Construction of a Specialized-Ribosome Vector for 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

Received October 19, 1990/Accepted March 5, 1991

An expression system utilizing specialized ribosomes has been constructed with β-galactosidase as the product. Ribosomes specific for *lacZ* mRNA are generated due to a mutation within the anti-Shine-Dalgarno region of a plasmid-borne 16S rRNA gene that is complementary to a mutation within the ribosome-binding site of *lacZ*. Hence, a subpopulation of ribosomes specific for translation of the cloned-gene mRNA is produced. Transcription of the *lacZ* gene is regulated by the *tac* promoter, while transcription of the mutated *rrnB* locus is controlled by the λp_L promoter. Batch experiments indicate that full induction of both operons (2 m*M* IPTG, 42°C) leads to maximal β-galactosidase activity per cell at levels 35% higher than that obtained using a wild-type ribosome expression system. Using a novel, site-directed mutagenesis technique, construction of the specialized ribosome vector is outlined, and the results of *lacZ* expression are presented as transcription of both the cloned-gene and the specialized-ribosome locus are induced.
Key words: ribosome vector • cloned-gene expression • *Escherichia coli*

INTRODUCTION

It is generally understood that gene expression is governed by the rate of transcription of the DNA locus, the stability of its mRNA, and the rate at which the transcript is translated.[7] Our group has been investigating the relative importance of these protein synthesis reactions in plasmid-based expression systems designed to produce recombinant protein. Using both an amplifiable-copy-number series of plasmids in which the copy number was varied from 0 to over 400[28] and a fixed-copy-number plasmid in which transcription was regulated over a 500-fold range by derepressing the *tac* promoter,[29] we have shown that synthesis of the cloned-gene mRNA by RNA polymerase does not limit protein expression (in the presence of a strong promoter). However, as cloned-gene expression is increased by amplifying copy number or by inducing transcription, the stability of the cloned-gene transcript decreases due to a 12- to 14-fold increase in the mRNA degradation rate constant.[28,29] Hence, the stability of the transcript becomes increasingly important at high expression levels.

Our results have also indicated that the total protein levels within the cell follow closely the steady-state concentrations of rRNA.[28,29] The total RNA concentration within the cell also decreased as the copy number increased in the amplified-copy-number experiments.[28] Both of these results indicate that there may be significant translational limitations in the bacterial cell as cloned genes are expressed.

Further evidence of translational limitations are supplied by the decrease in specific growth rate that occurs upon amplification of plasmid copy number and upon expression of cloned genes.[1,3] Since the ability of the cell to produce protein is linked closely to the specific growth rate (the synthesis of ribosomes varies as the square of the specific growth rate[12]), it appears the competition between the chromosome-directed, host metabolic activity and the plasmid-directed metabolic activity leads to a net reduction in the ability of the cell to translate cloned-gene transcripts.

Based on these results, it appeared reasonable to alter the protein synthetic capacity of the cell in order to possibly alleviate some of the translational limitations involved in expressing cloned genes. The methodology for altering the ribosomes was provided by H. A. de Boer and co-workers.

By studying the complementary relationship between the bases within the ribosome binding site (RBS) of a transcript and the anti-Shine-Dalgarno site (ASD) of the 3′ end of the 16S ribosomal RNA, de Boer et al. found that increasing homology between the mRNA and 16S rRNA by increasing the homologous region from 4 bases to 8 or 13 bases can lead to a reduction in translation.[5] This group then tested if complementarity was required at all for translation by mutating the RBS of the leukocyte interferon A gene such that it would hydrogen bond less with the wild-type sequence of the 3′ end of the 16S ribosomal RNA (ASD). The group constructed two types of mutations in the RBS of the leukocyte interferon A gene that diverge from the consensus RBS; these are shown as System IX and X of Table I. Using these RBS mutations, but retaining the wild-type 16S rRNA sequence, they found that expression of leukocyte interferon A gene was reduced to undetectable levels.[5] This indicates that wild-type ribosomes are unable to translate these mutant RBS regions.

To determine if the consensus SD region itself was necessary for translation or if complementation between

* To whom all correspondence should be addressed.

Biotechnology and Bioengineering, Vol. 38, Pp. 891–906 (1991)

CCC 0006-3592/91/080891-16$04.00

Table I. Specialized ribosome complementary mutations.

Mutation System	RBS sequence (mRNA)	ASD sequence (16S rRNA)	de Boer mutant ASD vectors (ref. 10)
Wild-type	5′ GGAGG 3′	5′ CCUCC 3′	pASDVIII-p_L
IX	5′ CCUCC 3′	5′ GGAGG 3′	pASDIX-p_L
X	5′ GUGUG 3′	5′ CACAC 3′	pASDX-p_L

a ribosome and transcript was necessary and sufficient for translation, the 16S rRNA gene of the *rrnB* locus was mutated in order to make ribosomes that would hydrogen bond to the mutated System IX and X RBS mutations of a cloned gene.[5] In effect, *specialized* ribosomes (Fig. 1) were created as a second kind of ribosome in the bacterial cell that preferentially translates the mRNA of the cloned gene since only this transcript has a RBS capable of hydrogen bonding completely to the mutated 16S rRNA of the mutant ribosomes. Using this system to express human growth hormone (hGH), they found that wild-type ribosomes were incapable of translating the mutant RBS of the hGH mRNA; however, specialized ribosomes were capable of producing active hGH.[5] In their specialized-ribosome system, the hGH gene with the mutant RBS is constitutively transcribed while transcription of the mutant 16S rRNA of the *rrnB* locus is under control of the λp_L promoter. Human growth hormone was produced at slightly less than 50% of the levels found within a construct which uses a wild-type RBS and does not produce specialized ribosomes.

In the specialized ribosome system, cloned-gene mRNA is translated by a specific pool of ribosomes that does not translate other cellular transcripts to a large extent.[10] Hence, the cloned-gene mRNA does not compete for wild-type ribosomes in the specialized ribosome system, and cloned-gene expression has the potential to be enhanced.[11] Specialized ribosomes may also increase the pool size of both types of ribosomes within the cell since it has been shown that the mutation in the 16S rRNA that generates specialized ribosomes short circuits the feedback inhibition mechanism responsible for regulation of ribosomes.[30] In this manner, specialized ribosomes may increase the total translational capacity of the cell.

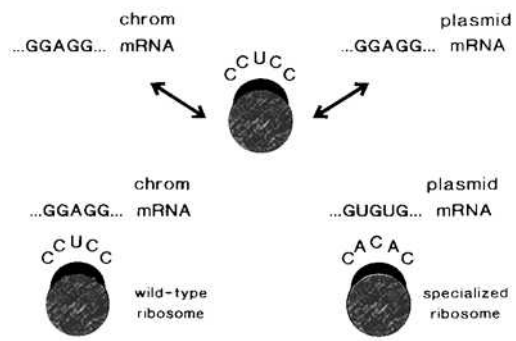

Figure 1. Schematic of specialized ribosomes. The upper portion shows competition between chromosomal mRNA and plasmid cloned-gene mRNA for a wild-type ribosome. The lower portion indicates affinity of wild-type and specialized ribosomes for their respective mRNA.

To study specialized ribosomes further, a specialized ribosome system has been constructed that produces β-galactosidase. Using a novel site-directed mutagenesis technique, we have constructed a single vector in which transcription of the β-galactosidase mRNA with its mutant System X RBS is induced by the addition of the lactose-analog IPTG, and transcription of the specialized 16S rRNA (and the rest of the *rrnB* locus) is controlled by increasing the fermentation temperature above 32°C. In this way, transcription and translation of the cloned-gene product (β-galactosidase) have been decoupled. This vector facilitates the study of the metabolic impact of enhancing transcription (without subsequent translation), and the impact of producing additional ribosomes on cellular metabolism. Segregational stability of the vector is maintained by the *parB* locus.[27] Construction of the specialized-ribosome vector is outlined, and β-galactosidase expression as a function of induction of the *lacZ* gene as well as induction of the mutant *rrnB* locus is presented.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Enzymes

Strain JM109 [*recA1, endA1, gyrA96, thi*$^-$, *hsdR17, supE44, relA1,* Δ(*lac,proAB*), {F′*traD36 proAB*$^+$ *laqI*^{q}*ZΔM15*}][31] with the λ lysogen NM1070 (*Wam403 Eam1100 lacZ*$^+$ *cI857 nin5 Sam100*)[16] was used to amplify plasmid pASDX-p_L (Fig. 2). This host was used because the *recA1* mutation reduces the chance of homologous recombination, and the temperature-sensitive *c*I857 repressor prevents transcription of this specialized ribosome locus when bacterial growth is maintained at 30°C. Transformation[15] of pASDX-p_L into strains which lack the *c*I repressor results in transformants with large

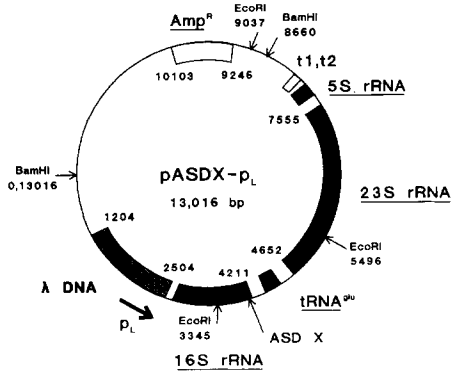

Figure 2. Plasmid pASDX-p_L (ref. 10) with mutated *rrnB* locus. The mutated 16S rRNA is transcribed (along with the whole *rrnB* locus) under control of the λp_L. ASDX indicates the System X mutation within the 16S rRNA (5′-CACAC-3′). Amp^r represents the gene which confers ampicillin resistance, and t1 and t2 are the strong, transcription termination sites of *rrnB*.

deletions occurring within pASDX-p_L (unpublished results). The plasmid was amplified using 170 μg/mL chloramphenicol and CsCl centrifugation as described below. During cell growth and plasmid amplification, ampicillin was present at 50 μg/mL.

For construction of pTKW201, BK6 [Δ(*lacIPOZ*)*C29, lacY*$^+$, *hsdR, galU, galK, strA', leuB6, trpC9830,* Δ(*srl-recA*)306::Tn*10*][27] was used as the host since it is a *recA*$^-$ strain; therefore, little homologous recombination between plasmid and chromosome could occur. The JM101-derivative K5716 [λ$^+$, Δ(*lac,proAB*), *supE,* {F'*traD36 proAB*$^+$ *laqI*^{q}ZΔ*M15*}][10] was used for the construction of pTKW413 since it is readily transformed and has a wild-type *c*I repressor.

Characterization of expression of β-galactosidase using the specialized ribosome system via temperature induction of the λp_L was performed in K12ΔH1 [ATCC No. 33767; K-12 M72, F$^-$, Sm^R, *lacZ*am, Δ*bio-uvrB,* (λ*Nam7, Nam53, c*I857, ΔH1)][22,32] and M5219 [M72, *lacZ*am, *trp*am, Sm^R, (λ*bio252, c*I857, ΔH1)].[22] The ΔH1 deletion includes lambda genes *cro-R-A-J-b2;* this mutation deletes about 80% of the lambda phage genome.[9] Two *recA*$^-$ versions of these strains were also transformed with the specialized ribosome vectors pTKW413 and pTKW430 to check expression of β-galactosidase: NO3203[8] was obtained as a *recA* version of M5219, and NCChE1 was constructed by inducing the Δ(*srl-recA*)306::Tn*10* deletion in strain K12ΔH1 with P1 *kc* transduction.[27]

Plasmid pTKW106, a pBR322-derivative, has been described previously[27,29] and is shown in Figure 3. It consists of a functional *lacZ* gene under control of the *tac* promoter, the *lacI*^{Q} gene for strong repression of the *tac* promoter, the APH gene (kan^r), and the *parB* locus to enhance segregational stability. This plasmid was used in construction of the specialized ribosome plasmid, and it was transformed into K12ΔH1 for use as a control.

Plasmid pASDX-p_L is shown in Figure 2 and was obtained in the form of miniscreen DNA from Herman de Boer. It was constructed[10] by placing the specialized ribosome locus (mutated *rrnB* locus) within plasmid pBR322 (Fig. 2). In this vector, the indigenous tandem promoters of the mutant *rrnB* locus have been replaced by the λp_L promoter such that specialized ribosome rRNA is produced when the temperature is shifted from 30 to 42°C in strains which harbor the *c*I857 repressor. The p_L promoter was also inserted so that antitermination and post-transcription processing of the rRNA locus occurs as in the wild-type system.[33] The p_L promoter lies at the end of the 1300 bp of inserted lambda DNA (approximately 35,583–36,883 bp using the number scheme of *Lambda II*[9]) that is adjacent to *rrnB.*

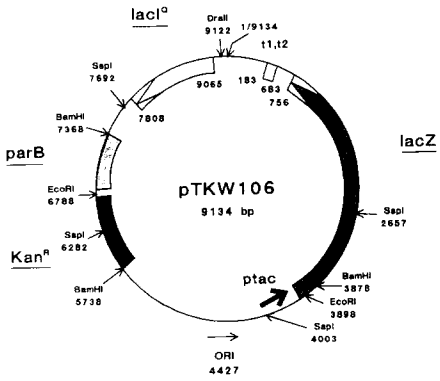

Figure 3. β-Galactosidase expression vector pTKW106. The chemically inducible *tac* promoter controls transcription from *lacZ,* the *parB* locus enhances segregational stability, and Kan^r represents the gene that confers resistance to kanamycin. The strong, transcription termination sites t1 and t2 are also present.

The restriction enzymes were purchased from Boehringer Mannheim and New England BioLabs; T4 polynucleotide kinase was obtained from New England Biolabs; T4 DNA polymerase, alkaline phosphatase, and ATP were bought from Boehringer Mannheim; and T4 DNA ligase was purchased from Bethesda Research Labs. The ultrapure dNTPs were obtained from Pharmacia.

Media and Fermentation Conditions

M9 minimal medium[23] containing 0.2 wt % glucose, 0.4 wt % casamino acids, and 0.0018 wt % tryptophan was used initially to express β-galactosidase in the specialized-ribosome strain. Subsequent batch fermentations in which β-galactosidase was expressed as a function of temperature and IPTG levels were performed with LB medium[23] containing 10 g Bacto tryptone, 5 g Bacto yeast extract, and 10 g NaCl per liter. Selection pressure was maintained for all fermentations with kanamycin (50 μg/mL).

The fermentations (21–25 mL) were conducted in 250-mL Erlenmeyer flasks shaken at 250 rpm in a New Brunswick or LabLine circulating air shaker. The optical density of the cell suspensions was monitored using a Shimadzu UV160U Spectrophotometer with a constant temperature cell set at 25°C and wavelength fixed at 600 nm.

For the β-galactosidase expression experiments, overnight cultures were inoculated from −40°C stocks and grown for 12–16 h at 30°C in LB-Kan at 250 rpm. To initiate each batch fermentation, 1–5 mL of the overnight culture were added to 20 mL of fresh medium, and the cells were grown at 30°C until the cell suspension reached an optical density (OD) of 0.2 (0.5–1.0 h). The fermentations were then shifted to the appropriate temperature (30–45°C), and grown for 8.68 h. In this way, the batch fermentations were conducted such that the cells were induced during the early exponential phase of growth at the same cell density. Five-milliliter samples were then taken, and the cells were placed on ice. Additionally, at the start and end of each fermentation, a 1.5-mL sample of cell suspension was taken to monitor the plasmid DNA stability, and

MacConkey–Kan plates were streaked to check for the *lacZ*$^+$ phenotype.

The experiments in which the time-course of β-galactosidase expression was followed differed from the above in that samples were removed over a 23-h period. Also, the cellular OD at the time of induction was as indicated.

β-Galactosidase Activity and Total Protein

The β-galactosidase assay has been described previously.[29] Briefly, 5–10 mL of each fermentation were centrifuged to form a cell pellet, and the supernatant was removed and placed on ice until assayed. The cells were resuspended and sonicated at 21 kHz for 1.33 min. The cell homogenate and supernatant were then assayed spectrophotometrically by following the color change in a Shimadzu UV160U Spectrophotometer with a constant temperature cell set at 25°C. The activity of the β-galactosidase was then calculated using Beer's law.[29]

Sigma Protein Kit No. 690 was used to analyze the total protein content of the cells of each fermentation. Two samples (7.5 μL) of the sonicated cellular homogenate of each fermentation that was used to determine β-galactosidase activity were analyzed and the results were averaged to determine the total cellular protein.

Plasmid Isolations and Horizontal Gel Electrophoresis

One liter of LB medium supplemented with kanamycin and chloramphenicol was used for the large-scale plasmid isolations. After centrifugation, the cells were lysed with lysozyme, and the cell wall and other insoluble material was precipitated using Triton X-100. RNA was removed from the supernatant with RNase and DNA was precipitated by adding PEG. Plasmid DNA was isolated from chromosomal DNA using CsCl ultracentrifugation (60,000 rpm). After precipitating with ethanol, the concentration of the DNA was then determined by its absorbance at 260 nm.

Rapid plasmid isolations were conducted using the protocol of Rodriguez and Tait.[23] Briefly, 1.5 mL cell suspension was spun at 1.5×10^4 rpm in a microfuge, washed and resuspended in sucrose solution, then lysed with lysozyme in the presence of RNase. Sodium acetate was used to precipitate chromosomal DNA, and the plasmid DNA was precipitated from the supernatant using 2-propanol. The pDNA was washed with 80% ethanol and resuspended in TE (10 m*M* tris, pH 7.6, and 1 m*M* EDTA).

Horizontal agarose gel electrophoresis was used to separate and identify plasmid DNA bands.[15] Gels were cast with 0.6 wt % agarose (Bio-Rad) and electrophoresed in TBE buffer (89 m*M* tris, 89 m*M* boric acid, 2.0 m*M* EDTA, pH 8.5) in the presence of 0.5 μg/mL EtBr for 500–1000 V h.

Specialized Ribosome Vector Construction

The specialized ribosome vector consists chiefly of the *lacZ* gene with a mutated Shine–Dalgarno region, and the *rrnB* locus with a complementary mutation in the 3′ end of the 16S portion (the anti-Shine-Dalgarno region). It was desired to have the *lacZ* gene under the direction of the *tac* promoter, and transcription of the *rrnB* locus to be directed by the λp_L promoter. In this manner, the level of transcription of *lacZ* is controlled by the addition of the inducer IPTG, and the level of specialized rRNA is controlled by derepressing the λp_L promoter due to inactivation of the chromosome-based, temperature-sensitive *c*I857 repressor. Hence, construction of the specialized ribosome vector involved mutating the RBS of *lacZ* in the stable vector pTKW106[27] to form pTKW201. The *rrnB* locus of pASDX-p_L was then placed within pTKW201 to form plasmids pTKW413 and pTKW430, the specialized ribosome vectors which differ in the orientation of the *rrnB* locus. The general cloning techniques that follow (T4 polynucleotide kinase reactions, T4 DNA polymerase reactions, dephosphorylation reactions, etc.) are derived from those presented by Hui et al.,[11] Maniatis et al.,[15] and Berger and Kimmel.[2]

pTKW201

The five-base pair mutation in the *lacZ* RBS of pTKW106 (which renders translation of this recombinant gene under the control of the specialized ribosomes) was created using a novel site-directed mutagenesis technique which is outlined in Figure 4. Due to the proximity of the multiple cloning site (MCS) in pTKW106 to the RBS of *lacZ* (42 bp downstream), a *Sma*I site within the MCS provided an unique opportunity to mutate the RBS of *lacZ*. Since two *Sma*I sites are present in pTKW106, the DNA was partially digested with *Sma*I (2.5 μg digested for 2 min with 3 units of enzyme) so that pTKW106 was predominantly cut once leaving approximately 50% of the DNA uncut and producing almost no plasmid DNA that had been restricted at both sites (verified through gel electrophoresis).

In order to expose a single-stranded section of the pDNA so that a synthetic 29-base oligomer could bind (containing the mutation in the RBS that forms the System X mutant RBS), the 3′ ends of the blunt *Sma*I site were digested with T4 DNA polymerase such that 120 nucleotides should have been removed according to Maniatis et al.[15] The 29 nt oligo (5′-AACAATTTCACA GTGTG AACAGCGATGAA-3′) was synthesized and purified at the NCSU Genetics Department Molecular Biology Center. After the oligomer was phosphorylated with T4 polynucleotide kinase, the 29 nt oligomer (Fig. 4) was added (molar ratio 1000:1 oligo to vector) to the pTKW106 vector that had been digested with *Sma*I and T4 DNA polymerase.

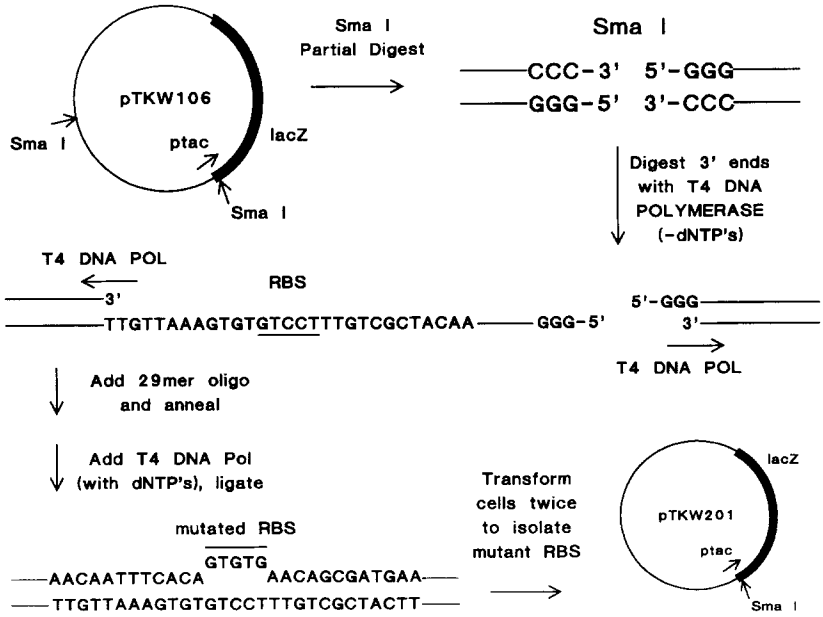

Figure 4. Construction of pTKW201.

The single-stranded gaps were then filled by adding T4 DNA polymerase and dNTPs. The T4 DNA polymerase was chosen over the Klenow fragment since T4 DNA polymerase does not strand-displace the oligomer during the fill-in reaction.[19]

Blunt-end ligation was carried out in the presence of dNTPs to prevent any T4 DNA polymerase that may be present from acting as an exonuclease. Ligation proceeded overnight at 12°C (low temperature) to avoid melting the oligo from the DNA template. The results of the ligation and partial digest reactions were checked by a electrophoresis using a 0.6 wt % agarose gel. The resulting plasmid pTKW201 is shown in Figure 4.

BK6 cells were made competent according to the method of Maniatis et al.,[15] and were transformed with 300 ng DNA in the ligation solution. Since the cells which are transformed with the mutant plasmid initially have one copy of both the mutant plasmid pTKW201 and the wild-type pTKW106 (Fig. 4), both wild-type and mutant transformants appear red on MacConkey agar plates. However, cells which replicate the mutant plasmid pTKW201 have a growth advantage when grown in the presence of 0.5 m*M* IPTG since the pTKW201 mRNA for β-galactosidase is not translated due to the mutation in the Shine-Dalgarno site.[5] Therefore, 100 μL of the transformation mixture was transferred to 10 mL of LB with kanamycin (50 μg/mL) and IPTG (0.5 m*M*) to induce expression of β-galactosidase in the wild-type BK6/pTKW106. The difference in growth rate between BK6/pTKW201 and BK6/pTKW106 is slightly less than 0.68–0.61/h, the respective growth rates of BK6/pTKW106 with and without 0.5 m*M* IPTG,[27] since transcription still occurs in BK6/pTKW201 although translation is prevented.

After 24 h, 100 μL of the LB-Kan-IPTG solution was diluted and spread on MacConkey–Kan plates. BK6/pTKW201 colonies were chosen as white colonies, and the pDNA was screened by a DNA miniscreen.[23] The plasmid DNA was cut with *Bam*HI and compared to *Bam*HI restricted pTKW106 DNA. Since the suitable transformants may still harbor some pTKW106, the pDNA was again obtained through a miniscreen and used to transform BK6. The BK6/pTKW201 cells were selected as white colonies resistant to kanamycin. In this manner, pTKW201 was obtained that was uncontaminated by pTKW106. A large-scale plasmid prep was used to acquire purified pTKW201 pDNA, and the plasmid DNA was subjected to restriction enzyme analysis using *Bam*HI, *Eco*RI, *Sca*I, *Hin*dII, *Ssp*I, *Eco*O109I, and *Sma*I. Horizontal gel electrophoresis was used to separate the DNA fragments. A restriction map of pTKW201 is shown in Figure 5.

pTKW413

An outline of the construction of the specialized ribosome vector pTKW413 is shown in Figure 6. The mutated ASD of the 16S rRNA gene and the rest of the *rrnB* locus of pASDX-p_L (Fig. 2) was isolated by restricting pASDX-p_L with *Bam*HI. This enzyme cuts pASDX-p_L at the two sites surrounding the *rrnB* locus (Fig. 2). The larger, 8660 bp fragment was isolated using a 0.6 wt % agarose horizontal electrophoresis gel and was made blunt by filling the restriction sites with T4 DNA polymerase.

The recipient plasmid pTKW201 which harbors the altered RBS of *lacZ* was cut at its unique *Eco*O109I site between the $lacI^Q$ and transcription terminators of *lacZ*. The ends of the linearized pTKW201 were then made blunt by T4 DNA polymerase in the presence of dNTPs.[15] In order to prevent religation of pTKW201, the blunt vector was dephosphorylated with alkaline phosphatase.[15]

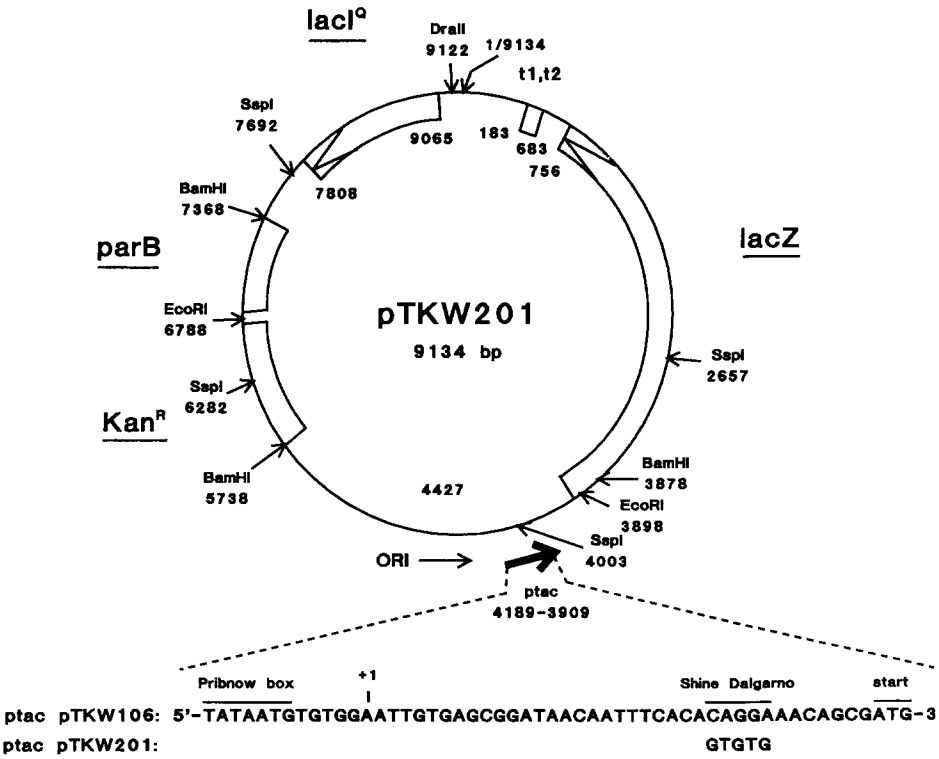

Figure 5. Restriction map of pTKW201. The mutated Shine–Dalgarno region of pTKW201 is compared to that of pTKW106. The first nucleotide of the mRNA is shown by +1.

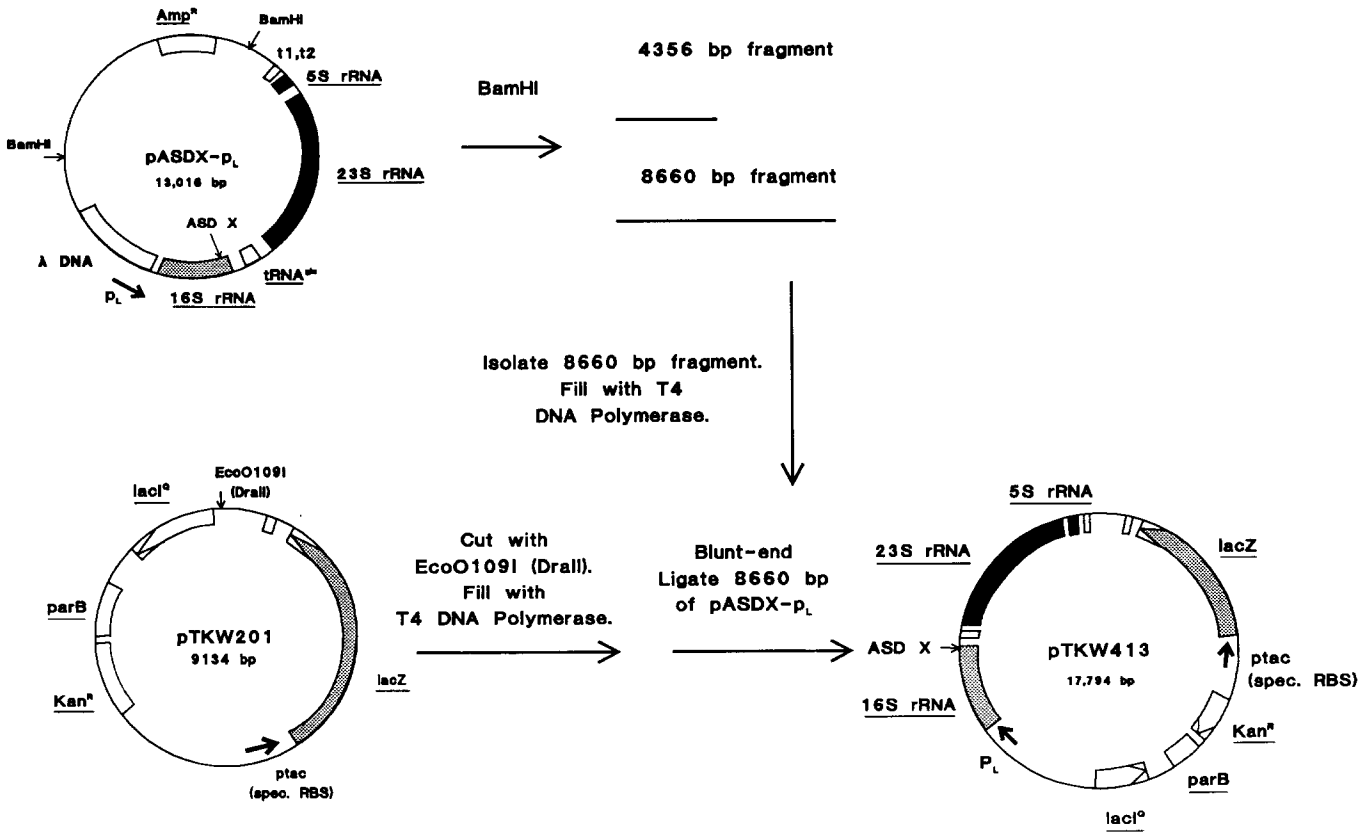

Figure 6. Construction of specialized ribosome vector pTKW413.

The 8660 bp fragment containing the specialized ribosome locus was blunt-end ligated to pTKW201 using a 10:1 molar ratio. The blunt 8660 bp fragment (0.8 pmol) was added to the dephosphorylated, blunt pTKW201 (0.08 pmol). The mixture was ligated for 16.5 h at 23°C.[23] These conditions fix the j/i ratio (the effective concentration of one end of a molecule relative to the other [j] over the total concentration of ends in the ligation mixture [i]) at 0.5 so linear concatamers should be formed preferentially.[23]

In order to obtain pTKW413 from the ligation mixture, the intermediate strain K5716 was used as the host since it was transformed more easily than the *c*I857-bearing strains K12ΔH1 and M5219. (Attempts to isolate pTKW413 from the ligation mixture with host strains K12ΔH1 and M5219 were unsuccessful.) Transformation was achieved by adding roughly 350 ng of ligation mixture to 200 μL of competent K5716 cells and pulsing the cells for 2 min at 42°C.[15] In order to reduce the stress on the recipient cells, the transformation solution was spread (100 μL) on LB-Kan plates rather than MacConkey–Kan plates (which induce transcription of the *lacZ* gene).

Kanamycin-resistant colonies were then scored using MacConkey–Kan plates: one plate was exposed to 42°C for 15 min then kept at 37°C overnight while another MacConkey–Kan plate was kept at 30°C. Suitable transformants were selected by choosing colonies which had a very slight red color and which grew slowly on MacConkey–Kanamycin agar at 37°C compared to 30°C. The slow growth is probably due to the presence of some specialized ribosomes formed as a result of incomplete repression of the p_L promoter by the single copy of the *c*I repressor on the chromosome. Colonies containing pTKW413 or pTKW430 were identified by rapid plasmid isolations followed by an *Eco*RI digest and gel electrophoresis.

A large-scale plasmid prep was used to obtain purified pDNA; this pDNA was characterized by cleaving with *Eco*RI, *Bam*HI, *Ssp*I, *Sac*I, *Xba*I, *Bst*EII, *Xba*I/*Bst*EII, *Kpn*I, *Eco*O109I, and *Sma*I and visualized using horizontal gel electrophoresis. The four bands of the *Eco*RI digest serve to establish the orientation in which the 8660 bp *rrnB* locus was inserted into pTKW201 (Fig. 7). Transformants were found with the 8660 bp fragment inserted in both orientations. The plasmid with the two sets of t1, t2 transcription terminators adjacent was numbered pTKW413, and the opposite orientation of the 8660 bp fragment was numbered pTKW430.

In order to characterize the specialized ribosome vectors, pTKW413 and pTKW430 were transformed into NO3203, M5219, K12ΔH1, and NCChE1. Plasmid DNA obtained from a rapid plasmid prep[23] of K5716/pTKW413 was transformed into each of these hosts using a 2-min pulse at 42°C.[15]

RESULTS

Since both specialized ribosome vectors pTKW413 and pTKW431 appeared to produce equivalent amounts of β-galactosidase (they differ solely in the orientation of the *rrnB* locus), pTKW413 was chosen for the remain-

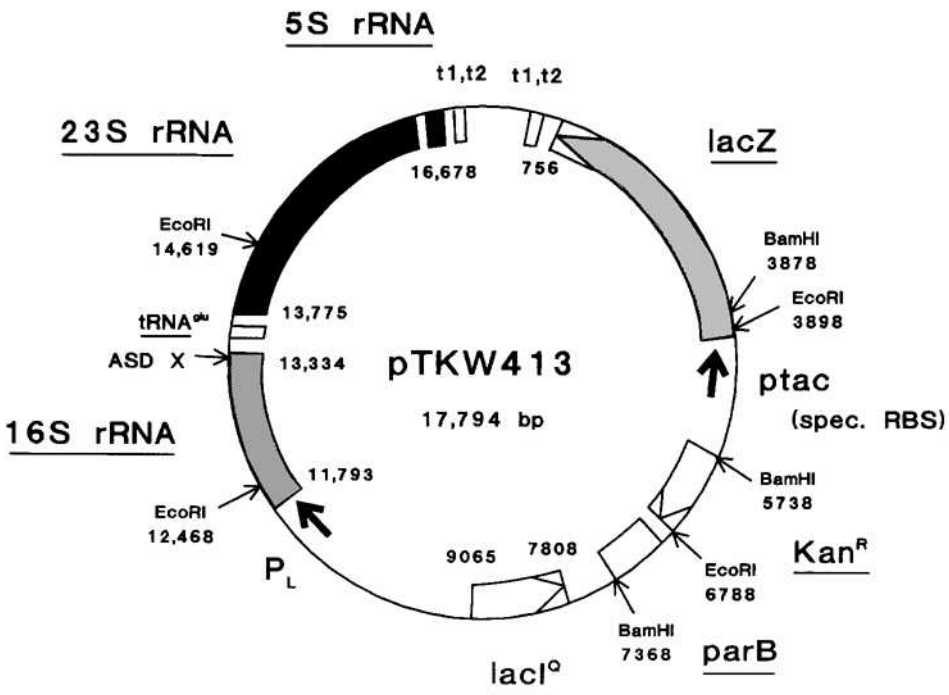

Figure 7. Specialized-ribosome vector pTKW413. The *lacZ* gene containing the mutated RBS (5′-GTGTG-3′) is transcribed from the chemically-inducible *tac* promoter, and the mutated 16S rRNA is transcribed from the λp_L which is temperature-inducible due to the presence of the temperature-sensitive, λ repressor *c*I857 in the host K12ΔH1.

ing studies. Using this plasmid, host K12ΔH1 appeared to produce slightly more β-galactosidase on MacConkey agar plates compared to strain M5219, so K12ΔH1/pTKW413 was chosen as the specialized ribosome strain.

Growth Rates and Expression in Minimal Medium

As *lacZ* is expressed in the specialized ribosome strain K12ΔH1/pTKW413, the growth rate of the bacterium decreases steadily in minimal medium. After shifting the temperature from 30 to 40°C and adding IPTG, the instantaneous growth rate decreases from 0.8 to 0.25 h^{-1} at IPTG concentrations of 0.04, 0.20, and 1.0 m*M* IPTG in the first 5.5 h of growth. During this induction period, the absorbance readings were very low ($A_{600\,nm}$ = 0.015–0.060), so growth was not limited by the nutrient or waste concentrations. Instead, induction of specialized ribosomes via the p_L promoter appears to have a profound effect on cellular metabolism in minimal medium. This appears consistent with the fact that cultures grown at 30°C and with 2.0 m*M* IPTG achieve steady growth rates of 0.73 h^{-1}; these cultures produce β-galactosidase mRNA in large quantities but the p_L promoter and its specialized-ribosome locus are repressed due to the low temperature.

In addition to a decrease in growth rate, significant cell lysis was seen after 20 h of expression of *lacZ* with minimal medium in K12ΔH1/pTKW413. The resulting intracellular and supernatant β-galactosidase activities are shown in Table II. In these experiments, batch cultures were grown in shake flasks at the indicated temperatures with the inducer concentration fixed at 2 m*M* IPTG. The supernatant β-galactosidase activity increased from 14 to 470% of the intracellular activity; therefore, complex media was used in the remaining experiments to limit cell lysis and to limit the impact of the production of specialized ribosomes on cellular metabolism. Cell lysis and decelerating growth rate have been reported by other researchers using the p_L promoter and minimal medium.[25] Hence, it is difficult to determine whether the specialized ribosomes or the p_L promoter cause the lysis and growth problems in minimal medium.

Table II. The β-galactosidase expression with K12ΔH1/pTKW413 in minimal medium with 2.0 m*M* IPTG.

Temperature (°C)	β-Galactosidase activity, (nmol ONPG cleaved/min AU)	
	Intracellular	Supernatant
30	77	11
40	234	146
42	365	1690

Effect of Specialized Ribosomes on Growth Rate in Complex Medium

To discern the impact of specialized ribosomes on cellular metabolism, the specific growth rate was measured as a function of induction of the specialized ribosome locus, *rrnB,* in complex (LB) medium. Since it is necessary to change the temperature to induce synthesis of the specialized ribosomes in this system, the specific growth rate of the host was determined under the same conditions as a control. By comparing the growth rates of the two strains, K12ΔH1 and K12ΔH1/pTKW413, the effect of temperature on the cellular components other than the specialized ribosome system and its p_L promoter is effectively removed since it should affect both strains to the same extent. Since no IPTG was added to either strain, there is essentially no β-galactosidase mRNA synthesized in the plasmid-bearing strain.

The respective growth rates at the indicated temperatures are presented in Table III. At each temperature from 30 to 42°C, the growth rate of the specialized ribosome strain is considerably less than that of the host (which contains only wild-type ribosomes). This indicates the specialized ribosomes influence cell growth to a large extent. Furthermore, the decrease in specific growth rate is greatest (32%) at 39.5°C. This corresponds closely to the maximum induction temperature of the p_L promoter (41°C) where it is expected production of specialized ribosomes is highest.

Since the specialized ribosomes significantly decrease the growth rate even at low temperatures, these results also suggest the p_L promoter is incompletely repressed (although some of the reduction in growth rate relative to the host is caused by the metabolic burden of plasmid replication). Additional results (described below) verify that the p_L promoter is repressed inefficiently (Table VI).

Time Course of β-Galactosidase Production: Specialized Ribosomes vs. Wild-Type Ribosomes

To further evaluate the specialized ribosome vector K12ΔH1/pTKW413, the time course of β-galactosidase expression was followed as transcription of the special-

Table III. Effect of specialized ribosomes on the specific growth rate (μ) in the absence of *lacZ* mRNA induction (0 m*M* IPTG) and in LB medium.

Temperature (°C)	Specific Growth Rate, μ (h)		Decrease in μ (%)
	K12ΔH1	K12ΔH1/pTKW413	
30	0.91	0.77	15
37	1.91	1.66	13
39.5	2.09	1.42	32
42	1.46	1.21	17

ized *rrnB* locus and *lacZ* are induced simultaneously. Production of β-galactosidase in the specialized ribosome system for this time-course experiment is shown in Figure 8 for cells grown at 42°C (to induce the λp_L promoter of *rrnB*) and in the presence of 2 m*M* IPTG (to induce the *tac* promoter of *lacZ*). On the ordinate, the enzyme activity has been normalized by the level of total cellular protein so that the results reflect the amount of active β-galactosidase enzyme each cell contains. Time on the abscissa represents the hours elapsed since the addition of the inducer IPTG (2 m*M*) and the simultaneous shift in temperature from 30 to 42°C.

It appears that after an initial lag in which the enzyme activity increases sluggishly, β-galactosidase production capacity increases linearly for approximately 7 h then reaches its maximum. The 3-h lag period probably reflects the time required to produce functional specialized ribosomes since *lacZ* mRNA has been shown to be produced without any measurable lag time after the addition of IPTG.[17] Additionally, in wild-type ribosome systems at all growth rates, it takes less than two minutes to produce active β-galactosidase enzyme after the addition of IPTG.[13,18] No lag period has been seen in our lab using wild-type ribosomes to express β-galactosidase. After the initial lag, it appears that the population of specialized ribosomes increases steadily which results in the synthesis of more β-galactosidase.

To verify these results, an additional experiment was conducted in which the same fermentation conditions were used except the initial cell density at time 0 was $A_{600\,nm} = 0.133$ rather than $A_{600\,nm} = 0.086$. The same trend as that shown in Figure 8 was obtained except the time at which the maximum enzyme production occurred was shifted forward to 9.3 h rather than 11.7 h. The same maximum value in β-galactosidase production was obtained. From these results, it was determined that subsequent fermentations should be conducted for roughly 9 h so that the cells would have enough time to produce specialized ribosomes and make β-galactosidase at appreciable levels. These results seem reasonable in that Okita et al.[20] report maximum product yields for the production of malaria antigens in complex medium occur after 4 to 7.3 h. These authors used an analogous host that contains the *c*I857 repressor and ΔH1 mutation in the lambda lysogen as well as temperature shifts to induce the λp_L promoter.

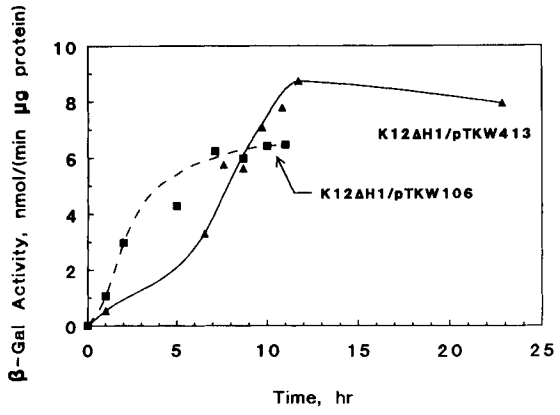

Figure 8. Time course of β-galactosidase production in K12ΔH1/pTKW413 (solid line, specialized ribosomes) and control K12ΔH1/pTKW106 (dashed line, wild-type ribosomes) at 2.0 m*M* IPTG and 42°C. Lines are drawn to indicate trends.

As a control for synthesizing β-galactosidase using wild-type ribosomes, the *lacZ* locus was expressed in strain K12ΔH1/pTKW106. Plasmid pTKW106 (Fig. 3) does not produce specialized ribosomes since it lacks the mutated *rrnB* locus, and the *lacZ* mRNA that is transcribed upon addition of IPTG has the wild-type RBS. The time course of β-galactosidase expression is plotted in Figure 8. At time zero, exponentially growing cells ($A_{600\,nm} = 0.200$) were shifted from 37 to 42°C, and IPTG was added (2 m*M*).

Initially, the concentration of β-galactosidase increases more rapidly in the wild-type system compared to the specialized-ribosome strain. After approximately 7.0 h at 42°C and 2.0 m*M* IPTG, the β-galactosidase activity in K12ΔH1/pTKW106 reaches a plateau at a concentration that is 25% less than that produced in the specialized ribosome strain (6.47 vs. 8.73 nmol/min μg protein). Therefore, specialized ribosomes appear more effective in producing β-galactosidase than the wild-type ribosomes. β-Galactosidase was also expressed at 37°C in K12ΔH1/pTKW106; the enzyme activity after 8.68 h was 5.8 nmol/min μg protein.

Optimal Induction of the Specialized Ribosomes

There is some uncertainty in the literature concerning the appropriate temperature conditions for optimal induction of the λp_L promoter.[20] For example, some authors suggest increasing temperature from 30 to 42°C for 15 min then reducing temperature to 37°C to induce transcription.[11] Others recommend increasing the temperature to 42°C for the remainder of the experiment after a suitable cell density has been obtained.[22,24]

To determine the appropriate time of exposure at 42°C for production of specialized ribosomes via derepression of the p_L promoter, a series of fermentations was conducted in which each culture was grown for 8.68 h in the presence of 1.0 m*M* IPTG; however, the length of time each culture was exposed to 42°C was varied from 0 to 8.68 h. After the initial exposure to 42°C, the cultures were shifted to 37°C for the remainder of the 8.68 h.

The results are shown in Table IV with the β-galactosidase activity reported per microgram of total protein. It appears that the maximum amount of β-galactosidase is synthesized if the culture is kept at 42°C for the entire time of induction. Exposure to 42°C for 1.5 h at the start of the fermentation leads to β-galactosidase synthesis at 75% of the maximum.

Table IV. The β-galactosidase expression in K12ΔH1/pTKW413 as a function of *initial* time at 42°C followed by a shift to 37°C (1.0 m*M* IPTG).

Time at 42°C (h)	β-Galactosidase activity (nmol ONPG cleaved/min μg protein)
0	1.90
0.25	1.57
0.75	2.05
1.50	4.61
8.68	6.05

An experiment was also performed to determine if β-galactosidase synthesis is affected by the time at which IPTG is added to the medium to induce *lacZ* mRNA transcription. Five cultures were exposed to 41°C for 8.68 h so that specialized ribosomes were continuously produced; however, IPTG (1.0 m*M*) was added at progressively later times to induce *lacZ* mRNA synthesis. The results are shown in Figure 9 and indicate that the largest amount of enzyme is synthesized when *lacZ* mRNA is transcribed for the duration of the experiment. Enzyme activity falls off linearly as the exposure to the chemical inducer decreases.

These results suggest that it may be possible to increase β-galactosidase production by inducing β-galactosidase mRNA synthesis continuously and shifting the culture from 37 to 42°C so they are exposed at 42°C for greater than 1.5 h. In this way, the cells are grown at the optimum temperature (37°C) for at least part of the fermentation; this may increase mRNA synthesis and its stability as well as decrease enzyme denaturation and degradation. To test this hypothesis, cultures were grown in the presence of 2 m*M* IPTG and shifted to 42°C after 0, 2, 4, or 6 h at 37°C during 8.68-h fermentations. The resulting β-galactosidase activities are presented in Table V. It is clear the highest level of β-galactosidase is synthesized when the cells are grown at 42°C continuously without any exposure to 37°C. In this way, specialized ribosomes and β-galactosidase mRNA are produced for the duration of the fermentation.

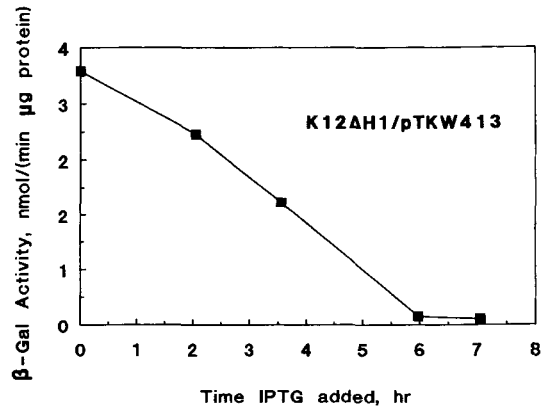

Figure 9. β-Galactosidase expression in K12ΔH1/pTKW413 as a function of time of IPTG addition (1.0 m*M*) at 41°C. Line drawn to indicate trend.

One additional experiment was conducted to determine the optimum conditions for expression of the specialized ribosomes: a culture of K12ΔH1/pTKW413 was exposed to 42°C for 15 min of each hour to fully induce the *rrnB* locus. The culture was kept at 37°C for the remaining 45 min of each hour while *lacZ* mRNA was continuously expressed by 2.0 m*M* IPTG. At the end of 8.68 h, the concentration of β-galactosidase was assayed at one-half the level found in cultures that were continuously exposed to 42°C. Therefore, to maintain β-galactosidase synthesis at near optimum levels, the remaining fermentations were conducted such that the cultures were grown continuously at the specified induction temperature, and the IPTG concentration was kept constant during the entire experiment.

β-Galactosidase Activity versus IPTG

In order to ascertain that β-galactosidase mRNA is indeed produced by induction of the *tac* promoter and to determine the impact of cloned-gene transcription in a simplified manner, a series of fermentations using K12ΔH1/pTKW413 was performed in which the inducer of transcription of β-galactosidase mRNA, IPTG, was varied from 0 to 10 m*M* while the temperature was kept constant and high. Under these conditions, specialized ribosomes are produced continuously during the fermentation. For all of these experiments, at time zero the temperature was changed from 30 to 42°C, and IPTG was added to the fermentation medium. The cells were grown for 8.68 h at 42°C, then the β-galactosidase activity was assayed. The results are presented in Figure 10 from 0 to 1.0 m*M* IPTG using a logarithmic scale.

Over the course of *lacZ* mRNA transcription induction, β-galactosidase enzyme activity varies over a 1300-fold range indicating that transcription induction has a profound effect on enzyme synthesis and that there is remarkably tight control of the *tac* promoter. The maximum in activity occurs essentially at 0.5 m*M* and is essentially unchanged by higher concentrations of IPTG: at 1.0, 2.0, 5.0, and 10.0 m*M* IPTG, the β-galactosidase activity was approximately constant at 6.0, 5.5, 5.2, and 5.9 nmol/min μg protein, respectively.

Most importantly, the range of inducibility is very narrow since the bulk of induction occurs between 0.0 and 0.5 m*M* IPTG. Chemostat studies using pTKW106, the parent plasmid which lacks the mutated *rrnB* lo-

Table V. The β-galactosidase expression in K12ΔH1/pTKW413 as a function of the time at which the cultures are shifted from 37 to 42°C (2.0 m*M* IPTG).

Time at 37°C (h)	β-Galactosidase activity (nmol ONPG cleaved/min μg protein)
0	5.62
2	2.09
4	1.84
6	1.59

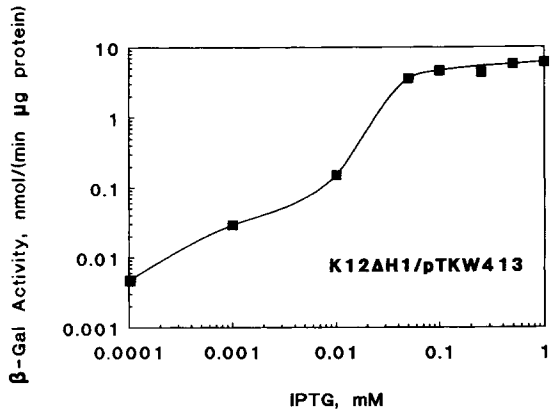

Figure 10. β-Galactosidase expression in K12ΔH1/pTKW413 as a function of IPTG at 42°C. The line is drawn to indicate trend.

cus, revealed that the *tac* promoter was inducible up to 7.5 m*M* IPTG in this wild-type ribosome system with the *lacI*^{Q} repressor on the plasmid.[29] Hence, in the specialized-ribosome strain, either further mRNA transcription occurs beyond 0.5 m*M* IPTG but there are not additional specialized ribosomes to translate the additional *lacZ* transcripts, or transcription at the *rrnB* locus under the direction of the p_L promoter limits further transcription at the *tac* promoter.

Plasmid pTKW413 was stable during the course of these experiments; no segregational or structural instability was seen. Furthermore, there was essentially no cell lysis since negligible β-galactosidase was found in the supernatant (less than 3% of intracellular activity at all IPTG concentrations).

β-Galactosidase Activity versus Temperature

Induction of functional specialized ribosomes was studied by inducing transcription of the *rrnB* locus: derepression of the p_L promoter by temperature shifts leads to the formation of mutant 16S rRNA that is incorporated into functional ribosomes.[10] These specialized ribosomes have high specificity for the β-galactosidase mRNA. In these experiments, IPTG levels were maintained high (2.0 m*M*) to ensure that *lacZ* mRNA concentrations were always elevated so translation of the β-galactosidase transcript would limit enzyme synthesis. The results are presented in Figure 11 where the enzymatic activity is plotted on a total protein basis.

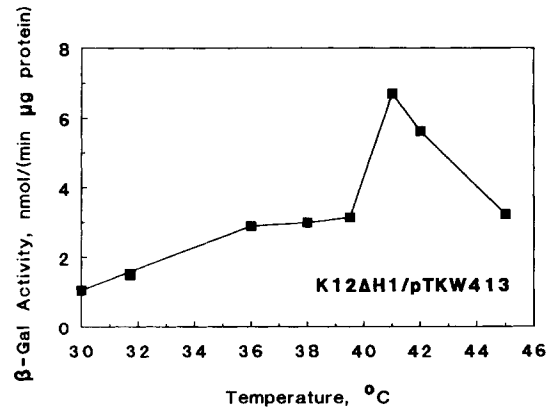

Figure 11. β-Galactosidase expression in K12ΔH1/pTKW413 as a function of temperature with 2.0 m*M* IPTG. The line is drawn to indicate trend.

It is clear that β-galactosidase activity reaches a sharp maximum at 41°C. This indicates that 41°C is the optimal temperature for induction of the p_L promoter for specialized ribosome synthesis. This result agrees well with the data of Okita et al. who showed induction of two malaria antigens is favored at 39.5 and 41°C when the λp_L promoter is used.[20]

As temperature is varied, β-galactosidase activity is inducible only over a 6.3-fold range. Since the maximum in activity of 6.7 nmol/min μg protein at 41°C agrees well with the maximum which occurred when temperature was fixed at 42°C and IPTG concentrations were varied (Fig. 10, 6.0 nmol/min μg protein), it appears the lack of inducibility is due to leakiness of the λp_L promoter at 30°C. The enzyme activity at 30°C and 2.0 m*M* IPTG is 225-fold higher than that which occurs when IPTG is not added to the fermentation and subsequently β-galactosidase mRNA is not produced (Fig. 10). The plasmid from which the specialized-ribosome plasmid is derived (pTKW106) has a copy number of approximately 84,[29] therefore, the p_L promoter on the specialized-ribosome vector is probably repressed inefficiently by the *C*I857 repressor which exists as only a single copy on the chromosome. This is substantiated by the results presented in the following section.

Plasmid pTKW413 was stable (segregationally and structurally) during the course of these experiments as evidenced by the plasmid DNA bands from mini-preps that were separated by agarose gel electrophoresis and by growth on MacConkey agar plates. The pDNA was obtained from fermentation samples taken at the end of each experiment. Essentially no cell lysis occurred since negligible β-galactosidase was found in the supernatant (less than 2.5% of intracellular activity) except at the extreme temperature of 45°C where extracellular β-galactosidase activity was 15% of the intracellular activity.

β-Galactosidase Expression: *c*I Repressor and Wild-Type Ribosomes

From the previous experiments it is clear that temperature control of β-galactosidase expression in the specialized ribosome system (p_L promoter) is not as tightly regulated as *ptac*-controlled transcription of *lacZ* since substantial β-galactosidase is made even at 30°C in the presence of 2.0 m*M* IPTG, and the growth rate of K12ΔH1/pTKW413 is reduced relative to the host alone even at low temperatures. It appears two explanations are possible: either wild-type ribosomes recognize the mutant RBS of *lacZ* mRNA made from pTKW413, or the p_L promoter of the specialized rRNA locus is incompletely repressed by the *c*I857 repressor at low tempera-

tures. If the *rrnB* locus is always transcribed to some extent due to incomplete repression of p_L, then specialized ribosomes are always present in the cell and are free to translate the *lacZ* mRNA whenever it is made.

To determine if β-galactosidase was produced due to recognition of the mutant RBS of *lacZ* by wild-type ribosomes, enzyme expression was checked in BK6/pTKW201 (Fig. 5). This vector contains the *lacZ* gene with the specialized ribosome RBS that is transcribed from the *ptac* promoter; therefore, the *lacZ* mRNA should not be recognized by wild-type ribosomes. Cells were grown for 8.68 h at 30°C in the presence of 2.0 m*M* IPTG so that transcription of the *lacZ* gene was maximum. As shown in Table VI, no detectable β-galactosidase is made under these conditions; therefore, the wild-type ribosomes are *completely* unable to recognize the mutant RBS of *lacZ*, even when there are many transcripts available for transcription. This agrees well with the results of de Boer's group which was unable to detect leukocyte interferon and human growth hormone in constructs with the same mutant RBS.[5] To ensure the stability of the plasmid during this experiment, the plasmid was isolated before and after expression, cleaved with *Eco*RI, and the bands were separated using horizontal electrophoresis. No structural instability was seen.

This result implies strongly that the p_L promoter is not fully repressed at low temperatures (30°C); therefore, transcription of the specialized ribosome rRNA and the formation of active specialized ribosomes occurs at low temperatures. This hypothesis was verified by comparing the extent β-galactosidase is expressed in K5716/pTKW413 and K12ΔH1/pTKW413. The main difference in these strains is that host K12ΔH1 has the temperature-sensitive (mutant) *c*I857 repressor whereas K5716 has the wild-type *c*I repressor. The results are shown in Table VI for expression after 8.68 h at 30°C and 2 m*M* IPTG. Since the β-galactosidase activity in the strain with the wild-type *c*I repressor (K5716) is 1/500 that of the temperature-sensitive strain (K12ΔH1), it is clear that the β-galactosidase activity seen at low temperatures is the result of incomplete repression of the p_L promoter by *c*I857. The mutation that causes the temperature-sensitive phenotype must render the repressor much less efficient. The relatively high copy number increases this effect since the large number of p_L promoters titrates the repressor pool. The stability of these plasmids during the course of the experiment was verified as with pTKW201 using a miniprep and electrophoresis.

These β-galactosidase expression results were also easily verified qualitatively by growing the three strains on MacConkey–Kan plates. As expected, K12ΔH1/pTKW413 colonies turned red after 12–24 h at 30°C due to inefficient repression of the p_L promoter while colonies of K5716/pTKW413 and BK6/pTKW201 did not turn red after 70 h.

Plasmid Stability

No *segregational* instability was encountered during the course of these experiments or during the plasmid constructions. It appears the *parB* locus on the specialized-ribosome plasmid successfully prevents plasmid-free segregants from taking over the fermentations by killing them as they arise.[27]

However, the specialized ribosome plasmid pTKW413 (Fig. 7) was not *structurally* stable for long periods since plasmid rearrangements and deletions were sometimes noticed after approximately two subcultures (44 hr) in LB–Kan in K5716 (wild-type *c*I). For this reason the fermentation medium was always inoculated from −40°C stocks, and the plasmids were checked at the start and end of each fermentation.

The structural instability appears to be due to the large regions of homology between the plasmid and the chromosome as well as the size of the plasmid. The *rrnB* locus (5480 bp), *lacZ* gene (3110), and $lacI^Q$ gene (1161 bp) of plasmid pTKW413 are all homologous with the corresponding loci on the chromosome.

To check if a reduction in homology leads to an increase in stability, plasmid pASDX-p_L (Fig. 2) was transformed into strain K12ΔH1, grown in LB-Ap, and its pDNA was monitored. This plasmid contains the p_L promoter and the specialized *rrnB* locus; however, it lacks both the $lacI^Q$ and *lacZ* loci (a reduction in homology of 4300 bp).

For three consecutive periods of 21 h, K12ΔH1/pASDX-p_L was grown in a 10-mL culture tube at 250 rpm and 30°C. At the end of each period, the pDNA was isolated using a mini-prep and checked by gel electrophoresis. A 10-μL sample of the turbid cul-

Table VI. The β-galactosidase expression as a function of *c*I repressor and wild-type ribosomes at 30°C and 2 m*M* IPTG.

Strain	β-Galactosidase activity (nmol ONPG cleaved/min μg protein)
BK6/pTKW201 (no specialized ribosomes)	0.000
K5716/pTKW413 (wild-type repressor *c*I)	0.002
K12ΔH1/pTKW413 (temperature-sensitive *c*I857)	1.026

ture was then diluted into 10 mL of fresh medium. During the three days of growth (which included both exponential and stationary phases), no structural instability of the plasmid was observed. Therefore, it appears the instability of pTKW413 is due to the larger homologous region.

Effect of *recA*

In an effort to reduce the structural instability of the plasmid, pTKW413 was placed into two hosts, NCChE1 and NO3203, which have a *recA* mutation in addition to the *c*I857 repressor. This *recA* mutation is a deletion which has been shown to reduce the ability of the cell to undergo homologous recombination with the chromosome by a factor of 3.6×10^4.[6] NCChE1 is isogenic with K12ΔH1 (the usual specialized-ribosome expression host) except for the *recA* mutation. NO3203 has a slightly different deleted lambda prophage than K12ΔH1 (see the Materials and Methods Section).

Using MacConkey plates at 30 and 37°C, both of these hosts had no detectable expression of β-galactosidase. These results were checked by expression of NCChE1/pTKW413 and NO3203/pTKW413 at 42°C for 8.68 h with 2.0 m*M* IPTG. Neither strain produced measurable quantites of β-galactosidase and cell lysis occurred in NO3203/pTKW413. It therefore appears that the $recA^+$ genotype is necessary for complete inactivation of the *c*I857 repressor for formation of specialized ribosomes.

The effect of the *recA* mutation on plasmid stability was also examined for NCChE1/pTKW413 and NO3202/pTKW413. After four serial dilutions in the presence of antibiotic selection pressure, pTKW413 underwent a structural mutation in both strains. Approximately 1.5×10^4 bp were deleted from pTKW413 which resulted in the formation of a 2600-bp plasmid that lacks any homology with the chromosome (*lacZ, rrnB,* and $lacI^Q$ deleted). Hence, the *recA* mutation did not increase plasmid stability significantly in these strains.

DISCUSSION

β-Galactosidase Expression with Specialized Ribosomes

A specialized ribosome vector pTKW413 has been constructed which produces *lacZ* mRNA with an altered RBS by inducing the *tac* promoter. Upon increasing temperature, this vector also transcribes a mutated *rrnB* locus which leads to production of a subpopulation of ribosomes that translate specifically the specialized *lacZ* mRNA. It has been shown that the specialized *lacZ* mRNA is not translated by wild-type ribosomes. This follows from the observation that β-galactosidase is not synthesized at detectable levels when the strain BK6/pTKW201 overproduces specialized *lacZ* mRNA. Since no specialized ribosomes are made in this strain, only wild-type ribosomes are present and they are incapable of recognizing the mutant RBS. Since induction of transcription of the specialized *rrnB* locus from the p_L promoter in K12ΔH1/pTKW413 leads to β-galactosidase synthesis, functional specialized ribosomes must be synthesized in this strain. This also indicates that the mutant 16S rRNA is correctly processed from the large *rrnB* transcript, and it is incorporated into specialized ribosomes that actively translate the specialized *lacZ* mRNA.

Since the largest concentration of enzyme is reached if the cells constantly transcribe mRNA (Fig. 9), high level expression of β-galactosidase requires the cell to continuously synthesize mRNA such that its concentration builds within the cell. Also, the linear decline in enzyme activity as mRNA production is delayed reveals that β-galactosidase mRNA concentrations probably increase linearly with time as transcription of *lacZ* is induced.

Furthermore, optimum synthesis of β-galactosidase occurs if the *rrnB* is induced constantly (with high temperatures); therefore, it appears that either cloned-gene expression with specialized ribosomes requires the synthesis of a large population of specialized ribosomes (indicating that the specialized ribosomes are inefficient in initiating translation of the cloned gene), or that it takes hours to produce a significant pool of specialized ribosomes in the cell. Since the half-life of rRNA is estimated as 10 h based on its degradation constant,[21] there should be very little turnover of the wild-type ribosomes within the cell. Hence, it may take hours to build a significant population of specialized ribosomes. This is supported by the fact that more β-galactosidase is synthesized if the culture is induced at 42°C for 2 h at the start of the experiment rather than for 2 h at the end (4.82 vs. 1.59 nmol/min μg protein).

The inability to synthesize quickly specialized ribosomes with the strong promoter p_L may indicate that production of additional ribosomal proteins may be a limiting factor or the r-proteins may preferentially bind to wild-type rRNA. If the specialized rRNA is unprotected by r-proteins, it may degrade almost as rapidly as it is synthesized.[8] This would cause its concentration to build slowly in the cell even in the presence of a strong promoter. Furthermore, transcription attenuation and termination may limit transcription of the *rrnB* operon[14] even if it is initiated rapidly by the p_L promoter. Additional experiments in which the level of specialized ribosomes and rRNA are quantitated are required to address these issues.

Comparison of Specialized-Ribosome System and the Wild-Type Expression System

Construction of the specialized ribosome vector pTKW413 was designed such that both the gap distance between the RBS and the start codon and the sequence of these intervening bases were not altered when the RBS of *lacZ* was mutated. It has been shown that both

the gap distance and the kinds of bases that reside between the RBS and the start codon have a profound effect on translation efficiency in *E. coli*.[4] Therefore, plasmid pTKW106 is an excellent control to determine the effect of the specialized ribosomes on the production of β-galactosidase in that it has the same gap distance and the same nucleotides in this region as the specialized-ribosome vector pTKW413. Therefore, the two vectors differ only in the sequence of their ribosome binding sites and the presence (or absence) of the specialized *rrnB* locus.

Using specialized ribosomes, β-galactosidase synthesis was enhanced by 35% compared to that produced in a strain which depends on wild-type ribosomes (Fig. 8). This indicates that the production of a subpopulation of ribosomes that specifically translate the cloned gene can lead to an increase in productivity. This enhancement is achieved by reducing the translational limitations that occur when cloned genes are expressed.[28,29]

Although the β-galactosidase activity attained using the specialized-ribosome strain K12ΔH1/pTKW413 is larger than that of the wild-type ribosome control (K12ΔH1/pTKW106), it is 9.3-fold less than the best β-galactosidase-expression strain in our lab, BK6/pTKW106. This strain is capable of producing 40–50 wt % of total cell protein as β-galactosidase at 37°C.[29]

The main reason for the difference in cloned-gene expression is the difference in the two hosts, BK6 and K12ΔH1. Upon IPTG addition to K12ΔH1, the entire chromosomal *lac* operon is expressed along with the specialized *lacZ* locus on plasmid pTKW413. Hence, along with active enzyme from the plasmid genes, inactive β-galactosidase (due to the amber mutation *lacZ*am), lactose permease (*lacY* product), and transacetylase (*lacA* product) are produced by the host. Since β-galactosidase is active as a tetramer,[23] the mutant, chromosome-derived polypeptides may also decrease activity by combining with the plasmid-derived polypeptides. The chromosome-based expression represents a large, additional metabolic burden which decreases the productivity of K12ΔH1 and leads to the lower enzyme activity. Host BK6 lacks completely the *lac* operon (due to the deletion Δ(*lacIPOZ*)*C29*); therefore, no inactive enzyme is made from the chromosome. Furthermore, because the specialized-ribosome strain requires cloned-gene expression at a higher temperature (42°C) to induce the *rrnB* locus, β-galactosidase enzyme activity may also be reduced due to higher enzyme denaturation and degradation, and reduced mRNA stability.

Since the specific six-base region used to create the specialized RBS (5′ GUGUG 3′) may not be the optimum sequence to initiate translation, it may be possible to further enhance β-galactosidase expression by optimizing the specialized-ribosome RBS and the complementary ASD region of *rrnB*. The nucleotides which surround the RBS should also be chosen such that they satisfy the emerging rules for strong translation initiation.[4,26] It is also possible that the stability of the *lacZ* mRNA may be affected by changing the RBS, or it may be decreased due to the stress imposed on the cell from transcription of the *rrnB* locus.[28,29] Therefore, subsequent experiments should investigate the mRNA stability as well as the quantity and translational efficiency of the specialized ribosomes.

The specialized ribosomes have also been shown to have a significant affect on cell metabolism since their production reduced the specific growth rate by 32% at 39.5°C in the absence of cloned-gene mRNA transcription (Table III). Therefore, in terms of producing a *production* vector that overcomes translational limitations, it may be wiser to focus research on the effect of increasing translation of the cloned-gene by optimizing the gap length and sequence of bases between the RBS and the start codon as well as focus on the sequence of the RBS (for wild-type ribosomes) rather than rely on specialized ribosomes. The increased metabolic burden specialized ribosomes place on the cell, and the long-term structural instability of the plasmid due to the ribosomal locus limits the utility of the specialized-ribosome vector as an expression system.

Plasmid Stability

The stability of plasmid pTKW413 in the batch experiments was checked by isolating the plasmid at the start and at the end of each batch fermentation, cutting with *Eco*RI, and comparing the fragments to DNA molecular-weight standards. Plasmid stability was checked in all these experiments and did not occur during the length (8.68 h) of these experiments used to characterize β-galactosidase expression. Furthermore, no segregational instability was seen in any of the experiments. However, structural instability in pTKW413 was seen often in fermentations which lasted 45 h or more.

Mutations in the rRNA loci are very difficult to isolate since they affect cell metabolism directly and can be lethal.[11] Therefore, structural instability such as deletions and rearrangements occur frequently if the rRNA mutation is not suppressed by using a regulatable promoter.[11] It appears that the structural instability of pTKW413 is caused by an inability of the *c*I857 repressor of K12ΔH1 to fully repress the lambda p_L promoter of the specialized-ribosome vector. This is supported by the observation that the *c*I857 repressor is 1/500 as effective as the *c*I repressor at 30°C in repressing specialized ribosome synthesis (Table VI). The growth rate of the specialized-ribosome strain was also reduced relative to the host at low temperatures when β-galactosidase was not expressed, indicating leakiness of the p_L promoter. Furthermore, the plasmid pASDX-p_L (which contains the specialized ribosome locus but lacks *lacI*^{Q} and *lacZ*) did not show any instability after 64 h (three subcultures, LB–Kan) in a strain that contains the wild-type *c*I repressor (K12ΔH1). However, if this plasmid

is placed within a host which lacks the *c*I repressor (HB101), extreme structural instability is observed (large deletions). Therefore, incomplete repression of p_L by the *c*I857 repressor seems to be the cause of the instability in that cells that lack the specialized-ribosome locus are constantly favored due to the difference in growth rate.

The inefficient repression is probably caused by the large number of p_L promoters that arise from the high copy number. This situation might be remedied by placing the *c*I857 repressor on the plasmid itself. This technique has been effective with the *lacI*^{Q} repressor and the *tac* promoter.[29]

Even though plasmid pTKW413 appeared unstable in *recA* strains, it is likely that the *recA*$^+$ genotype of the host strain K12ΔH1 increases the structural instability of the specialized-ribosome plasmid pTKW413 due to the great deal of homology between the plasmid and the chromosome. However, the RecA protein appears to be required by the cell to fully derepress *c*I857 for expression of *rrnB* since β-galactosidase expression was negligible in two hosts which contained *c*I857 and the *recA* mutation. Perhaps the cell requires the RecA protease to inactivate *c*I857 so that the lambda p_L promoter can be fully derepressed. It may therefore be advantageous to use a *recA* host and a different promoter to induce the specialized *rrnB* locus. It also seems prudent to reduce homology between the chromosome and the plasmid by expressing only the specialized 16S rRNA and removing the remainder of the *rrnB* locus. Homology between the plasmid and chromosome can be further reduced by deleting the *lac* operon from the chromosome.

CONCLUSIONS

A second class of *E. coli* ribosomes, specialized ribosomes, have been used successfully to translate preferentially a cloned-gene transcript. A single plasmid has been constructed in which the rate of transcription of *lacZ* mRNA with a specialized RBS is regulated by the *tac* promoter and in which transcription of mutant *rrnB* rRNA can be controlled with the p_L promoter.

Initial experiments using this specialized-ribosome vector have shown that production of specialized ribosomes significantly affects the growth of the cell in both minimal and complex medium, and that the specialized-ribosome locus is structurally unstable after exponential growth for about 60 generations. Additionally, the optimal induction strategy for production of β-galactosidase was investigated and shown to involve constant exposure to high temperature to induce transcription fully at the specialized ribosome locus as well as high, constant induction of *lacZ* mRNA transcription. In effect, the greatest enzyme production occurs if both loci are transcribed continuously at their maximum rates. Under these conditions, the specialized ribosomes enhanced β-galactosidase activity by 35% over the wild-type control. Furthermore, the *tac* promoter was regulated tightly by the addition of IPTG (1300-fold); however, the p_L promoter was not controlled tightly due to inefficient repression by *c*I857.

By comparing the time course of β-galactosidase production in the specialized ribosome and wild-type systems, it was found that a lag in β-galactosidase synthesis occurs when specialized ribosomes are used. Furthermore, as has been previously shown with leukocyte interferon A and human growth hormone,[7] the Shine–Dalgarno region is absolutely required for translation of β-galactosidase with wild-type ribosomes since no translation of specialized *lacZ* mRNA occurs without it, even with full induction of transcription of mRNA with strong *tac* promoter.

Since the specialized *lacZ* mRNA is not recognized by wild-type ribosomes , the specialized-ribosome vector can be used to study the impact of strong mRNA transcription on cellular metabolism in the absence of translation of the transcript. Furthermore, the impact of production of a subpopulation of ribosomes that are specific for the cloned-gene message can be investigated. Hence, cloned-gene transcription has been decoupled from translation.

Studying ribosomes and their interaction with cloned-gene mRNA appears worthwhile in order to investigate possible translational bottlenecks which occur when recombinant proteins are produced at high concentrations. The specialized ribosome vector constructed in this work represents a significant tool for investigating both transcription and translation independently in bacterial cells.

This work was supported in part by the North Carolina Biotechnology Center (Grant 86-G-00722) and by a stipend from the Southeastern Regional Fellowship Program (for TKW). We would like to thank Marcel Brink and Dr. Herman de Boer for providing plasmid pASDX-p_L and strain K5716, N. Padukone for donating strain JM109-NM1070, and Loan Nu and Dr. Masayasu Nomura for providing strains M5219 and NO3203. We are also grateful for the stimulating ideas provided by Stuart Thomas in regard to cloning strategies, and for the advice of Dr. Anna Hui and Dr. Mark Conkling.

References

1. Bentley, W. E., Mirjalili, N., Anderson, D. C., Davis, R. H., Kompala, D. S. 1990. Plasmid-Encoded Protein: The Principal Factor in the 'Metabolic Burden' Associated with Recombinant Bacteria. Biotechnol. Bioeng. **35**: 668.
2. Berger, S. L., Kimmel, A. R. (eds.) 1987. Guide to Molecular Cloning Techniques. Academic Press. Orlando, FL.
3. Betenbaugh, M. J., Beaty, C., Dhurjati, P., 1989. Effects of Plasmid Amplification and Recombinant Gene Expression on the Growth Kinetics of Recombinant *E. coli*. Biotechnol. Bioeng. **33**: 1425.
4. de Boer, H. A., Hui, A., Comstock, L. J., Wong, E., Vasser, M. 1983. Portable Shine-Dalgarno Regions: A System for a Systematic Study of Defined Alterations of Nucleotide Sequences within *E. coli* Ribosome Binding Sites. DNA. **2**: 231.

5. de Boer, H., Ng, P., Hui, A. 1985. Synthesis of Specialized Ribosomes in *Escherichia coli.* In: R. Calendar and L. Gold (eds.) Sequence Specificity in Transcription and Translation. Alan R. Liss, New York.
6. Csonka, L. N., Clark, A. J. 1979. Deletions Generated by the Transposon TN*10* in the *srl recA* Region of the *Escherichia coli* K-12 Chromosome. Genetics. **93**: 321.
7. von Gabain, A., Belasco, J. G., Schottel, J. L., Chang, A. C. Y., Cohen, S. N. 1983. Decay of mRNA in *Escherichia coli:* Investigation of the Fate of Specific Segments of Transcripts. Proc. Nat. Acad. Sci. **80**: 653.
8. Gourse, R. L., Takebe, Y., Sharrock, R. A., Nomura, M. 1985. Feedback Regulation of rRNA and tRNA Synthesis and Accumulation of Free Ribosomes After Conditional Expression of rRNA Genes. Proc. Nat. Acad. Sci. **82**: 1069.
9. Hendrix, R.W., Roberts, J.W., Stahl, F.W., Weisberg, R. A. (eds.) 1983. Lambda II. Cold Spring Harbor Laboratory. Cold Spring Harbor, NY.
10. Hui, A., de Boer, H. A., 1987. Specialized Ribosome System: Preferential Translation of a Single mRNA Species by a Subpopulation of Mutated Ribosomes in *Escherichia coli.* Proc. Nat. Acad. Sci. **84**: 4762.
11. Hui, A., Jhurani, P., de Boer, H. A. 1987. Directing Ribosomes to a Single mRNA Species: a Method to Study Ribosomal RNA Mutations and Their Effects on Translation of a Single Messenger in *Escherichia coli.* In: R. Wu and L. Grossman (eds.) Methods in Enzymology Vol. 153: Recombinant DNA Part D. Academic Press, San Diego.
12. Jinks-Robertson, S., Gourse, R. L., Nomura, M. 1983. Expression of rRNA and tRNA Genes in *Escherichia coli:* Evidence for Feedback Regulation by Products of rRNA Operons. Cell. **33**: 865.
13. Kepes, A. 1963. Kinetics of Induced Enzyme Synthesis: Determination of the Mean Life of Galactosidase-Specific Messenger RNA. Biochemica et Biophysica Acta. **76**: 293.
14. Kingston, R. E., Chamberlin, M. J. 1981. Pausing and Attenuation of in vitro Transcription in the *rrnB* Operon of *E. coli.* Cell. **27**: 523.
15. Maniatis, T., Fritsch, E. F., Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Lab. Cold Spring Harbor, NY.
16. Midgley, C. A., Murray, N. E. 1985. T4 Polynucleotide Kinase; Cloning of the Gene (*pse*T) and Amplification of Its Product. EMBO Journal. **4**: 2695.
17. Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Lab. Cold Spring Harbor, NY.
18. Neidhardt, F. C. 1987. *Escherichia coli* and *Salmonella typhimurium:* Cellular and Molecular Biology. American Society for Microbiology. Washington, D.C.
19. Nossal, N. G. 1974. DNA Synthesis on a Double-Stranded DNA Template by the T4 Bacteriophage DNA Polymerase and the T4 Gene 32 DNA Unwinding Protein. J. Biol. Chem. **249**: 5668.
20. Okita, B., Arcuri, E., Turner, K., Sharr, D., Del Tito, B., Swanson, J., Shatzman, A., Zabriskie, D. 1989. Effect of Induction Temperature on the Production of Malaria Antigens in Recombinant *E. coli.* Biotechnol. Bioeng. **34**: 854.
21. Peretti, S.W., Bailey, J. E. 1986. Mechanistically Detailed Model of Cellular Metabolism for Glucose-Limited Growth of *Escherichia coli* B/r-A. Biotechnol. Bioeng. **28**: 1672.
22. Remaut, E., Stanssens, P., Fiers, W. 1981. Plasmid Vectors for High-Efficiency Expression Controlled by the p_L Promoter of Coliphage Lambda. Gene. **15**: 81.
23. Rodriguez, R. L., Tait, R. C. 1983. Recombinant DNA Techniques. An Introduction. Benjamin/Cummings. Menlo Park, CA.
24. Shatzmant, A. R., Rosenberg, M. 1986. Efficient Expression of Heterologous Genes in *Escherichia coli.* Ann. NY Acad. Sci. **478**: 233.
25. Siegel, R., Ryu, D. D.Y. 1985. Kinetic Study of Instability of Recombinant Plasmid pPLc23*trpAl* in *E. coli* Using Two-Stage Continuous Culture System. Biotechnol. Bioeng. **27**: 28.
26. Stanssens, P., Remaut, E., Fiers, W. 1985. Alterations Upstream from the Shine-Dalgarno Region and Their Effect on Bacterial Gene Expression. Gene. **36**: 211.
27. Wood, T. K., Kuhn, R. H., Peretti, S.W. 1990. Enhanced Plasmid Stability Through Post-Segregational Killing of Plasmid-Free Cells. Biotech. Tech. **4**: 36.
28. Wood, T. K., Peretti, S.W. 1990. Depression of Protein Synthetic Capacity Due to Cloned-Gene Expression in *E. coli.* Biotechnol. Bioeng. **36**: 865.
29. Wood, T. K., Peretti, S.W. 1991. Effect of Chemically-Induced, Cloned-Gene Expression on Protein Synthesis in *E. coli.* Biotechnol. Bioeng. in press.
30. Yamagishi, M., de Boer, H. A., Nomura, M. 1987. Feedback Regulation of rRNA Synthesis. A Mutational Alteration in the Anti-Shine-Dalgarno Region of the 16S rRNA Gene Abolishes Regulation. J. Mol. Biol. **198**: 547.
31. Yanish-Perron, C., Viera, J., Messing, J. 1985. Improved M13 Phage Cloning Vectors and Host Strains: Nucleotide Sequences of the M13mp18 and pUC19 Vectors. Gene. **33**: 103.
32. Yanofsky, C., Franklin, N. 1979. Construction of Plasmid Cloning Vehicles that Promote Gene Expression from the Bacteriophage Lambda p_L Promoter. Gene. **5**: 59.
33. Young, R. A., Steitz, J. A. 1978. Complementary Sequences 1700 Nucleotides Apart Form a Ribonuclease III Site in *Escherichia coli* Ribosomal Precursor RNA. Proc. Nat. Acad. Sci. **75**: 3593.