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The Effects of Gratuitous Heterologous Gene Expression on the Amplification of ColE1-related Plasmids in Cultures of *Escherichia coli* BL21 (DE3) in the Presence of Sub-inhibitory Levels of Chloramphenicol

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The use of chloramphenicol (CAM) in the amplification of ColE1-derived plasmids is commonly employed and its mechanisms of action are well documented. Plasmid amplification following inhibition of chromosomal DNA replication can also be achieved through IPTG-induced gratuitous over-expression of heterologous genes. In this experiment, the additive effects of IPTG induction of the *fre* gene and sub-inhibitory chloramphenicol treatments on plasmid pES1 amplification in *Escherichia coli* BL21 (DE3) were examined. Bacterial cultures were grown in sub-inhibitory concentrations of chloramphenicol alone or in combination with pre-treatment of IPTG. Total DNA was purified by phenol:chloroform:isoamyl alcohol extraction and plasmid concentration was determined using agarose gel assay and line densitometry. ColE1-related plasmid amplification was found to be enhanced 1-2 fold in cultures subjected to simultaneous treatment of chloramphenicol and IPTG compared to those treated with the antibiotic alone. No plasmid amplification was observed under low levels of IPTG treatment alone and the possible effects of active transcription due to IPTG induction on ColE1-related plasmid amplification was not observed.

Chloramphenicol (CAM), a bacteriostatic antibiotic that reversibly binds to the 50S ribosomal subunit, interferes with fundamental ribosomal functions such as peptidyltransferase (PTase) activity during elongation, binding and movement of ribosomal substrates (peptidyl-tRNA) through PTase center and translational termination (19), effectively halting protein synthesis and bacterial growth. Under the treatment of chloramphenicol, it was discovered that Colicinogenic factor E1 (ColE1)-derived plasmids continue to replicate in a semi-conservative fashion (4). Such plasmids could be amplified by as much as 125-fold over the normal level of 24 copies per *Escherichia coli* cell (4). The effect arises because the inhibition of translation leads to an immediate block of the initiation of *oriC*-dependant chromosomal DNA replication which depends on active protein synthesis (1, 18). In contrast, replication initiation of plasmids containing ColE1-related origins (pMB1, p15A) is less dependent on permanent translation and active protein synthesis. The replication initiation factor, DnaA, has been suggested as a limiting factor that is essential during oriC-dependent DNA replication, and such molecules are not believed to be involved in the replication initiation of ColE1-like plasmids (8). Moreover, the ColE1 origin is dependent on RNA molecules and Polymerase I for replication, the latter of which is found in abundance within the bacterial cell (1). As a result, ColE1-related plasmids replicate continuously in the presence of chloramphenicol with substantial increase in plasmid copy number long after chromosomal replication has ceased.

Amplification of ColE1-related plasmids containing recombinant genes has also been observed in *E. coli* following IPTG induction of ColE1-derived plasmids controlled by the tac promoter (18). The underlying mechanism of such amplification was proposed to be related to the relative reduction of other protein synthesis and inhibition of chromosomal replication caused by over-expression of the heterologous genes (18), a process similar to plasmid amplification following chloramphenicol treatment. Gratuitous over-expression and accumulation of recombinant heterologous genes, whose protein products serve no essential function in the cell, perturbs the intracellular balance of protein pools and leads to a cumulative breakdown of ribosomal RNA with loss of protein synthesis capacity (5). It was also suggested that a direct participation of uncharged tRNA molecules and the alarmone of the stringent response, guanosine tetraphosphate (ppGpp), both of which are abundant during amino acid starvation, could affect plasmid replication (18). This could be achieved through their sequence homology to the RNA molecules (RNAI, RNAII) responsible for modulating replication initiation of ColE1-related plasmids and their potential

roles in primer formation for plasmid replication (18, 21). Furthermore, a recombinant mRNA molecule that proceeds over its terminator can also act as a primer for plasmid replication (3). Therefore, ColE1-related plasmid copy number should be enhanced with high expression of heterologous genes under simultaneous treatment with chloramphenicol. Such amplification should result from the additive effects of the inhibition of translation and chromosomal replication and possible mechanisms modulating the initiation of plasmid replication that are associated with active transcription or stringent response.

In the present study, plasmid pES1 containing the heterologous NAD (P)H: flavin oxidoreductase (*fre*) gene was used to determine the effects of over-expression of IPTG-inducible heterologous gene on the efficiency of plasmid amplification in the presence of sub-inhibitory levels of chloramphenicol. Bacterial cultures were treated with concentrations of chloramphenicol alone or in combination with IPTG prior to antibiotic treatment in order to study the effects of transcription and translation on plasmid copy number. Treatment with IPTG induces the production of highly specific T7 RNA polymerase from the host chromosome of *E. coli* BL21 (DE3), which in turn actively transcribes *fre* gene from the T7 promoter located on pES1 followed by over-expression of target protein products. The plasmid copy number per genome was expected to increase with strong induction of the heterologous *fre* gene under simultaneous treatment with chloramphenicol. Our results indicated higher levels of plasmid amplification under high level induction of a heterologous gene and chloramphenicol treatment simultaneously. Furthermore, strong induction of transcription of pES1 alone had no observable amplification effects on plasmid copy number, excluding the direct positive influence of transcription of a heterologous gene on plasmid replication and the amplification of ColE1-related plasmids.

MATERIALS AND METHODS

Strains and Media: *E. coli* BL21 (DE3) cells were transformed with pES1 (20) isolated from *E. coli* DH5α cells. Both strains were supplied from the MICB 421 culture collection at the University of British Columbia. Modified Luria Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 10 g NaCl and 2 g glycerol per litre medium) was used to grow cultures under experimental conditions. Modified LB broth supplemented with kanamycin (50 μg/ml) was used for the growth of overnight cultures.

Introduction of pES1 into *E. coli* BL21 (DE3): Extraction of pES1 from *E. coli* DH5α was performed using GeneJET plasmid miniprep kit (Cat. # K0502, Fermentas) following manufacturer's instructions (6). Electro-competent *E. coli* BL21 (DE3) were prepared following Bio-Rad's Gene Pulse electroporator manual (2). Two vials of competent *E. coli* BL21 (DE3) were thawed on ice. Next, 72 μg and 144 μg of pES1 extracted from *E. coli* DH5α was added to each tube and mixed gently. The mixture was incubated on ice for 1 minute before transferring to a pre-chilled 0.2 cm electroporation cuvette (Bio-Rad, Mississauga, ON). The cells were electroporated using the "Ec1" setting on the Gene Pulser electroporator (Bio-Rad). Immediately after the pulse, 1 ml of modified LB broth was added to the cuvette. The mixture was gently transferred to a fresh microfuge tube using Pasteur pipette. The cells were allowed to recover on shaking platform at 220 rpm, 37°C, for 1 hour. Then, the electroporated cells were plated on modified LB agar plates with 50 μg/ml kanamycin in 50 μl, 100 μl, and 200 μl plating volumes. The plates were incubated at 37°C overnight to allow growth of successfully transformed *E. coli* BL21 (DE3).

Confirmation of pES1 Transformation: pES1 plasmids isolated from *E.coli* DH5α and *E.coli* BL21 (DE3) cells via GeneJET Plasmid Miniprep kit (Cat. # K0502, Fermentas) as previously described. A_{260} and A_{280} readings were taken to determine DNA concentration. Restriction endonuclease *BglI* (Cat. # R0143L, New England Biolabs Ipswich, MA) was utilized at 4-5 units/μg DNA as previously described (11). Digest mixtures were incubated at 37°C for 1 hour. Four microlitres of 6X gel loading buffer (GLB; 0.5 g bromophenol blue, 0.5 g xylene cyanol, 30 ml glycerol, 170 ml dH_2O) were added to stop reaction.

Cell Preparation: Five millilitres of LB was inoculated with *E.coli* B21 (DE3) cells containing pES1 plasmid and incubated overnight at 37°C on a tube roller. Subsequently, 2 flasks with 57 ml of Modified LB broth were each inoculated with 3 ml of the overnight culture, 0.2 mM IPTG was added to one flask; both flasks were incubated in a 37°C shaking water bath for 30 minutes. The turbidity at 660 nm before and after incubation was measured to confirm culture growth. Each culture was then divided equally between 3 flasks. Chloramphenicol was added at concentrations of either 4 or 20 μg/ml to both IPTG treated and untreated sets of samples; these cultures were incubated for 18 hours in the 37°C shaking water bath. The culture without chloramphenicol and IPTG treatment was used as experimental control. The turbidity of the 18-hour cultures was measured at harvest. Volumes of culture equivalent to one ml of a 1.2 OD_{660} culture were transferred to sterile microcentrifuge tubes and spun for 2 minutes at 10,000 rpm in a microfuge to pellet the cells. The supernatants were discarded. The pelleted cells were lysed and used for total bacterial DNA isolation.

Bacterial DNA Isolation and Purification: Total DNA isolation of experimental cultures was performed as previously described (1). Each cell pellet was resuspended in 400 μl of TE buffer (50 mM Tris/50 mM EDTA, pH 8.0). Eight microlitres of 50 mg/ml lysozyme solution in TE was added to each tube and the mixtures were incubated at 37°C for 30 minutes. Four microlitres of 10% sodium dodecyl sulfate (SDS) and 8 μl of 15 mg/ml proteinase K was added to each tube, and each mixture was incubated at 50°C for 50 minutes. Proteinase K was then heat-inactivated at 75°C for 10 minutes. RNA was digested by the addition of 2 μl of 10 mg/ml RNase solution with subsequent incubation at 37°C for 30 minutes. Next, 425 μl of phenol:chloroform:isoamyl alcohol (25:24:1) was added, followed by vigorous vortex action for 20 seconds. The lysate was sedimented for 5 minutes at 10,000 xg. The upper aqueous phase was transferred to a fresh microfuge tube using a wide-opening pipette tip (regular tip cut about 0.5 cm from the tip using a clean blade). Four hundred microlitres of chloroform were added to the new tube, followed by vigorous vortex action for 20 seconds. The mixture was spun for 5 minutes at 10,000 xg. The resulting clear supernatant (upper aqueous phase), containing purified total DNA, was transferred to a clean microfuge and the purified DNA was stored at -20°C until further analysis.

Determination of Band Identity: Pure genomic DNA, pure plasmid as well as total isolated DNA were separately treated with *BglI* as described above and ran on a 0.8% Seakem® Gold agarose (Cambrex, Charles City, IA) for comparison of banding patterns.

Agarose Gel Electrophoresis and Imaging: Protocol adapted from Begbie *et al* (1). TE buffer (0.301 g Tris Base, 0.93 g EDTA, 50 ml distilled water) was used to make two-fold dilutions of the purified bacterial samples. Twenty microlitres of the experimentally

induced and restriction digest samples were mixed with 4 µl of 6X GLB and were run through 0.8% Seakem® Gold agarose. In addition 20 µl of λ HindIII Ladder (Cat. # 15612-013, Invitrogen) was added to each gel. The gels were run in 1x TAE buffer (0.484 g Tris base, 0.114 ml glacial acetic acid, 0.037 g EDTA, pH 8.0, in 100 ml dH_2O) at 100 V until the bromophenol blue was approximately 2 cm from the bottom of the gel. The gel was then soaked in ethidium bromide bath (0.2 µg/ml) for 20 minutes then destained in a dH_2O bath for 1 hour. Images of the gel were taken using the AlphaImager Version 4.1 (AlphaInnotech, USA). The intensity (integrated area under the peaks) of the bands was determined by the 1D-Multi (Line Densitometry) program provided in the AlphaImager software package. Auto background was used for background subtraction.

Determination of Plasmid Copy Number: Data reduction as previously described (1, 13) was not necessary in this experiment. The problem of peak area non-linearity was only encountered in samples with high total DNA concentration obtained from high OD of cells used for total DNA isolation. Instead of substituting individual DNA peak areas obtained via data reduction (1, 13), the ratio of total plasmid DNA (pDNA) and genomic DNA (gDNA) was determined using % area values obtained from the AlphaImager 1D-Multi line densitometry program for 1/2 dilution samples and employed in the calculation of plasmid copy number according to the following modification of a previously published formula (13):

$$\text{plasmid copy number per genome (PCN)} = \frac{\text{size of chromosomal DNA (4640kbp) x total pDNA (original)}}{\text{size of plasmid DNA (6049bp) x total gDNA}}$$

$$\text{plasmid copy number per genome (PCN)} = \frac{\text{size of chromosomal DNA (4640kbp) x \% pDNA area (modified)}}{\text{size of plasmid DNA (6049bp) x \% gDNA area}}$$

Assay Recovery and Visualization: To determine interference from genomic DNA on plasmid absorbance area three samples were run alongside on an agarose gel; gDNA spiked with 12 ng of pES1, a control sample of unspiked gDNA and 12 ng pure pES1. The plasmid area of the spike following DNA isolation was divided by the 12 ng of pure pES1 plasmid to determine interference due to genomic DNA.

RESULTS

Evidence for pES1 plasmid transformation from *E.coli* DH5α to *E.coli* BL21 (DE3): Plasmid DNA extracted from both *E.coli* DH5α and pES1 transformed *E.coli* BL21 (DE3) were the same molecular weight (Fig. 1). Identical banding patterns in untreated and *BglI* restriction endonuclease treated plasmid DNA was also observed (Fig. 1) suggesting the plasmid pES1 was successfully transformed.

Recovery/Genomic Interference and Assay Sensitivity: To determine plasmid recovery using the total DNA isolation procedure documented by Pushovna *et al* (13), pure pES1 plasmid DNA was used to spike two samples, one prior to DNA isolation and one following DNA isolation. The percent recovery was found to be 51.6 % (data not shown). Genomic interference was found to be negligible, since the presence of genomic DNA when running a given amount of plasmid DNA was found to decrease the amount of plasmid visualized by 1.54% (Fig 2). Given that two-fold doubling dilutions were not performed for this study, the assay sensitivity was correlated to the minimum PCN quantified for one of the samples. The minimum quantifiable PCN was found to be 14 plasmids.

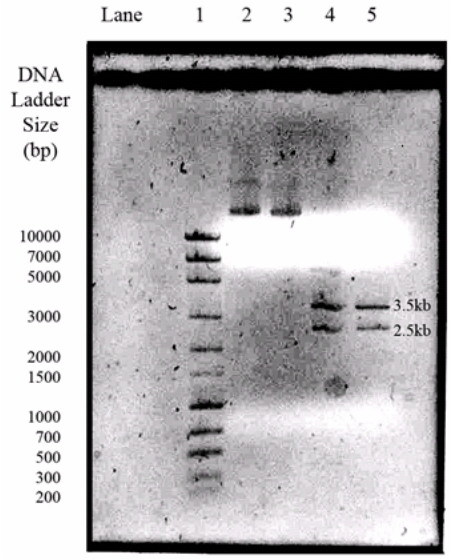

FIG 1. Evidence for transformation of *E.coli* BL21 (DE3) with pES1 plasmid. pES1 was isolated from electroporated *E. coli* BL21 (DE3) and was digested with *BglI* (NEB) . The samples were run on 0.8% agarose gel at 85 V for 75 minutes in 1x TAE buffer. Lane 1: Mass Ruler Express DNA Ladder Mix Forward (Cat. # SM1283, Fermentas); lane 2: undigested pES1 (*E. coli* BL21 (DE3)); lane 3: undigested pES1 (*E. coli* DH5α); lane 4: digested pES1 (*E. coli* BL21 (DE3)); lane 5: digested pES1 (*E. coli* DH5α).

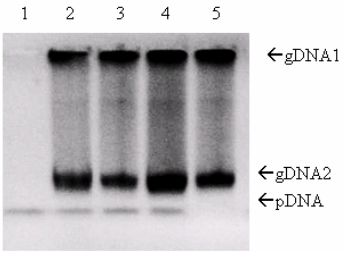

FIG 2. Genomic interference and spike recovery analysis. The samples were run at 85 V on 0.8% agarose gel for 75 minutes in 1x TAE. Lane 1: 12 ng of pure pES1; lane 2: DNA from *E. coli* BL21 (DE3) spiked with 12 ng of pES1 before extraction; lane 3-4: DNA from *E. coli* BL21 (DE3) spiked with 12 ng of pES1 after extraction; lane 5: DNA extracted from *E. coli* BL21 (DE3) without pES1 plasmid.

Determination of plasmid copy number per chromosome equivalent using sub-inhibitory levels of chloramphenicol and IPTG: Transformed *E. coli* BL21 (DE3) cells with pES1 plasmid were subjected to experimental conditions under treatments of IPTG and chloramphenicol in modified LB broth for 20 hours, after which total DNA was isolated for each sample. Electrophoresis results (Fig. 4) indicate adequate

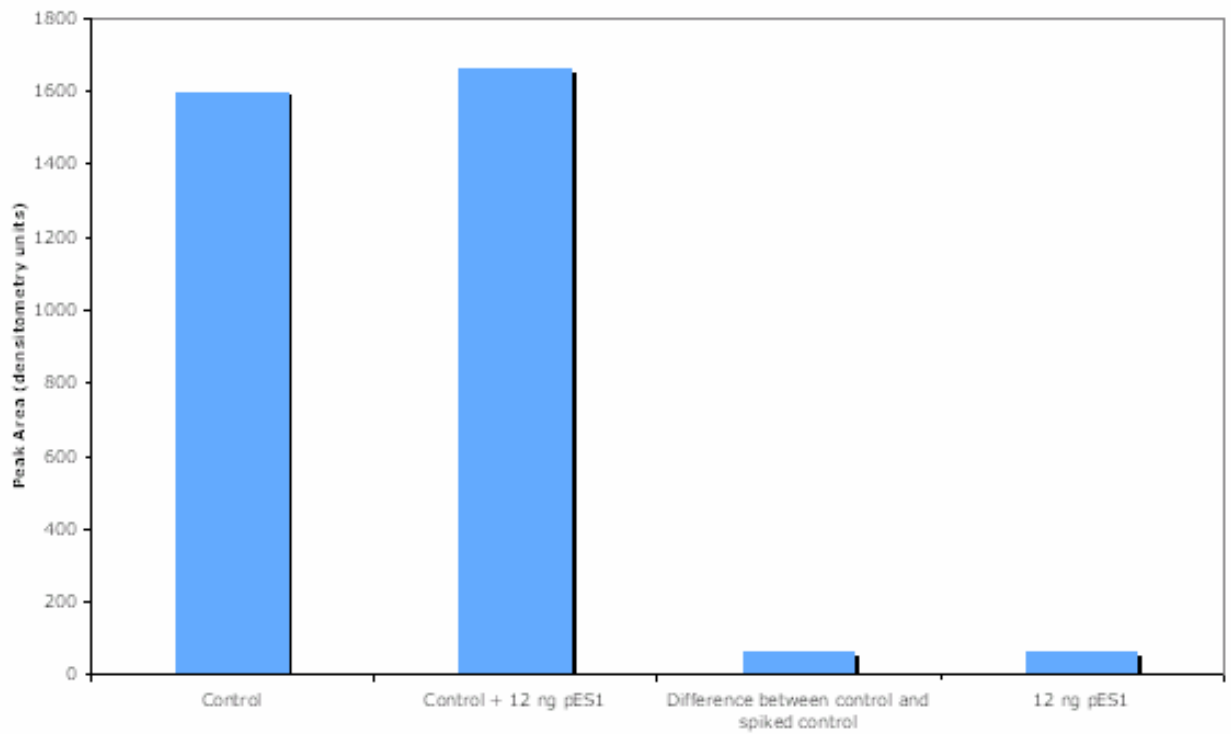

FIG 3. Effects of Genomic DNA interference on the visualization of plasmid pES1. Genomic interference was calculated by subtracting the area of the control sample from the backspiked sample which was subsequently compared to the area of pure plasmid (pES1) DNA. DNA extracted from *E. coli* BL21 (DE3) without pES1 plasmid was used as control. Peak areas were measured using 1D-Multi Line Densitometry by Alpha Innotech for the assay and accuracy control samples.

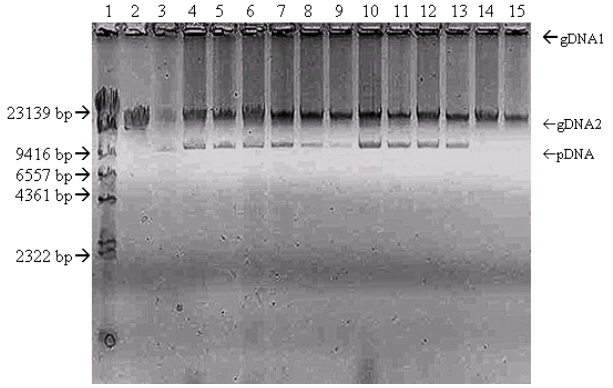

FIG 4. The effects of simultaneous treatments of sub-inhibitory levels of chloramphenicol and IPTG compared with chloramphenicol alone. Total DNA was extracted from all experimental *E. coli* BL21 (DE3) with pES1 cultures. Samples were run at 106 V on 0.8% agarose gel for 90 minutes in 1x TAE. Lane 1: λ HindIII ladder (Cat. # 15612-013, Invitrogen); lane 2: λ ladder; lane 3: Blank; lane 4: No IPTG, CAM 20 µg/ml; lane 5: 1/2 dilution of lane 4; lane 6: No IPTG, CAM 4 µg/ml; lane 7: 1/2 dilution of lane 6; lane 8: No IPTG, no CAM (control); lane 9: 1/2 dilution of lane 8; lane 10: 0.2 mM IPTG, CAM 4 µg/ml; lane 11: 1/2 dilution of lane 10; lane 12: 0.2 mM IPTG, CAM 20 µg/ml; lane 13: 1/2 dilution of lane 12; lane 14: 0.2 mM IPTG, no CAM; lane 15: 1/2 dilution of lane 14.

resolution and separation of pDNA and gDNA bands to independently measure the respective peak areas using line densitometry (Fig. 5). Due to the uncertainty in identifying the middle band (Fig. 4) as genomic DNA, plasmid copy number is calculated using three different approaches (Table 1). Discrepancies among estimated plasmid copy number (PCN) were observed. However, the PCN results demonstrate a general observable trend of increasing PCN with chloramphenicol treatment in all situations regardless of whether the middle band in Fig. 4 is identified and regarded as genomic DNA. The amplification factors in all three situations are also within comparable range with and without IPTG induction. On average, there is a 2-fold increase in PCN following the addition of sub-inhibitory chloramphenicol in the absence of IPTG induction with no significant difference in PCN between concentrations of 4 µg/ml and 20 µg/ml. Samples subjected to chloramphenicol treatments following IPTG induction showed 1-2 fold higher increase in PCN compared to those treated with the antibiotic alone. No plasmid peak area was detected with the AlphaImager 1D-Multi densitometry program for samples treated with IPTG alone. Corresponding plasmid bands were also not visible in gel electrophoresis (Fig 4). Plasmid DNA concentration in this culture sample could be too low for detection and no significant plasmid amplification over normal cellular levels was detected. PCN is listed as zero for such samples (Table 1).

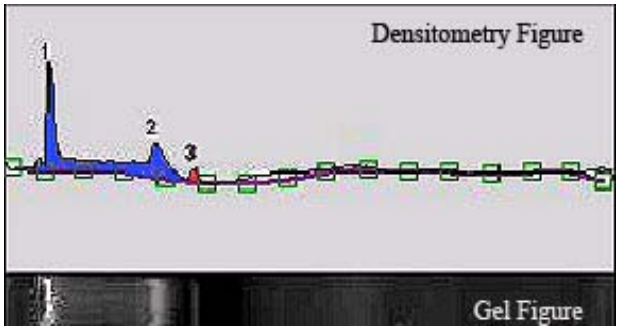

FIG 5. Densitometry peak analysis of extracted total DNA from *E. coli* BL21 (DE3) with pES1 under treatment of 0.2mM IPTG and 4 µg/ml CAM. The first two peaks picked up are considered to be gDNA, and the third peak is pDNA. The interconnected rectangular boxes represent randomly selected auto-background peak shoulders for baseline estimation. The bottom portion represents corresponding gel lane generating the densitometry results on top.

Table 1. pES1 plasmid copy number (PCN) per chromosome equivalent and amplification under treatment of sub-inhibitory concentrations of chloramphenicol before and after induction of heterologous *fre* gene. PCN is calculated using integrated scan data obtained from the Alphamager 1D-Multi line densitometry program of 2-fold dilution samples for different assumptions of gDNA bands to determine whether precise gDNA identification is essential in the estimation of plasmid copy number.

Chemical Treatment	pDNA vs. Total gDNA	pDNA vs. gDNA2	pDNA vs. gDNA1
No induction			
No CAM (control)	26	42	71
4ug/ml CAM	47	70	145
20ug/ml CAM	52	76	160
Amplification Factor	1.8-2.0	1.7-1.8	2.0-2.3
0.2mM IPTG induction			
No CAM (control)	0	0	0
4 µg/ml CAM	86	127	270
20 µg/ml CAM	63	93	198
Amplification Factor	2.4-3.3	2.2-3.0	2.8-3.8

pDNA – plasmid DNA
gDNA – genomic DNA
AF – amplification factor is calculated using the PCN obtained from the sample without chemical treatment as the control in each case.

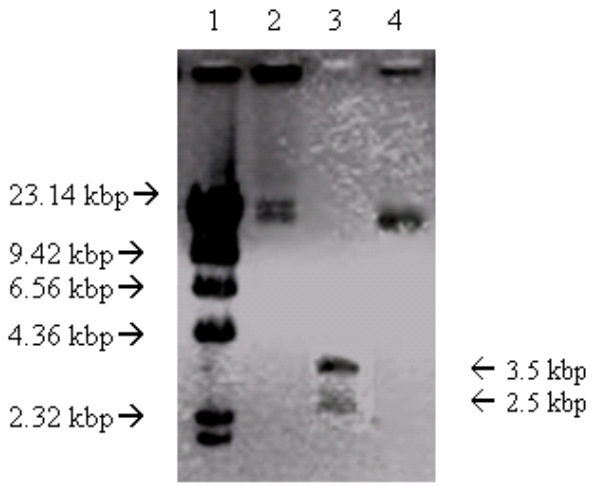

FIG. 6 Restriction digest assay for plasmid and chromosomal DNA. Total *E. coli* BL21 (DE3) DNA, pES1 and genomic DNA samples were digested with *BglI* for 1 hour. Samples were run at 106 V on 0.8% agarose gel for 90 minutes in 1x TAE. Lane 1: λ HindIII ladder (Cat/ # 15612-013, Invitrogen); lane 2: Total DNA (from 0.2 mM IPTG, CAM 4 µg/ml culture); lane 3: pES1; lane 4: genomic DNA.

Identification and confirmation of gDNA band 2 with *BglI* restriction digests: Identification of the gDNA band 2 (Fig. 4) was determined through a *BglI* restriction endonuclease digest as described in materials and methods. The banding patterns of digested total isolated DNA, digested pure chromosomal DNA, digested pure pES1 plasmid and undigested pure chromosomal DNA samples were compared via agarose gel electrophoresis. The digested total DNA (Fig. 6; lane 2) and digested pure chromosomal DNA (Fig 6; lane 4) showed a similar banding pattern to the undigested pure chromosomal DNA sample (Fig 6; lane5), the major difference being a lower concentration of DNA, possibly implying the samples were cut but not to completion and the resulting fragments either ran off the gel or were present at levels lower than the sensitivity of the assay. The pure pES1 digest resulted in the expected banding pattern (Fig 6; lane 3) suggesting complete digestion.

DISCUSSION

Consistent with previous studies (1), our results showed that treatment with chloramphenicol led to an increase in the plasmid copy number of ColE1-derived plasmids. This observation might be due to relief of the stringent response via a decrease in the global regulator, guanosine tetra-phosphate (ppGpp). Since binding of chloramphenicol to ribosome blocks aa-tRNA association and translation elongation (19), it can be hypothesized that chloramphenicol is able to inhibit the synthesis of ppGpp as RelA cannot become activated if binding of uncharged tRNA molecules is prevented.

Congruent with this rationale, it has indeed been observed in earlier studies, that ppGpp formation was inhibited by chloramphenicol treatment (9).

Analogous to the effects of chloramphenicol, the level of ppGpp is found to decrease following over-expression of heterologous recombinant gene products (18), which is also associated with an increased plasmid copy number. Also similar to chloramphenicol treatment (4), chromosomal replication was found to be inhibited 2 hours post induction (18); however, a direct effect of the IPTG during cellular stress response has been excluded (18). Following IPTG induction, ppGpp synthesis is inhibited through the depletion of the ribosomal pool required for the activation of Rel A. It is therefore postulated, that replication of ColE1-related plasmids is under negative regulation by ppGpp. This hypothesis is further supported by the discovery that ColE1 plasmids in Rel A mutants (Rel A^-) that are unable to synthesize ppGpp are amplified to similar levels after amino acid starvation as after chloramphenicol treatment with normal cells (7, 15). In addition, ColE1-related plasmid amplification was not observed in cells containing Rel A that were able to synthesize ppGpp under amino acid starvation (7, 15).

Since RNAI and RNAII are the main regulatory elements in the replication initiation of ColE1 plasmids (7), ppGpp could be a participant in the primer formation of plasmid replication through direct interaction with these RNA molecules. Such interactions could act to hamper plasmid replication in the presence of ppGpp so that a decrease in ppGpp levels seen during chloramphenicol treatment and IPTG induction would have an opposite effect; however, this proposal has not been proven. Based on the hypothesis and experimental results from this study, it is likely that chloramphenicol addition and IPTG induction follow the same mechanism of action in the amplification of plasmid copy number.

In contrast, stringent response with elevated levels of ppGpp during amino acid starvation has also been implicated in an increase in ColE1-related plasmid copy number (4, 7). It was proposed that initiation of plasmid replication could be controlled by interactions between RNAI, a molecule responsible for suppression of primer formation during plasmid replication initiation, and uncharged tRNA molecules that contain sequence homologies to RNAI (7, 21). The inhibitory effect of RNAI on primer formation may have been reduced with elevated levels of uncharged tRNAs that are typically observed in bacterial cells following amino acid starvation. This explanation could partly account for the amplification results obtained in this study since, depending on the extent of gratuitous protein production, a strong expression of heterologous protein products could eventually diminish intracellular amino acid pools leading to amino acid starvation.

The PCN results in this study demonstrated that plasmid pES1, pre-constructed by cloning *fre* gene into a high level protein expression system that contains a ColE1-related origin (pMB1), could be amplified multiple folds with the treatment of sub-inhibitory chloramphenicol as established by the works of previous researchers (1). In this study, the PCN of the ColE1-related plasmid was found to be further amplified when the antibiotic treatment is coupled with IPTG that indirectly induces the production of gratuitous heterologous proteins. However, in contrast to previous experimental findings, no amplification was observed in *E. coli* cultures induced with IPTG alone. It was demonstrated that plasmid amplification was not solely related to the amount of induced protein products but rather to observed growth inhibition that occurred as a result of IPTG-induced stringent response (18). Furthermore, accumulation of gratuitous protein was found to be associated with a progressive decrease in cellular growth rate which eventually ceased at the same time that protein accumulation reached a plateau (5). Cellular growth inhibition as a requirement for plasmid amplification would be expected since ppGpp, the aforementioned negative regulator of ColE1 plasmid replication, is normally induced during growth inhibition by nutrient starvation. Since the turbidity of the culture treated with IPTG alone reached a significantly high level (OD_{660} of 5), similar to that of the control sample, it is believed that no growth inhibition occurred as a result of IPTG treatment. Such observations indicate that gratuitous protein levels induced in the sample were not significant enough to perturb normal cellular balance and initiate ribosome destruction that would eventual lead to growth inhibition. It should be noted that the concentration of IPTG used to induce plasmid amplification in previous experiments (5, 18) were at least 5-folds higher than the concentration employed in this study. The considerably lower IPTG concentration may have failed to fully induce gratuitous protein production from the high-expression plasmid system that could have potentially reached 50% of total cellular protein content. Since IPTG is needed both to induce the production of T7 polymerase from the host chromosome and the target *fre* gene production from pES1, a higher level of IPTG may be needed to result in observable plasmid amplification following extensive gratuitous protein production and eventual growth inhibition. Furthermore, the results suggest active transcription of the pES1 plasmid by IPTG induction has no significant effect on plasmid copy number, in contrast to a previous observation that mRNA could act as primer for plasmid replication (3). In accordance with our interpretations of the results obtained in this study, the direct influence of heterologous gene transcription on the amplification of plasmid copy number was not considered as a

potential explanation for the observed effects of IPTG by Teich et al (18). However, it is not clear whether the lowered IPTG concentration resulted in a lower level of transcription that contributed to this observation by influencing primer formation.

Given the known effects of chloramphenicol and IPTG on plasmid copy number when tested independently, it was hypothesized that simultaneous treatment with chloramphenicol and high level induction would further enhance plasmid amplification in comparison to chloramphenicol treatment alone. The results obtained in Table 1 support this hypothesis. It was postulated above that chloramphenicol and high level induction likely work to amplify PCN through similar mechanisms. Consequently, the effects of the antibiotic and the inducer may have been additive such that simultaneous treatment with chloramphenicol and IPTG led to the observed augmentation in PCN level compared to treatment with either alone. Although IPTG concentrations in these samples were also low, the results indicate that enhanced levels of PCN would be observed as long as the effects discussed previously that contributed to plasmid amplification are strengthened by the addition of chloramphenicol. Growth inhibition was observed in samples treated with chloramphenicol compared to the control, a condition that was previously stated as a prerequisite for increasing PCN. Such inhibition may account for plasmid amplification that was not detected with IPTG induction alone. Furthermore, IPTG induction that led to multiple folds of plasmid amplification and maximum induction using the pET-30 high expression system were only observed after several hours of incubation in previous studies (12, 18). Since cultures were only induced with IPTG for half an hour before chloramphenicol addition in this study, the amplification effects observed might not have been optimal.

In this study, the sample treated with IPTG in the absence of the antibiotic exhibited plasmid levels below the detection limit. It should be noted that, since the volume used for total DNA isolation was correlated between samples according to cellular mass rather than cell number, the total cell number taken for this sample could have been the lowest among all experimental samples. Without the addition of chloramphenicol, T7 RNA polymerases are continuously induced and protein products are steadily synthesized from the *fre* gene. The highly active and selective nature of T7 RNA polymerase for the T7 promoter means that almost all cellular resources are devoted to target gene production such that *fre* gene products can comprise up to 50% of the total cellular protein within a few hours of IPTG induction (12). The size of the bacterial cells under such elevated levels of gratuitous protein expression could be multiple times that of normal cells. The might explain the absence of plasmid bands in the sample treated with IPTG alone as the cell number could be lower as compared to cells in the control sample, both of which had similar optical density (OD=5.0) and would account for concentrations of plasmid DNA that are below the limit of detection without significant levels of plasmid amplification.

There was no significant difference in PCN between the two sub-inhibitory levels of chloramphenicol treatment for samples treated with the antibiotic alone (Table 1). The turbidity of the overnight culture treated with 20ug/ml chloramphenicol was only 0.1OD lower than the culture treated with 4ug/ml at the time of harvest. The difference in the extent of growth inhibition by the antibiotic between the two culture samples was therefore not apparent. These results suggest that, beyond a certain sub-inhibitory concentration of chloramphenicol, further increase in the amount of the antibiotic has little effect on bacterial growth until the concentration reaches inhibitory levels, at which point cellular growth ceases completely. In contrast, cultures treated with 20ug/ml of chloramphenicol along with IPTG induction showed an observable decrease in PCN compared to those treated with 4ug/ml of chloramphenicol. This effect could be due to a faster and more complete shut down of protein synthesis following a higher level of chloramphenicol addition such that IPTG induction of gratuitous gene expression in the sample was halted more immediately. Since the effects of chloramphenicol and IPTG are postulated to be additive, a termination of protein over-expression through the addition of higher levels of chloramphenicol would have limited the extent of plasmid amplification in this respect.

The PCN per chromosomal equivalent for the control sample, calculated using the ratio of pDNA to the sum of gDNA1 and gDNA2 (Fig. 4), corresponds most closely with the normal copy number of pBR322 derived (pES1) plasmids at 15-20 copies per bacterial cell (13). Hence the PCN results obtained using this method are considered better representations of true plasmid copy number (Fig. 4). However, since there are typically more than one chromosome equivalents per bacterial cell with the number varying with different culture media, a direct comparison was not possible (4, 18). Consequently, plasmid copy number per *cell* could not be precisely estimated from the calculated PCN. Moreover, PCN may have been under or over-estimated, depending on the technical errors encountered during total DNA isolation procedures, gel electrophoresis and line densitometry.

For the purpose of this study and throughout the analysis, the upper bands appearing on the agarose gel corresponding to a DNA fragment of approximately 20 kb in size and the DNA band at the very top of the gel are presumed to be genomic DNA, while the lowest

band visualized are taken to be open circular 6049 bp pES1 plasmid (Fig 4). The intensity and thickness of the presumptive gDNA band is consistent amongst all samples, regardless of treatment and induction, implying that the origin of this DNA is resistant to amplification by chloramphenicol treatment, serving as further evidence of the genomic origin of this band. The identity of the plasmid band could be confirmed by the spike recovery gel results (Fig. 2), in which the plasmid band within the spiked samples ran to locations corresponding to those thought to be plasmid bands in Fig. 4 and to locations corresponding to bands in pure plasmid samples (data not shown). The identity of the middle band in Fig 4, on the other hand, is more difficult to confirm, as it could be extensively fragmented genomic DNA or concatomer of plasmid DNA. If such DNA bands were fragments of genomic DNA, it would have been completely digested with restriction enzyme *BglI* and the dense band would be transformed into a broad smear spanning the lanes of the gel. However, if two lower molecular weight bands were observed at locations corresponding to approximately 3.5 kb and 2.5 kb as observed in Fig. 1 upon pES1 digestion with *BglI*, then this would suggest that the middle band previously assumed to be genomic DNA was, in fact, of plasmid origin (1, 12). Restriction digest results with *BglI* in this study could not confirm with confidence the identity of this DNA band. A smear of fragments was not observed, nor was the band converted to lower molecular weights that would implicate plasmid DNA. However, the presence of such bands was also observed in samples with pure chromosomal DNA isolated from untransformed *E. coli* BL 21 (DE3) (Fig 2, lane 5). It should also be noted that the intensity of the DNA bands was significantly reduced after *BglI* digest, indicating the occurrence of digestion. This observation, coupled with the absence of bands which would implicate the presence of plasmid DNA, serves as evidence for the genomic origin of the middle band. In this study, the identity of this particular band did not affect the general trend observed in plasmid amplification. However, in experiments where plasmid copy number needs to be precisely determined, it becomes critical for such confirmation since discrepancies exist among the estimated PCN (Table 1). Furthermore, under sub-inhibitory treatments of chloramphenicol, it is extremely unlikely that plasmid DNA would be amplified to an extent that would generate bands with intensities comparable to that of genomic DNA left in the well. The persistence of a thick band corresponding to 20 kb in these lanes is believed to be due to limiting concentrations of the restriction enzyme that resulted in incomplete digestion since conditions were optimized prior to digestion and *BglI* is not sensitive to methylation, ruling out sub-optimal enzyme activity as a possible explanation for the observed results.

The results of the present experiment show that ColE1-related plasmids can be amplified to a higher level under treatment with chloramphenicol and IPTG concentrations that induce gratuitous protein production as compared to chloramphenicol treatment alone. We postulate that the two seemingly different methods of plasmid amplification follow the same molecular mechanism of action to increase plasmid copy number and that their effects are believed to be additive. Furthermore, active transcription of the heterologous gene induced under conditions of lone IPTG treatment did not have an observable amplification effect on plasmid copy number.

FUTURE EXPERIMENTS

The basis of the above analysis relied heavily upon the findings and assumptions made by earlier work on the related subject matter (1, 13). In particular, assumptions regarding the location and identity of bands corresponding to genomic (gDNA) versus plasmid DNA (pDNA) were adopted in order to explain the phenomena observed in this study. Attempts to validate these assumptions through the employment of digest assays failed, forcing the perpetuation of uncertainty regarding the true identity of bands alleged to represent gDNA.

Further experimentation should seek to confirm the true identity of such bands prior to continuing with studies utilizing the plasmid quantification method reported by Pushova *et al.*(13). One method for validation involves excising and eluting the putative gDNA band from an agarose gel (17). The excised DNA can be used to PCR amplify 16S rRNA genes which are found only on the bacterial genome but not on a plasmid. The presence of amplified products on an agarose gel at the appropriate molecular weight would confirm that the excised DNA was, in fact, genomic. A second method for validation involves the Southern Blotting technique and the use of a labeled probe for 16S rRNA. The visualization of a band at the molecular weight corresponding to the putative gDNA would confirm its identity as such.

Other results obtained through this study may be significantly altered by tweaking the protocol to buffer for drawbacks associated with the DNA isolation and quantification method and to optimally resolve the effects of various treatments. Reviewing Table 1, it is surprising to discover that samples treated with IPTG alone lack observable plasmid using the densitometry method for PCN quantification, considering that previous research reported plasmid amplification to levels that are comparable to those following chloramphenicol treatment. (18) This result may be due

to the fact that levels of IPTG used to induce cells were too low (0.2 mM) and that the densitometry method is not sensitive enough to recognize less than 14 plasmids. Accordingly, future experiments should use a concentration of 1 mM IPTG, similar to that used by Teich *et al.* in their experiments using IPTG induction for the amplification of ColE1 related plasmids (18). In addition, it would be advised for future groups to induce cells for longer periods of time with IPTG prior to treatment with chloramphenicol in order to enhance the observable effects of IPTG on plasmid copy number.

The inability of densitometry to detect less than 14 plasmids combined with the variation in total DNA extracted and the low extraction efficiency of 51.6% call the accuracy of this method for the quantification of plasmid copy number into question. Such findings suggest that future studies should seek alternative methods in order to minimize sources of experimental error and improve sensitivity. Ideally, the use of real-time PCR will prevent the loss of plasmid observed in this study while increasing the sensitivity of plasmid detection and consequently enhancing the accuracy of plasmid quantification.

Finally, taking the results of this study and the existing literature collectively, it was hypothesized that ppGpp acts as a negative regulator of plasmid amplification, such that low levels of this alarmone are responsible for the observed amplification upon treatment with IPTG and chloramphenicol. Although this direct relationship has not yet been proven, the relationship between ppGpp and plasmid amplification, specifically its interactions with RNA I or II, should be explored in future studies.

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