of British Columbia. The wild-type DH5 $\alpha$  was used as the ampicillin sensitive strain, while the DH5 $\alpha$  containing plasmid pBR322 was used as the ampicillin resistant strain.

**Media.** All strains were grown in M9 minimal media (6 g/liter Na<sub>2</sub>HPO<sub>4</sub>, 3 g/liter KH<sub>2</sub>PO<sub>4</sub>, 0.5 g/liter NaCl, 1 g/liter NH<sub>4</sub>Cl, 1 mM MgSO<sub>4</sub>, pH 7.1), supplemented with 0.4% (w/v) glucose, 1  $\mu$ g/ml thiamine, and 50  $\mu$ g/ml leucine to fulfill auxotrophic growth conditions (1, 10).

**Growth of *E. coli* NF536As19.** An overnight culture of NF536As19 was prepared and grown at 30°C in a shaking water bath with mild aeration at 75 rpm. The following day, the overnight culture was used to inoculate 250 ml of fresh media to a starting OD<sub>460</sub> of 0.15, and was incubated for an hour, at 30°C and 100 rpm, to let the cells grow out of lag phase. The culture was then split (time,  $t=0$ ) into separate flasks, with 50 ml going to each of the 30°C and 37°C water baths (done in duplicate) at 100 rpm. Culture turbidity was measured with a Spectronic 20D spectrophotometer at 460nm.

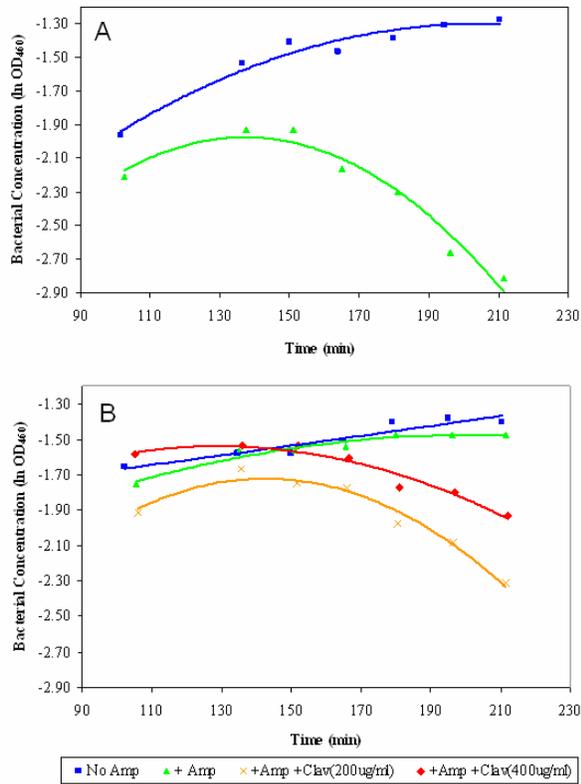
**Effectiveness of ampicillin and clavulanic acid.** Overnight cultures of DH5 $\alpha$  and DH5 $\alpha$  containing plasmid pBR322 were prepared and grown at 37°C in a shaking water bath with mild aeration at 75 rpm. The following day, the overnight cultures were used to inoculate 40 ml (for DH5 $\alpha$ ) and 80 ml (for DH5 $\alpha$  containing plasmid pBR322) of fresh media to a starting OD<sub>460</sub> of 0.15, and were incubated for an hour, at 30°C and 100 rpm, to let the cells grow out of lag phase. The cultures were then split (time,  $t=0$ ) into separate flasks: 10 ml of the DH5 $\alpha$  culture into each of two separate flasks, and 10 ml of the DH5 $\alpha$  containing plasmid pBR322 culture into each of four separate flasks. Immediately, ampicillin (Sigma A9518, 100 mg/ml stock) was added to one flask with DH5 $\alpha$  and three of the flasks with DH5 $\alpha$  containing plasmid pBR322, to obtain a final

concentration of 100  $\mu$ g/ml. At the same time potassium clavulanate (Sigma P3494-100MG, 10 mg/ml stock), a  $\beta$ -lactamase inhibitor, was added to two DH5 $\alpha$  containing plasmid pBR322 flasks with ampicillin, to obtain a final concentration of 200 and 400  $\mu$ g/ml, respectively. All six cultures were incubated in a 37°C water bath at 125 rpm, and monitored for ampicillin induced lysis via OD<sub>460</sub> readings.

**Stringent response and  $\beta$ -lactamase.** An overnight culture of NF536As19 was prepared and grown in a test tube at 30°C in a 40 rpm tube roller. The following day, the overnight culture was used to inoculate a flask of 65 ml of fresh media to a starting OD<sub>460</sub> of 0.2 (time,  $t=0$ ), and was incubated at 30°C and 200 rpm in a shaking water bath, until sufficient growth was observed via OD<sub>460</sub> measurements. The culture was then split, with 10 ml going into each of six separate flasks. Three of the flasks were incubated at 30°C while the other three were incubated at 37°C. After 20 minutes, ampicillin (Sigma A9518, 100 mg/ml stock) was added to two flasks at each temperature to obtain a final concentration of 100  $\mu$ g/ml. At the same time, potassium clavulanate (Sigma P3494-100MG, 10 mg/ml stock) was added to one of the flasks with ampicillin at each temperature, to obtain a final concentration of 400  $\mu$ g/ml. Ampicillin induced lysis and tolerance was monitored via OD<sub>460</sub> readings.

## RESULTS

**Bacterial growth curves.** To evaluate the behavior of strain NF536As19, growth was observed at 30°C and 37°C. Figure 1 shows that the bacteria grew significantly faster at the lower temperature, where both the observed growth rate and final concentration observed at 30°C were approximately 1.6x higher than at 37°C. The doubling time at 30°C was around 115



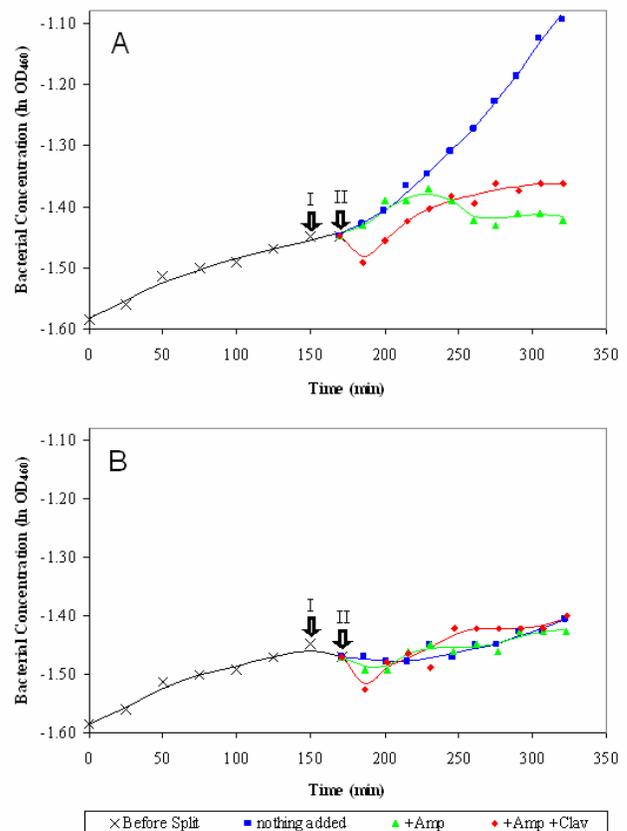
**FIG. 2** The effect of ampicillin and clavulanic acid on the growth of ampicillin-sensitive *Escherichia coli* DH5 $\alpha$  (A) and ampicillin-resistant *Escherichia coli* DH5 $\alpha$  containing plasmid pBR322 (B), grown in M9 minimal media with shaking at 37°C. Overnight culture was diluted to a starting OD of 0.15 at time t=0, and stock ampicillin was added to a final concentration of 100  $\mu$ g/ml. Due to technical error, OD readings before 100 min were not reliable and thus not plotted. Amp - ampicillin, Clav - clavulanic acid.

minutes, whereas at 37°C the cells grew much slower, and did not double during the assay, indicating a double time of over 120 min, which is more than 3x longer than the doubling time at 30°C, indicating stringent response was successfully induced. Furthermore, comparing the growth of NF536As19 only (no additions) at 30°C and 37°C (Fig. 3A and 3B), growth at 37°C continued at a slow, steady pace, whereas at 30°C, growth was much faster. In fact, after ~200 min, the growth at 30°C was almost 4x higher than that at 37°C, again confirming the potential induction of stringent response due to deprivation of charged valinyl-tRNA. Further support for this was provided since the final bacterial concentrations obtained the following day at 30°C was almost 1.5x higher than that of the 37°C culture (Fig. 4).

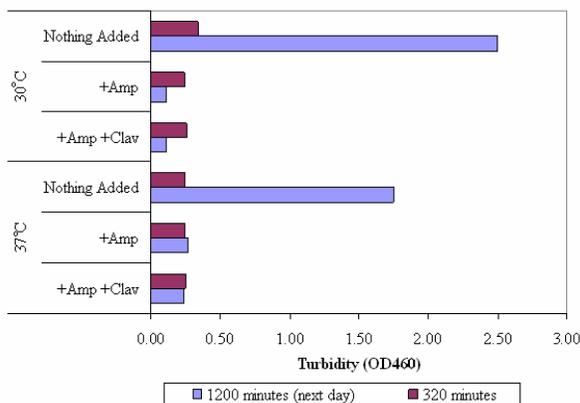
In determining the ampicillin-induced lysis of ampicillin sensitive DH5 $\alpha$ , the lag phase appeared to last ~100 min, before which a definitive trend was not observable (Fig. 2A). The same applied for the

experiment with the ampicillin resistant DH5 $\alpha$  containing plasmid pBR322 (Fig. 2B). With the NF536As19 experiment, the lag time appeared to be ~50 min (Fig. 3A and 3B).

Interestingly, the doubling time of NF536As19 initially observed at 30°C (Fig. 1) was ~7.5x that of the initial NF536As19 growth between 0 min and 150 min when nothing was added at 30°C (Fig. 3A and 3B), indicating growth was significantly slower in the latter case. This was unexpected because theoretically both conditions are the same, and should show similar growth rates and curves. However, at about 230 min, the growth rate of NF536As19 at 30°C increased quickly but was still slower than the original NF536As19 curve, with a doubling time only ~2x that of NF536As19 in figure 1. Overall, growth of NF536As19 observed was significantly slower in the latter experiment.



**FIG. 3** The effect of clavulanic acid on the partial ampicillin resistance of *Escherichia coli* NF536As19, under stringent response, grown in M9 minimal media with shaking at 30°C (A) or 37°C (B). From 0 to 150 minutes all samples grew in the same flask at 30°C. Cultures were split at (I) 150 minutes, and stock ampicillin and clavulanic acid were added to a final concentration of 100  $\mu$ g/ml and 400  $\mu$ g/ml respectively at (II) 170 minutes. Amp - ampicillin, Clav - clavulanic acid.



**FIG. 4** Comparison of bacterial concentrations between samples of *Escherichia coli* NF536As19 under various conditions at 320 minutes (see figure 3), and the following day at 1200 minutes. Amp - ampicillin, Clav - clavulanic acid.

**Ampicillin-induced lysis.** In order to evaluate the effectiveness of ampicillin, strains DH5 $\alpha$  (ampicillin sensitive) and DH5 $\alpha$  containing plasmid pBR322 (ampicillin resistant) were grown in the presence of ampicillin (Fig. 2A and 2B, respectively). The growth curves of both strains grew the fastest and to the highest concentrations in the absence of ampicillin. In figure 2A, the ampicillin-sensitive bacteria grew with or without ampicillin present, but the no ampicillin curve started to level off, whereas the ampicillin curve dropped significantly almost at a constant rate starting around 140 min, with a death rate almost 2x faster than the growth rate of the bacteria in no ampicillin. Also, the final bacterial concentration in the ampicillin-treated culture was around 4.5x lower than in the culture lacking ampicillin. As expected for the DH5 $\alpha$  containing plasmid pBR322 strain, ampicillin had minimal effects on the growth curves, as shown by the strikingly similar growth curves of no ampicillin and ampicillin (Fig. 2B). Also, these two samples have both starting and final concentrations that are in the same range.

**Clavulanic acid-enhanced lysis.** Ampicillin-resistant DH5 $\alpha$  containing plasmid pBR322 was grown in the presence of ampicillin and clavulanic acid, to explore the effect of clavulanic acid on the ampicillin resistance. The two samples with ampicillin and varying concentrations of clavulanic acid showed initial culture growth which leveled off and with both curves showing the start of cell lysis occurring at approximately the same time around 140 min (Fig. 2B). Due to technical problems with the spectrophotometer, growth before 110 min was not accurate, and the most reliable trends were observed after 135 min. After 135 min, both curves appeared similar and began to drop at continually increasing death rates. In fact, the two

curves appeared to have quite similar death rates, but they could not be quantitatively compared since the death rates were not constant. However, the lower clavulanic acid concentration appeared to show a slightly higher death rate than the higher concentration, but both concentrations were effective.

Compared to the rise in both the untreated control curve and the ampicillin-treated curves, the drop in the clavulanic acid-treated curves were strikingly different as they drop, whereas the no ampicillin and ampicillin curves rise (Fig. 2B). In addition, the lower clavulanic acid concentration culture had a starting concentration lower than that of the no ampicillin and ampicillin cultures, whereas that of the higher clavulanic acid concentration culture was higher than the starting concentrations of the no ampicillin and ampicillin cultures. Importantly, as expected, the addition of ampicillin and clavulanic acid together killed ampicillin-resistant bacteria, whereas the addition of ampicillin alone did not.

**Stringent response-induced ampicillin resistance.**

In order to evaluate the effect of stringent response on the ampicillin sensitivity of NF536As19, this strain was grown in ampicillin at 30°C and 37°C (Fig. 3). After ampicillin addition, the growth curves at both temperatures were strikingly similar as they both continued at the original slow growth rate. The curves show seemingly large fluctuations between the data points, but this is consistent in the curves with ampicillin and ampicillin and clavulanic acid at both temperatures. However, it appeared as though the ampicillin curve at 30°C had a short spurt of growth between 200 min and 250 min, which leveled off afterwards.

Noteworthy was that there was a 4-fold difference between the growth of the untreated control culture and the ampicillin culture at 30°C, showing the bactericidal effect of ampicillin on NF536As19 in the absence of stringent response. This was further supported by the ~23-fold difference in the bacterial concentrations of these two cultures at 30°C at 1200 min (Fig. 4). However, at 37°C, the growth rates of the untreated control culture and the ampicillin culture were remarkably similar, indicating ampicillin had minimal effect in cells undergoing stringent response. Interestingly, at 1200 min, the final bacterial concentration of the untreated control culture was ~6.5x higher than the ampicillin culture, indicating growth was significantly faster in the untreated control culture at 37°C. Overall, there was a notable difference between the ampicillin cultures at 30°C and 37°C, where the final bacterial concentration at 37°C was almost 2.5x higher than that at 30°C, indicating more cells survived ampicillin treatment under stringent response.

**Effect of clavulanic acid on stringent response-induced ampicillin resistance.** To evaluate the effect of clavulanic acid on the ampicillin killing, NF536As19 was also grown in ampicillin and clavulanic acid at 30°C and 37°C (Fig 3). After the splitting of cultures and the addition of the drugs, the clavulanic acid-containing cultures at both temperatures showed temporary, yet significant drops in turbidity. At 37°C, slight dipping was more consistent as it occurred for all three samples, however, the dips in the clavulanic acid cultures were by far the most prominent. In addition to the similar dipping, the general clavulanic acid curves at both temperatures were remarkably similar to each other as they both started to grow at almost identical rates, and then somewhat leveled off. Furthermore, at both temperatures, the clavulanic acid curves were quite similar to the corresponding ampicillin only curves. However, after the dips in the clavulanic acid curves, at both temperatures, the clavulanic acid cultures grew quickly and surpassed the ampicillin curves. Since the same results were obtained at both temperatures, this indicates that at 37°C with stringent response, the clavulanic acid had minimal effect on the effectiveness of ampicillin. The results at 1200 min (Fig. 4) provide further support as the final bacterial concentrations of the clavulanic acid cultures at both temperatures are almost identical to those of the ampicillin cultures at the respective temperature. Similar to the difference in the final bacterial concentrations of the ampicillin cultures at 30°C and 37°C, the final bacterial concentration of the clavulanic acid cultures at 37°C was slightly over 2x higher than that at 30°C, again indicating that the clavulanic acid did not alter the bacteria's responses to ampicillin.

## DISCUSSION

Given how increasingly widespread  $\beta$ -lactamase enzymes are in the microbial world, and the observation that their expression can be induced during stringent response (4,8,9), the intent of this experiment was to test if the observed resistance to ampicillin shown by NF536As19 under stringent response (1) could have been partially due to the presence of an induced  $\beta$ -lactamase, or if the resistance was due indirectly to the inhibitory effects of stringent response on cell wall synthesis.

Growth experiments performed at 30°C and 37°C showed significantly slower growth of the NF536As19 culture grown at 37°C relative to the culture grown at 30°C (Fig. 1). NF536As19 has a temperature-sensitive biosynthetic amino acid gene, *valS* and at 37°C, the gene becomes inactivated, resulting in a depletion of that amino acid and a subsequent induction of stringent response through RelA synthesis of (p)ppGpp (1). The actions of (p)ppGpp results in changed gene expression

which allows the bacteria to continue to grow during the amino acid shortage, however, under these conditions the growth rate is slower (2). Therefore, our results verify the temperature sensitivity of the strain, and suggest that our experimental conditions should have successfully induced stringent response.

The ampicillin-sensitive strain of DH5 $\alpha$  lysed upon treatment with ampicillin, showing that our ampicillin stock was functional (Fig. 2A) and thus any bacteria capable of growth in that ampicillin must have a resistance mechanism. As expected for an ampicillin resistant strain, DH5 $\alpha$  containing plasmid pBR322, was able to continue to grow in the presence of ampicillin at a rate similar to its growth rate without ampicillin (Fig. 2B). This observation confirmed that DH5 $\alpha$  containing plasmid pBR322 produced a  $\beta$ -lactamase capable of hydrolyzing the  $\beta$ -lactam ring of ampicillin, allowing it to survive and grow in the presence of ampicillin.

When grown in the presence of both ampicillin and the  $\beta$ -lactamase inhibitor, clavulanic acid, the previously resistant DH5 $\alpha$  containing plasmid pBR322 became sensitive to ampicillin and rapidly died off (Fig. 2B). This confirms our expectation that addition of clavulanic acid would progressively inactivate the  $\beta$ -lactamases produced by the resistant strain, preventing the degradation of the ampicillin and resulting in ampicillin-induced cell lysis (6). The two concentrations of clavulanic acid showed similar trends, however the observation that the lower concentration of clavulanic acid resulted in slightly faster lysis was unexpected. Clinically, clavulanic acid has always been prescribed at a dose of 125 mg and there is little research on the effects on efficacy that different concentrations of clavulanic acid may have (7). Higher levels of the  $\beta$ -lactam antibiotic amoxicillin have been shown to inhibit the absorption of clavulanic acid (7), however, since the ampicillin concentration was kept constant in both cultures, this was probably not a factor in our experiment. Finally, it should be mentioned that given the two cultures treated with clavulanic acid had different bacterial concentrations at the time of the addition, it is difficult to estimate the ratio of clavulanic acid to cell each culture actually had. However, despite the slight difference in rates, sensitivity to ampicillin was restored at concentrations of both 200  $\mu$ g/ml and 400  $\mu$ g/ml, showing that clavulanic acid is effective at inhibiting  $\beta$ -lactamase-induced ampicillin resistance in DH5 $\alpha$  containing plasmid pBR322.

Having established the effectiveness of our ampicillin and  $\beta$ -lactamase inhibitor, experiments were then performed to test if a  $\beta$ -lactamase could be partially responsible for the ampicillin resistance exhibited by NF536As19 during stringent response (1). Cultures were grown at 30°C and then subjected to two different temperatures and various treatments (Fig. 3A

and Fig. 3B). The apparent drop in OD by all three cultures immediately after the split at 37°C is likely an effect of the transfer of the cultures to new flasks (Fig. 4B). Immediately after the splitting of the cultures and addition of treatments, the two cultures treated with both ampicillin and clavulanic acid appeared to die off rapidly (Fig. 3A and Fig. 3B). That the exact same trend occurred in both clavulanic acid containing cultures makes it less likely that it was due to an error in the splitting of the cultures. Two other reasons could perhaps account for the drop in OD after the addition of ampicillin and clavulanic acid. Firstly, the cultures were diluted by the addition of ampicillin and clavulanic acid, which would cause a drop in the measured OD. However, this decrease would be very slight due to the relatively small volume added to the cultures. Secondly, it is possible that clavulanic acid interacts with the bacterial cell surface and could change how the cell surface refracts light, which would affect the amount of light transmitted. It is unclear if this drop also occurred for the experiment involving DH5 $\alpha$  containing plasmid pBR322 (Fig. 2B) since spectrophotometer problems meant our results immediately after the addition of treatments at time = 0 min were unreliable. From time 200 to 300 minutes, the 37°C culture treated with ampicillin and clavulanic acid appeared to grow faster than the culture with just ampicillin or no treatment (Fig. 3B). This increased growth was unexpected given our expectations that the inhibitor would either have no effect if no  $\beta$ -lactamase were present, or would increase ampicillin induced death if it were present. One possible explanation is that in the absence of  $\beta$ -lactamase, clavulanic acid may have other unknown interactions with other cell components which could perhaps alter growth. Furthermore, previous studies have also shown that clavulanic acid appears to cause cells to become irregular shaped with greater volume (7), a change which, if gradual, may give the appearance of increasing cell concentration when measuring turbidity with a spectrophotometer. Therefore, it is possible that our turbidity readings may not accurately represent cell concentrations.

The apparent fluctuations in concentration seen throughout the experiment are likely a result of the normal fluctuations of spectrophotometer readings being magnified due to the fact that our data covers a very small range of ODs on account of the slow growth of the bacteria. This slow growth rate of NF536As19 seen in figures 3A and 3B, compared to its growth rate in figure 1, is because the cultures were probably still in lag phase in figure 3, whereas in figure 1 the cultures were already in log phase. The reason for this is because the cultures in figure 1 were able to grow longer before OD readings were taken, than those in figure 3.

Despite the fluctuations and unexpected observations after the addition of treatments, the long term trends are quite clear. At 30°C the untreated control culture continued to grow while the cultures treated with ampicillin, and ampicillin plus clavulanic acid grew slower (Fig. 3A) and eventually lysed overnight (Fig. 4), indicating that NF536As19 is sensitive to ampicillin at 30°C. Both the ampicillin treated culture and the ampicillin with clavulanic acid cultures showed similar growth trends and ended up at similar concentrations after 1200 minutes (Fig. 4), which suggests that clavulanic acid had no effect on ampicillin-induced lysis. This indicates that no  $\beta$ -lactamase was present at this temperature, however this data alone is not sufficient to rule out the possibility of a  $\beta$ -lactamase not inactivated by clavulanic acid (12), or the possibility of one being induced during stringent response (4,8,9).

In contrast to the trends observed at 30°C, at 37°C the control culture and the two ampicillin containing cultures showed similar growth trends to each other (Fig. 3B), suggesting ampicillin resistance. The spectrophotometer readings of the cultures taken the next day showed this even more dramatically as the ampicillin treated cultures, both with and without clavulanic acid, at 30°C lysed, while the corresponding ampicillin-treated cultures at 37°C did not lyse, showing that stringent response provides long-term resistance to ampicillin-induced lysis (Fig. 4). This result confirms previous observations that NF536As19, when induced at 37°C to enter stringent response, exhibits partial resistance to ampicillin (1).

From these results, it does not appear that the inhibitor affected the observed partial resistance during stringent response. At 37°C, the culture treated with ampicillin and clavulanic acid grew similarly to the ampicillin only culture (Fig. 3B), and by 1200 minutes was at a concentration that was only slightly less than the ampicillin only culture (Fig. 4). The difference between the two cultures was insignificant. Thus, these results appear to show that the presence of the inhibitor did not lessen the resistance of NF536As19 to ampicillin. However, there are some limitations to the conclusions that can come from this observation. Firstly, there are different types of  $\beta$ -lactamases, and clavulanic acid functions only against certain variants, and thus it is possible that NF536As19 produces a  $\beta$ -lactamase which is resistant to clavulanic acid (12). Secondly, as shown previously, it is possible that ampicillin resistance can be explained as an indirect result of the effects of stringent response on cell wall synthesis (11). Finally, our results do not eliminate the possibility of the stringent response ampicillin resistance observed in other strains of bacteria being partly due to the presence of a  $\beta$ -lactamase.

Given that stringent response has been implicated in the resistance of such clinically relevant bacteria as *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* (3,13), it is beneficial to learn if bacterially encoded resistance mechanisms, such as  $\beta$ -lactamases, can sometimes contribute to stringent response-induced resistance to antibiotics. The findings that  $\beta$ -lactamase, an enzyme which can provide antibiotic resistance, is induced during stringent response (4,8,9) makes this study even more important since knowing whether or not enzymatic resistance plays a role could have implications for the most appropriate response in dealing with bacteria which exhibit antibiotic resistance when in stringent response (3,13).

In conclusion, the results of our experiment rule out the hypothesis that NF536As19 ampicillin resistance during stringent response involves classes of  $\beta$ -lactamases inactivated by clavulanic acid. This suggests that the resistance may be due to the inhibition of cell wall synthesis which occurs during stringent response, or possibly to the induction of  $\beta$ -lactamase inhibitors not inactivated by clavulanic acid.

#### FUTURE EXPERIMENTS

The purpose of this experiment was to investigate the mechanism of the ampicillin resistance observed in *E. coli* NF536 under stringent response, and more specifically, to determine whether it involved direct ampicillin degradation by  $\beta$ -lactamase enzymes. We were able to rule out the presence of some types of  $\beta$ -lactamases by seeing no response to clavulanic acid, but perhaps a more thorough way of testing for the presence of a wider variety of  $\beta$ -lactamase gene classes would be through the use of molecular techniques such as Northern blots, or through PCR and gene sequence analysis using  $\beta$ -lactamase-specific primers. These methods are more sensitive and direct than turbidity measurements. Alternatively, different types of  $\beta$ -lactamase inhibitors, such as tazobactam (12), which are effective against different classes of bacterial  $\beta$ -lactamases could also be tested in a modification of our experimental procedure.

If  $\beta$ -lactamase genes are identified in this strain by any of the above methods, their regulation and expression should be studied, for example by using DNA protection assays to identify the transcriptional regulators that turn them on during stringent response. It would be interesting to determine whether the alarmone (p)ppGpp or other stringent response-related proteins are involved.

#### REFERENCES

1. **Bozorg, S., J. Gooderham, and T. Halim.** 2005. Activation of a partial stringent response protects amino acid-deprived *Escherichia coli* from ampicillin-induced lysis. *J.Exp.Microbiol.Immunol.* **7**:36-42.
2. **Chatterji, D., N. Fujita, and A. Ishihama.** 1998. The mediator for stringent control, ppGpp, binds to the  $\beta$ -subunit of *Escherichia coli* RNA polymerase. *Genes Cells* **3**:279-287.
3. **Digby, F.W. and V. Mizrahi.** 2006. Tuberculosis chemotherapy: the influence of bacillary stress and damage response pathways on drug efficacy. *Clin. Microb. Rev.* **19**:558-570.
4. **Henderson, T.A., K. D. Young, S. A. Denome, and P. K. Elf.** 1997. AmpC and AmpH, proteins related to the class C beta-lactamases, bind penicillin and contribute to the normal morphology of *Escherichia coli*. *J. Bacteriol.* **179**:6112-21.
5. **Ishiguro, E.E. and W.D. Ramey.** 1976. Stringent control of peptidoglycan biosynthesis in *Escherichia coli* K-12. *J. Bacteriol.* **127**:1119-1126.
6. **Ishii, Y., M. Galleni, L. Ma, J.M. Frere, K. Yamaguchi.** 2007. Biochemical characterization of the CTX-M-14  $\beta$ -lactamase. *Int J Antimicrob Agents.* **29**:159-64.
7. **Navarro, A.M.** New formulations of amoxicillin/clavulanic acid: a pharmacokinetic and pharmacodynamic review. *Clin. Pharmacokinet.* 2005. **44**:1097-1115
8. **O'Connor, K., and D. Zusman.** 1999. Induction of  $\beta$ -lactamase influences the course of development in *Mycococcus xanthus*. *J. Bacteriol.* **181**:6319-6331.
9. **Park, J. T.** 1995. Why does *Escherichia coli* recycle its cell wall peptides? *Mol. Microbiol.* **17**:421-426.
10. **Ramey, W.D., and J. Sibley.** 2006. Microbiology 421 Manual of Experimental Microbiology. Department of Microbiology and Immunology, UBC.
11. **Rodionov, D.G. and E.E. Ishiguro.** 1995. Direct correlation between overproduction of guanosine 3',5'-bispyrophosphate (ppGpp) and penicillin tolerance in *Escherichia coli*. *J Bacteriol.* **177**:4224-4229.
12. **Sulton, D., D. Pagan-Rodriguez, X. Zhou, Y. Liu, A.M. Hujer, C.R. Bethel, M.S. Helfand, J.M. Thomson, V.E. Anderson, J.D. Buynak, L.M. Ng and R.A. Bonomo.** Clavulanic Acid Inactivation of SHV-1 and the Inhibitor-resistant S130G SHV-1  $\beta$ -Lactamase. *J. Biol. Chem.* 2005. **280**:35528-35536.
13. **Viducic, D., T. Ono, K. Murakami, H. Susilowati, S. Kayama, D. Hirota, and Y. Miyake.** 2006. Functional Analysis of *spoT*, *relA* and *dkxA* Genes on Quinolone Tolerance in *Pseudomonas aeruginosa* under Nongrowing Condition. *Microbiol. Immunol.* **50**:349-357.
14. **Wendrich, T.M., G. Blaha, D.N. Wilson, M.A. Marahiel, K.H. Nierhaus.** 2002. Dissection of the mechanism for the stringent factor RelA. *Mol. Cell.* **10**:779-788.