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Effects of the stringent response on pBR322 plasmid copy number in *Escherichia coli* strains

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This experiment attempted to study the replication control of pBR322 during stringent growth in *E. coli*. If the stringent response is responsible for the control of pBR322 copy number, an amino acid-starved stringent *E. coli* strain would be expected to decrease plasmid replication, whereas relaxed mutant strains would exhibit amplification of pBR322. In this experiment, the growth rate of an *E. coli relA*$^+$ wild-type strain was reduced when the stringent response was stimulated through inhibition of valyl-tRNA synthetase. As expected, the isogenic *E. coli relA*$^-$strain exhibited similar growth kinetics to the control. However, spectrophotometric quantification, gel electrophoresis band assessment, and a minimum inhibitory concentration assay using tetracycline showed no significant difference in plasmid copy number between the two strains during stringent growth. Both displayed a decrease in pBR322 copy number when deprived for the amino acid valine, leading us to conclude that other mechanisms besides the stringent response are involved in regulating plasmid copy number.

Plasmid pBR322 is one of the most widely used replicating vectors in DNA recombinant technology. A ColE1-type plasmid, regulation of pBR322 replication initiation is mediated by two plasmid transcripts (RNA II and RNAI) and one protein (Rom; 1). The RNA II transcript is a pre-primer that folds into a secondary structure and hybridizes near the plasmid origin of replication (1). Cleavage of the hybrid by RNaseH generates the mature primer, initiating replication by DNA polymerase I. RNA I, an anti-sense RNA, is a negative regulator of pBR322 replication. It binds complementary to RNA II, inhibiting hybrid formation between the RNA II pre-primer and its DNA template, and thereby preventing plasmid replication initiation (1). The Rom protein is a second negative regulatory element which stabilizes RNA I-RNA II binding (1).

Various areas of plasmid DNA research involve preparations of substantial quantity of plasmid DNA. An approach commonly used to amplify plasmid DNA yields is the use of chloramphenicol, a translation inhibitor, at high inhibitory concentrations (3). Recent studies using sub-inhibitory levels of chloramphenicol have also attained increased plasmid yields (3,5). Unlike the disruptive effects of chloramphenicol on chromosomal replication, plasmids containing relaxed origin of replication do not depend on cellular protein synthesis and thus are not affected, allowing continued plasmid replication even when genomic DNA replication halts (4,5). The disruptive effects of chloramphenicol are not limited to the translation process. Chloramphenicol also binds to the ribosomal A-site, preventing entry of tRNAs and inhibiting activation of RelA. As a result, formation of ppGpp is blocked (10). This has great implications, as ppGpp is the key alarmone involved in the bacteria stringent response (2,4).

Stringency is induced in response to nutritional stresses–eg. amino acid deprivation (2). Activation of the stringent response results in physiological changes in the bacterial cell, allowing the cell to survive difficult environmental conditions. These changes include general decreases in the biosynthetic reactions in the cell, shut down of stable RNA (tRNA, rRNA) synthesis, and greater rates of degradation of unnecessary proteins to supply building blocks necessary for cell maintenance and stress defense (2,4).

In this study, we attempted to elucidate the effects of the stringent response on pBR322 plasmid copy number by using valine temperature-sensitive *E. coli* strains NF536As19 (*relA*$^+$) and its isogenic counterpart NF537As19(*relA*$^-$) harboring pBR322. We hypothesize a marked increase in pBR322 concentrations in amino-acid starved relaxed *E. coli*, similar to responses observed during chloramphenicol treatment.

MATERIALS AND METHODS

Bacterial strains. *E. coli* DH5α was used in the isolation of pBR322. Isogenic *E. coli* strains NF536As19 (*relA*$^+$, *valS*^{ts}, *leu*$^-$) and NF537As19 (*relA*$^-$, *valS*^{ts}, *leu*$^-$) were used to conduct stringency experiments. All bacterial strains courtesy of the UBC Department of Microbiology and Immunology.

Growth media. Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 10 g NaCl, 2 g glucose per liter) and Luria-Bertani (LB) agar (LB broth with the addition of 15 g agar) were used to support the growth of bacteria throughout the experiment. Ampicillin (Sigma; 100 mg/mL stock, prepared in distilled H_2O) and tetracycline (OmniPur; 15 mg/mL stock, prepared in 50% ethanol) were prepared and sterilized through a Millipore filter of pore size 0.22 μm (Cat.# GSWP02500). Antibiotics were stored at –20°C and added to media as necessary.

pBR322 plasmid isolation. Twenty milliliters of overnight culture of *E. coli* DH5α was prepared and incubated at 30°C under mild aeration (200 rpm). Two milliliters of the overnight culture was used to isolate plasmid pBR322 using the Invitrogen PureLink HQ Mini Plasmid Purification Kit (Cat.# K2100-01) following the manufacturers instructions.

Preparation of electrocompetent cells. Two-and-a-half milliliters of an overnight culture of *E. coli* NF536As19 and *E. coli* NF537As19 were inoculated into two flasks containing 250 mL LB broth. The cells were cultured at 30°C overnight at mild aeration (300 rpm). When cultures reached an optical density of 0.5O D_{600}-0.7 OD_{600}, they were chilled on ice for 20 mins, and were subsequently centrifuged using a Beckman JA-14 rotor (model J2-21) at 4000 x g for 15 mins at 4°C. The pellet was resuspended in 250 mL, 125 mL, and 10 mL of ice cold 10% glycerol respectively. Each pellet was centrifuged at 4000 x g for 15 mins at 4°C between each resuspension. Each pellet was finally resuspended in 1mL of ice cold 10% glycerol.

Electroporation of *E. coli* NF536As19 and *E. coli* NF537As19. Electroporation of *E. coli* NF536As19 and *E. coli* NF537As19 cells was performed using a BioRad MicroPulser. A mixture containing 50 µL of freshly prepared electrocompetent cells and 2 µL of purified pBR322 plasmid was mixed in a BioRad Gene Pulser Cuvette (Cat.# 165-2088). With the settings on the MicroPulser at "Ec2", the cells were subjected to a short 2.5 kV electric pulse. 1 mL of LB broth was immediately added to the electroporated cells, and the cells were allowed to recover at 30°C under mild moderate aeration (300 rpm) for 1 hour. Various volumes (10 µL, 50 µL, 100 µL, and 300 µL) of the transformants were plated onto LB agar plates containing 100 µg/mL ampicillin.

pBR322 copy number under stringent conditions. Overnight cultures of the transformed *E. coli* NF536As19 and *E. coli* NF537As19 were prepared in 20 mL of fresh LB broth supplemented with 20 µg/mL ampicillin; overnight cultures were incubated at 30°C at mild aeration (200 rpm). Seven milliliters of the overnight *E. coli* NF536As19 culture was transferred to each of two flasks containing 43 mL of fresh LB broth supplemented with 20 µg/mL ampicillin; duplicate flasks for *E. coli* NF537As19 were similarly set up. All four flasks were incubated at 30°C at mild aeration (200 rpm) until they reached an optical density between 0.5 OD_{500}-0.6 OD_{600}. Stringency was induced by transferring one flask of each strain into a 42°C water bath. A control culture for each strain was maintained at 30°C. A sample was obtained from each flask when the temperature shift was executed, and 4 hours after the temperature shift. Samples were diluted in fresh LB broth such that a 3 mL volume of the respective sample would have an optical density of 0.55 OD_{600}. The 3 ml diluted samples were subjected to plasmid purification using the Invitrogen PureLink HQ Mini Plasmid Preparation Kit (Cat.# K2100-01), and eluted into 50 µL of elution buffer. Plasmid concentrations were quantified spectrophotometrically by reading the absorbance of the sample at 260 nm using a Beckman Coulter DU530 Life Science UV/Vis Spectrophotometer. All samples were later subject to DNA agarose gel electrophoresis.

Minimum inhibitory concentration of tetracycline. One-hundred microliter samples from each of the experimental conditions–*E. coli* NF536As19 at 30°C, *E. coli* NF536As19 at 42°C, *E. coli* NF537As19 at 30°C, *E. coli* NF537As19 at 42°C–were inoculated into a series of test tubes containing 5 mL of LB media supplemented with increasing concentrations of tetracycline–0µg/mL, 10 µg/mL, 50 µg/mL, 100 µg/mL, 500 µg/mL, 1000 µg/mL, 3000 µg/mL. All tubes were incubated overnight at 30°C at mild aeration (200 rpm). Cell growth was determined spectrophotometrically by measuring the optical density at 600 nm using a Beckman Coulter DU530 Life Science UV/Vis Spectrophotometer.

EcoRI restriction digestion. To confirm the identity of our plasmid isolate, an aliquot of the putative pBR322 plasmid sample was subject to restriction digest with EcoRI. Ten microliters of plasmid DNA was combined with 2 uL 10x React 3 buffer, 7 uL H_2O, and 1 uL EcoRI (GibcoBRL, Cat.# 15202-013). The reaction was allowed to proceed at 37°C for 2 hours.

Gel electrophoresis and densitometry. Ten microliters of each purified plasmid samples, and the entire digested sample were subject to agarose gel electrophoresis. Each sample was loaded with 2 µL of 6x loading buffer (0.5 g bromophenol blue, 0.5 g xylene cyanol, 30 mL glycerol, 170 mL H_2O) onto a 0.8% tris-borate EDTA (TBE; 10.8 g tris-base, 5.5 g boric acid, 16 mL 0.5 M EDTA pH 8.0 per liter) agarose gel. To allow for estimations of plasmid quantities by densitometry, a gradient of pBR322 was established by loading known amounts of pure pBR322 (concn. 39.2 µg/mL)–20 µL (0.78 µg), 10 µL (0.39 µg), 5 µL (0.20 µg), 1 µL (0.04 µg). A 1 kb Plus DNA Ladder (Invitrogen, Cat.# 10787-018) was used to approximate the size of the DNA bands. The gel was run in 1x TBE buffer at 120 V for 2 hours. The gel was subsequently stained with ethidium bromide (0.2 µg/mL) for 20 mins, and visualized using AlphaImager software (Alpha Innotech). Cumulative band intensity was determined using the 1D-Multi Line Densitometry program provided in the AlphaImager software. Plasmid samples were assumed to contain negligible amounts of genomic DNA and other impurities, and therefore, the intensity of all visible bands was considered.

RESULTS

Isolation of pBR322 from *E. coli* DH5α. The identity of plasmid DNA isolated from *E. coli* DH5α was confirmed. A 10 µL aliquot of the extracted plasmid sample from the *E. coli* NF537As19 culture grown at 42°C and sampled at time 0 was subject to restriction digestion by EcoRI. This sample is thought to be representative of the plasmid content of all other samples as it contains the most diverse banding pattern, maximizing the possibility of other DNA moieties in the sample. The entire digested sample was subjected to electrophoresis resulting in a sole expected band of approximately 4.3 kb; the absence of any other bands suggested a successful isolation of high purity pBR322 plasmid DNA (FIG. 3, lane 14).

Growth of *E. coli* and induction of the stringent response. Exponential growth of *E. coli* NF536As19 ($relA^+$) and the isogenic strain *E. coli* NF537As19 ($relA^-$) grown under permissive conditions (30°C) was observed between 0 and 4 hours (data not shown; FIG. 1). The induction of stringent control in *E. coli* NF536As19 ($relA^+$), by means of valine deprivation via the inactivation of valyl-tRNA synthetase (ValS), resulted in a marked decrease in the growth rate of these cells; a virtual cessation of growth was observed (FIG. 1, NF536As19–42C). A similar attempt at inactivation of valyl-tRNA synthetase (ValS) of *E. coli* NF537As19 ($relA^-$), via a temperature shift to the non-permissive temperature of 42°C, did not alter the observed pattern of growth (FIG. 1, NF537As19–42C).

Quantification of isolated pBR322 plasmid DNA by spectrophotometry. The absorbance of isolated plasmid samples were measured at 260 nm. Based on the conversion factor 1 A_{260} = 50 µg/mL, the DNA concentration of each sample was calculated (FIG. 2). Both *E. coli* strains grown under permissive conditions (30°C) did not exhibit any significant changes in their plasmid DNA concentration. Both *E. coli* NF536As19

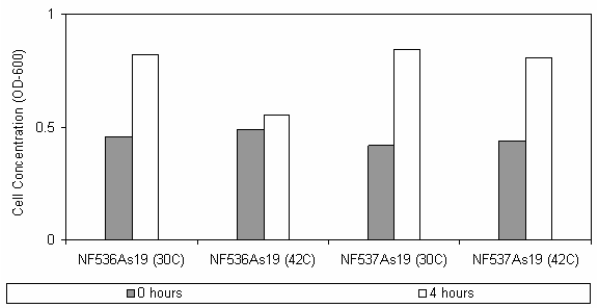

FIG. 1. Growth of *E. coli* NF536As19 and NF537As19 at 30°C or 42°C for 4 hours.

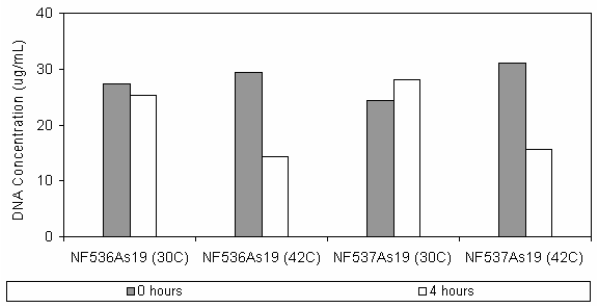

FIG. 2. Effects of growth on the yield of pBR322 plasmid isolated from cultures grown at permissive (30°C) and non-permissive (42°C) temperatures for 4 hours. All samples were extracted from experimental cultures adjusted to 0.55 OD_{600}. Plasmid concentrations were determined spectrophotometrically.

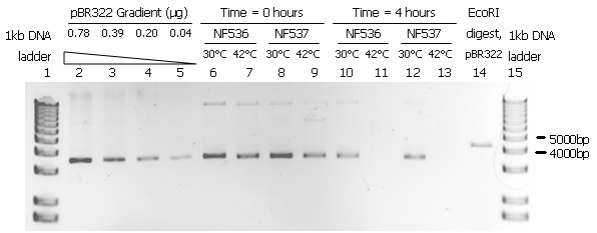

FIG. 3. Analysis of pBR322 plasmid content of *E. coli* NF536As19 (NF536) and *E. coli* NF537As19 (NF537) grown under permissive (30°C) and non-permissive (42°C) conditions using electrophoresis. Ten microliter samples of the isolated pBR322 plasmid were run on a 0.8% TBE DNA agarose gel.

(*relA*$^+$) and *E. coli* NF537As19 (*relA*$^-$) strains stabilized at an approximate concentration 26 µg/mL plasmid DNA, possibly indicating the minimum amount of pBR322 plasmids required to efficiently survive at the supplied concentration of ampicillin (20 µg/mL). A 50% reduction in plasmid DNA concentration was observed after 4 hours when strains were grown at 42°C (FIG. 2). Absorbance values at 280 nm were also obtained, and an A_{260}/A_{280} was calculated, to determine the purity of the isolated samples. All samples were confirmed to contain no significant protein contamination (data not shown).

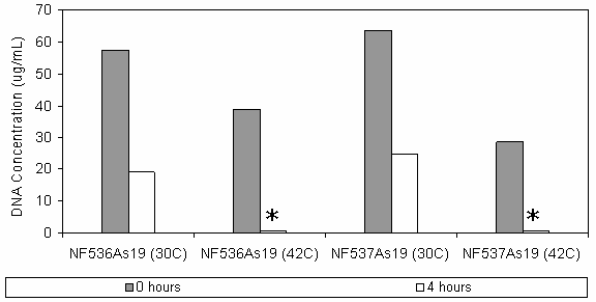

FIG. 4. Densitometric determination of pBR322 plasmid concentration of samples extracted from *E. coli* NF536As19 and *E. coli* NF537As19 grown in permissive (30°C) and non-permissive (42°C) conditions. 1D-line densitometry performed on all visible bands in a lane (see FIG. 3). *, no bands detected.

Quantification of plasmid DNA by gel electrophoresis and densitometry. Isolated plasmids from each sample were subject to agarose gel electrophoresis in conjunction with known amounts–0.78 µg, 0.39 µg, 0.20 µg, and 0.04 µg–of plasmid pBR322 (FIG. 3). Densitometry readings were performed on each lane of the agarose gel, and the sum of all band intensities were accumulated; all bands appearing on the agarose gel were assumed to be plasmid, as confirmed by an EcoRI restriction digest of a representative sample. Densitometry readings performed on each sample showed a 3-fold and 2-fold decrease in plasmid copy number in *E. coli* NF536As19 (*relA*$^+$) and *E. coli* NF537As19 (*relA*$^-$) grown for 4 hours at 30°C, respectively (FIG. 4). Although a drop in plasmid concentration was evident, levels of pBR322 in cells grown for an additional 4 hours at 30°C equilibrated at approximately 22 µg/mL. This value is not significantly different from the equilibrium value obtained from spectrophotometry readings (26 µg/ml), supporting the notion of a minimal copy number of pBR322 must be maintained in the cell for its efficient survival at experimental ampicillin concentrations (20 µg/mL). Levels of pBR322 in *E. coli* NF536As19 (*relA*$^+$) and its isogenic strain *E. coli* NF537As19 (*relA*$^-$) grown for 4 hours at 42°C fell below the limits detectable by densitometry (Fig. 3, Fig. 4).

Determination of the minimum inhibitory concentrations of tetracycline. Growth of *E. coli* strains NF536As19 (*relA*$^+$) and NF537As19 (*relA*$^-$) at 30°C was slightly inhibited at 50 µg/mL tetracycline (MIC < 500 µg/mL; FIG. 5). The same strains grown at 42°C displayed a marked reduction in growth at 50 µg/mL tetracycline (MIC < 50µg/mL; FIG. 5). Growth of all experimental cultures was noticeably inhibited at increased concentrations of tetracycline (1000 µg/mL and 3000 µg/mL; data not shown).

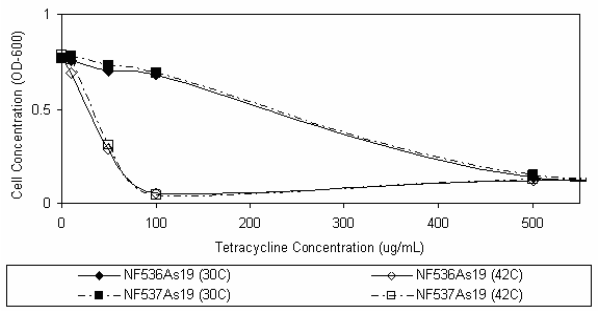

FIG. 5. Survival of *E. coli* strain NF536As19 and the isogenic strain NF537As19 experimentally grown in permissive (30°C) and non-permissive (42°C) conditions. Cell concentration determined for cultures grown at increasing concentrations of tetracycline. All growth in the presence of tetracycline carried out at 30°C.

DISCUSSION

This study examined the effects of the stringent response on the replication of plasmid pBR322 in an *E. coli* NF536As19 (*relA*$^+$, *valS*^{ts}, *leu*$^-$) and the isogenic *E. coli* NF537As19 (*relA*$^-$, *valS*^{ts}, *leu*$^-$) genetic background. A decrease in the concentration of plasmid pBR322 in *E. coli* NF536As19 (*relA*$^+$) was observed under stringent conditions as expected (FIG. 2). Previous studies have also shown a reduction in pBR322 concentrations in cells subjected to stringent conditions (7). However, we were surprised to find a similar decrease in the concentrations of pBR322 in relaxed *E. coli* NF537As19 (*relA*$^-$).

To assess if the observed results are a consequence of unknown technical issues, the pBR322 plasmid copy number was also assessed by differing means. Isolated plasmid was visualized on an agarose electrophoresis gel in conjunction with varying known concentrations of plasmid pBR322 (FIG. 3). The decrease in plasmid copy number in *E. coli* NF536As19 and the isogenic *E. coli* NF537As19 grown at 42°C can be inferred by the loss of the expected 4.3 kDa band from lane 11 and lane 13 respectively. Band intensity on the gel was quantitatively analyzed using densitometry. Although there are discrepancies between the obtained plasmid concentration values, possibly due to inherent errors present in both measures, the general pattern of both sets of data is similar–there is a marked reduction in plasmid concentration in *E. coli* grown at 42°C when compared to their respective strain grown at 30°C (FIG. 2, FIG. 4).

Consistent with our spectrophotometry results and our gel electrophoresis results, similar trends were observed from the indirect quantification of pBR322 plasmid copy number using a MIC assay. Given that pBR322 encodes a tetracycline resistance gene, it is expected that cells harboring more copies of pBR322 would be more efficient at producing the efflux machinery necessary for tetracycline resistance, and hence survive at higher tetracycline concentrations. Indeed, increases in tetracycline sensitivity was observed in cells subjected to 42°C treatment (MIC < 50μg/mL) compared to those that remained at 30°C (MIC < 500μg/mL), regardless of their *relA* phenotype (FIG. 5).

These data are contrary to several other studies in the literature that have shown an increase in pBR322 synthesis in *E. coli* strains displaying a relaxed response (7,8). It was previously believed that the differences in pBR322 copy number in stringent and relaxed *E. coli* strains was due to a reduction in the synthesis of RNA II, thereby reducing the frequency of replication initiation (7). ppGpp, a key alarmone in the stringent response, binds to the RNA polymerase complex and inhibits transcription at many promoters (14). Based on this model, replication of pBR322 would be inhibited in stringent cells, but not in relaxed mutants that do not produce ppGpp. However, Lin-Chao and Bremer have shown that promoters $p_{RNA\ I}$ and $p_{RNA\ II}$ are not under stringent control (11,12).

The current model of pBR322 replication in stringent conditions does not rely on the actions of ppGpp; instead, it focuses on the Rom protein and uncharged tRNAs. The antisense mRNA, RNA I, is produced to negatively regulate replication initiation by hybridizing with RNA II (1). Yavachev and Ivanov propose that the availability of RNA I to hybridize to RNA II is regulated by the levels of uncharged tRNA in the cell (16). RNA I is thought to interact with uncharged tRNA molecules in the cell that possess complementary sequences (8,14,16). The increased abundance of uncharged tRNA in relaxed cells, compared to stringent cells, quarantines a greater fraction of the available RNA I molecules, thereby allowing for increased replication (2,8,14,16).

This model, however, does not account for the observed decrease in copy number of plasmid pBR322 in both stringent *E. coli* NF536As19 (*relA*$^+$) and relaxed *E. coli* NF537As19 (*relA*$^-$). Therefore, we propose some slight modifications to this model to account for our observed results. Wegrzyn (14) has suggested that there are regions on uncharged tRNAs to bind both RNA I and RNA II. If this is the case, uncharged tRNA molecules in the cell can equally isolate both RNA I and RNA II. The enhancing effects of the isolation of RNA I on replication initiation would be offset by the isolation of RNA II, leaving the relative concentrations of RNA I and RNA II in the cell unchanged.

It has been demonstrated that stringent control induced by the deprivation of different amino acids may result in differential effects on the replicons of ColE1-type plasmids (9,15). Therefore, another possible explanation for the observed similarities between stringent and relaxed *E. coli* on the copy

number of plasmid pBR322 is that valyl-tRNA does not have the ability to bind RNA I or RNA II. The inability of RNA I or RNA II to bind to uncharged valyl-tRNA would leave the relative concentrations of RNA I and RNA II unchanged, thereby maximizing the amount of RNA I to hybridize to RNA II. This would result in RNA I levels similar to that of unstarved cells, where uncharged tRNA levels are minimal. Various studies examining the effects of stringency on plasmid replication control have induced the stringent response by depriving cells of a variety of amino acids; the most common include leucine (7,13), isoleucine (8,9), and arginine (8,9). However, few have examined the plasmid replication control in response to valine deprivation.

Discrepancies between our observed results, and results present in the literature may also be compounded by the use of different *E. coli* backgrounds in each study. We employed *E. coli* NF536As19 (*relA*$^+$) and *E. coli* NF537As19 (*relA*$^-$) in our study of stringency. However, other groups who have studied replication control of pBR322 under stringent conditions have employed *E. coli* CP78 and CP79 (7,8,13), the *E. coli* K12 prototrophic strain MG1655 and its isogenic *ΔrelA251::kan* derivative (8), the *E. coli* B/rA strain RL331T (12), and several others; no known studies have investigated stringent control on pBR322 plasmid replication in the *E. coli* NF536As19 and the isogenic *E. coli* NF537As19 background. Wegrzyn (14) has found that the observed differences in plasmid pBR322 replication control under stringency can, at least partially, be ascribed to the different genetic backgrounds of the host strains employed.

The binding of both RNA I and RNA II to uncharged tRNA, or the inability of both regulatory RNAs to bind to uncharged tRNA still does not explain the decrease in plasmid concentration, and hence plasmid copy number. This can be explained by a reduced transcription of both RNA I and RNA II during amino acid deprivation conditions (12). The reduction in RNA II transcription would limit the available pool of RNA II to initiate replication of pBR322.

Drawing solid conclusions from our data proved difficult, due to unforeseen shortcomings in our experiment. A notable issue arising in our experiment is the growth of the *E. coli* NF537As19 strain at the non-permissive temperature of 42°C. This suggests that the strain may have exhibited a reversion mutation to a temperature insensitive phenotype, as temperatures of 42°C should have inactivated valyl-tRNA synthetase preventing the biosynthesis of any proteins that contain valine, and thereby disrupt cell growth. If in fact a reversion mutation to a temperature insensitive phenotype was observed, induction of the stringent response in *E. coli* NF537As19 (*relA*$^-$) may not have succeeded, thereby eliminating an essential negative control for this experiment.

However, hypotheses can still be inferred from our results. Yield of plasmid pBR322 appears to be unaffected by valine deprivation or growth. If the *E. coli* NF537As19 strain used reverted to a temperature insensitive phenotype, then these cells grown at 42°C would not have been deprived of valine. Comparison with the *E. coli* NF536As19 strain grown at the non-permissive temperature of 42°C, depriving these cells of valine, reveals similar levels of plasmid pBR322 in the cell. Therefore, valine deprivation does not appear to have an affect on pBR322 plasmid copy number. Growth also seems to have negligible affect on plasmid copy number, as differences in the ability of *E. coli* NF536As19 and *E. coli* NF537As19 to grow was not reflected as differences in pBR322 plasmid copy number.

Temperature also appears to play a role in the replication control of pBR322; higher temperatures appear to have an inverse affect on the ability of plasmid pBR322 to replicate. Riethdorf et al. have demonstrated that the amplification of pBR322 is temperature dependent (13). Maximal amplification of plasmid pBR322 under stringent conditions is observed at 32°C, with amplification decreasing to a negligible amount by 37°C (13). Therefore, it is proposed that in the attempt to induce stringency, via a temperature shift to 42°C, the replication of pBR322 has essentially ceased.

Physiological changes of the cell induced by stringency may also play a role in the decrease of pBR322 copy number. Increased innate antibiotic resistance due to changes in the composition of the cell wall, and a reduction in growth rate in cells under stringent control would reduce the selective pressure for higher pBR322 copy numbers (6). Also, physiological changes due to the possible induction of heat shock proteins as a result of temperature differences may be confounded with the results observed; this makes drawing conclusions difficult as differences in plasmid copy number can also be attributed to possible temperature effects (as discussed above).

In conclusion, replication regulation of pBR322 does not appear to be solely controlled by the stringent response, as both *relA*$^+$ and *relA* mutant strains responded similarly to temperature-induced valine deprivation. Plasmid copy number of pBR322 was observed to decrease during amino acid deprivation in both stringent and relaxed strains. We believe that other mechanisms, possibly acting synergistically with stringency, are involved in the regulation of plasmid pBR322 during amino acid deprivation.

FUTURE EXPERIMENTS

It has been suggested that replication control of plasmid pBR322 in stringent conditions is affected by temperature, and the specific amino acid deprivation condition employed in the experiment. Therefore, to study these effects on the replication of pBR322, it is possible to exploit the *leu*⁻ phenotype of *E. coli* NF536As19 and *E. coli* NF537As19. pBR322 replication should be considered in leucine-starved cells. Leucine deprivation eliminates temperature as a confounding variable in the experiment, as it is possible that cells may have reacted to the increase in temperature in a manner that is independent of the stringent response. Employing leucine deprivation also examines the effects of different amino acid deprivation conditions on the replication control of pBR322.

A further difficulty we encountered when trying to relate our results to those observed in the literature is the differences in the genetic background of the cells used in stringency experiments. Discrepancies in the literature can, at least partially, be accounted for by the different strains of cells used. Future studies on the stringent control of pBR322 replication should be carried out in an *E. coli* CP78 and *E. coli* CP79 background, as it appears more prevalent in the literature.

The methods we used to quantify plasmid copy number yielded conflicting results; the A_{260} spectrophotometry readings and gel electrophoresis band densitometry values do not appear to correspond well. Inherent errors in spectrophotometric readings and densitometry, as well as limitations on the resolution of these methods, may have compounded to result in more significant deviations. Therefore, a more sensitive assay is required to quantify plasmid concentration. A common method is the incorporation of [^{3}H]thymidine to quantify DNA replication. When the stringent response is induced, the [^{3}H]thymidine would mostly accumulate in relaxed replicating plasmids, since chromosomal replication is quickly arrested. Measuring the amount of [^{3}H]thymidine incorporated into each strain may therefore be a more reliable method to reflect pBR322 copy number in the cells.

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REFERENCES

1. **Balbas, P, and F. Bolivar.** 2004. Back to basics: pBR322 and protein expression systems in E. coli. Methods Mol. Biol. **267:**77–90.
2. **Baracchini E., and H. Bremer.** 1988. Stringent and growth control of rRNA synthesis in *Escherichia coli* are both mediated by ppGpp. J. Biol. Chem. **263:**2597–2602.
3. **Begbie, S., G. Dominelli, K. Duthie., J. Holland, and M. Jitratkosol.** 2005. The effects of sub-inhibitory levels of chloramphenicol on pBR322 plasmid copy number in *Escherichia coli* DH5α cells. J. Exp. Microbiol. Immunol. **7:**82–88.
4. **Chang, D-E., D.J. Smalley, and T. Conway.** 2002. Gene expression profiling of *Escherichia coli* growth transitions: an expanded stringent response model. Mol. Microbiol. **45:**289–306.
5. **Frenkel, L., and H. Bremer.** 1986. Increased amplification of plasmids pBR322 and pBR327 by low concentrations of chloramphenicol. DNA **5:**539–544.
6. **Gilbert, P., P.J. Collier, and M.R.W. Brown.** 1990. Influence of growth rate on susceptibility to antimicrobial agents: biofilms, cell cycle, dormancy, and stringent response. Antimicrob. Agents Chemother. **34:**1865–1868.
7. **Hecker, M., A. Schroeter, and F. Mach.** 1983. Replication of pBR322 DNA in stringent and relaxed strains of *Escherichia coli*. Mol. Gen. Genet. **190:**355–357.
8. **Herman, A., A. Wegrzyn, and G. Wegrzyn.** 1994. Regulation of replication of plasmid pBR322 in amino acid-starved *Escherichia coli* strains. Mol. Gen. Genet. **243:**374–378.
9. **Herman, A., A. Wegrzyn, and G. Wegrzyn.** 1994. Differential replication of plasmids during stringent and relaxed response in *Escherichia coli*. Plasmid **32:**89–94.
10. **Liang, S.T., Y.C. Xu, P. Dennis, and H. Bremer.** 2000. mRNA composition and control of bacterial gene expression. J. Bacteriol. **182:**3037–3044.
11. **Lin-Chao, S., and H. Bremer.** 1986. Effect of *relA* function on the replication of plasmid pBR322 in *Escherichia coli*. Mol. Gen. Genet. **203:**150–153.
12. **Lin-Chao, S., and H. Bremer.** 1986. Effect of the bacterial growth rate on replication control of plasmid pBR322 in *Escherichia coli*. Mol. Gen. Genet. **203:**143–149.
13. **Riethdorf, S., A. Schroeter, and M. Hecker.** 1989. RelA mutation and pBR322 plasmid amplification in amino acid-starved cells of *Escherichia coli*. Genet. Res. **54:**167–171.
14. **Wegrzyn, G.** 1999. Replication of plasmids during bacterial response to amino acid starvation. Plasmid **41:**1–16.
15. **Wróbel, B., and G. Wegrzyn.** 1997. Differential amplification efficiency of pMB1 and p15A (ColE1-type) replicons in *Escherichia coli* stringent and relaxed strains starved for particular amino acids. Microbiol. Res. **152:**251–255.
16. **Yavachev, L., and I. Ivanov.** 1988. What does the homology between *E. coli* tRNAs and RNAs controlling ColE1 plasmid replication mean? J. Theor. Biol. **131:**235–241.