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Growth Rate-Dependent Accumulation of RNA from Plasmid-Borne rRNA Operons in *Escherichia coli*

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Inadequate regulation of the expression of additional plasmid-borne rRNA operons in *Escherichia coli* was exaggerated at slow growth rates, resulting in increases of approximately 100% for RNA concentration and 33% for doubling time. These observations are consistent with the hypothesis that multiple rRNA operons constitute a metabolic burden at slow growth rates.

Most prokaryotic genes are present as a single copy on the chromosome. Exceptions to this generality include the rRNA genes, which are frequently organized into an operon. The number of rRNA operons can range from 1 to as many as 13 copies per chromosome (19). This variation and the absence of any obvious correlation between rRNA operon copy number and organismal phylogeny (19) led us to investigate the potential adaptive significance of rRNA operon copy number.

It has been assumed that the number of rRNA operons is directly proportional to maximal growth rates (13). Although an organism with one rRNA operon may not be able to achieve the maximum growth rates that are obtainable by an organism with a higher multiplicity of rRNA operons, the deletion of one or two rRNA operons in *Escherichia coli* has a marginal effect on maximal growth rate (4, 8). The inactivation of single or multiple rRNA operons in *E. coli* does, however, influence the time that is required for a shift to faster growth rates upon the encounter of more favorable growth conditions (5). The enhanced capacity for a rapid response to favorable growth conditions suggests that multiple rRNA operons may be an evolutionary adaptation and an advantage to organisms which experience fluctuating growth conditions (6, 19). A potential trade-off for this enhanced capacity is the synthesis of superfluous rRNA and ribosomes under constant, slow growth conditions. While transcription of the rRNA operons is proportional to growth rate in moderate- to fast-growing cells, this relationship does not exist at slow growth rates (6, 10). Under slow growth conditions in a chemostat, the presence of "excess" ribosomes has been observed in populations of *E. coli* (15).

The potential disadvantages of multiple rRNA operons in *E. coli* were investigated in this study by increasing rRNA gene dosage with plasmid-borne rRNA operons. *E. coli* B/r which had been propagated for 2,000 generations in glucose minimal medium (16) was selected for this study to minimize changes in growth rate due to adaptation to laboratory growth conditions. Cells were transformed (17) with plasmids derived from pKK3535, which contains the *rrnB* operon from *E. coli* (3). Plasmid pEZ211 contains the functional *rrnB* operon (Fig. 1A) and was made by digesting pKK3535 with the restriction endonucleases *Sgr*AI and *Mun*I (Boehringer Mannheim, Indianapolis, Ind.) to remove the upstream λ promoter P_{L57} (14). Sequence determination of this region revealed that a fragment of 1,539 bp was removed, apparently due to star activity of *Sgr*AI. This and subsequent digestions left overhanging ends, which were polished with T4 DNA polymerase (New England Biolabs, Inc., Beverly, Mass.) and religated with T4 DNA ligase (Boehringer Mannheim). Restriction, polymerase, and ligation reactions were performed according to manufacturers' protocols. The rRNA promoters P1 and P2 were deleted in a second plasmid, pEZ200, by digesting pKK3535 with the restriction endonucleases *Sgr*AI and *Bgl*II (Fig. 1B).

Growth rates for each transformant were determined by measuring the change in optical density at 600 nm as a function of time. Growth rate experiments were initiated by inoculating 5 ml of Luria broth (LB) (17) from culture stocks preserved at −80°C. Following overnight incubation at 37°C, triplicate conditioning cultures were inoculated (1:100) in 5 ml of test medium. The test medium consisted of modified Davis minimal medium (DM) (16) or LB. DM and LB were both amended with 200 μg of ampicillin per ml when they were used to grow transformants. DM was further amended with either 1 mg of sodium acetate (Ac), sodium acetate plus 40 μg (each) of 10 essential L-amino acids (Sigma Chemical Co., St. Louis, Mo.) per ml (Ac-AA), 1 mg of glucose per ml (Glc), or glucose plus amino acids (Glc-AA). LB was amended with 1 mg of glucose per ml (LB-Glc). Aliquots (0.5 ml) of the conditioning cultures in mid-exponential growth phase were used to inoculate triplicate 50-ml cultures of specific test medium. All liquid cultures were aerated via vigorous shaking (≥200 rpm) at 37°C. To determine if differences in growth rates were correlated with the accumulation of RNA, concentrations of RNA were determined by a scaled-down orcinol assay (7) for at least three replicate cultures of the host strain and transformants carrying either pEZ200 or pEZ211 grown in each test medium. Since RNA concentrations are proportionate to growth rate (2), comparisons of RNA concentrations were made after normalization of the data to specific growth rate.

Effects attributed to carriage of the pEZ plasmid backbone and promoterless rRNA operon were assessed by comparing the growth of pEZ200 transformants with that of the host strain. There was no consistent difference between the growth rates of transformants carrying the control plasmid pEZ200 and the host strain (mean = 96%; Table 1). Similarly, the presence of pEZ200 had little effect on the concentration of RNA relative to that of the host strain on any medium (mean = 95%; Table 2).

There was, however, a dramatic effect on both growth rate and cellular RNA concentration when the rRNA promoters

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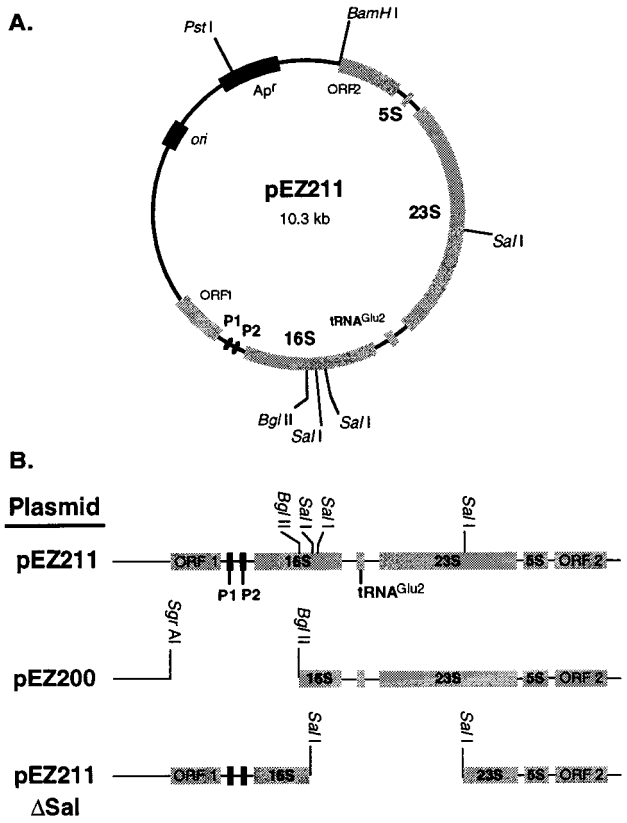

FIG. 1. (A) Map of pEZ211 showing the 6.9-kb *rrnB* operon of *E. coli* cloned into pBR322. (B) Linearized representation of *rrnB* insert in pEZ211, removal of *rrn* promoters P1 and P2 in pEZ200, and internal deletion in pEZ211ΔSal. ORF, open reading frame; Glu2, glucose 2.

were present on the plasmid-borne rRNA operon (pEZ211). At slower growth rates (with Ac, Ac-AA, and Glc), cells with pEZ211 grew an average of 33% more slowly than those with the control plasmid, pEZ200 (Table 1). Along with slower growth rates, extra rRNA operons resulted in normalized RNA concentrations which were at least 100% higher than those of the control (Table 2). Transformants carrying pEZ211 had not only higher concentrations of RNA per unit volume (fg/fl) but also more RNA per cell (Fig. 2).

The accumulation of RNA in slowly growing *E. coli* cells may be due to constitutive expression of the rRNA operons from

TABLE 1. Specific growth rates of the host strain and transformants carrying pEZ200 or pEZ211 grown in different media

Medium	Specific growth rate [μ (h^{-1})[a]]			Relative growth rate[b]	
	Host strain	pEZ200	pEZ211	pEZ200/ host strain	pEZ211/ pEZ200
Ac	0.41 (0.01)	0.35 (0.02)	0.23 (0.01)	85	66
Ac-AA	0.66 (0.01)	0.67 (0.04)	0.45 (0.00)	102	67
Glc	1.09 (0.01)	0.99 (0.05)	0.66 (0.04)	91	67
Glc-AA	1.19 (0.02)	1.15 (0.05)	0.98 (0.07)	97	85
LB-Glc	1.60 (0.01)	1.71 (0.18)	1.45 (0.31)	107	85

[a] Values are the sample means of at least three replicate cultures. Sample standard deviations are in parentheses.
[b] Relative growth rate is the ratio of specific growth rates depicted as a percentage (i.e., multiplied by 100).

TABLE 2. Concentrations of RNA of the host strain and transformants carrying pEZ200 or pEZ211 grown in different media

Medium	Concn of RNA (fg/fl)[a]			Relative RNA concn (normalized to μ)[b]	
	Host strain	pEZ200	pEZ211	pEZ200/ host strain	pEZ211/ pEZ200
Ac	26.8 (2.1)	25.0 (1.7)	33.3 (3.9)	109	202
Ac-AA	40.2 (4.9)	40.1 (3.4)	54.1 (8.7)	98	201
Glc	56.2 (3.6)	44.8 (2.5)	74.0 (9.8)	88	248[c]
Glc-AA	79.5 (8.1)	72.1 (5.8)	81.4 (5.8)	94	132
LB-Glc	122.5 (2.8)	115.7 (5.6)	122.4 (3.4)	88	125

[a] Values represent the sample means of at least three replicate cultures. Sample standard deviations are in parentheses. Cell volumes (femtoliters) of 5% formalin-fixed cells were determined with a Counter Channelizer (Coulter Electronics, Inc., Miami, Fla.).
[b] Ratio of normalized RNA concentrations (divided by μ on respective growth medium; Table 1) depicted as a percentage.
[c] Normalized RNA concentration of transformants carrying pEZ211 was 218% of that of the host strain, suggesting that the determination of RNA concentration for cells containing pEZ200 and growing on Glc was anomalously low.

the P2 rRNA promoter (9, 18). Since rRNA synthesis is linked to the expression of ribosomal proteins and other genes, the overproduction of rRNA would have the effect of diverting limiting resources to the production of unnecessary ribosomes, resulting in a decreased growth rate (10–12). The additional RNA in transformants carrying pEZ211 does not appear to be due to an increase in plasmid copy number, because in comparable experiments with a slightly different host strain and rRNA operon-containing plasmid constructs, Baracchini and Bremer (1) reported a 41% decrease in growth rates and only slight differences in the plasmid copy number at various growth rates.

The effect of extra rRNA operons on both growth rate and cellular RNA concentration was diminished when cells were grown in media which led to faster growth rates (Glu-AA and

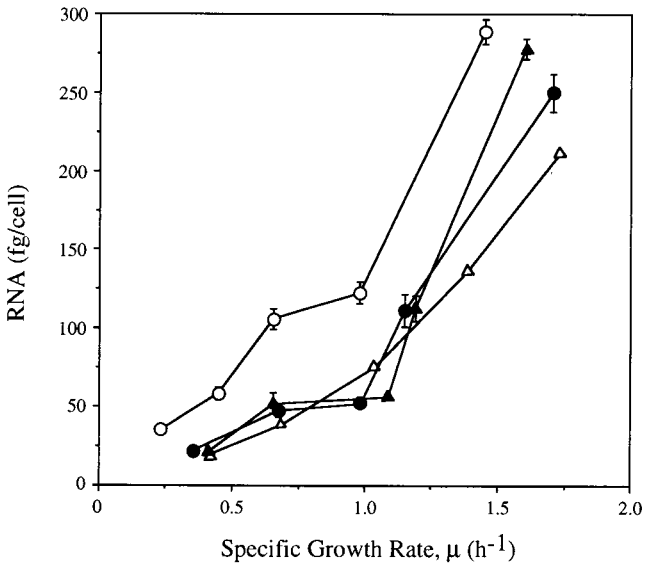

FIG. 2. Cellular RNA content (fg/cell) as a function of growth rate [μ (h^{-1})] for the evolved host strain (▲) and transformants with pEZ200 (●) or pEZ211 (○) compared with previously published values for *E. coli* B (△) (2). Error bars represent sample standard deviations.

TABLE 3. Specific growth rates for pEZ211ΔSal transformants in different media

Medium	Specific growth rate [μ (h^{-1})] for pEZ211ΔSal[a]	Relative growth rate for pEZ211ΔSal/pEZ200[b]
Ac	0.38 (0.04)	108
Ac-AA	0.60 (0.05)	88
Glc	0.82 (0.03)	83
Glc-AA	1.14 (0.18)	99
LB-Glc	1.62 (0.15)	95

[a] Same as footnote *a* in Table 1.
[b] Same as footnote *b* in Table 1.

LB-Glu). Transformants carrying pEZ211 grew 15% more slowly than those with pEZ200 (Table 1) and had an average of 29% more RNA per unit volume when the data were normalized to specific growth rate (Table 2). The smaller difference between the RNA concentrations of the pEZ200 and pEZ211 transformants suggests a more effective means of compensation for the additional rRNA operons at higher growth rates. The mechanism of compensation is most likely transcriptional regulation of the operons (12).

Nomura and colleagues (12) made direct measurements of rRNA synthesis rates with similar plasmid constructs at faster growth rates and reported that cells were able to down regulate rRNA synthesis from all rRNA operons within the cell as a result of feedback inhibition. The growth rate of these populations was reduced by 10%. This reduction was attributed to an imbalance in tRNA pools resulting from the increased expression of tRNAs located on the plasmid-borne operon. The rRNA synthesis rate in these cells was 19% higher than that in the control when data were normalized to growth rate (12), suggesting that modest RNA overproduction may have also contributed to the reduced growth rate.

A third plasmid (pEZ211ΔSal) was used to investigate the possibility that the observed changes in growth rate were due to the sequestration of RNA polymerase by the numerous promoters of the rRNA operons. The nonfunctional rRNA operon of pEZ211ΔSal was developed by digesting pEZ211 with *Sal*I, resulting in the deletion of a majority of the structural RNA genes (Fig. 1B). There was no consistent deviation in the doubling times of cells expressing nonfunctional rRNA (pEZ211ΔSal transformants) relative to that for the control (Table 3), suggesting that the presence of the promoters alone is not sufficient to explain the changes in growth rate. Cells carrying pEZ211ΔSal were elongated, frequently occurred in chains, and tended to form aggregates at slower growth rates. Although the altered cell morphology had no obvious effect on growth rate estimation, cell enumeration was ineffective via particle counting or direct microscopy. As a result, the concentration of RNA could not be determined for cells with pEZ211ΔSal.

Conclusions. The presence of extra rRNA operons in a population of *E. coli* resulted in overproduction of RNA and decreased growth rates. These effects were exaggerated at slower growth rates, suggesting that regulation of rRNA synthesis was overwhelmed at slow growth rates. The metabolic expense associated with ribosome overproduction when nutrient availability is low may be compensated for by the capacity to rapidly shift up growth rate in response to an influx of nutrients. The potential advantage of a rapid shift in growth rate may seldom be realized in stable, low-nutrient environments in which the diversion of limited resources toward the production of excess ribosomes would clearly be disadvantageous. An understanding of the advantages and disadvantages of multiple rRNA operons in different environmental conditions should provide insight into the selective pressures that influence rRNA copy number.

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REFERENCES

1. **Baracchini, E., and H. Bremer.** 1991. Control of rRNA synthesis in *Escherichia coli* at increased *rrn* gene dosage. J. Biol. Chem. **266:**11753–11760.
2. **Bremer, H., and P. P. Dennis.** 1987. Modulation of chemical composition and other parameters of the cell by growth rate, p. 1527–1542. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
3. **Brosius, J., A. Ullrich, M. A. Raker, A. Gray, T. J. Dull, R. R. Gutell, and H. F. Noller.** 1981. Construction and fine mapping of recombinant plasmids containing the *rrnB* ribosomal RNA operon in *Escherichia coli*. Plasmid **6:** 112–118.
4. **Condon, C., S. French, C. Squires, and C. L. Squires.** 1993. Depletion of functional ribosomal RNA operons in *Escherichia coli* causes increased expression of the remaining intact copies. EMBO J. **12:**4305–4315.
5. **Condon, C., D. Liveris, C. Squires, I. Schwartz, and C. L. Squires.** 1995. rRNA operon multiplicity in *Escherichia coli* and the physiological implications of *rrn* inactivation. J. Bacteriol. **177:**4152–4156.
6. **Condon, C., C. Squires, and C. L. Squires.** 1995. Control of rRNA transcription in *Escherichia coli*. Microbiol. Rev. **59:**623–645.
7. **Daniels, L., R. S. Hanson, and J. A. Phillips.** 1994. Metabolism: chemical analysis, p. 536–537. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C.
8. **Ellwood, M., and M. Nomura.** 1980. Deletion of a ribosomal ribonucleic acid operon in *Escherichia coli*. J. Bacteriol. **143:**1077–1080.
9. **Gourse, R. L., H. A. deBoer, and M. Nomura.** 1986. DNA determinants of rRNA synthesis in *E. coli*: growth rate dependent regulation, feedback inhibition, upstream activation, and antitermination. Cell **44:**197–205.
10. **Gourse, R. L., T. Gaal, M. S. Bartlett, J. A. Appleman, and W. Ross.** 1996. rRNA transcription and growth rate-dependent regulation of ribosome synthesis in *Escherichia coli*. Annu. Rev. Microbiol. **50:**645–677.
11. **Gourse, R. L., Y. Takebe, R. A. Sharrock, and M. Nomura.** 1985. Feedback regulation of rRNA and tRNA synthesis and accumulation of free ribosomes after conditional expression of rRNA. Proc. Natl. Acad. Sci. USA **82:**1069–1073.
12. **Jinks-Robertson, S., R. L. Gourse, and M. Nomura.** 1983. Expression of rRNA and tRNA genes in *Escherichia coli*: evidence for feedback regulation by products of rRNA operons. Cell **33:**865–876.
13. **Jinks-Robertson, S., and M. Nomura.** 1987. Ribosomes and tRNA, p. 1358–1385. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
14. **Kingston, R. E., R. R. Gutell, A. R. Taylor, and M. J. Chamberlin.** 1981. Transcriptional mapping of plasmid pKK3535: quantitation of the effect of guanosine tetraphosphate on binding to the *rrnB* promoters and a λ promoter with sequence homologies to the CII binding region. J. Mol. Biol. **146:** 433–449.
15. **Koch, A. L.** 1971. The adaptive responses of *Escherichia coli* to a feast and famine existence. Adv. Microb. Physiol. **6:**147–217.
16. **Lenski, R. E., M. R. Rose, S. C. Simpson, and S. C. Tadler.** 1991. Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. Am. Nat. **138:**1315–1341.
17. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., p. 1.74–1.84. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Sarmientos, P., and M. Cashel.** 1983. Carbon starvation and growth rate-dependent regulation of the *Escherichia coli* ribosomal RNA promoters: differential control of dual promoters. Proc. Natl. Acad. Sci. USA **80:**7010–7013.
19. **Schmidt, T. M.** 1997. Multiplicity of ribosomal RNA operons in prokaryotic genomes, p. 221–229. *In* F. J. deBruijn, J. R. Lupski, and G. M. Weinstock (ed.), Bacterial genomes: physical structure and analysis. Chapman and Hall, New York, N.Y.