The EMBO Journal vol.11 no.11 pp.4175–4185, 1992

Comparison of the expression of the seven ribosomal RNA operons in *Escherichia coli*

Ciaran Condon, Jennifer Philips, Zheng-Yuan Fu, Craig Squires and Catherine L.Squires[1]

Department of Biological Sciences, Columbia University, New York, NY 10027, USA
[1]Corresponding author

Communicated by M.Grunberg-Manago

We have compared the expression of the seven ribosomal RNA operons (*rrn*) of *Escherichia coli* and their responses to a variety of physiological and genetic perturbations. We used a set of *rrn* promoter fusion constructs in their native chromosomal positions to examine effects of chromosomal location on *rrn* operon expression and the same set of fusions on lambda lysogens to assay intrinsic promoter strengths independent of chromosome context. In its native chromosomal location, expression of the *rrnH* operon was significantly lower than expected. This effect was not attributable to weak promoter activity and was dependent on the growth medium. The *rrnE* operon had reduced promoter activity relative to the other ribosomal operons in minimal medium and thus appears to have abnormal growth rate regulation. The ribosomal RNA operons showed varied responses to amino acid starvation; expression of *rrnD* was inhibited most. There was only a slight increase in *rrn* transcription in response to a temperature shift (30°C to 42°C) and the differences between individual operons was very small. The *rrnG* operon showed a significantly lower response than the other ribosomal RNA operons to a depletion of the *rrn* transcription activator, Fis, and thus appears to have decreased Fis-mediated transactivation. Finally, the chromosomal fusion strains were used to study the effect on growth rate of inactivating each *rrn* operon. In fast growth conditions, loss of certain *rrn* operons caused subtle decreases in growth rate on complex medium.

Key words: Fis/heat shock/*rrn* promoter fusions/stringent control

Introduction

The number of ribosomal RNA gene copies in different organisms varies greatly; bacteria possess from one to 10 *rrn* operons per genome (Bott *et al.*, 1984; Hui and Dennis, 1985; La Fauci *et al.*, 1986), while there are hundreds or even thousands of ribosomal transcription units per eukaryotic genome (Long and Dawid, 1980). Although it has been commonly assumed that these multiple gene copies are functionally identical, in one instance it has been shown that the parasite *Plasmodium* encodes two different 18S rRNAs; one gene is expressed predominantly in the mammalian host stage of the parasite's life cycle, while expression of the other gene is greater in the mosquito host (Gunderson *et al.*, 1987). Could such 'dedicated' rRNA genes possibly be a more general phenomenon, or is the multiplicity of *rrn* operons primarily a mechanism for maintaining the 'right' amount of rRNA in the cell? To pursue this question in a model system, we have studied how each of the seven *Escherichia coli rrn* operons is expressed using reporter gene fusions. It has been previously shown that deletion of one of the seven operons in *E.coli* or one of the 10 *B. subtilis rrn* operons had no observable influence on cell growth rates or physiology, suggesting that neither organism requires its full complement of *rrn* operons (Ellwood and Nomura, 1980; Widom *et al.*, 1988). On the other hand, the persistence of seven or 10 operons in these bacteria suggests some long-term advantage to the organism of retaining its full complement of *rrn* genes. Such an advantage could be the ability to adapt efficiently to different growth conditions, or it might, for example, be advantageous for particular operons to make ribosomes with specific cellular functions. Although distinct developmental stages do not exist in *E.coli*, we may still ask if there is a unique requirement for any of the seven operons under particular physiological conditions.

The seven *rrn* operons in *E.coli* are located in non-contiguous sites around the chromosome (Ellwood and Nomura, 1982; Figure 1) and all are transcribed in the same direction in which the chromosome is replicated. All operons have approximately the same organization: tandem promoters, P_1 and P_2–16S–spacer–23S genes–5S genes–terminator region (Jinks-Robertson and Nomura, 1987). Four of the operons contain tRNA Glu_2 in their spacer regions (*rrnB, C, E* and *G*) and three contain tRNA Ala_{1B} plus tRNA Ile_1 (*rrnA, D* and *H*). Some *rrn* operons also have one or two tRNAs following the 5S gene; notably,

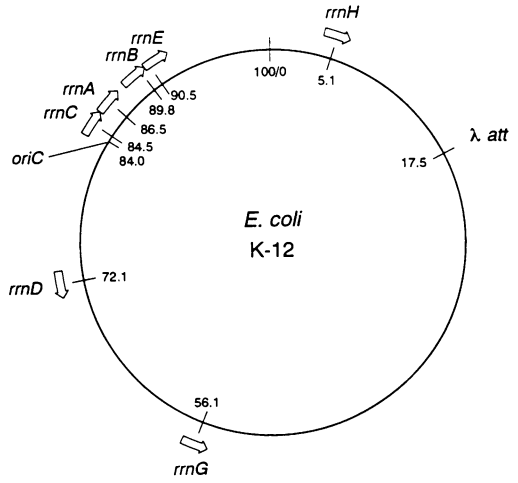

Fig. 1. Location in minutes of the ribosomal RNA operons (*rrn*) and lambda attachment site (λ*att*) on the *E.coli* chromosome. Arrows indicate the direction of transcription. The origin of chromosomal replication, *oriC*, is shown at 84.0 min.

rrnC has the cell's only copy of tRNA Trp in this position. In addition to differences in types and numbers of tRNAs encoded, the operons also contain sequence heterogeneities. These occur both within the genes themselves (Carbon *et al.*, 1979; Shen *et al.*, 1982) and within the control (Jinks-Robertson and Nomura, 1987; Plaskon and Wartell, 1987), and spacer regions (Harvey *et al.*, 1988). The significance of these heterogeneities is unknown, but it is possible that they might cause differences in regulation or function of the stable RNAs produced.

Ribosomal RNA promoters are among the strongest known, accounting for more than half the cell's transcriptional activity at high growth rates (Bremer and Dennis, 1987), and they are subject to a complex set of overlapping control mechanisms. The best studied is the *rrnB* operon promoter region. The *rrnB* P_1 promoter is activated 20- to 30-fold *in vivo* by a sequence element known as the *u*pstream *a*ctivation *r*egion (UAR; Gourse *et al.*, 1986). This consists of factor-dependent and factor-independent subregions (Josaitis *et al.*, 1990; Ross *et al.*, 1990; Leirmo and Gourse, 1991), both containing highly bent DNA (Gourse *et al.*, 1986; Plaskon and Wartell, 1987). The factor-dependent region stimulates *rrn* expression 10- to 20-fold upon binding the Fis protein (Ross *et al.*, 1990), first identified as a factor promoting inversion reactions in phage Mu (Koch and Kahmann, 1986) and in *Salmonella* (Johnson and Simon, 1985). Ribosomal RNA operons have three consensus Fis binding sites upstream of the P_1 promoter, although actual Fis binding has only been demonstrated for *rrnB* (Ross *et al.*, 1990). The factor-independent domain is rich in A−T bases and accounts for the remainder (2- to 4-fold) of upstream activation by the UAR (Leirmo and Gourse, 1991). The extent of A−T-rich sequences varies from one operon to the next, with *rrnD* having the highest A−T content (Plaskon and Wartell, 1987). However, there has been no demonstration as yet of a link between A−T content, DNA bending and promoter strength for the ribosomal RNA operons.

It has been shown that *rrn* P_1 promoters are also subject to stringent control; when an uncharged tRNA binds to the ribosome, a rapid accumulation of guanosine tetraphosphate (ppGpp) ensues, causing inhibition of transcription from ribosomal promoters (for review, see Cashel and Rudd, 1987). The sequence elements important for stringent control map in and around the −10 and −35 hexamers. In addition to stringent control and upstream activation, the *rrnB* P_1 promoter is regulated as a function of growth rate, such that *rrn* expression is kept proportional to the square of the growth rate (Nomura *et al.*, 1984). The exact mechanism of growth rate regulation remains unresolved.

Interdigitated with *rrn* P_1 promoters is a consensus sequence for a σ^{32} heat shock promoter (R.L.Gourse, personal communication), but no direct demonstration of physiological significance has yet been shown for this promoter. The *rrnB* P_2 promoter is a lower level, constitutive promoter, thought to ensure a basal level of rRNA transcription during periods when P_1 is turned off (Sarmientos *et al.*, 1983). Finally, downstream of *rrn* P_2 are sequences resembling the antitermination *nut* site of phage lambda, which allow RNA polymerase to maintain a high degree of processivity through Rho-dependent terminators (Li *et al.*, 1984). Cryptic Rho-dependent terminators have been found within the 16S structural gene (Aksoy *et al.*, 1984b).

We wished to see whether all ribosomal RNA operons were expressed and regulated similarly or whether chromosomal location or the sequence heterogeneities observed in the control regions might result in differential promoter activity under particular physiological conditions. To address these questions we constructed two sets of *E.coli* strains. The first consisted of each of the seven ribosomal RNA promoter regions fused to the chloramphenicol acetyl transferase gene (CAT) and crossed into the chromosome at their respective operon's sites (Figure 2). These strains allowed us to study previously characterized patterns of *rrn* regulation in the native chromosomal context. We measured CAT activity from the chromosomal fusions in both complex and minimal media and in response to stringent control, heat shock and a mutation in the *fis* gene. The second set of strains consisted of the same fusions on lambda lysogens. These lysogen fusions allowed measurement of inherent promoter strength independent of chromosomal location and were assayed on both complex and minimal media. It is clear that the seven ribosomal RNA operons are neither expressed nor regulated equally, but rather that expression of individual ribosomal RNA operons can be affected differently by both chromosomal location and physiological conditions.

We also wished to determine the effect of inactivating each of the seven *rrn* operons. Ellwood and Nomura (1980) deleted the *E.coli rrnE* gene and found that this caused no adverse consequences to the cell. Growth rates of several *rrnE* deletion strains were identical to that of the wild type in both complex and minimal media. In mixing experiments, the deletion strain was not lost in >100 generations of competitive growth with its parent. The *rrnE* operon contains one of the four spacer region tRNA Glu_2 genes (Jinks-Robertson and Nomura, 1987). Thus, it is also possible to lose at least one gene specifying this tRNA species without affecting cell growth. We wished to do a similar study of the other ribosomal RNA operons. Strains with the *rrn* P_1P_2−CAT reporter constructs in place of their respective chromosomal genes were used as null mutants for each of the seven *rrn* operons. In mixed cultures with the parental strain over many generations, the *rrnE* deletion strain grew similarly to the wild type in both complex and minimal media, consistent with the results of Nomura. However, this observation was not the general case for all ribosomal RNA operons, particularly on complex medium. In general, strains lacking ribosomal RNA operons were slightly out-competed by the parental strain in complex medium. In minimal medium, no major deficiencies were observed; in fact some mutant strains appeared to grow slightly faster than the parental strain.

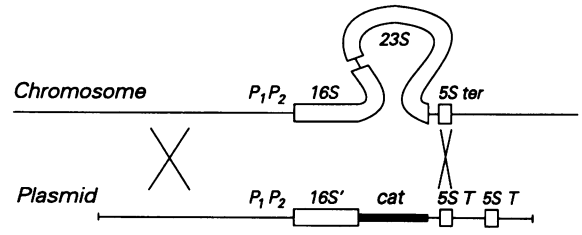

Fig. 2. Scheme for gene replacement by the *rrn* P_1P_2−CAT fusions. P_1 and P_2 are the tandem *rrn* operon promoters and *ter* is the termination region. Open blocks indicate the location and extent of the 16S, 23S and 5S genes. *T* is the *rrnB* t_1t_2 termination region. The diagram depicts a double crossover event between the linearized plasmid and the chromosome, exchanging the wild type operon for the fusion construct. The downstream recombination event can occur at either of the two plasmid-borne 5S genes.

Results

It is generally assumed that the seven ribosomal RNA operons of *E.coli* are functionally equivalent. We have designed a reporter gene system to study whether sequence heterogeneities noted within the control regions and structural genes are physiologically significant. We have fused a large fragment (3.5 to 8 kb) containing the upstream control region plus promoters of each operon to a promoterless CAT gene, and put these constructs back on the chromosome either in their native chromosomal locations or as lysogens in the lambda *att* site. We have assayed these fusions under fast and slow growth conditions, and in response to heat shock, amino acid starvation and a mutation in the *fis* gene. We have also looked at the effect on growth rate of inactivating each of the seven rRNA operons. There was no absolute requirement for any single *rrn* operon under the conditions tested. However, it is clear from this study that while the seven *E.coli* operons are transcribed and regulated similarly, some have distinct responses to particular physiological and genetic perturbations.

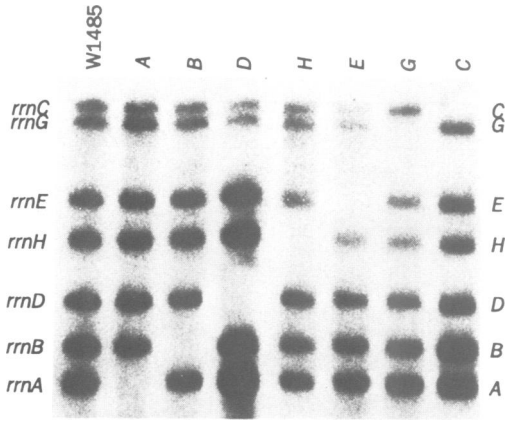

Fig. 3. Southern analysis of strains carrying the various chromosomal *rrn* P_1P_2−CAT fusions described here. Shown are *Bam*HI and *Pst*I total digests of chromosomal DNA probed with a ^{32}P labelled portion of 16S DNA. Strains carrying the fusion constructs lack the characteristic band of the wild type *rrn* operon. W1485 is the parent strain and letters over each lane indicate the particular *rrn*-inactivated strain.

Construction of rrnP$_1$P$_2$−CAT chromosomal fusions

Each of the chromosomal ribosomal RNA operons (Figure 1) was replaced by a homologous copy that had its promoter region fused to a promoterless CAT gene. Gene replacement was achieved by fragment transformation of a *recBC sbcB* host (Figure 2; Arps and Winkler, 1987). We then used P1 transduction to move the chromosomal chloramphenicol marker into the wild type strain, W1485. Each of the seven ribosomal RNA operons lies on a *Bam*HI−*Pst*I fragment of a unique size, described previously by Boros *et al.* (1979), and Hill and Harnish (1981). Chromosomal DNA was isolated as described in Materials and methods and was digested with *Bam*HI and *Pst*I. The replacement of the desired *rrn* operon by the *rrn* P_1P_2−CAT fusion was confirmed by Southern blot analysis. The probe used was specific to a region of the 16S gene removed in the construction and thus, the band characteristic of that operon should be absent from the blot. Figure 3 is a Southern blot of chromosomal digests showing each of the chromosomal fusion strains.

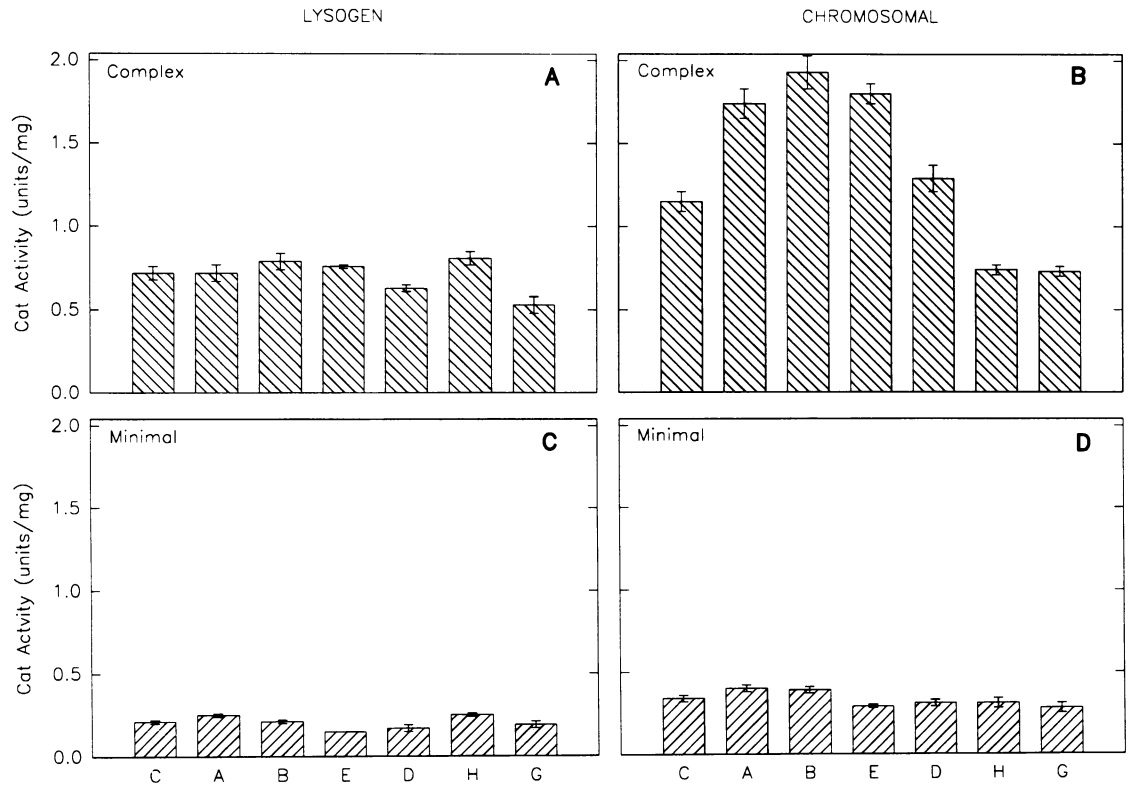

Fig. 4. CAT activities (units per milligram protein) of the lysogen and chromosomal fusions on complex and minimal media. Operon fusions are arranged in order of increasing distance from the origin of replication. **A.** Lysogen fusions assayed on LB glucose. **B.** Chromosomal fusions assayed on LB glucose. **C.** Lysogen fusions on assayed M9 glucose. **D.** Chromosomal fusions assayed on M9 glucose. Results are the average of four independent assays. Standard deviation for *rrnE* lysogen on minimal medium is 0.003.

rrn expression in complex medium

Strains carrying the *rrn* promoter fragments fused to the CAT gene on lambda lysogens were assayed for CAT activity in LB glucose medium. This allowed measurement of inherent promoter strength independent of chromosomal location. The levels of expression varied over a 1.5-fold range (see Figure 4A). The *rrnB* and *H* operons had the highest promoter activity; *rrnE, rrnC* and *rrnA* had intermediate strength while the *rrnD* and *rrnG* operons had significantly lower promoter activity.

Because fast growing *E.coli* cells have multiple replication forks, effectively resulting in a higher copy number of genes close to the origin of replication, gene dosage should contribute to the CAT activity measured in fusions in their normal chromosomal locations. Consistent with this prediction, a much higher range of expression was seen (2.6-fold; Figure 4B) and the level of expression from each operon was, in general, reflected by its distance from the origin of replication, *oriC*. The *rrnC* and *rrnH* operons, however, gave significantly lower expression than would be expected by this criterion (see Discussion).

rrn expression in minimal medium

With the significant exception of *rrnE*, the hierarchy of expression of the *rrn* P_1P_2−CAT lysogen fusions in minimal medium correlated well with that on complex medium (compare Figure 4A and C). The range of promoter activities was also similar (1.6-fold). *rrnA* and *rrnH* had greatest promoter activity, *rrnB* and *rrnC* were of intermediate strength and *rrnG*, *rrnD* and *rrnE* had the weakest promoter activity. The relative decrease in activity of *rrnE* in minimal medium resulted in a high derepression ratio (LB glucose/M9 glucose) for this operon; 5.1, whereas the other operons were within the range 2.8−3.8.

In its normal chromosomal location, expression of the *rrnE* operon was also low in minimal medium (Figure 4D). The derepression ratio for *rrnE* was 6.2 compared with 4.4 and 5.0 for *rrnA* and *rrnB* respectively. Otherwise, the hierarchy of expression from this set of fusions in minimal medium also correlated well with that on LB medium (Figure 4B), i.e. the CAT activities reflected distance from the origin of replication. A much narrower range of expression was measured (1.4-fold compared with 2.6-fold), due to dampening of the gene dosage effect on minimal medium.

Response of rrn promoters to stringent control

Ribosomal RNA P_1 promoters are essentially switched off upon the accumulation of uncharged tRNAs in the cell (Sarmientos *et al.*, 1983). We examined the relative stringent responses of each of the seven ribosomal RNA operons using the chromosomal fusion system. We looked at the effect of serine hydroxamate-induced amino acid starvation on the synthesis of CAT mRNA from the ribosomal RNA promoters. All seven operons were clearly stringently regulated (Figure 5). However, the extent of the responses to amino acid starvation varied significantly; *rrnD* showed the strongest effect (3.0-fold decrease) and *rrnB, rrnC* and *rrnH* had similar, weak effects (1.9-fold decrease).

Effect of heat shock on rrn expression

It has been noted (J.T.Newlands and R.L.Gourse, personal communication) that consensus heat shock promoters are interdigitated with the *rrn* P_1 promoters and they have shown that an RNA polymerase−σ^{32} complex can bind to and transcribe *rrnB in vitro*. Thus, the chromosomal *rrn* P_1P_2−CAT constructs were assayed for CAT activity before and 10 min after a shift from 30°C to 42°C. Most

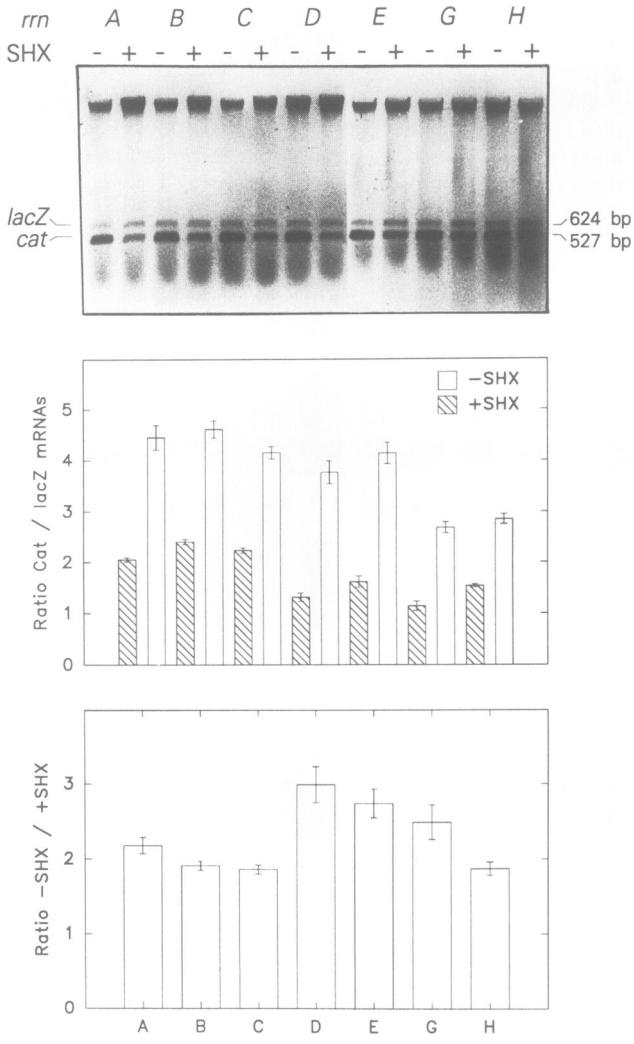

Fig. 5. Effect of amino acid starvation on CAT mRNA levels from the chromosomal fusions; quantitative S1 and T1 nuclease analysis. **Top panel.** Negatives of ethidium bromide stained gels showing stringent control of each of the seven ribosomal RNA operons. *lacZ* mRNA from carrier cells was used as an internal standard. SHX, serine hydroxamate. **Center panel.** Ratio of CAT to *lacZ* mRNAs for each of the chromosomal fusions before and after addition of SHX. **Bottom panel.** Ratio of −SHX to +SHX from above. Results are the average of two independent mRNA isolations, each assayed twice.

Table I. Effect of heat shock on expression of *rrn* promoter fusions

Fusion	CAT activity at 30°C (units/mg protein)	CAT activity at 42°C (units/mg protein)	Ratio (42°C/30°C)
rrnA	0.53 (0.09)[a]	0.55 (0.07)	1.04
rrnB	0.52 (0.02)	0.56 (0.06)	1.08
rrnC	0.51 (0.06)	0.54 (0.06)	1.06
rrnD	0.44 (0.06)	0.45 (0.05)	1.02
rrnE	0.47 (0.07)	0.48 (0.08)	1.02
rrnG	0.32 (0.02)	0.35 (0.02)	1.09
rrnH	0.39 (0.04)	0.39 (0.04)	1.00

[a]Standard deviations in parentheses.

operons gave a very slight increase (< 10%) in CAT activity in response to the temperature shift (see Table I). A similar effect on CAT mRNA was seen for each of the operons in response to a 5 min temperature shift, whereas in control experiments with a known heat shock gene, *clpB* (Squires *et al.*, 1991), an ~10-fold increase in mRNA levels was noted (data not shown). Thus, a large peak of synthesis, characteristic of a heat shock response for other genes, was not observed for the ribosomal RNA operons.

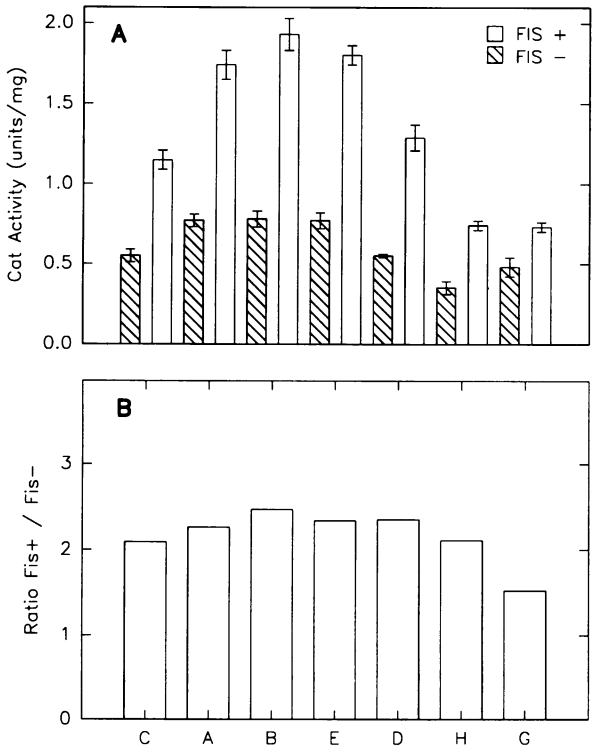

Fig. 6. Effect of Fis on CAT activities of chromosomal fusions in complex medium. **A.** CAT activities measured in Fis^+ (open bars) and in Fis^- (hatched bars) strains. Values for the Fis^+ strains are taken from Figure 4B. **B.** Ratio of Fis^+ to Fis^- from above. Operon fusions are arranged in order of increasing distance from the origin of replication. Results are the average of four independent assays.

Effect of Fis on rrn expression

Strains carrying the chromosomal CAT fusions were transduced to *fis*$^-$ using kanamycin as a selectable marker (see Materials and methods). The resulting strains were assayed for CAT activity in LB glucose medium (Figure 6). The *fis* mutation caused a 2.1- to 2.5-fold decrease in CAT activity for six of the ribosomal operons, but significantly less inhibition (1.5-fold) in the case of *rrnG*.

Effect of rrn inactivation on growth rate

The *rrn* P_1P_2−CAT chromosomal fusions also served as null mutants for each of the seven operons. We were therefore able to exploit these strains to examine the effect on growth rate of inactivating each ribosomal RNA operon. By direct measurement of growth rate, however, we were unable to see a significant difference between the doubling times of the wild type and strains with a single inactivated operon. A much more sensitive method to detect subtle differences in growth rates is to follow the fate of a mutant in a mixed culture with its parent over many generations. Strains carrying the *rrn* P_1P_2−CAT fusions in place of their respective wild type operon were mixed in 1:1 ratios with W1485 in chemostats and their relative proportions followed for 140 generations. The chemostats were run at a rate that allowed maximum growth of the wild type, W1485 (k = 2.6/h). Strains with one operon inactivated could be identified by their resistance to chloramphenicol. In LB glucose, strains lacking one *rrn* operon were generally out-competed by the wild type; the rate ranging from relatively fast ($k = -4.0\times10^{-3}$/h; Δ*rrnA* and Δ*rrnB*) to no significant effect (Δ*rrnC* and Δ*rrnG*) (Figure 7 and Table II). In minimal glucose medium (M9), these mutant strains were significantly more competitive than on complex medium and, in fact, strains lacking *rrnA, C, G* and *H* appeared to have a slight advantage ($k = 2.0\times10^{-3}$/h) over

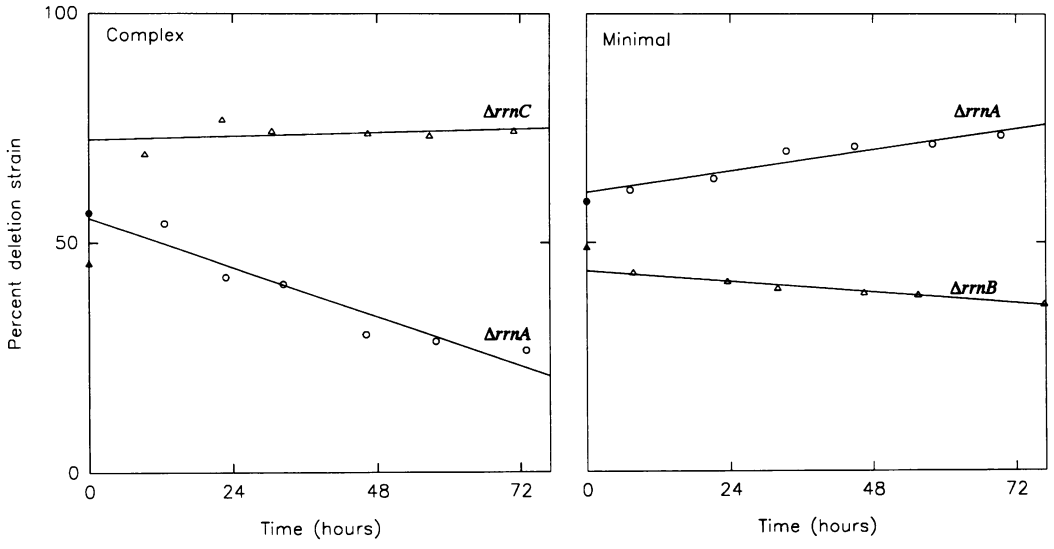

Fig. 7. Effect of *rrn* inactivation on growth rate in complex and minimal media. Chromosomal fusion strains were mixed in equal proportions with the wild type, W1485 and the relative proportion of each strain was followed by virtue of the resistance of the fusions to chloramphenicol. **Left Panel.** Effect of inactivating the *rrnA* and *rrnC* operons on competitive growth on LB glucose. **Right Panel.** Effect of inactivating *rrnA* and *rrnB* on competitive growth on M9 glucose. See Table II for summary of rate of loss for each of the fusion strains. Note: first data point (closed symbol) is omitted in the calculation of the slope as the fusion strains differed in their rate of recovery from the stationary phase of the inoculum.

the parent under these conditions. Possible reasons for this will be discussed later.

Discussion

It is generally assumed that there is little variation in the primary sequence of the ribosomal RNA genes in *E.coli*. This is because the restriction maps of the 16S and 23S genes of the seven operons are very similar (Boros *et al.*, 1979; C.Squires, unpublished data) and because of the large number of ribosomal proteins that depend on the tertiary structure of ribosomal RNAs for correct assembly into ribosomes. However, only the *rrnB* operon sequence has been reported in its entirety (Brosius *et al.*, 1981) and this operon is used to represent *E.coli* in the generation of speciation trees by 16S rRNA comparisons (Woese and Olsen, 1986) and as the paradigm scaffold for *E.coli* ribosome structure modelling (Noller and Nomura, 1987). Micro-sequence heterogeneities are, however, known to exist between the seven operons within the 16S and 23S genes (Carbon *et al.*, 1979; Shen *et al.*, 1982) and some of the control regions associated with the promoters have a significant amount of variation (Figure 8; Jinks-Robertson and Nomura, 1987; Plaskon and Wartell, 1987). To address the question of whether these sequence heterogeneities were physiologically significant and ask whether some of these variations would lead to differences in function or regulatory patterns, we constructed two sets of fusion strains. One was a set of strains each containing one of the seven ribosomal RNA promoter regions fused to the CAT gene and crossed into the chromosome at their respective operon's sites. These strains allowed us to measure *rrn* expression in the native chromosomal context. They also allowed us to evaluate the functional redundancy of individual *rrn* operons by virtue of the fact that the wild type operon is replaced by the fusion construct. The second set of strains consisted of the same fusions on lambda lysogens, which allowed measurement of inherent promoter strength independent of chromosomal location. Assay of these strains under a variety of physiological conditions has yielded a number of interesting and important observations.

Table II. Rate of loss of chromosomal fusion strains in mixed culture with W1485

Fusion	LB glucose ($\times 10^{-3}$/h)	M9 glucose ($\times 10^{-3}$/h)
rrnA	−4.0 (0.9)[a]	2.0 (0.8)
rrnB	−4.0 (0.6)	−1.0 (0.1)
rrnC	0.3 (0.0)	2.0 (0.1)
rrnD	−1.0 (0.1)	−1.0 (0.9)
rrnE	−1.0 (0.3)	−0.4 (0.1)
rrnG	−0.4 (0.8)	2.0 (0.5)
rrnH	−2.0 (0.4)	2.0 (0.7)

[a]Standard deviations in parentheses.

rrn expression on complex medium

Although none of the *E.coli* ribosomal RNA promoters (P_1 or P_2) match the consensus promoter sequence (Figure 8), they are among the strongest known and account for more than one-half of total *E.coli* transcription at high growth rates (Bremer and Dennis, 1987). Their strength is largely due to activation by two *cis* elements in the UAR, between −48

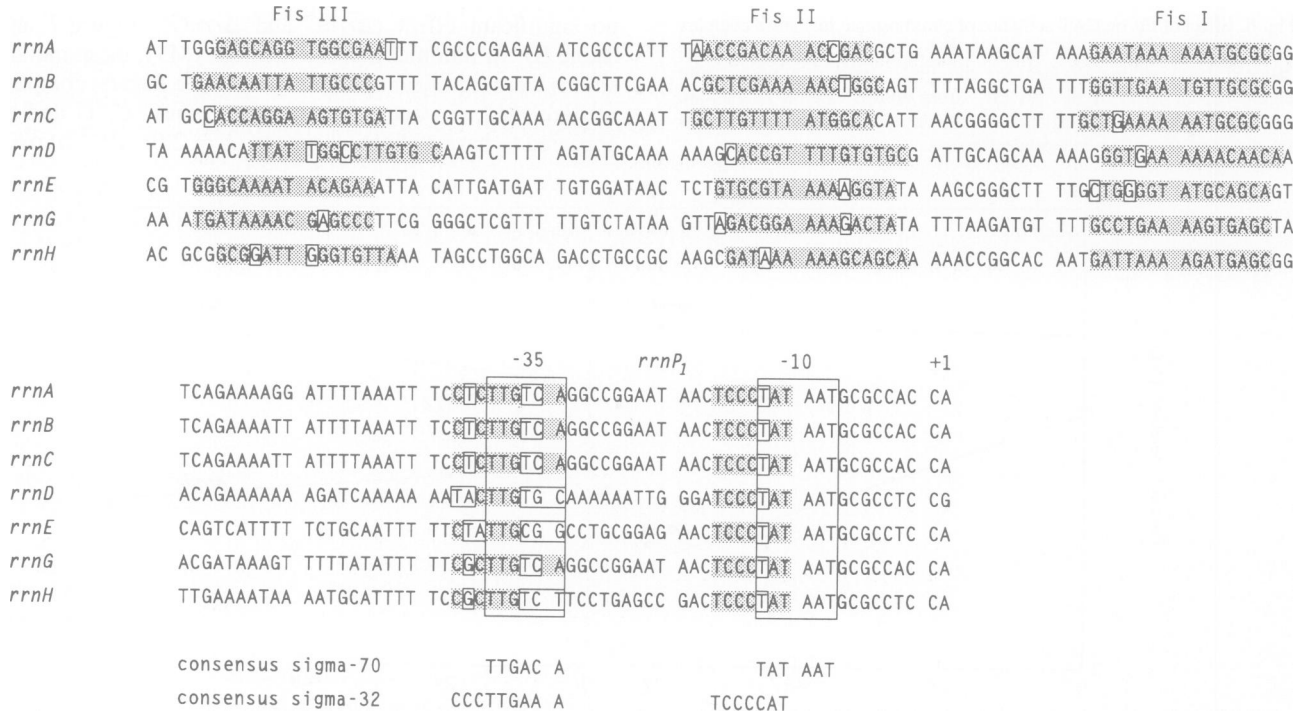

Fig. 8. Sequences of the seven ribosomal RNA P_1 promoter regions. Sequences are from Nakahigashi and Inokuchi (1990; *rrnA*), Brosius *et al.* (1981; *rrnB*), Holben *et al.* (1985; *rrnC*), Young and Steitz, (1979; *rrnD* and *rrnH*), deBoer *et al.* (1979) and Aiba and Mizobuchi (1989; *rrnE*) and Shen *et al.* (1982; *rrnG*). The 5′ sequences of the *rrnD* and *H* operons are from this laboratory and their upstream sequence data have been deposited in the EMBL Data Library under the accession numbers X67682 and X67217, respectively. The Fis binding sites (consensus: G/T--PyPu--A/T--PyPu--C/A; Hubner and Arber, 1989) are stippled in the top portion of sequence and the potential heat shock promoters are stippled in the lower portion. Non-consensus nucleotides are in open boxes. The −10 and −35 regions (large open boxes) and consensus sequence of the σ^{70} promoters are shown in addition to the heat shock σ^{32} consensus.

and −154 relative to P_1, both of which contain bent DNA (Gourse *et al.*, 1986; Plaskon and Wartell, 1987). The more upstream region from P_1 contains three potential binding sites for the Fis protein, which contribute 10- to 20-fold to the activation (Ross *et al.*, 1990). The contribution of Fis to the expression of individual *rrn* operons will be discussed later. The second region consists of a long A−T stretch that bends DNA in the absence of any protein factor. Plaskon and Wartell (1987) have carried out a DNA curvature score analysis of a series of strong *E.coli* promoters. Five of the six highest scores were *rrn* P_1 promoters, with *rrnD* P_1 (12.3) and *rrnG* P_1 (9.2) having close to twice the scores of *rrnH, B* and *C* P_1 (~5.6). Of the *rrn* P_2 promoters, *rrnA* P_2 and *rrnC* P_2 (1.2) had twice the curvature score of *rrnE, G* and *H* P_2 (0.6). In our experiments, however, the hierarchy of promoter strengths of the lysogen fusions in LB glucose (Figure 4A) did not reflect the hierarchy predicted by this analysis. Although a similar range of differences in promoter strengths was observed (1.5-fold), the *rrnD* and *rrnG* operons actually had the weakest and the *rrnH* and *B* operons the greatest intrinsic promoter strengths. Thus, it is clear that factors in addition to A−T-mediated DNA curvature are important in governing *rrn* promoter activity.

In addition to intrinsic promoter strength, it seemed likely that chromosomal location relative to the origin of replication would affect expression of individual ribosomal RNA operons, particularly under fast growth conditions. In rapidly growing cells, the time taken to replicate the whole *E.coli* chromosome is much greater than the time taken for cell division. To overcome this problem, *E.coli* starts new rounds of replication before earlier replication forks have reached the terminus of replication and newly divided cells inherit chromosomes that are already partially replicated (von Meyenburg and Hansen, 1987). Thus, the *rrnC, A, B* and *E* operons, which are close to the origin of chromosomal replication *oriC*, have a higher copy number than the more distally located *rrnD, G* and *H* operons (see Figure 1). By theoretical calculation, there should be an ~2-fold difference between the expression of *rrnG* and the *rrnC, A, B, E* cluster. We expected to see this copy number effect reflected in the assays of the *rrn* P_1P_2−CAT fusions in their normal chromosomal locations. The range of activities (2.6-fold) agreed with the theoretical range (~2-fold) and with the notable exceptions of the *rrnC* and *rrnH* constructs, the hierarchy of CAT activities from these fusions on complex medium correlated well with the relative chromosomal locations of each of the ribosomal operons (Figure 4B).

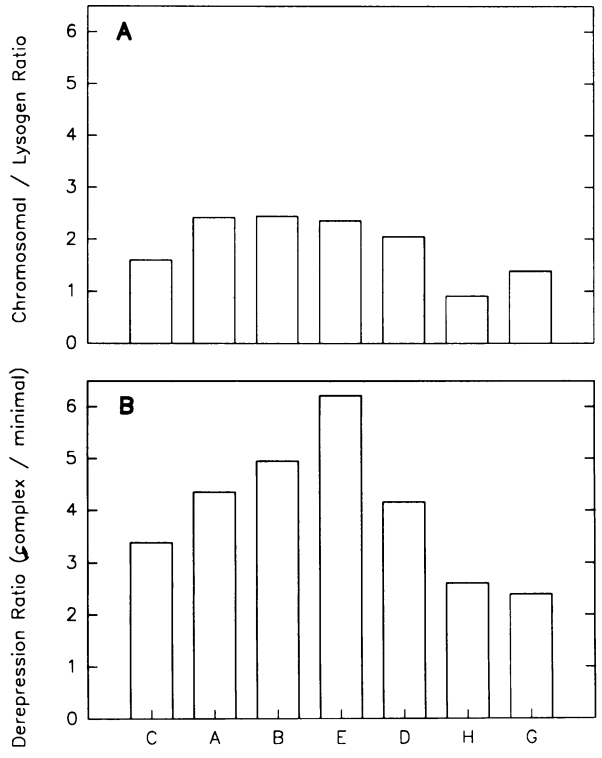

Fig. 9. Ratios of CAT activities calculated from Figure 4. **A.** Ratio of expression of chromosomal to lysogen fusions in LB glucose. **B.** Derepression ratios (LB Glucose/M9 Glucose) of chromosomal fusions. Fusions are arranged in increasing distances from the origin of replication.

The *rrnC* promoter fusion (0.5 min from *oriC*) had significantly lower activity than the *rrnA, B* or *E* constructs (2.5, 5.8 and 6.5 min from *oriC* respectively). However, when we measured *rrnC*−CAT mRNA levels directly in the course of the stringent control experiments (see Figure 5), it was apparent that *rrnC* was transcribed similarly to *rrnA, B* and *E*. Thus, there appeared to be a problem with translation of the CAT message in the *rrnC* fusion. Immediately 3′ of *rrnC* 23S rRNA is the only copy of a tryptophanyl tRNA gene on the *E.coli* chromosome. Furthermore, the 220 codon CAT mRNA contains five tryptophan codons, almost twice the average tryptophan content in most *E.coli* proteins (Zhang and Zubay, 1991). We therefore explored the possibility that the *rrnC*−CAT construct might interfere with expression of the downstream tryptophanyl tRNA. The decreased expression was 70% relieved by the overexpression of $tRNA^{Trp}$ from plasmid pCDS-110 (Rojiani, 1989; data not shown) suggesting that $tRNA^{Trp}$ limitation for the translation of the CAT message was indeed the cause of this anomaly.

Expression of the *rrnH* operon was affected by its chromosomal location in a growth medium dependent way. On complex medium, the *rrnH* fusion was expressed ~10% better on the lysogen than in its chromosomal site, which is 12.4 min closer to *oriC* (compare Figure 4A and B). However, this effect was not apparent in minimal medium. Since the *rrnH* operon is highly expressed in both complex and minimal media at the lambda *att* site, it is clear that the lower expression of *rrnH* in its native chromosomal location is not due to an intrinsic property of the *rrnH* promoter. This suggests a growth medium dependent regulation of chromosome structure occurs in the region of the *rrnH* operon. The effect is best seen when the ratio of chromosomal to lysogen fusions is calculated for complex medium (Figure 9A). We are currently investigating ways to examine the nature of this unusual effect.

rrn expression on minimal medium

On minimal M9 medium, much narrower ranges of expression were seen (Figure 4C and D). The total CAT activity from the chromosomal fusions was about one-quarter of that on complex medium (compare Figure 4B and 4D) and presumably because *E.coli* has fewer replication forks in slow growth conditions, the effect of chromosomal location on gene expression was significantly decreased. The reduction of the gene dosage effect (85%) was slightly greater than the theoretical calculation (60%) of Ellwood and Nomura (1982). The hierarchy of CAT activities from the chromosomal fusions was for the most part similar to that

obtained with complex medium. However, expression of *rrnE* was significantly lower than anticipated (Figure 4D), resulting in a high derepression ratio for *rrnE* (expression on LB/expression on M9; Figure 9B). In the case of *rrnE*, it is clear that the inhibition is at the level of promoter activity because the *rrnE* P_1P_2−CAT fusion was also poorly expressed on lambda lysogens in minimal medium (Figure 4C). Thus, it appears that the *rrnE* promoter is down-regulated more than other *rrn* promoters in slow growth conditions and may therefore be an interesting, naturally existing variant with which to study growth rate regulation.

Response of rrn promoters to stringent control

The accumulation of uncharged tRNA causes an abrupt shut down of stable RNA (rRNA and tRNA) synthesis in *E.coli*, a phenomenon known as the stringent response (for review see Cashel and Rudd, 1987). This effect is mediated through the accumulation of the nucleotide alarmone ppGpp, synthesized by the *relA* gene product and causes decreased transcription initiation at susceptible promoters. In the ribosomal RNA operons, the −35 and G−C rich discriminator (nucleotides −8 to −1) regions of P_1, but not P_2, have been implicated in stringent control. Amino acid analogues, such as serine hydroxamate, can be used to induce a starvation response for their corresponding amino acids by inhibiting tRNA aminoacylation (Merlie and Pizer, 1973). We have compared the effect of serine hydroxamate on the expression of each of the chromosomal *rrn*−CAT fusions. All seven operons were stringently regulated, exhibiting a 2- to 3-fold decrease in expression upon amino acid starvation (Figure 5). This is consistent with the decrease seen with the plasmid-contained *rrnA* operon by Cashel and co-workers in similar experiments (Sarmientos *et al.*, 1983). The *rrnD* and *rrnE* operons had the strongest responses, which is interesting in light of the fact that these operons also have the most variation in the −35 and discriminator regions of *rrn* P_1 (see Figure 8). While transcript stability is undoubtedly also affected by the addition of serine hydroxamate (Sarmientos *et al.*, 1983) the relative contribution to each of the CAT fusions should be equal as the fusion point is the same for the seven operons (position 612 of the 16S gene). Thus, the differences noted are valid measurements of the stringent control of each of the *rrn* promoters and not likely to be differential effects on mRNA stability.

Effect of heat shock on rrn transcription

E.coli rapidly accumulates a set of ~17 heat shock proteins within 1−2 min of a sudden shift up in temperature (for a review, see Neidhardt *et al.*, 1984). Peak synthesis occurs 5−10 min after induction, after which synthesis declines to a level characteristic of the new temperature. The magnitude of the induction is dependent on the magnitude of the temperature shift and can be as high as 10-fold. The genes for each of these proteins have consensus sequences for the heat shock RNA polymerase, $E\sigma^{32}$. Sequences highly homologous to consensus σ^{32} recognition sequences interdigitated with each of the *rrn* P_1 promoters have been noted by J.T.Newlands and R.L.Gourse (personal communication). This group has shown core RNA polymerase binds to these sequences in *rrnB* in the presence of σ^{32} and can initiate transcription *in vitro*. All seven operons have six out of seven consensus nucleotides in the −10 region (TCCCTAT versus TCCCCAT). The *rrnA, B, C* and *G* operons have the closest match (six out of nine) to the σ^{32} consensus in the −35 region (see Figure 8). The *rrnH* promoter has five out of nine consensus nucleotides and *rrnD* and *E* have only four. It is interesting to note that the gene immediately upstream of the *rrnG* operon, *clpB*, is also a heat shock gene (Squires *et al.*, 1991). Preliminary experiments suggested there might be significant readthrough the *clpB* transcription terminator into the *rrnG* operon (C.Squires, unpublished data). We wished to see if the ribosomal RNA operons would show a typical heat shock response following a temperature upshift (30°C to 42°C) and if so, whether all operons would respond equally. The chromosomal fusion strains were assayed on slow growth medium (M9 + casamino acids) so any increases should be more easily seen (Table I). Although operons with the closest match to consensus showed the greatest induction, only subtle increases (<10%) in specific CAT activity occurred and differences between each of the operons were minor. Furthermore, expression of the *rrnG* operon does not appear to be significantly affected by the 10-fold heat induction of the upstream *clpB* gene. These results suggest that a large peak of rRNA synthesis probably does not occur upon heat shock, consistant with observations by Zengel and Lindahl (1985). (Measurement of the *rrn* P_1P_2−CAT mRNAs from each of the fusions after 5 min heat shock also confirmed this result; data not shown). Thus, though it is clear that *rrn* expression is slightly increased following a temperature shift, none of the operons show a classical heat shock response. However, we do not know the relative contributions of σ^{70} and σ^{32} to the *rrn* expression measured. Under heat shock conditions, the σ^{32} promoter may be predominant, ensuring continued *rrn* expression at elevated temperatures. Our experiments were not designed to distinguish between these possibilities.

Effect of fis mutation on rrn expression

The Fis protein binds to three sites upstream of the *rrnB* P_1 promoter, causing a 10- to 20-fold activation of gene expression *in vitro* (Ross *et al.*, 1990). The other six operons also have three potential Fis binding sites, G/T--PyPu--A/T--PyPu--C/A (Figure 8; Verbeek *et al.*, 1990). However, the homology to consensus varies significantly from site to site and from operon to operon; *rrnB* and *rrnC* appear to have the closest total match and *rrnD* the worst. To see whether the seven ribosomal RNA operons were differentially activated by Fis, we measured the expression of the *rrn* P_1P_2−CAT fusions in a *fis* mutant. The 10- to 20-fold decrease in expression seen *in vitro* may not be seen *in vivo* because the decrease is partly masked by derepression of the feedback inhibition system. All of the operons had 2.1- to 2.5-fold decreased expression in the *fis*$^-$ strain except *rrnG*, which was only affected 1.5-fold (Figure 6). Thus, the *rrnG* promoter region is either activated to a lesser extent by Fis than the other operons or can be derepressed better. The *rrnG* promoter sequence contains three potential Fis binding sites that do not appear significantly different from those of the *rrnA, E,* or *H* operons (see Figure 8). On the other hand, the *rrnG* lysogen fusion gave lowest activity on LB glucose medium (Figure 4A) supporting the idea that Fis may have decreased affinity for this promoter. We are currently designing experiments to distinguish the relative

contribution of derepression and Fis-mediated activation to the expression of the *rrnG* operon.

Effect of rrn inactivation on growth rate

The *rrn* P_1P_2−CAT chromosomal fusions replace their respective wild type *rrn* operons and can thus be used to study the effect of inactivating individual operons on growth rate. Ellwood and Nomura (1980) showed it was possible to delete *rrnE* without deleterious consequence to growth rate on either complex or minimal media. In mixed cultures with the wild type strain for 72 h, the *rrnE* deletion showed no effect on growth in minimal medium and was only very slowly out-competed by the wild type (-1.0×10^{-3}/h) over this time period, consistent with Nomura's observations (Table II). However, this was not the general case for the other six operons. In complex medium, strains inactivated for *rrnA* and *rrnB* were outcompeted at a significantly faster rate (-4.0×10^{-3}/h) and strains lacking *rrnC* or *rrnG* had no defect at all (Figure 7 and Table II). Besides the *rrnA* and *rrnB* deletions, there was no obvious correlation between the copy number of the operon inactivated (due to chromosomal location) and the ability to compete with the wild type. To our surprise, in minimal medium, strains lacking *rrnA, C, G* or *H* were slightly more competitive than the parental strain (2.0×10^{-3}/h). Ribosomal RNA expression is slightly derepressed in these strains because they have only six *rrn* operons. Thus, strains lacking the *rrnA, C, G* and *H* operons appear to have overshot in their derepression resulting in increased ribosome synthesis and consequently, faster growth rates. If this is so, it is interesting to speculate that the derepression, which compensates for the lack of a ribosomal RNA operon, may occur in a step-like fashion rather than as a smooth linear function. Alternatively, the increased growth rates on minimal medium may be the result of a better balance between the number of ribosomes and the potential to synthesize protein. In a wild type strain in minimal medium, there is a tendency for translational capacity to exceed the internal amino acid supply and the increased production of ppGpp in minimal medium reflects this slight amino acid deficit. In strains lacking a ribosomal RNA operon, the relative excess of ribosomes over amino acids may be decreased, resulting in a slightly more efficient system and faster growth rates. A second interesting observation was that some of the deletion strains were altered in their rates of reinitiating growth from stationary phase. For this reason, the first data point was omitted in the calculation of rates of loss of these strains in the competition assay. This effect is exacerbated as more

Table III. Bacterial strains, plasmids and phage

Designation	Relevant characteristics	Source/reference
Bacterial strains		
JC7623	*arg*$^-$ *ara*$^-$ *his*$^-$ *leu*$^-$ *pro*$^-$ *recB21 recC22 sbcB15 thr*$^-$	Arps and Winkler (1987)
MG3442	F$^-$ *imm4* lysogen *his* Δ(*O-J-bio chl*A)	M.Gottesman
RJ1617	*fis767* Δ*lac*X74 *ara*D139 Δ*ara−leu7637 gal*U *gal*K *str*A	Johnson *et al.* (1988)
W1485	wild type	C.Yanofsky
W1485 ΔA to ΔH	*rrnA* P_1P_2−CAT to *rrnH* P_1P_2−CAT	This paper
W1485 Fis$^-$ ΔA to ΔH	*rrnA* P_1P_2−CAT to *rrnH* P_1P_2−CAT *fis*	This paper
W1485 λ:*rrnA*P_1P_2−CAT to λ:*rrnH*P_1P_2−CAT	single lysogens	This paper
Plasmids		
pKK232-8	CAT expression vector	Brosius (1984)
pLC19-3	*rrnA* operon cloned in colE1	Clarke and Carbon (1976)
pLC22-36	*rrnC* operon cloned in colE1	Clarke and Carbon (1976)
pLC16-6	*rrnD* operon cloned in colE1	Clarke and Carbon (1976)
pLC23-30	*rrnG* operon cloned in colE1	Clarke and Carbon (1976)
pKB6-8	*rrnA* P_1P_2 from pLC19-3 in pKK232-8	Berg (1988)
pKB2-4	*rrnC* P_1P_2 from pLC22-36 in pKK232-8	Berg (1988)
pKB4-1	*rrnD* P_1P_2 from pLC16-6 in pKK232-8	Berg (1988)
pKB3-5	*rrnG* P_1P_2 from pLC23-30 in pKK232-8	Berg (1988)
pJF1	*rrnB* P_1P_2 from λ534 in pKK232-8	This laboratory
pJF3	*rrnE* P_1P_2 from λ531 in pKK232-8	This laboratory
pJF2	*rrnH* P_1P_2 from λ124 in pKK232-8	This laboratory
pCDS-110	Asp and Trp tRNAs under control of P_{tac}	Rojiani *et al.* (1989)
Phage		
λD69	*imm21* lambda cloning vector	Mizusawa and Ward (1982)
λtet35	*imm21* λD69:: '*bla tet* CAT' recombination vector	This laboratory
λB446	*imm21 b515 att24 int*$^+$ helper phage	M.Gottesman
λG345	*imm4 int red*	M.Gottesman
λ534	*rrnB* operon cloned in λ2001	Kohara *et al.* (1987)
λ531	*rrnE/D* hybrid operon cloned in λEMBL4	Kohara *et al.* (1987)
λ124	*rrnH* operon cloned in λEMBL4	Kohara *et al.* (1987)
SUM6(+)	624bp *Hin*cII *lacZ* fragment in M13 mp7	Aksoy *et al.* (1984a)
mpcat10(+)	527bp *Rsa*I CAT fragment in M13 mp18	This laboratory

operons are sequentially inactivated (C.Condon, C.Squires and C.L.Squires, manuscript in preparation), suggesting that a full complement of *rrn* operons allows more efficient adaptation to changing nutrient environments.

Summary

We have compared the expression of the seven ribosomal RNA operons in *E.coli* under a variety of conditions to gain insight into the stable preservation of multiple *rrn* copies in organisms throughout evolution. None of the seven *E.coli* operons were essential for logarithmic growth on either minimal or complex media and all of the operons were expressed under each of the stress conditions tested: amino acid starvation, heat shock and a mutation in the *fis* gene. Although the expression of each of the rRNA operons under a number of different conditions was for the most part similar, a number of interesting differences were highlighted: *rrnH* showed growth medium-dependent regulation of its chromosomal context; *rrnE* had unusual growth rate control of its promoter region; operons varied in their response to amino acid starvation; and the *rrnG* operon had unusual Fis activation. Thus, it seems unlikely that 'dedicated' functions exist for any of the seven ribosomal RNA operons, at least for these conditions tested, and the persistence of multiple *rrn* operons in *E.coli* may reflect a selective advantage in the ability to adapt efficiently to changing growth conditions.

Materials and methods

Bacterial strains and plasmids

The strains of *E.coli*, plasmids and phage used in this paper are described in Table III. Plasmid DNA was isolated by the method of Birnboim and Doly (1979).

Construction of rrn P_1P_2–CAT fusion plasmids

Each of the *rrn* promoter fragments, except *rrnE*, was purified as a blunt *Sma*I fragment and cloned into the *Sma*I site of pKK232-8 (Brosius, 1984). The *rrnE* promoter fragment was a *Sma*I–*Pst*I fragment, blunted and cloned into pKK232-8 cut with *Sma*I. The promoter fragments ranged in size from 3.5 kb (*rrnA* and *rrnE*) to ~7.8 kb (*rrnC*) and were purified from either the Clarke and Carbon (1976) plasmids pLC19-3 (*rrnA*), pLC22-36 (*rrnC*), pLC16-6 (*rrnD*), pLC23-30 (*rrnG*) or the Kohara *et al.* (1987) λ phage λ534 (*rrnB*), λ531 (*rrnE*), λ124 (*rrnH*).

Fragment transformation of recBC sbcB strain JC7623

The promoter–CAT fusions were crossed onto the *E.coli* chromosome by fragment transformation of JC7623 *recBC sbcB* (Arps and Winkler, 1987). Plasmids containing the *rrn* promoter regions fused to CAT–5S–t_1t_2 were cleaved with *Bgl*I to obtain linear DNA, except the *rrnC* construct, which was cut with *Cla*I. The restricted DNA was used to transform *E.coli* JC7623 made competent by modifications of the method of Cohen *et al.* (1972; Figure 2). Cells were incubated in ice cold 5 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$ for 10 min. After centrifugation, the cells were resuspended in ice cold 5 mM Tris–HCl, 5 mM $MgCl_2$ and 100 mM $CaCl_2$, and incubated on ice for a further 25 min. The cells were then centrifuged and resuspended in one-tenth volume of the Tris–Mg–Ca buffer. Transformants were selected on chloramphenicol-containing plates (25 μg/ml) and screened for absence of plasmid DNA. Antibiotics were purchased from Sigma.

P1 transductions

P1 *vir* lysates were made of the JC7623 strains successfully transformed with the linear plasmids. Lysates were used to transduce W1485 to chloramphenicol resistance as described by Miller (1972). Resistant clones were prepared for Southern blot analysis of total chromosomal DNA. *Fis::kan* derivatives of the chromosomal fusion strains were made using a P1 *vir* lysate grown on RJ1617 *fis767*. This strain has most of the *fis* gene replaced by a gene for kanamycin resistance (Johnson *et al.*, 1988).

Preparation of chromosomal DNA

Chromosomal DNA was prepared for Southern analysis of ribosomal operons using modifications of the method of Grimberg *et al.* (1989). The chromosomal DNA was incubated at 65°C for 3–4 h in the presence of 1 mg/ml proteinase K. DNA was then extracted with an equal volume of phenol and precipitated with 30 μl 3 M NaOAc + 0.1 mM EDTA and 1 ml (−20°C) 95% ethanol. Pellets were dried and resuspended in 200 μl TE buffer (25 mM Tris–HCl, pH 7.5, 0.1 mM EDTA).

Southern blot analysis

Chromosomal DNA was digested to completion with the restriction endonucleases *Bam*HI and *Pst*I and electrophoresed on 0.8% agarose gels. The DNA was blotted onto Hybond-N nylon filters (Amersham) according to the manufacturer's instructions. Hybridization with P^{32}-oligonucleotide probes was as described by Southern (1975). The template for synthesis of the radiolabelled 16S probe was made by employing PCR technique between two synthetic primers: 5′-AACCTGGGAACTGCATCTG-3′ and 5′-TGAATCACAAAGTGGTAAGCC-3′ at positions 619 and 1459 of the 16S gene, respectively. The template for PCR was *E.coli* chromosomal DNA. Using the same two primers, this 853 bp fragment was used as a template for the incorporation of [α-^{32}P] dATP by Sequenase™ (United States Biochemical Corporation) to make the radiolabelled probe. Excess, unincorporated radionucleotides were removed by purification in NACS PREPAC columns (Bethesda Research Laboratories, Gaithersburg, MD).

Construction of rrn lysogen fusions

The *rrn* P_1P_2–CAT constructs on plasmids were recombined onto a lambda phage designed for this purpose, λtet35. λtet35 (H.Schreiner, unpublished) was derived by cloning a ~4 kb *Bst*YI fragment into the *int* gene of λD69 (Mizusawa, 1982). This fragment has a gene for tetracycline resistance cloned between a portion of the β-lactamase (*bla*) gene and a portion of the CAT gene. Thus, homology is provided for a double recombination event between the *bla* and CAT genes that are present on both the plasmid and phage. Such a double crossover results in transfer of the intervening plasmid sequences to the phage, loss of the tetracycline resistance gene and gain of ampicillin and chloramphenicol resistance by the phage. λtet35 lysates made on strains carrying the *rrn* P_1P_2–CAT plasmids were mixed with equal amounts of helper phage, λB446 *int*$^+$ and allowed to infect 10 mM $MgSO_4$ washed W1485 cells for 10 min at room temperature. Dilutions of this mix were plated on ampicillin- (100 μg/ml) chloramphenicol- (5 μg/ml) and fusaric acid-containing plates (12 μg/ml; Sigma) to select for tetracycline sensitivity. Tetracycline sensitive strains have been shown to be resistant to lipophilic chelating agents, such as fusaric acid (Bochner *et al.*, 1980). The resulting strains were then examined for single copy number lambda lysogens (see below).

Test for single copy lysogens

Single copy lambda lysogens can be distinguished from strains containing several tandem phage by the number of *cos* sites on the chromosome. We used the following method to detect multiple *cos* sites: the *rrn* P_1P_2–CAT lysogens (*imm21*), were infected with the *imm4 int red* phage, λG345. The resulting lysates were plated on a lawn of the *imm4* lysogen, MG3442. Multiple tandem lysogens have several resident *cos* sites and these can be cut out of the chromosome by the *ter* system of the heteroimmune infecting phage, λG345. The resulting lysate thus contains a large number of intact *imm21* phage which can form plaques on an *imm4* lawn. Single lysogens, however, have only one resident *cos* site and will not be packaged during superinfection by another phage. In this case, the *imm4* lysate will not contain any heteroimmune phage and will not form plaques on an *imm4* lawn.

CAT assays of rrn promoter strengths

Strains were grown in 30 ml LB glucose or M9 minimal medium to OD_{420} of 0.65. Cells were put on ice and duplicate 10 ml samples were centrifuged (4°C) at 10 000 r.p.m. in a JA17 rotor and resuspended in 3 ml 50 mM Tris–HCl pH 8.0. Cells were spun down again and resuspended in 200 μl 20 mM Tris–HCl pH 8.0, 10 mM EDTA for preparation of cell extracts. Extracts were made by sonication for 10 s in a salt–ice bath. CAT assays were carried out as described by Shaw (1975). Protein microassays were done using the Biorad Protein Assay Kit II according to the manufacturer's instructions. CAT activities were calculated as units per milligram of protein.

Assay of stringent control

Strains were grown to OD_{420} of 0.65 on LB glucose medium. The stringent response was induced by the addition of serine hydroxamate (1 mg/ml; Sigma). After 20 min further incubation RNA was isolated by modification of the method of Sarmientos *et al.* (1983). 25 ml of culture were added to 10 ml of 90–100°C lysis buffer (1% SDS, 100 mM NaCl and 8 mM EDTA) for 5 min. (0.5 ml of carrier cells containing an IPTG-induced *lacZ* plasmid were added as an internal control for lysis). 25 ml water-saturated phenol were added while the lysis mixture was still hot. Samples were then placed on a 37°C orbital shaker for 20 min. RNA was ethanol precipitated

and resuspended in 5 ml 0.3 M NaOAc, pH 5.2. RNA was phenol extracted twice at 65°C, ethanol precipitated and resuspended in 250 μl DEPC treated H_2O. Typically, 720 μg of RNA was hybridized with 10 μg single stranded DNA in 0.3 M NaCl; 1 mM EDTA; 25 mM Tris−HCl, pH 8.0 for 1 h at 65°C. The single stranded DNAs used were a 527 bp *Rsa*I fragment of the CAT gene cloned in M13 mp18 and a 624 bp fragment of the *lacZ* gene cloned in M13 mp7. Ribonuclease T1 (360 units; BRL or Sigma) and S1 (700 units; BRL) digestion followed for 1 h at 42°C. Samples were ethanol precipitated and run on 2% agarose gels. Gels were photographed on Polaroid 665 film and the negatives recovered according to the manufacturer's instructions. Relative amounts of CAT/*lacZ* messages before and after serine hydroxamate addition were calculated using a Bio Image Visage110™ densitometer (Millipore).

Assay of heat shock

Cells were grown at 30°C to OD_{420} of 0.6 in M9 glucose medium supplemented with 0.05% casamino acids. 10 ml were harvested for CAT assay as described above. The remainder was transferred to 42°C for 10 min, before a further 10 ml were withdrawn for assay. The effect of heat shock was quantified by calculating the ratio of CAT activity at 42°C to that at 30°C.

Steady state mixed culture experiments

Strains were grown overnight in either LB glucose or M9 minimal media (Miller, 1972). They were then inoculated (1:1000 dilution) in 1:1 mixtures with the parent strain, W1485, in chemostats. Flow rate was 4.35 ml/min, volume was 100 ml in LB glucose. In M9 medium, the flow rate was 2.0 ml/min, volume 100 ml. The mixed culture was streaked on YT plates in duplicate every ~12 h for ~72 h. 100 colonies were picked from each of these plates to YT plates with 25 μg/ml chloramphenicol to monitor the relative proportion of each strain.

Acknowledgements

We wish to thank the following people for sending us strains used in these experiments: E.Goldman, M.Gottesman, R.Johnson and H.Schreiner. We also thank M.Gottesman for suggesting how to test for single copy lambda lysogens; R.L.Gourse for pointing out heat shock promoter sequences and for sharing unpublished information; and B.Ross, for sequencing *rrn* promoter regions. This work was supported by grant GM24751 from the National Institutes of Health.

References

Aiba,A. and Mizobuchi,K. (1989) *J. Biol. Chem.*, **264**, 21239−21246.
Aksoy,S., Squires,C.L. and Squires,C. (1984a) *J. Bacteriol.*, **157**, 363−367.
Aksoy,S., Squires,C.L. and Squires,C. (1984b) *J. Bacteriol.*, **159**, 260−264.
Arps,P.J. and Winkler,M.E. (1987) *J. Bacteriol.*, **169**, 1061−1070.
Berg,K.L. (1988) Ph.D. thesis. Columbia University, New York, NY.
Birnboim,H.C. and Doly,J. (1979) *Nucleic Acids Res.*, **7**, 1513−1523.
Bochner,B.R., Huang,H-C., Schieven,G.L. and Ames,B.N. (1980) *J. Bacteriol.*, **143**, 926−933.
Boros,I., Kiss,A. and Venetianer,P. (1979) *Nucleic Acids Res.*, **6**, 1817−1830.
Bott,K., Stewart,G.C. and Anderson,A.G. (1984) In Hoch,J.A. and Ganesan,A.T. (eds) *Syntro Conference on Genetics and Biotechnology of Bacilli*. Academic Press, Inc., New York. pp. 19−34.
Bremer,H. and Dennis,P.P. (1987) In Neidhardt,F.C., Ingraham,J.L., Low,K.B., Magasanik,B., Schaechter,M. and Umbarger,H.E. (eds), *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*. American Society for Microbiology, Washington, DC, pp. 1527−1542.
Brosius,J. (1984) *Gene*, **27**, 151−160.
Brosius,J., Dull,T., Sleeter,D. and Noller,H. (1981) *J. Mol. Biol.*, **148**, 107−127.
Carbon,P., Ehresmann,C., Ehresmann,B. and Ebel,J.P. (1979) *Eur. J. Biochem.*, **100**, 399−410.
Cashel,M. and Rudd,K.E. (1987) In Neidhardt,F.C., Ingraham,J.L., Low,K.B., Magasanik,B., Schaechter,M. and Umbarger,H.E. (eds), *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*. American Society for Microbiology, Washington, DC, pp. 1410−1438.
Clarke,L. and Carbon,J. (1976) *Cell*, **9**, 91−99.
Cohen,S., Chang,A. and Hsu,L. (1972) *Proc. Natl. Acad. Sci. USA*, **69**, 2110−2114.
deBoer,H.A., Gilbert,S.F. and Nomura,M. (1979) *Cell*, **17**, 201−209.
Ellwood,M. and Nomura,M. (1980) *J. Bacteriol.*, **143**, 1077−1080.
Ellwood,M. and Nomura,M. (1982) *J. Bacteriol.*, **149**, 458−468.
Grimberg,J., Maguire,S. and Belluscio,J. (1989) *Nucleic Acids Res.*, **17**, 8893.
Gourse,R.L., deBoer,H.A. and Nomura,M. (1986) *Cell*, **44**, 197−205.
Gunderson,J.H., Slogin,M.L., Wollett,G., Hollingdale,M., de la Cruz,V.F., Waters,A.P. and McGutchan,T.F. (1987) *Science*, **238**, 933−937.
Harvey,S., Hill,C.W., Squires,C. and Squires,C.L. (1988) *J. Bacteriol.*, **170**, 1235−1238.
Hill,C.W. and Harnish,B.W. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 7069−7072.
Holben,W.E., Prasad,S.M. and Morgen,E.A. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 5073−5077.
Hubner,P. and Arber,W. (1989) *EMBO J.*, **8**, 577−585.
Hui,I. and Dennis,P.P. (1985) *J. Biol. Chem.*, **260**, 899−906.
Jinks-Robertson,S. and Nomura,M. (1987) In Neidhardt,F.C., Ingraham,J.L., Low,K.B., Magasanik,B., Schaechter,M. and Umbarger,H.E. (eds.) *Escherichia coli* and *Salmonella typhimurium: Cellular and Molecular Biology*. American Society for Microbiology, Washington, DC, pp. 1358−1385.
Johnson,R.C. and Simon,M.I. (1985) *Cell*, **41**, 781−789.
Johnson,R.C., Ball,C.A., Pfeffer,D. and Simon,M.I. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 3484−3488.
Josaitis,C.A., Gaal,T., Ross,W. and Gourse,R.L. (1990) *Biochim. Biophys. Acta*, **1050**, 307−311.
Koch,C. and Kahmann,R. (1986) *J. Biol. Chem.*, **261**, 15673−15678.
Kohara,Y., Akiyama,K. and Isono,K. (1987) *Cell*, **50**, 495−508.
La Fauci,G., Widom,R.L., Jarvis,E.D. and Rudner,R. (1986) *J. Bacteriol.*, **165**, 204−214.
Leirmo,S. and Gourse,R.L. (1991) *J. Mol. Biol.*, **220**, 555−568.
Li,S.C., Squires,C.L. and Squires,C. (1984) *Cell*, **38**, 851−860.
Long,E.O. and Dawid,I.B. (1980) *Annu. Rev. Biochem.*, **49**, 727−764.
Merlie,J.P. and Pizer,L.I. (1973) *J. Bacteriol.* **116**, 355−366.
Miller,J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 201−205 and 431−433.
Mizusawa,S. and Ward,D.F. (1982) *Gene*, **20**, 317−322.
Nakahigashi,K. and Inokuchi,H. (1990) *Nucleic Acids Res.*, **18**, 6439.
Neidhardt,F.C., VanBogelen,R.A. and Vaughn,V. (1984) *Annu. Rev. Genet.*, **18**, 295−329.
Noller,H.F. and Nomura,M. (1987) In Neidhardt,F.C., Ingraham,J.L., Low,K.B., Magasanik,B., Schaechter,M. and Umbarger,H.E. (eds), *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*. American Society for Microbiology, Washington, DC, pp. 104−125.
Nomura,M., Gourse,R.L. and Baughman,G. (1984) *Annu. Rev. Biochem.*, **53**, 75−117.
Plaskon,R.R. and Wartell,R.M. (1987) *Nucleic Acids Res.*, **15**, 785−796.
Rojiani,M.V., Jakubowski,H. and Goldman,E. (1989) *J. Bacteriol.*, **171**, 6493−6502.
Ross,W., Thompson,J.F., Newlands,J.T. and Gourse,R.L. (1990) *EMBO J.*, **9**, 3733−3742.
Sarmientos,P., Sylvester,J.E., Contente,S. and Cashel,M. (1983) *Cell*, **32**, 1337−1346.
Shaw,W.V. (1975) *Methods Enzymol.*, **43**, 737−755.
Shen,W-F., Squires,C. and Squires,C. (1982) *Nucleic Acids Res.*, **10**, 3303−3313.
Southern,E.M. (1975) *J. Mol. Biol.*, **98**, 503−517.
Squires,C.L., Pedersen,S., Ross,B.M. and Squires,C. (1991) *J. Bacteriol.*, **173**, 4254−4262.
Verbeek,H., Nilsson,L., Baliko,G. and Bosch,L. (1990) *Biochim. Biophys. Acta*, **1050**, 302−306.
von Meyenburg,K. and Hansen,F.G. (1987) In Neidhardt,F.C., Ingraham,J.L., Low,K.B., Magasanik,B., Schaechter,M. and Umbarger,H.E. (eds) *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*. American Society for Microbiology, Washington, DC, pp. 1555−1577.
Widom,R.L., Jarvis,E.D., La Fauci,G. and Rudner,R. (1988) *J. Bacteriol.*, **170**, 605−610.
Woese,C.R. and Olsen,G.J. (1986) *Sys. Appl. Micro.*, **7**, 161−177.
Young,R.A. and Steitz,J.A. (1979) *Cell*, **17**, 225−234.
Zengel,J.M. and Lindahl,L. (1985) *J. Bacteriol.*, **163**, 140−147.
Zhang,S. and Zubay,G. (1991) In Setlow,J.K. (ed.), *Genetic Engineering: Principles and Methods*. Plenum Press, New York, Vol. 13, pp. 73−113.

Received on April 27, 1992; revised on July 10, 1992