Depression of Protein Synthetic Capacity Due to Cloned-Gene Expression in *E. coli*

Thomas K. Wood and Steven W. Peretti
Department of Chemical Engineering, North Carolina State University, Raleigh, North Carolina 27695-7905

Recombinant bacterial systems exhibit limited capacities for heterologous protein production. As seen with this and other systems, cloned-gene protein production reaches an upper limit despite further increases in gene dosage. A series of closely related plasmids which contain mutations affecting their copy number has been used to investigate the macromolecular impediments to enhanced recombinant protein production. Within a common host, HB101, the level of the ampicillin resistance gene, *bla*, was varied using five plasmids which differ solely in their replication machinery. Separate fermentations were conducted in which the plasmid copy number was varied from 0 to over 400 while the specific growth rate was fixed at 0.6 h^{-1} for each chemostat cultivation. The effects of constitutive expression of the *bla* gene as copy number was elevated were then determined using pulse-labelling and RNA–DNA hybridizations. Specifically, the steady-state level, synthesis rate, and stability of β-lactamase messenger RNA and ribosomal RNA were determined as a function of copy number. The results indicate that as copy number rises, both β-lactamase mRNA synthesis rates and steady-state mRNA levels increase. Therefore, β-lactamase production in these strains does not appear to be limited by the level of β-lactamase mRNA. However, as the copy number was amplified, the stability of rRNA decreased to the point that steady-state levels of rRNA decreased. These data indicate that a limitation develops within the translational capacity of the cell at high levels of cloned-gene expression. The results suggest that strategies designed to enhance recombinant protein expression should include manipulation of translation as well as transcription.

INTRODUCTION

Bacterial cells have successfully been used to produce both eucaryotic and procaryotic proteins using genetic engineering techniques.[1] With strong promoters and gene amplification, recombinant products have been produced in quantities as high as 35% of the total cellular protein for bacterial cells.[2,3] However, there appears to be a maximum for protein production in these systems. Seo et al.[4] have observed that β-lactamase production increases initially in a linear fashion with increasing copy number, yet the level of this enzyme reaches a maximum as copy number increases still further. In addition, work in this lab (unpublished) has shown that β-galactosidase production initially increases linearly with the level of transcription, but synthesis of this enzyme reaches a maximum when high levels of the inducer IPTG are added. For both types of expression, it remains to be determined what cellular macromolecules dictate the maximum level of recombinant protein synthesis.

At high expression levels, protein synthesis may be limited by the level of mRNA for the recombinant protein or by the translational machinery of the cell. The level of mRNA for the cloned gene is a function of both its synthesis rate and its stability. The synthesis rate is controlled by the strength of the promoter as well as by the concentration of RNA polymerase. For a fixed promoter strength, one plausible bottleneck in the synthesis of recombinant protein would be a shortage of this enzyme within the cell. However, it has been shown that RNA polymerase is abundant at all growth rates. At high growth rates, no more than 80% of the total enzyme is actively used and at low growth rates only 25% is used.[5]

To study further the effects of RNA polymerase levels on the rate of rRNA synthesis, Nomura et al.[6] developed an inducible RNA polymerase synthesizing system. In this system, the chromosomal genes for RNA polymerase core enzyme ($\alpha_2\beta\beta'$) were supplemented by placing additional genes for the polymerase subunits on two plasmids under lac promoter/operator control. After induction with IPTG, the steady-state concentration of assembled and active RNA polymerase was two-fold that of the wild-type levels. Nomura et al. found that the synthesis rate of total mRNA increased twofold and reflected the increase in the RNA polymerase concentration. However, the synthesis rate of total protein and rRNA did not change. In an analogous fashion, an under-producing RNA polymerase system was also constructed.[6] They found that decreasing the cellular concentration of RNA polymerase decreases the synthesis rate of total mRNA; however, the synthesis rate of rRNA and total protein was not altered, as before.

The investigators concluded that the rate of rRNA synthesis is not limited by the amount of RNA polymerase, but mRNA synthesis is limited by the concentration of this enzyme. Also, the synthesis rate of total protein is not determined by the synthesis rate of total mRNA. Nomura et

al. speculated that mRNA is synthesized in excess for most proteins, and that the cell degrades mRNA after utilizing only one half of its capacity.

Recombinant protein production may also be limited by mRNA stability. The average half-life of mRNA is 1.3 min, and it varies from 0.5 to 20 min depending on the transcript.[7,8] The half-life of the mRNA of the reference protein β-lactamase used in these experiments is 3.0 min.[9] To study the stability of the β-lactamase transcript, Cohen et al. developed an assay which determined the decay rates of specific regions of the mRNA.[9] Their results indicated that the β-lactamase transcript is degraded rapidly once decay is initiated, and few degradation intermediates accumulate.[9]

To further investigate mRNA decay, Cohen et al.[7] introduced stop codons within the transcript to see the effect of ribosome coverage. Their results indicated that ribosome coverage of an *initial* boundary region of mRNA for the β-lactamase transcript (first 56 codons) affects transcript stability; rapid decay occurs if translation is halted before the ribosome reaches the end of this boundary. However, premature translation beyond this early site does not affect the stability of the translated upstream region or the untranslated downstream region.[7]

The maximum in cloned-gene production may also be constrained by deficiencies within the protein machinery of the cell. Even though ribosomal RNA is generally considered to be stable, degradation of rRNA occurs at all growth rates in *E. coli*.[10] The half-life of this "unstable" rRNA is similar to the half-life of mRNA.[10]

Gausing has found that rRNA is synthesized in excess at all growth rates. The rate of rRNA transcription (normalized by the protein content of the cell) increases 15-fold as the specific growth rate (μ) increases from 0.07 to 1.0 h^{-1}.[5,10] However, the stability of this newly synthesized rRNA varies as a function of cell growth rate. Ribosomal RNA is degraded rapidly in slowly growing cells where rRNA is synthesized in excess of ribosomal proteins; 70% of the transcribed rRNA is degraded as it is made at $\mu = 0.07$ h^{-1}.[10] Ribosomal RNA is apparently more stable at higher growth rates, as only 10% of de novo synthesized rRNA is degraded at $\mu = 1.0$ h^{-1}.[8,10]

In light of the need to further elucidate the macromolecular limitations of high-level expression of cloned-gene products, this research investigates the effect of gene amplification on the bacterial cell. Specifically, the synthesis rates and steady-state levels of β-lactamase mRNA and rRNA were studied as a function of copy number to determine the metabolic impact of recombinant protein production.

Escherichia coli HB101 was chosen as the host strain for this research because it is the best-characterized bacterium, and it is a common host for protein expression. In order to study the effect of gene dosage on this host bacterium, it was decided to vary the copy number of a plasmid that contains a constitutive gene for a reference protein. The copy-number mutant plasmids developed by Moser et al. were suitable since five plasmids (pDM246, pDM247, pRSF1050, pDM248, and pFH118) were available which

had mutations within their replication machinery.[11-13] The plasmids are closely related since they are derived from the same vector, and the replication mutations cause the copy number of each plasmid to be different within a common bacterial host. Each plasmid carries a single copy of the ampicillin-resistance *bla* gene which codes for β-lactamase.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The host chosen for these studies is the *Escherichia coli* bacterium HB101, a hybrid K12/B strain.[14] HB101 contains the following genetic markers:[14,15] F$^-$ *leu*B6 *pro*A2 *thi*1 *rec*A13 *ara*14 *lac*Y1 *gal*K2 *xyl*5 *mtl*1 *rps*L20 λ^- *sup*E44 *hsd*S20 $r_B^- m_B^-$. Hence, the strain is a leucine, proline, and thiamine auxotroph, and DNA transformed into the strain cannot undergo homologous recombination.

The gene dosage of a recombinant, reference protein, β-lactamase, was varied by using a copy-number mutant plasmid series. Each strain in this series consists of the HB101 host and one of the following pMB1-derived vectors: RSF1050, pFH118, pDM246, pDM247, or pDM248.[11] All five vectors are closely related and contain one copy of the gene that codes for the enzyme β-lactamase (*bla* gene). Each of the vectors has a different copy number within HB101 due to mutations within the plasmid genes that control plasmid replication. The copy numbers as determined by Seo et al.[4] and Moser et al.[11] in LB medium and the minimum inhibitory concentration (MIC) of ampicillin as determined by Dennis[16] are shown in Table I.

The plasmids in the series are relaxed, non-transmissible vectors derived from pMB8.[11] The different copy numbers of the plasmids were induced by manipulating the gene dosage of the wild-type inhibitor of replication, RNA I, as well as by manipulating a mutant form of this inhibitor.[11-13]

The identities of the five different copy-number mutant plasmids were verified through gel electrophoresis of the plasmid DNA in the following manner. Cells from each of the five strains were lysed with SDS and treated with lysozyme. Following treatment with RNase, the plasmid DNA was separated from chromosomal DNA using CsCl density gradient centrifugation. The plasmid DNA from each of the five strains was then cleaved with the restriction endonucleases *Bam*HI and *Eco*RI. The resulting linear

Table I. Copy-number mutant plasmid series.[4,11,16]

Vector	Copy number	MIC amp (μg/mL)
pDM247	12	1000
pDM246	24	1500
RSF1050	60	2500
pDM248	122	2900
pFH118	408	7700

DNA was visualized with a 0.6 wt % agarose gel and the restriction fragments were compared to verify the identity of each plasmid.

Hybridization Vectors

In order to quantitate the steady-state levels and synthesis rates of ribosomal RNA, the ribosomal genes within plasmid pKK3535 were utilized.[17] This plasmid contains the rrnB locus which is one of the seven E. coli ribosomal operons. This locus contains the wild-type 16S, 23S, and 5S rRNA genes as well as the glutamine tRNA gene. A 7.5-kb BamHI fragment containing this locus has been placed within the cloning vector pBR322 to construct pKK3535 (11.86 kb) by Brosius et al.[17]

The source of the β-lactamase DNA used to quantitate the steady-state levels and synthesis rates of β-lactamase mRNA was the high copy number plasmid pFH118 within the host HB101.[11] The bla gene is contained within the HaeII fragment on this plasmid that contains a unique BamHI site.[12]

Media and Chemostat Cultivation

M9 minimal medium containing 0.2 wt % glucose supplemented with 0.4 wt % casamino acids, 0.1 wt % thiamine, and ampicillin (Na salt) was used as the growth medium in the chemostat.[18] The amount of ampicillin added to the medium was 10% of the MIC for each strain (Table I). In this manner, the same selection pressure was placed on all five plasmid-bearing strains. Additionally, silicon antifoam was added at a concentration of 44 μg/mL to prevent foaming.[19]

The bacterial strains were grown at a dilution rate of 0.6 h^{-1} in a 3.0-L Applikon ADI 1020 Bioprocessor and Fermentor. This chemostat has a working volume of 2.5 L but was filled to 1.0 L for these studies. The Bioprocessor was used to control pH at 7.0 ±0.05 by addition of 3M NaOH. At this molarity, the volume of base that is added represents a very small fraction of the feed. The Bioprocessor maintained the temperature at 37.0 ±0.1°C in the reactor by controlling a water bath that circulates water through a glass jacket on the exterior of the reactor. A dissolved oxygen probe was used to monitor oxygen levels within the reactor and to assure the cells were growing aerobically. Air was supplied to the reactor using a porous metal sparger in order to maintain high oxygen transfer rates. The air flow rate was determined with a rotameter and maintained constant at 122 mL/min for all cultivations. This rate was chosen to ensure that the dissolved oxygen level remained above 65% saturation and to limit foaming. Agitation was constant at 800 rpm. The absorbance values of the reactor effluent were monitored on-line with a Bausch and Lomb Spec 12 UV spectrophotometer.

The reactor was inoculated with 1–20 mL of a batch, overnight LB-Ap culture. The cell density was allowed to increase as the reactor operated in batch mode until the reactor was visibly turbid, then the reactor was operated as a chemostat. The continuous culture within the reactor was assumed to have achieved steady-state after passage of five residence times[19] and when the absorbance readings from the spectrophotometer were invariant.

The number of viable cells at steady-state was determined by diluting the reactor samples and plating on LB and LB-Ap plates (five plates each). Plasmid segregational instability, if present, was indicated by larger cell numbers on LB plates relative to the number of cells on LB-Ap plates.

Total Protein and Total RNA

The total protein content of the steady-state cells was analyzed by the Sigma Protein Kit No. 690. This kit is based upon a modification of the biuret and Lowry methods. The cellular protein level was quantified by diluting a thawed 1-mL chemostat cell sample with 5 mL phosphate buffer (0.1M Na$_2$HPO$_4$ and 0.1M KH$_2$PO$_4$, pH 7.0) containing 1 mM phenylmethylsulfonylfluoride (PMSF) as a protease inhibitor. The cells were sonicated for 1 min at 21 kHz with an Artek Sonic Dismembrator model 300 set at 30%, and 200 μL of the cellular homogenate was analyzed according to the Sigma protocol.

For isolation of RNA, the glassware was rendered RNase-free according to the procedures of Berger and Kimmel.[20] All glassware except Corex centrifugation tubes were washed and then heat-treated for at least three hours at 250°C. The Corex centrifugation tubes were immersed in 0.1 wt % diethylpyrocarbonate (DEPC) overnight and then autoclaved. Virgin plastic items such as pipettes and microcentrifuge tubes were assumed to be RNase-free and were handled only with gloves. Solutions not containing Tris buffer were also treated with DEPC overnight and then autoclaved. Solutions containing Tris buffer were formulated without Tris and treated with DEPC. After autoclaving, Tris stock solution (made from distilled water that had been treated with DEPC) was added. Each solution was then reautoclaved.

Using the RNase-free equipment, pulsed-labeled and unlabeled RNA were isolated from 10 mL chemostat culture (for each) according to the protocol of Dennis and Nomura[21] which has been slightly modified by the addition of 200 mM vanadyl–ribonucleoside complexes (VRCs) obtained from New England Biolabs. The VRCs are specific inhibitors of RNase which act by forming non-dissociating reaction complexes (transition state analogs) with the RNase.[20]

Briefly, the isolation involved lysing the cells with SDS, extracting three times with redistilled phenol, and precipitating with ethanol. The RNA was centrifuged and resuspended in Tris/azide/EDTA buffer; the phenol was removed by ether extractions. The DNA and protein were removed be DNase and pronase treatments. After additional phenol extractions and another ethanol precipitation, the RNA was resuspended in 2X SSC buffer (0.3M NaCl and 30 mM sodium citrate). The phenol was removed again by three ether extractions, and the resulting total RNA for each chemostat culture was quantified by averag-

ing the spectrophotometrically determined concentrations of the pulse-labeled and nonlabelled RNA samples. An absorbance of 1.0 at 260 nm was assumed to be equal to 50 μg RNA/mL.[21] A Shimadzu UV160U Scanning Spectrophotometer with a constant temperature cell set at 25°C was used for this measurement.

β-Lactamase Activity

The iodometric assay[22,23] was used to determine the steady-state level of β-lactamase in the samples. The number of moles of penicillin which react is proportional to the reaction rate which, for excess substrate concentrations (compared to the Michaelis constant), is equal to the maximum reaction rate, v_{max}. Also, v_{max} is proportional to the total β-lactamase enzyme level at the start of the reaction. Hence, knowledge of the proportionality constant between v_{max} and the total enzyme concentration (determined in an experiment in which the enzyme concentration is known) allows the active enzyme concentration to be calculated once v_{max} is determined by the assay.

The steady-state sample was stored at $-40°C$ until the sample was sonicated by taking 1 mL cell suspension and diluting with 5 mL phosphate buffer (with PMSF to prevent protein degradation). The appropriate duration of sonication was found from experiments in which cells were sonicated for periods to 2 min; the enzyme activities obtained indicated that sonication for 1 min was suitable for releasing most of the β-lactamase. Therefore, the cells were sonicated for 1 minute at 21 kHz using an Artek Dismembrator model 300 set at 30%. The cell debris was removed by centrifugation at 1.5×10^4 rpm for 30 min.

The iodometric assay was performed at 30°C by taking 2.5 mL of the supernatant and mixing with 0.5 mL of a 16 mM benzylpenicillin solution for a period of 15–60 s, depending on the enzyme concentration.[22,23] The reaction was terminated before the substrate concentration was reduced significantly by adding the buffered I_2 reagent (8 mM I_2, 60 mM KI, and 0.464M NaOAc, pH adjusted to 4.0 with glacial acetic acid), and the solution was allowed to stand for 5 min at 30°C. The absorbance at 540 nm was then recorded with a Bausch & Lomb Spec 21 UV Spectrophotometer. A blank consisting of 0.5 mL benzylpenicillin and 2.5 mL phosphate buffer was also assayed. The β-lactamase activity in the assay tube was calculated as

$$v_{max} = \frac{\mu mol \ penicillin \ degraded}{min \ mL}$$

$$= \frac{(A_{Blank} - A_{Sample})}{(A_{Blank})} \times 40 \ \mu mol \ I_2$$

$$\times \left(\frac{1 \ mol \ penicillin}{4 \ mol \ I_2}\right) \times \frac{1}{[T(min)]3.0 \ mL} \quad (1)$$

In order to convert the enzyme activities to concentrations and to ascertain if the iodometric assay for β-lactamase was yielding initial, maximum reaction rates that follow classical Michaelis–Menten kinetics, the reaction

rate (activity) for purified β-lactamase was investigated. The source of the β-lactamase was *Bacillus cereus* (Sigma, EC 3.5.2.6). Eight samples varying in initial enzyme concentration from 0.78 to 7.8 pmol/mL were reacted with penicillin G substrate. Figure 1 presents these results.

From the slope of the graph, the turnover rate, k_3, for this enzyme was calculated as 2880 molecules/s at 30°C. This value compares well with the value listed as 2000 molecules/s in Stryer.[24] Using this value, the β-lactamase activities (reaction rates) can be converted to active concentrations of enzyme since for Michaelis–Menten kinetics,

$$[\beta\text{-lactamase}] = \frac{v_{max}}{k_3} \quad (2)$$

Isolation of DNA Loci for Hybridizations

Using a large-scale plasmid DNA prep (2 L), pFH118 and pKK3535 (in host HB101) were each amplified using chloramphenicol and purified using CsCl utracentrifugation. Plasmid pKK3535 contains the ribosomal *rrnB* loci necessary for the hybridization experiments. The plasmid DNA was cut in 260-μg sample sizes/gel with the restriction enzyme *Bam*HI to yield a 7500-bp fragment that includes the 16S, 23S, and 5S ribosomal RNA units and a small 4300-bp fragment. The two bands were separated by horizontal gel electrophoresis using a 0.6 wt % agarose gel. The correct band was determined based on size using a molecular-weight ladder, cut from the gel, and electrophoresed in a dialysis bag according to the method of Maniatis et al.[24a] to separate the gel and the DNA. The ethidium bromide used to visualize the DNA fragment was removed with an equal-volume, isoamyl alcohol extraction, and the DNA was precipitated with ethanol. The DNA containing the desired locus was then dissolved in a TE buffer and frozen at $-40°C$.

The high copy-number plasmid pFH118 contains the β-lactamase gene required to quantify the level of mRNA$_{\beta\text{-lac}}$. A double digest is required to harvest the β-lactamase gene from this plasmid.[19] There are eight fragments generated
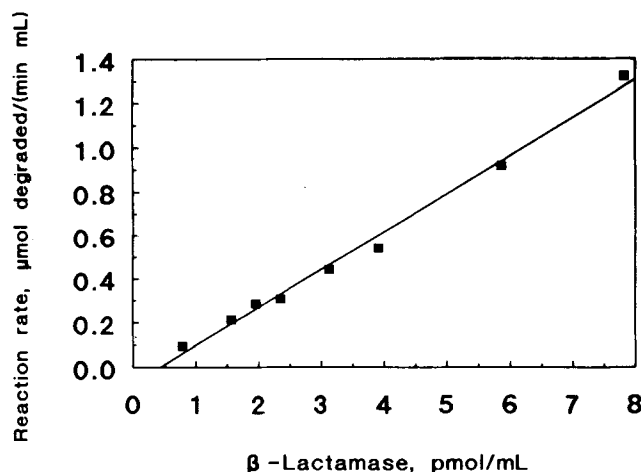

Figure 1. The β-lactamase turnover rate, k_3. Linear regression line is shown.

by cutting with the restriction enzyme HaeII. To determine which of the fragments contains the β-lactamase gene, an additional digest was performed with the restriction enzyme BamHI which has a unique site within the fragment that contains the β-lactamase gene. By comparing a sample of the single digest using HaeII with that of a double digest using HaeII and BamHI, the appropriate fragment (1.8 kb) can be discerned as the only fragment which advances (due to its smaller size) in a 1% agarose gel. This band was then removed from the gel, and the β-lactamase gene was isolated in an analogous fashion as was described above for the rRNA locus. Successful isolations of both the β-lactamase gene fragment and the ribosomal locus fragment were verified by electrophoresis in a 0.6 wt % gel, and the DNA concentrations were determined spectrophotometrically.

Synthesis Rates of rRNA and β-Lactamase mRNA

The synthesis rates of both rRNA and β-lactamase mRNA were found by pulse-labelling with ^{3}H-labeled uracil (20 μCi/mL). To a 10-mL sample from a steady-state chemostat culture, 200 μL of 1 mCi/mL [5,6-^{3}H] uracil (ICN, 45 Ci/mmol) were added to the sample. After a 2-min pulse, 12 mL of 4°C extinction buffer were added to prevent further cell metabolism, including further incorporation of the radioactively-labeled uracil.[25] The RNA was then isolated from the cells as previously outlined.

In order to quantify the amount of rRNA and β-lactamase mRNA, these transcripts must be separated from the total, cellular RNA mixture by hybridization with DNA specific for each of these RNA species. To reduce hybridization noise, extraneous DNA was removed from the probes as described above so that only the desired genes remained. Only RNA that has been transcribed from the same locus will then hybridize.

The isolated genes were bound to nitrocellulose following the method of Kafatos et al.[26] The double-stranded DNA fragments were denatured and any RNA present was degraded by adding 0.1 volume of 3M NaOH and incubating for 1 h at 65°C.[20] The DNA (2.5 μg of DNA/dot) was then bound to 0.45-μm nitrocellulose filter paper (S & S BA85) using a Schleicher and Schuell Minifold II Slot-blotter. Triplicates consisting of three identical dots of DNA were used for each chemostat cultivation. Background counts were found using duplicates of nonhomologous salmon testes DNA for each cultivation. The filter paper was baked for one hour at 80°C in a vacuum oven to bind permanently the DNA to the nitrocellulose.

The bound, nonradioactive DNA was probed by the total cellular RNA that had been pulse-labeled following the hybridization techniques of Berger and Kimmel,[20] Yamamoto et al.,[25] and Maniatis et al.[24a] The hybridization fluid contained heterologous RNA (yeast RNA, 1 mg/mL) to prevent random, nonspecific annealing of the probe (mRNA or rRNA) to the bound DNA. Yeast RNA covered all of the nonspecific sites, since it was present in great excess; subsequently, background noise was decreased. Likewise,

heterologous DNA (salmon testes DNA, 100 μg/mL) served to adhere to the bound DNA in a nonspecific manner preventing background noise. SDS (0.1 vol %) was present in the hybridization fluid to block the nitrocellulose as a detergent, and Denhardt's protein solution (5×) blocked the nitrocellulose as well. Formamide (50 vol %) was added to the hybridization fluid to decrease the melting temperature of the DNA–RNA hybrid and to preserve the nitrocellulose. Polyethylene glycol (PEG, 10 vol %) increased the rate of mixed-phase hybridizations by excluding the probe from the solution; therefore, it increased the apparent concentration of the probe. The hybridization buffer also contained 5× SSPE buffer (0.9M NaCl, 50 mM NaH$_2$PO$_4$, 5 mM EDTA, pH 7.4).

For prehybridization, the nitrocellulose dot containing bound DNA was excised from the slot-blot filter with a scalpel and placed in a microcentrifuge tube containing 300 μL hybridization fluid and incubated at 42°C for at least 1 h. Radioactively labeled RNA that had been heat denatured for 2 min at 80°C was added to the tube to begin hybridization, and the DNA and RNA were hybridized for 60–70 h at 42°C. Since the RNA concentrations were different in each chemostat cultivation, the RNA was diluted with TE buffer so that an equal volume of RNA solution was added to each microcentrifuge tube.

After hybridizing, the nitrocellulose paper was washed three times with wash No. 1 (1× SSPE with 0.1 vol % SDS) for 15 min at 60–65°C and once with wash No. 2 (0.1× SSPE with 0.1 vol% SDS). The filter was rinsed twice with 2× SSC to remove the SDS and incubated in the presence of 5.0 μg/mL RNase A in 2× SSC for 1 h at 37°C to eliminate background binding of the RNA probe to the filter. The filter was then rinsed with wash No. 1.

The radioactivity of each filter was determined by a Packard 1500 Tri-Carb Liquid Scintillation Analyzer. In order to get consistent counts, the nitrocellulose was first dissolved with 1.0 mL ethylene glycol monoethylether (Fisher Cellosolve) for 15 min in a liquid scintillation vial and then 5.0 mL of Fisher Scintiverse II liquid scintillation cocktail were added. As a result, the sample that was counted was homogeneous, and the tritium was released from the paper before it was counted.

Excess hybridizations studies were performed to ensure that the genes contained within the DNA on the nitrocellulose were always in excess of the transcripts contained within the total cellular RNA. DNA must be in excess because every transcript that shares homology with the gene, both labeled and unlabeled, must be able to bind to the DNA. To determine the amount of DNA that would always be in excess, 3.4 μg β-lactamase DNA obtained as a 1810-bp fragment of plasmid pFH118 (8454 bp) was bound to nitrocellulose. Cellular RNA from strain HB101/ pDM248 (copy number 120) grown in a chemostat cultivation was chosen since it has a high, stable copy number; therefore, large amounts of mRNA for β-lactamase would be present. If there is excess DNA for this strain, it was reasoned that there would be excess DNA for all of the other strains.

Cellular RNA in the amount of 2–100 µg was hybridized against the bound β-lactamase DNA. The same RNA was also hybridized against 3.4 µg salmon testes DNA; this DNA served as a nonhomologous control for determining background counts. Duplicates were used for both types of DNA. The response remained linear throughout this range, so a conservative threshold of less than (50 µg RNA)/(3.4 µg β-lactamase DNA) was chosen as the amount of probe to use during the hybridizations with the chemostat samples).

Similarly, in order to carefully determine the synthesis rate of rRNA, the ribosomal DNA bound to nitrocellulose must be in excess of that in the cellular RNA so all of the rRNA, both labeled and unlabeled, will be bound. To determine the amount of DNA that would always be in excess, 1.4 µg DNA containing the rrnB ribosomal locus was bound to nitrocellulose. This DNA was obtained as a 7.5-kb fragment of plasmid pKK3535 (11.7 kb). Cellular RNA from strain HB101 grown in a chemostat cultivation was chosen since this strain has the largest level of rRNA. If there is excess DNA for this strain, it was reasoned that there would be excess DNA for all of the other strains.

Cellular RNA was hybridized against the bound DNA in amounts of 0.1–100 µg RNA (a 1000-fold range). The same RNA was also hybridized against 1.4 µg salmon testes DNA; this DNA served as a nonhomologous control for determining background counts. Duplicates were used for both types of DNA. From the linear relationship obtained from 0.1 to 2.0 µg RNA added, a conservative threshold of less than (1.75 µg RNA)/(1.4 µg ribosomal DNA) was chosen. Above this level of RNA, the bound DNA becomes saturated with RNA, preventing an accurate measure of rRNA.

Steady-State Levels of rRNA and β-Lactamase mRNA

The steady-state levels of rRNA and β-lactamase mRNA were found by hybridizing bound, nonradioactive, cellular RNA to DNA that had been radioactively labeled using random oligomers (oligolabeling). The total cellular RNA was derived from a 10-mL sample of a chemostat culture at steady state. This sample was taken and immediately added to 12 mL of 4°C extinction buffer, which killed the cells. The cellular RNA was then isolated as previously described. During hybridization, the cellular RNA was bound to the nitrocellulose filter, and radioactive DNA specific for either rRNA or β-lactamase was used as the probe to determine the steady-state levels of the transcripts.

The DNA containing either the rrnB locus or the β-lactamase gene was isolated as described above using restriction enzymes and horizontal gel electrophoresis. The genes were then oligolabeled using the protocol of the Boehringer Mannheim Random Primed DNA Labeling Kit (1004 760). For each reaction, 750 ng DNA was labeled with 9.5 µL deoxycytidine 5′-triphosphate tetrasodium salt [5-³H] (ICN, 1 mCi/mL, 19 Ci/mmol). The ethanol in the dCTP

[5-³H] solution was removed before the reaction was started by vacuum drying until the liquid was gone (20 min). The reaction was carried out for 24 h at 37°C.

The nonradioactive, cellular RNA was bound to Schleicher and Schuell nitrocellular (BA85) according to the manufacturer's protocol. The RNA was diluted in TE buffer and 0.6 volume of 20× SSC (3M NaCl, 0.3M sodium citrate, pH 7.0) was added along with 0.4 volume of 37% formaldehyde. The RNA secondary structure was relaxed by incubating for 15 min in this solution at 60°C. Triplicate dots of RNA were then formed by applying three identical samples of RNA for each chemostat cultivation to the slot blotter. Nonhomologous yeast RNA was used to determine the background counts. The RNA was bound permanently to the nitrocellulose paper by heating at 80°C for 1 h in vacuo. The RNA dots were removed from the filter paper using a scalpel and placed in a microcentrifuge tube with 300 µL hybridization fluid to begin prehybridization. Prehybridization lasted for at least 1 h at 42°C.

The radioactively labeled DNA was denatured by incubating with 0.1 vol of 3.0M NaOH at 37°C for 5 min. The oligolabeled DNA was then added to the microcentrifuge tube containing the nitrocellulose and hybridized to the bound RNA for 60–70 h at 42°C. Randomly adherent probe DNA was removed by decanting the hybridization fluid and washing three times with wash No. 1 for 15 min at 25°C; these washes for bound RNA were less stringent than for bound DNA. The nitrocellulose was then washed once at 42°C for 15 min with wash No. 2. Each nitrocellulose dot was then counted in the liquid scintillation counter as described earlier.

To accurately determine total amounts of each RNA species, it was necessary that there be more radioactively labeled DNA than RNA transcripts on a molar basis so that every transcript for rRNA or β-lactamase mRNA bound to the filter has a radioactively labeled gene attached when it is counted. The excess experiments show this criteria is satisfied for β-lactamase mRNA if more than 1.0 µg oligolabeled DNA is used to probe 15.0 µg RNA. To be conservative, the hybridizations for determining total levels of β-lactamase mRNA used 0.49 µg DNA/4.8 µg RNA.

Similarly, a linear response can be expected for the ribosomal RNA levels if more than 1.0 µg DNA is used for every 1.25 µg RNA. The actual oligolabeled DNA and cellular RNA used were 0.335 µg DNA/0.25 µg RNA.

Degradation Constants from Transcription Rate Equations

In order to discern the pertinent experimental quantities to measure for the study of the stability of macromolecules, a material balance on a single bacterial cell is written. For any nondiffusible macromolecule j in a continuously stirred tank reactor (CSTR) containing cells of constant density (mass of cell/volume of cell), the material balance becomes[27]

$$\frac{dC_j}{dt} = r_f - r_d - \mu C_j \tag{3}$$

where C_j is the concentration of the macromolecule j (mol j/cell volume); t is time; r_f is the rate of formation of the macromolecule; r_d is the rate of degradation of the macromolecule; and μ is the specific growth rate. The last term represents the dilution of intracellular components due to growth and the subsequent expansion of the biotic phase.[27]

This equation can be applied specifically to the mRNA of the β-lactamase gene, $mRNA_{\beta\text{-lac}}$, in a chemostat at steady state by assuming the rate of degradation is proportional to the concentration of mRNA within the cell:[28]

$$r_{f,\,mRNA_{\beta\text{-lac}}} = k_{d,\,mRNA_{\beta\text{-lac}}}[mRNA]_{\beta\text{-lac}} + \mu[mRNA]_{\beta\text{-lac}} \tag{4}$$

Solving for the degradation constant, the equation becomes

$$k_{d,\,mRNA_{\beta\text{-lac}}} = \frac{r_{f,\,mRNA_{\beta\text{-lac}}}}{[mRNA]_{\beta\text{-lac}}} - \mu \tag{5}$$

The specific growth rate, μ, is fixed by the dilution rate of the reactor system; therefore, it is fixed by the experimenter.[29] Thus, if the rate of formation of the $mRNA_{\beta\text{-lac}}$ can be determined experimentally, as well as the steady-state level of the message, the degradation rate parameter can be determined.[20] This monomolecular decay rate indicates the stability of mRNA and reveals the extent to which the degradation rate is affected by gene dosage.

Similarly, rRNA was analyzed using the same technique. The equation governing its stability is:

$$k_{d,\,rRNA} = \frac{r_{f,\,rRNA}}{[rRNA]} - \mu \tag{6}$$

RESULTS

Copy Number

The chemostat experimental results are plotted as a function of the steady-state copy number of the reactor culture; however, the relative plasmid levels have not been verified within this lab for M9 minimal medium. Instead, the values shown are based on the copy number determinations made by two independent labs using LB medium. Both Seo et al.[4] and Moser and Campbell[11] determined the copy numbers for these copy-number mutants using LB medium and host HB101. The relative amounts of plasmid DNA within each strain are most important for these results, and this relative level is unlikely to change significantly in different media due to the genetic differences in these plasmids which control their copy number.[4]

β-Lactamase Protein

These experiments originated from the premise that although the gene dosage of the recombinant protein is increased, some limitation in transcription or translation reduces the yield of recombinant protein. The data shown in Figure 2 verify this hypothesis; β-lactamase activity on a
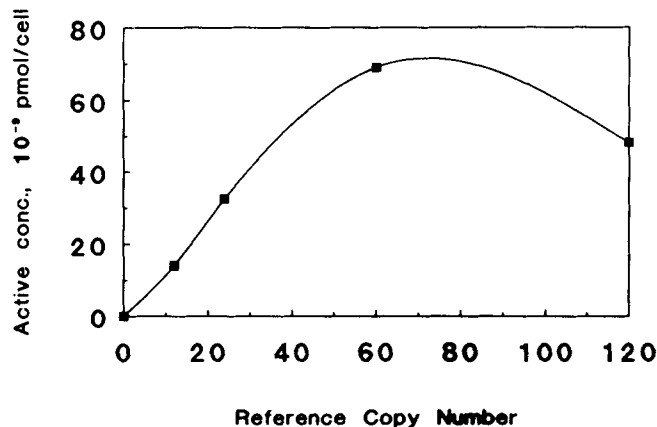
Figure 2. Effect of copy number on β-lactamase activity. The activity shown for copy number 120 has taken into account plasmid instability by dividing the measured activity by the plasmid-bearing cells (70%). Line drawn to indicate trend.

cellular basis reaches a plateau after initially increasing linearly with copy number. The linear portion of the curve mirrors the increase in copy number. Strain HB101/RSF1050 with its copy number of 60 has fivefold the active β-lactamase as the low copy-number strain HB101/pDM247 (copy number 12). The activity shown reflects the enzyme activity present in both the cell and medium. Because of the anomalous curve in the data, the activity of β-lactamase in each chemostat culture was measured three times; in each case the same results were obtained.

β-Lactamase mRNA

Synthesis Rates of β-Lactamase mRNA

Table II summarizes the results of pulse-labelling steady-state cells with ^{3}H-uracil. The disintegrations per minute (dpm) shown are proportional to the synthesis levels of β-lactamase mRNA for each of the strains studied. Care was taken to ensure that the hybridizations were executed under conditions in which the nonlabeled DNA was in excess of the labeled RNA probe.

A monotonic increase in the synthesis rate of β-lactamase mRNA occurred as copy number was amplified (Table II). From these results it appears that mRNA production increases but reaches a plateau for copy numbers 12–120. However, there seems to be a dramatic increase in

Table II. Synthesis rates of β-lactamase mRNA

Strain (HB101/)	Copy number	mRNA synthesis [10^{-6} dpm/(cell · min)]
pDM247	12	1.48
pDM246	24	3.17
pRSF1050	60	—
pDM248	122	3.73
pFH118	408	274.3 (est.)

mRNA production as copy number increases from 122 to 408.

These results are indicative of relaxation of transcription attenuation.[19] The high-copy-number strains, due to the large number of plasmids in these cells, have more promoters. Also, the additional amount of plasmid DNA represents an increase of 28 or 90% of the chromosomal DNA for the copy-number 122 and 408 strains, respectively. Either of these changes in the genome of the bacterial cell may titrate molecules which limit transcription for the lower copy number strains. For example, the *nusA* protein,[30] or an analogous regulator of transcription, may be titrated by the large increase in the number of promoters and active RNA polymerase molecules at those sites. Therefore, β-lactamase mRNA production is no longer attenuated and synthesis increases.

Steady-State Levels of β-lactamase mRNA

Steady-state levels of β-lactamase mRNA were determined using DNA-RNA hybridizations in which β-lactamase DNA was oligolabeled with ^{3}H-deoxycytidine 5'-triphosphate and used to detect the level of β-lactamase mRNA in a sample of nonlabeled RNA derived from steady-state cells. The disintegrations per minute (dpm) shown in Figure 3 are proportional to the steady-state level of β-lactamase mRNA for each of the strains studied. As before, excess hybridization experiments were conducted to ensure that the hybridizations were executed under conditions in which the oligolabeled DNA was in excess of the bound, nonlabeled RNA.

The steady-state levels of β-lactamase mRNA levels increase as copy number increases as shown in Figure 3. As with the mRNA synthesis rates for β-lactamase, there is a corresponding large increase in mRNA as copy number is increased from 122 to 408 and the level of message does not appear to have reached an upper limit. This large increase in β-lactamase mRNA was independently confirmed by using nick translation to radiolabel β-lactamase DNA to determine the steady-state levels of β-lactamase mRNA.
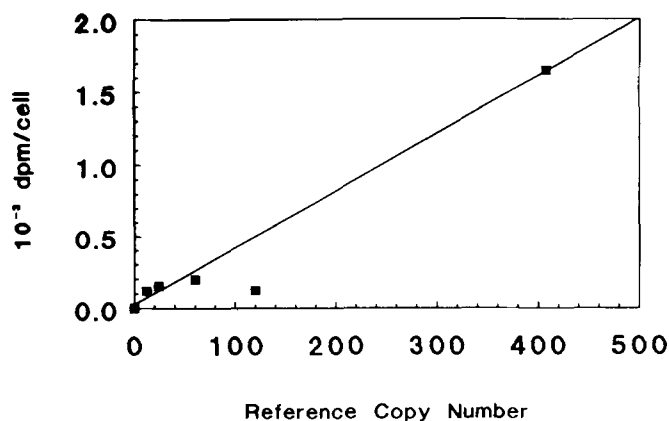

Figure 3. Effect of copy number on the steady-state level of β-lactamase mRNA. Line drawn to indicate trend.

β-Lactamase mRNA Degradation Constant

The β-lactamase mRNA synthesis rates and steady-state levels for each chemostat cultivation can be combined and used to determine the degradation constant, $k_{d,\,mRNA_{\beta\text{-lac}}}$, for this transcript. As shown in eq. (5), $k_{d,\,mRNA_{\beta\text{-lac}}}$ can be calculated for each strain by dividing the synthesis rate $(r_{f,\,mRNA_{\beta\text{-lac}}})$ by the steady-state level of the transcript $([mRNA]_{\beta\text{-lac}})$ and subtracting the specific growth rate μ which was 0.6 h^{-1} for all the chemostat cultivations.

The synthesis rates (dpm/min cell) and steady-state levels (dpm/cell) determined from hybridization experiments are both proportional to the moles of transcripts referred to in eq. (3). Although the two proportionality constants required for the conversion from dpm to moles are different and reflect the hybridization conditions of each experiment, each proportionality constant is fixed for all the strains since all the chemostat cultivations were analyzed at the same time in each hybridization experiment. Therefore, the two constants can be combined to create a single constant. This conversion constant has been arbitrarily set such that the degradation constant for the lowest copy strain is exactly 21/h, an average value found in the literature for the degradation of mRNA.[31] The resulting $k_{d,\,mRNA_{\beta\text{-lac}}}$ for each strain is shown in Table III.

Table III indicates that the β-lactamase degradation constant increases monotonically as copy number increases from 12 to 408, i.e., the mechanism of degradation is becoming more active as both the synthesis rate and steady-state level of β-lactamase mRNA increase.

Also shown in Table III is the transcription efficiency for β-lactamase, $\eta_{mRNA_{\beta lac}}$, defined as:[19]

$$\eta_{mRNA_{\beta lac}} = \frac{r_{f,\,mRNA_{\beta\text{-lac}}}}{G_{\beta lac}} \tag{7}$$

where $G_{\beta\text{-lac}}$ is the number of genes for β-lactamase per cell. Parameter $G_{\beta\text{-lac}}$ is determined by applying the formula of Cooper and Helmstetter[32] to determine the number of chromosomes per cell (1.5 for $\mu = 0.6/h$) and then multiplying by the number of plasmids per chromosome (copy number) for each strain. The transcription efficiency is useful as it reflects the amount and activity of RNA polymerase in the cell.[33] It also incorporates the availability of ribonucleotides and other cofactors.[33]

From the data shown, $\eta_{mRNA_{\beta\text{-lac}}}$ initially decreases from copy number 12 to 122 indicating that transcription is less efficient as the number promoter sites increases. However, the efficiency of transcription increases dramatically for

Table III. The β-lactamase mRNA degradation constants

Strain (HB101/)	Copy number	$k_{d,\,mRNA_{\beta\text{-lac}}}$ (h^{-1})	$\eta_{mRNA_{\beta lac}}$ (10^{-8} dpm/min · gene)
pDM247	12	21	8.2
pDM246	24	36	8.8
pRSF1050	60	—	—
pDM248	122	53	2.0
pFH118	408	291	45.0

the high copy number strain. This somewhat anomalous behavior suggests that either the number of RNA polymerase molecules which form transcription complexes initially is limiting, or that the RNA chain elongation rate is reduced at first and then increases at high copy numbers.

Ribosomal RNA

Synthesis Rates of rRNA

The synthesis rates of rRNA were found by pulse-labelling steady-state cells with ^{3}H-uracil: the measured disintegrations per minute (dpm) shown in Figure 4 are proportional to the synthesis rates of ribosomal RNA for each of the strains studied. Excess hybridization experiments were conducted to ensure that the hybridizations were executed under conditions in which the nonlabeled rrnB DNA used to quantify the level of rRNA transcripts was in excess of the labeled RNA.

For the cells grown in a chemostat, the ribosomal RNA synthesis rate increased as copy number increased as shown in Figure 4. The rate of increase is most dramatic initially as copy number is increased from 0 to 60; the rate of synthesis appears to level off as copy number increases from 60 to 120.

The number of genes for each strain that code for rRNA is independent of copy number since the growth rate and therefore the average chromosome level is constant for all the strains. Hence, any difference in the cell-specific synthesis rates is the result of changes in transcription efficiency for the rRNA operon.

It is reasonable to assume that the increasing metabolic burden of recombinant protein production results in a decrease in the level of free, nontranslating ribosomes, since these ribosomes are actively engaged in recombinant protein production. As shown in Figure 4, the cell responds by enhancing the synthesis of rRNA in an effort to replace its depleted pool of ribosomes. This response is largest at low copy numbers, which implies that the cell is reaching
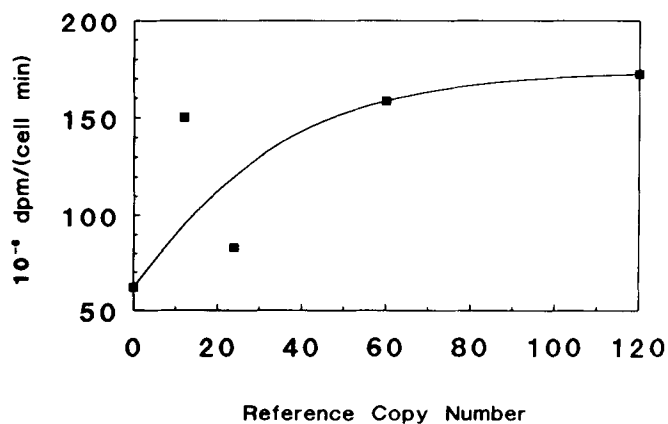
its maximum in production of rRNA as copy number increases.

Steady-State Levels of rRNA

Steady-state levels of ribosomal RNA were determined using DNA–RNA hybridizations in which the DNA probe was oligolabeled with ^{3}H-deoxycytidine 5'-triphosphate and used to detect the level of rRNA in a sample of nonlabeled RNA derived from steady-state cells. The disintegrations per minute (dpm) shown in Figure 5 are proportional to the steady-state level of rRNA for each of the strains studied. Based on excess hybridization experiments, the hybridizations were executed under conditions in which the oligolabeled rrnB DNA was in excess of the bound, nonlabeled RNA so that all of the rRNA would be detected.

Although the synthesis rate of rRNA increased with copy number, the data shown in Figure 5 indicate that the steady-state level of ribosomal RNA actually decreases with increasing copy number. The copy number 60 data point for steady-state rRNA is ignored in the line which indicates the downward trend in Figure 5 because the same downward trend is shown more clearly in the total, steady-state RNA values of Figure 7. Since total RNA consists chiefly of rRNA, the two curves should have the same trend. Additionally, total RNA levels are more readily measured experimentally than steady-state rRNA values, which are the result of lengthy DNA isolation, labelling, and hybridization procedures. The decrease in steady-state levels of rRNA shown in Figure 5 was also independently confirmed by using nick translation.

This indicates that the bacterial cell produces an increasing amount of rRNA which is very labile. It can be inferred that the increased synthesis in ribosomal RNA must not be complemented by an increase in the synthesis of ribosomal protein. Therefore, stable, active ribosomes are not manufactured. The unprotected rRNA then degrades in a manner analogous to that of mRNA.[34] This decrease in rRNA levels as copy number and gene expression increase supports the hypothesis that the primary detrimental effect

Figure 4. Effect of copy number on the synthesis rate of rRNA. Line drawn to indicate trend.
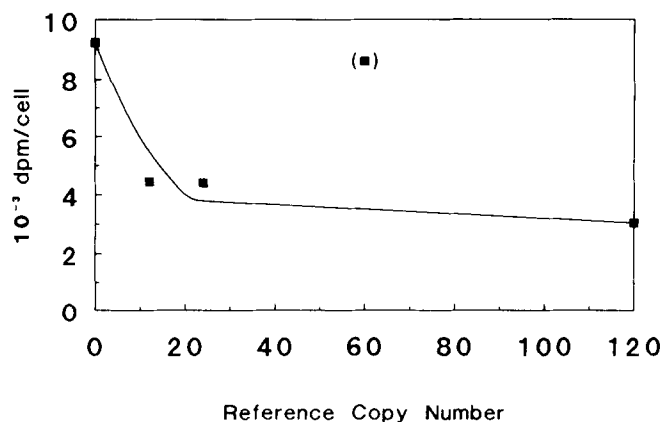

Figure 5. Effect of copy number on the steady-state level of rRNA. Line drawn to indicate trend.

on cell metabolism caused by plasmid gene expression is the reduction of the cell's protein synthesis capability.

Ribosomal RNA Degradation Constant

As in the β-lactamase case, the rRNA synthesis rates and steady-state levels for each chemostat cultivation can be combined and used to determine the degradation constant, $k_{d,rRNA}$, for this transcript. As shown in eq. (6), $k_{d,rRNA}$ can be calculated for each strain by dividing the synthesis rate ($r_{f,rRNA}$) by the steady-state level of the transcript ([rRNA]) and subtracting the specific growth rate μ. Additionally, the conversion constant between the hybridization experiments has been set such that the degradation constant for the plasmid-free host cultivation is 0.07/h, an average value found in the literature for degradation of stable RNA.[31] The resulting $k_{d,rRNA}$ for each strain is shown in Figure 6.

As copy number increases, there is an increase in the degradation constant for rRNA. Therefore, as with β-lactamase mRNA, the degradation process becomes more active as the metabolic load of producing recombinant protein rises.

Total RNA Levels

To determine the total RNA level for each chemostat culture, RNA was isolated from two 10-mL steady-state culture samples. The lower curve in Figure 7 presents the average of the two samples at each copy number for fermentations conducted at 0.6 h^{-1}, and shows the effect of increasing the recombinant gene dosage on the level of total RNA in the bacterial cell. A clear trend is discernible: on a cellular basis, the total level of RNA decreases as copy number increases.

Since approximately 85% of total RNA is rRNA in exponentially growing cells with specific growth rates greater than 0.5 h^{-1},[8] these results serve as an independent check on the steady-state ribosomal RNA results shown in Figure 5. Since the total amount of RNA is decreasing, these
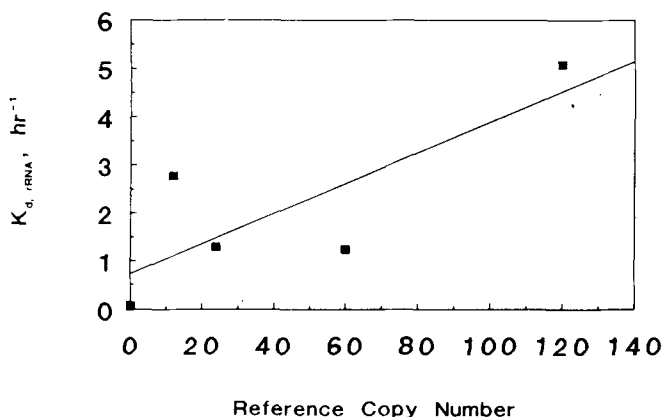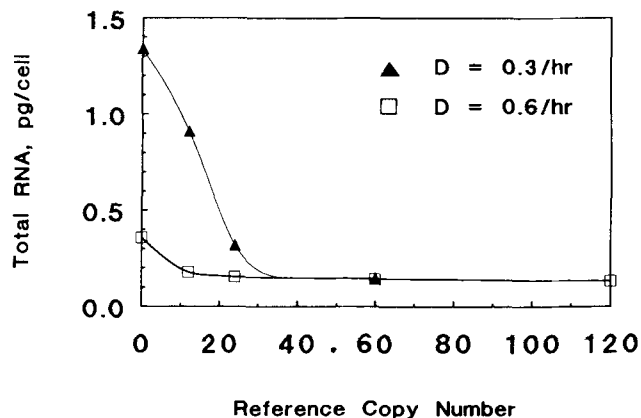

Figure 7. Effect of copy number on total RNA levels. The upper curve (▲) presents the results of chemostat cultures with a specific growth rate of 0.3 h^{-1} while the lower curve (□) presents the results of chemostat cultures with a specific growth rate of 0.6 h^{-1}. Lines are drawn to indicate trends.

results again suggest a decrease in the translational capacity of the recombinant cell as copy number increases.

In order to make sure this trend was correct, total RNA levels were also measured for chemostat cultures grown at specific growth rates of 0.3 h^{-1}. These results are shown in the upper curve of Figure 7. The same trend was again established; as copy number increases, the level of rRNA decreases within the cells. However, instead of the three-fold drop in RNA levels seen at 0.6 h^{-1}, the total RNA levels decreased by an order of magnitude. Furthermore, the same final level of RNA was reached at high copy numbers.

Total Protein Levels

The total protein results are shown in Figure 8 as a function of copy number. Each point represents the average of two separate protein determinations. The copy number 120 data point has been normalized using the plasmid-free host strain results to eliminate the effect of the plasmid-free cells. A downward trend in total protein level as copy number increases is evident.
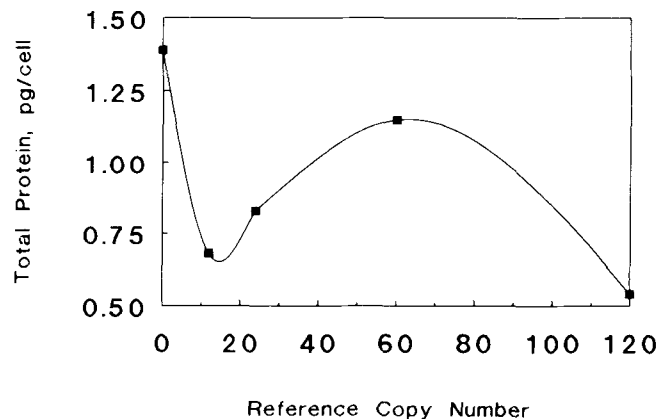

Figure 6. Ribosomal RNA degradation constants. Linear regression line shown.

Figure 8. Effect of copy number on total protein levels. Line drawn to indicate trend.

These results can be combined with the β-lactamase enzyme level results to show that active β-lactamase production varied as a function of copy number from 0.04% (copy number 12) to 2.0% of the total protein/cell (copy number 408). This expression level should be compared to the 30% level seen by other investigators using a tac promoter which is a 17-fold stronger promoter.[2,35] The results also indicate that the plasmid-free strain is the most efficient producer of *total* bacterial protein.

DISCUSSION

The six strains used in this work form a model system for varying gene dosage by varying copy number since each plasmid codes for a single β-lactamase gene which has a constitutive promoter. Discrete levels of gene dosage were obtained simply in the reactor by using each of the five strains with plasmids and by using the plasmid-free host. Because the DNA inserts are small, the plasmids in the system are roughly the same size and are basically identical in all aspects except replication control. This leads to the conclusion that the differences in transcription and translation of the reference protein, β-lactamase, and the variation in transcription of rRNA, can be attributed to the increase in gene dosage. Using this system, it is not necessary to vary the gene dosage through runaway replication, which requires changes in temperature to change the copy number.[36] Altering the temperature would induce many changes in the cellular chemistry, and these changes could mask those due solely to gene dosage.

Further, this system has the advantage that the only major proteins expressed from the plasmid are those required for plasmid replication and β-lactamase. Since the β-lactamase serves as both the reference protein and selectable marker, the analysis is not complicated by the addition of another prominent protein. Panayotatos[37] has shown that expression of a selectable marker (kanamycin) can decrease the expression of other plasmid-coded proteins by 50%.

Plasmid Stability

Plasmid instability for the two high-copy-number plasmids pDM248 (copy number 120) and pFH118 (copy number 410) was noted in the chemostat cultivations. The other three plasmids with lower copy numbers were completely stable. Sixty-nine percent of the chemostat population consisting initially of strain HB101/pDM248 harbored plasmids at steady-state while only 2% of the steady-state chemostat population of strain HB101/pFH118 was plasmid-bearing. This instability occurred in the presence of ample selection pressure (10% MIC). Apparently, a commensal relationship is established between the plasmid-bearing and plasmid-free population. Cells harboring the plasmid secrete β-lactamase at levels which allow the faster-growing, plasmid-free population to exist in the chemostat. Plasmid instability in this system has also been reported by other investigators.[4,19]

Because of this instability, the highest copy number values for the β-lactamase mRNA synthesis rate and the β-lactamase mRNA steady-state level are only estimates. However, β-lactamase mRNA is only produced by plasmid-bearing cells. Since the relative number of plasmid-bearing cells was determined at steady-state, the number of β-lactamase transcripts per cell was simply determined by dividing the hybridization results by the known number of plasmid-bearing cells.

Steady-State β-Lactamase Protein Levels

As shown in Figure 2, the initial increase in β-lactamase levels is proportional to copy number. However, at copy number 120, there is a decline in the enzyme activity. Such a decline has been reported before for these copy-number mutant strains.[19] In general, cloned gene product formation should not keep pace with gene dosage but instead reach a plateau.[4] The decline in β-lactamase level seen in these results does not preclude the existence of such a plateau because only enzyme activity has been measured. A great deal of the recombinant protein produced by the high-copy-number strains may be degraded or inactive. For example, since β-lactamase is known to form inclusion bodies in *E. coli* at high expression levels,[2] the concentration of β-lactamase within the cell for copy number 120 and above may reach the level that causes the formation of inclusion bodies or even a precipitate. If this is the case, β-lactamase production in the cell would not be adequately quantified solely by its activity.

Further, proteolytic enzymes may be produced at higher rates in the higher copy-number cells due to the additional metabolic stress of producing the recombinant protein. This proteolysis would be analogous to the increase in degradation that has been reported due to the stress of carbon starvation in chemostat cultures for proteins with intermediate stability.[38] This hypothesis can be checked by isolating the cellular protein through SDS-PAGE, transferring to nitrocellulose, and probing with an antibody specific for β-lactamase. The number and intensity of the β-lactamase bands found as copy number increases should indicate the effect of increased protein expression on the degradation process.

Ribosomal RNA Synthesis and Degradation

The ribosomal synthesis rates increased three-fold on a cellular basis as copy number increased from 0 to 120 as shown in Figure 4. Moreover, the total level of ribosomal RNA decreased by a factor of two for the same copy number range (Fig. 5). This decrease in rRNA is verified independently by the drop in total RNA levels shown in Figure 7. At these growth rates, total RNA is approximately 85% rRNA[8] so the decrease in total RNA by a factor of 2.7 reflects a decrease in the cellular ribosomal RNA pool. Additionally, total RNA levels from chemostat cultivations at dilution rate 0.3/hr decreased by a factor of 9 as

copy number increased from 0 to 60 (Fig. 7). These results indicate that although ribosomal RNA is being synthesized more rapidly at higher copy numbers, it is not incorporated into stable ribosomes. Instead, an increasing fraction of the ribosomal RNA is degraded at a rate that increases with copy number. The elevated degradation rate at higher copy numbers is demonstrated by the higher magnitude of the degradation constant for rRNA shown in Figure 6. Apparently, the degradation process is more active at higher copy numbers.

The threefold increase in the transcription rate of rRNA is certainly plausible based on the results of Gausing which indicate that *E. coli* can increase rRNA transcription at least by a factor of 2.5 to 4.0 beyond that required for exponential growth in these experiments.[5,10] Gausing's results also suggest that rRNA degradation may be linked to protein production;[10] therefore, overproduction of the recombinant protein may be triggering degradation of the rRNA.

Degradation of rRNA has been observed by Gourse et al.[34] when rRNA was produced in excess of ribosomal protein using a conditional rRNA expression vector. He postulated that the rRNA was not protected by protein and was subsequently degraded as if it were mRNA. In an analogous manner, it is possible that over-production of β-lactamase causes a shortage of ribosomes to develop since the cell is translating a great deal of recombinant protein. The shortage of ribosomes causes the cell to respond by increasing rRNA and ribosomal protein synthesis, but it is possible that ribosomal protein synthesis lags behind rRNA synthesis due to the utilization of the ribosomes for heterologous protein formation. Because ribosomes are not assembled (due to the shortage of ribosomal protein) and the pool of active ribosomes is decreasing due to the increasing translation load, rRNA synthesis continues to increase with increasing copy number. It appears that only ribosomes (*assembled* rRNA and r-protein) are capable of regulating rRNA synthesis by inhibiting transcription of rRNA.[34,39,40] As a result, rRNA synthesis continues and the newly synthesized rRNA is left unprotected. Subsequently, the rRNA is degraded at a rate that increases with copy number.

A deficiency in ribosomal protein synthesis is not the only possible explanation for the accelerated decay of rRNA. Any factor that prevents the formation of ribosomes from rRNA and ribosomal protein could be responsible for this decay.

It is interesting to note that the decrease in total RNA levels and the decrease in steady-state rRNA levels mirror the reported decrease in specific growth rate of the strains used in these experiments. Seo and Bailey[4] have reported that the specific growth rate of HB101 with the plasmids used in this study decreases monotonically from 0.79/h for HB101 (0 copy number) to 0.66/h for HB101/pDM248 (copy number 120) in M9C minimal media. Since the number of ribosomes per unit protein increases linearly with growth rate,[8,10] these results are expected: the ribosome level decreases causing the growth rate of the bacterium to decrease as well.

β-Lactamase Messenger RNA Synthesis and Degradation

The synthesis rates of β-lactamase shown in Table II indicate that mRNA synthesis increases monotonically with copy number. However, there is a burst of synthesis of mRNA at the highest copy number. The 75-fold increase in β-lactamase mRNA synthesis as copy number increased from 120 to 408 agrees well with a 100-fold increase reported in Peretti et al.[19] for the same host and plasmid system. Perhaps an inhibitor of transcription is titrated by the large increase in gene dosage at the high copy number which allows synthesis of mRNA to increase.

Since the synthesis rate of β-lactamase mRNA is increasing, the steady-state levels should rise unless degradation rates increase dramatically. The data in Figure 3 indicate that steady-state β-lactamase mRNA levels increase almost linearly with increasing copy number. There is a 13-fold increase in the steady-state level of β-lactamase mRNA as copy number increases from 120 to 408. This increase agrees well with the nine-fold increase found by Peretti et al.[19] using the same host/plasmid system.

Due to the increase in both β-lactamase mRNA synthesis rates and steady-state levels, the level of β-lactamase mRNA keeps pace with the increase in gene dosage. Hence, protein production is probably not limited at the level of transcription.

Analysis of the degradation constant for β-lactamase mRNA indicates that although the steady-state level of mRNA increases with copy number, the transcripts are degraded at an accelerated rate at high expression levels. This is shown by the 20-fold increase in the magnitude of the degradation constant as the copy number increases from 12 to 408. Hence, the impact of the large burst of mRNA synthesis is reduced by an increase in the mRNA decay rate.

Analysis of the efficiency of transcription of β-lactamase mRNA shown in Table III shows that the efficiency passes through a minimum as copy number increases, with the result that the transcription efficiency is high at elevated levels of expression. This suggests that the RNA polymerase levels or the fraction of RNA polymerase molecules involved in transcription increase with copy number.[19] Also, it may indicate that attenuation of transcription is relieved at high copy numbers.[19] Effector molecules may be titrated by the large number of promoters allowing transcription to proceed more rapidly.[19]

Therefore, the plateau reached in recombinant protein production does not appear to be a result of a deficiency in transcription. Instead, the increase in efficiency indicates that some other aspect of gene expression beyond transcription, such as the level of active ribosomes, limits recombinant protein production in *E. coli*.

These results agree well with those found by Nomura which suggest that mRNA production is in excess.[6] Therefore it appears that the limitation in recombinant-protein production for β-lactamase lies within the translation machinery of the cell and not within mRNA synthesis. In ef-

fect, these amplified copy number results extend the earlier results of Nomura to recombinant systems.

E. coli protein production has not been enhanced by increasing RNA polymerase levels[6] or by efforts directed at enlarging the wild-type ribosome population since feedback regulation prevents accumulation of wild-type ribosomes.[34] However, the data presented here suggest that it may be possible to enhance recombinant protein synthesis by manipulating the ribosome population of the cell. Using mutant ribosomes, it is possible to create a powerful subpopulation of ribosomes which translate solely the recombinant mRNA.[41]

These specialized ribosomes differ from wild-type proteins in that they have mutations in the anti-Shine-Dalgarno site of the 16S subunit.[41] This mutation is constructed to match a base change in the ribosome binding site of the cloned gene mRNA. One of the main advantages of this system is that the mutations in the specialized ribosome prevent feedback regulation of their own synthesis.[42] Therefore, specialized ribosomes should enhance the translational capacity of the cell by increasing the specificity of the ribosomes for the cloned-gene product and by increasing the pool of ribosomes within the cell.

CONCLUSIONS

The experimental data shown here suggest that the translational machinery of the bacterial cell limits recombinant protein production in *E. coli*. The β-lactamase mRNA synthesis and steady-state levels both increase with copy number, indicating transcription is not limiting protein synthesis. Also, for both β-lactamase mRNA and rRNA, degradation rates increase with increasing copy number.

In contrast to the β-lactamase mRNA results, decreasing steady-state ribosomal RNA levels and decreasing total RNA levels with increasing copy number imply that limitations develop within the translational machinery of the bacterial cell. These data suggest that the fundamental reason for the decrease in growth rate as protein expression increases is the decrease in the ribosome population within the cell.

References

1. A. Wyke, *Chem. Eng. Prog.*, **84**, 16 (1988).
2. G. A. Bowden and G. Georgiou, *Biotechnol. Prog.*, **4**, 97 (1988).
3. E. Remaut, P. Stanssens, and W. Fiers, *Gene*, **15**, 81 (1981).
4. Jin-Ho Seo and J. E. Bailey, *Biotechnol. Bioeng.*, **27**, 1668 (1985).

5. K. Gausing, "Regulation of Ribosome Biosynthesis in *E. coli*," in *Ribosomes: Structure, Function, and Genetics*, G. Chambliss, G. R. Craven, J. Davies, K. Davis, L. Kahan, and M. Nomura, Eds. (University Park Press, Baltimore, 1980).
6. M. Nomura, D. M. Bedwell, M. Yamagishi, J. R. Cole, and J. M. Kolb, "RNA Polymerase and Regulation of RNA Synthesis in *Escherichia coli*: RNA Polymerase Concentration, Stringent Control, and Ribosome Feedback Regulation," in *RNA Polymerase and the Regulation of Transcription*, W. S. Reznikoff et al., Eds. (Elsevier, New York, 1987).
7. G. Nilsson, J. G. Belasco, S. N. Cohen, and A. von Gabain, *Proc. Nat. Acad. Sci.*, **84**, 4890 (1987).
8. J. L. Ingraham, O. Maaloe, and F. C. Neidhardt, *Growth of the Bacterial Cell* (Sinauer Associates, Sunderland, MA, 1983).
9. A. von Gabain, J. G. Belasco, J. L. Schottel, A. C. Y. Chang, and S. N. Cohen, *Proc. Nat. Acad. Sci.*, **80**, 653 (1983).
10. K. Gausing, *J. Mol. Biol.*, **115**, 335 (1977).
11. D. R. Moser and J. L. Campbell, *J. Bacteriol.*, **154**, 809 (1983).
12. F. Hefron, M. So, and B. J. McCarthy, *Proc. Nat. Acad. Sci.*, **75**, 6012 (1978).
13. F. Hefron, P. Bedinger, J. J. Champoux, and S. Falkow, *Proc. Natl. Acad. Sci.*, **74**, 702 (1977).
14. H. W. Boyer and D. Roulland-Duossoix, *J. Mol. Biol.*, **41**, 459 (1969).
15. P. H. Pouwels, B. E. Enger-Valk, and W. J. Brammar, *Cloning Vectors. A Laboratory Manual* (Elsevier, Amsterdam, 1983).
16. K. Dennis, F. Srienc, and J. E. Bailey, *Biotechnol. Bioeng.*, **27**, 1490 (1985).
17. J. Brosius, A. Ullrich, M. A. Raker, A. Gray, T. J. Dull, R. R. Gutell, and H. R. Noller, *Plasmid*, **6**, 112 (1981).
18. R. L. Rodriguez and R. C. Tait, *Recombinant DNA Techniques. An Introduction* (Benjamin/Cummings, Menlo Park, CA, 1983).
19. S. W. Peretti and J. E. Bailey, *Biotechnol. Bioeng.*, **34**, 902 (1989).
20. S. L. Berger and A. R. Kimmel, Eds., *Guide to Molecular Cloning Techniques* (Academic, Orlando, 1987).
21. P. P. Dennis and M. Nomura, *J. Mol. Biol.*, **97**, 61 (1975).
22. T. Sawai, I. Takahashi, and S. Yamagishi, *Antimicrob. Agents Chemother.*, **13**, 910 (1978).
23. M. G. Sargent, *J. Bacteriol.*, **95**, 1493 (1968).
24. L. Stryer, *Biochemistry*, 2nd ed. (Freeman, San Francisco, 1981).
24a. T. Maniatis, E. F. Fritsch, and J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982).
25. T. Yamamoto, S. Yamagata, K. Horii, and S. Yamagishi, *J. Bacteriol.*, **150**, 269 (1982).
26. F. C. Kafatos, C. W. Jones, and A. Efstratiadis, *Nucl. Acids Res.*, **7**, 1541 (1979).
27. A. G. Fredrickson, *Biotechnol. Bioeng.*, **18**, 1481 (1976).
28. S. B. Lee and J. E. Bailey, *Biotechnol. Bioeng.*, **26**, 1372 (1984).
29. J. E. Bailey and D. F. Ollis, *Biochemical Engineering Fundamentals* (McGraw-Hill, New York, 1986).
30. J. Greenblatt, *Can. J. Biochem. Cell Biol.*, **62**, 79 (1984).
31. M. M. Domach, S. K. Leung, R. E. Cahn, G. G. Cocks, and M. L. Shuler, *Biotechnol. Bioeng.*, **26**, 203 (1984).
32. S. Cooper and C. E. Helmstetter, *J. Mol. Biol.*, **31**, 519 (1968).
33. S. W. Peretti and J. E. Bailey, "Transcription from Plasmid Genes, Macromolecular Stability and Cell Specific Productivity in *E. coli* Carrying Copy Number Mutant Plasmids," Ph.D dissertation, California Institute of Technology, Pasadena, CA, 1986.
34. R. L. Gourse, Y. Takebe, R. A. Sharrock, and M. Nomura, *Proc. Nat. Acad. Sci.*, **82**, 1069 (1985).
35. M. Brunner and H. Bujard, *EMBO J.*, **6**, 3139 (1987).
36. M. J. Betenbaugh, V. M. diPasquantonio, and P. Dhurjati, *Biotechnol. Bioeng.*, **29**, 1164 (1987).
37. N. Panayotatos, *Gene*, **74**, 357 (1988).
38. A. C. St. John, K. Jakubas, and D. Beim, *Biochim. Biophys. Acta*, **586**, 537 (1979).
39. R. L. Gourse, R. A. Sharrock, and M. Nomura, "Control of Ribosome Synthesis in *Escherichia coli*," in *Structure, Function, and*

Genetics of Ribosomes, B. Hardesty and G. Kramer, Eds. (Springer-Verlag, New York, 1985).

40. S. Jinks-Robertson, R. L. Gourse, and M. Nomura, *Cell*, **33**, 865 (1983).

41. A. Hui and H. A. de Boer, *Proc. Nat. Acad. Sci.*, **84**, 4762 (1987).

42. M. Yamagishi, H. A. de Boer, and M. Nomura, *J. Mol. Biol.*, **198**, 547 (1987).