Reversion rates in a *leuB* auxotroph of *Escherichia coli* K-12 correlate with ppGpp levels during exponential growth

Barbara E. Wright and Michael F. Minnick

Author for correspondence: Barbara E. Wright. Tel: +1 406 243 6676. Fax: +1 406 243 4184.
e-mail: bewright@selway.umt.edu

Division of Biological
Sciences, The University of
Montana, Missoula,
MT 59812-1002, USA

Two isogenic strains of *Escherichia coli* K-12 differing only in *relA*, as well as two *spoT* transductants of the *relA*[−] strain, were examined with respect to ppGpp levels and reversion rates of a *leuB*[−] allele under nine different conditions. A positive correlation was established between reversion rates and the steady-state concentration of ppGpp during exponential growth. The *leuB* genes from two *leuB*[−] strains (isogenic except for *relA*) were cloned and sequenced and found to contain a single mutation, namely, a C-to-T transition at nucleotide 857. This mutation resulted in a serine-to-leucine substitution at amino acid residue 286 of the LeuB protein. PCR products that encompassed the *leuB* lesion were generated from 53 revertants and then sequenced. Of these revertants, 36 were found to contain nucleotide substitutions that would result in a serine (wild type), valine or methionine at amino acid residue 286 of LeuB, and nearly all of them exhibited generation times similar to wild type. Seventeen of the analysed revertants were found to be suppressors that retained the encoded leucine at residue 286. The majority of the suppressor mutants exhibited generation times that were significantly longer than wild type.

Keywords: stringent response, ppGpp, mutation rates, starvation

INTRODUCTION

The complex of metabolic events triggered by amino acid starvation in *Escherichia coli* is called the stringent response (reviewed by Cashel & Rudd, 1987). This response is marked by an immediate burst in the accumulation of ppGpp, which acts to reduce the rate of rRNA and tRNA synthesis and to stimulate the rate of synthesis of 'nutritional stress' proteins such as amino acid biosynthetic enzymes. The synthesis of ppGpp is primarily controlled by ppGpp synthase I (the *relA* gene product) and requires mRNA, ribosomes and codon-specified uncharged tRNA bound in the ribosomal A site. The activity of the ppGpp degradative enzyme, (p)ppGpp 3'-pyrophosphohydrolase (the *spoT* gene product) is also regulated by starvation conditions (Fiil *et al.*, 1972; Gallant *et al.*, 1972). This enzyme, known as ppGpp synthase II, may be bifunctional, i.e. capable of catalysing either synthesis or degradation of ppGpp (Fehr & Richter, 1981; Xiao *et al.*, 1991). Clearly, an increase in ppGpp levels can result from a higher rate of synthesis or from an inhibition of degradation. Evidence indicates that cellular ppGpp levels are regulated by the

charging of total tRNA species, as a function of the intracellular concentrations of all amino acids (Friesen *et al.*, 1975). The ppGpp concentration adjusts the levels of biosynthetic enzymes relative to the supply of all the amino acids in the external environment, whereas operon-specific attenuator mechanisms respond to the need for specific amino acids (Stephens *et al.*, 1975; Winkler *et al.*, 1978). In *Salmonella typhimurium* regulation of the expression of the *his* operon by ppGpp is independent of the operon-specific attenuator mechanism that does not involve ppGpp. There is a good correlation between *his* operon expression and ppGpp levels under steady-state growth conditions (Rudd *et al.*, 1985; Shand *et al.*, 1989; Stephens *et al.*, 1975; Winkler *et al.*, 1978, 1979) and various lines of *in vivo* and *in vitro* evidence indicate that the effect on *his* expression occurs primarily by transcription initiation at the *his* promoter (Riggs *et al.*, 1986; Stephens *et al.*, 1975). The *leu* operon appears to be regulated like the *his* operon, i.e. by both attenuation (Burns *et al.*, 1966) and ppGpp, as indicated in this and related studies (Wright, 1996, 1997).

Recent investigations (Wright, 1996) have examined the

effect of the stringent response on reversion rates of point mutations in two isogenic strains of *E. coli* differing only in *relA*. Reversion rates of the *leuB*⁻ and *argH*⁻ alleles in strain CP78 were significantly higher than in the isogenic counterpart, CP79 (*relA2*). A positive correlation was established between reversion rates and the synthesis of ppGpp during the classical stringent response that occurs within seconds as growth deviates from the exponential phase in amino-acid-limited cultures. The distribution of revertants in mutation rate experiments indicated that most of the mutations occurred during this period of diminishing growth, i.e. the time between the end of exponential growth and the cessation of growth. However, mutations also occurred during exponential growth, especially when the rate of exponential growth was inhibited, for example by serine hydroxamate, which provokes the stringent response as a competitive inhibitor of seryl-tRNA (Cashel & Rudd, 1987; Metzgar *et al.*, 1989; Shand *et al.*, 1989). Under these conditions the mean revertant colony counts on positive plates were abnormally high (data not shown), indicating that the mutation occurred during the exponential growth phase of that culture. According to Cashel & Rudd (1987), effects associated with the classical stringent response also operate during exponential growth to a lesser extent and all relate to the metabolism of ppGpp. Therefore, one goal of the present investigation was to document a possible correlation between mutation rates and the steady-state concentrations of ppGpp during exponential growth, through the use of various strains and culture conditions that produce different levels of cellular ppGpp. To this end, two *spoT* alleles known to accumulate high ppGpp levels were transduced into CP79 and these strains were grown with and without serine hydroxamate. The CP78 strain was also grown with γ -glutamyl leucine, which apparently served as a poor source of leucine, thereby imposing leucine starvation during exponential growth. The results indicate a positive correlation between reversion rates and ppGpp concentrations during exponential growth.

Another goal of these investigations was to sequence and define the lesion in the *leuB* mutant gene as well as to sequence its revertants, as reversion rates of this mutant allele are being used as a model system to test the hypothesis that enhanced mutation rates result from derepression provoked by amino acid starvation and ppGpp accumulation (Wright, 1997).

METHODS

Strains. The genotypes of the *E. coli* strains used in this study are summarized in Table 1. To obtain strains with altered ppGpp levels, *spoT* alleles were transduced using P1 *vir* into the CP79 *relA2* strain, producing strains (Table 1) that grew more slowly than the CP79 *relA2* host. Strains with such *spoT* alleles are known to grow more slowly in *relA1* backgrounds than in Δ *relA* hosts, and cells with null alleles in both the *relA* and *spoT* genes are multiple auxotrophs (Sarubbi *et al.*, 1988; Xiao *et al.*, 1991). This suggested that *relA2* is not a deletion but is a leaky mutation, like *relA1*. To confirm this, *relA2* was replaced in the CP79 Δ *spoT207* strain by Δ *relA251* (Table 1).

As suspected, a multiple auxotroph was formed, confirming that *relA2* is similar to *relA1*.

Growth conditions for mutation rate determinations. Cells were grown at 37 °C for 42–48 h in minimal medium consisting of 40 mM glycerol, 50 mM sodium phosphate buffer, pH 6.5, 1.0 g (NH₄)₂SO₄ l⁻¹, 1.0 g MgSO₄ l⁻¹, 5.0 mg thiamine l⁻¹, 1.0 mM threonine, 0.15 mM leucine, 0.3 mM arginine and 0.3 mM histidine. When growth was limited by leucine, the amino acid was present at 0.025 mM; γ -glutamyl leucine was used at 0.06 mM. Cells grown in the presence of 1.5 mM serine hydroxamate and excess leucine (0.04 mM) were washed prior to plating, to prevent growth on the plates due to carry over of leucine. The 'zero' method of Luria & Delbrück (1943) was used to determine mutation rates. In a typical experiment, a large culture inoculated with cells from a 7-h-old nutrient agar plate at a cell density of about 5×10^4 ml⁻¹ was prepared and 1.5 ml aliquots were distributed into 40 2-cm-diam. test tubes which were shaken at a 45° angle at 37 °C for 24–48 h, depending upon the growth rate of the strain. Each entire culture was then spread onto selective plates. Tenfold dilutions in buffered saline were made of a few identical cultures and the OD₅₅₀ value read to select two typical cultures for appropriate dilutions to determine total viable cell numbers on nutrient agar plates. If the two viable counts differed by more than 25%, the experiment was discarded. The equivalence of viable counts and total cell numbers was assured based on a standard curve comparing the OD₅₅₀ reading to viable counts using exponentially growing cells. Revertant colonies first appear on selective plates about 40 h after plating and final counts were made at 65–78 h. Mutation rates were estimated by the 'zero' method (Luria & Delbrück, 1943) according to the expression $MR = (-\ln 2) (\ln P_0/N)$, where P_0 is the proportion of cultures with no revertants, and N is the total number of cells per culture.

Determination of steady-state levels of ppGpp. Cultures were grown at 37 °C in the above medium with the exceptions that (1) leucine was present at 0.1 mM, to achieve exponential growth during the ³²P labelling period, and (2) the phosphate buffer was replaced by 40 mM MES buffer, pH 6.5, and the KH₂PO₄ concentration was lowered to 0.5 mM to dilute added ³²P to a minimal extent. To analyse ppGpp concentration during exponential growth, the 16 h cultures were centrifuged and resuspended to an OD₅₅₀ of 0.03–0.25 (depending on their growth rate) in fresh medium with or without 1.5 mM serine hydroxamate or substituting 0.06 mM γ -glutamyl leucine for 0.1 mM leucine. Aliquots (1 ml) were removed and their phosphate concentrations determined (Sigma diagnostics, procedure no. 670). Aliquots (1.0–1.5 ml) were removed to 20 ml glass vials and ³²P (New England Nuclear) added to a final concentration of 100–300 μ Ci ml⁻¹ (3.7×10^6 – 1.1×10^7 Bq ml⁻¹). Aliquots (15 ml) were transferred to nephelo culture flasks and OD₅₅₀ was monitored every 30–60 min to determine the rate of exponential growth. After 4 h of labelling with ³²P, samples were removed and prepared for polyethylenimine (PEI) cellulose chromatography. To ensure that the ppGpp pool was saturated with ³²P, a second sample was removed for analysis at 4.5 h. Regardless of the generation time of the culture, the values at 4.5 h and 4.0 h were comparable, indicating saturation of the ppGpp pool. Aliquots of each culture (50 μ l) were pipetted into cold microcentrifuge tubes containing 17 μ l 23.6 M formic acid (final concn 6 M), mixed and freeze-thawed twice in dry ice/ethanol and a 37 °C water bath. After centrifugation, 2 and 5 μ l aliquots were spotted onto washed PEI cellulose plates. A 5 μ l aliquot of 10 mM GTP prepared in the same manner was spotted as a standard. Ascending chromato-

Table 1. Genotypes of *E. coli* strains used

Strain	Genotype	Source
CP78	F ⁻ <i>thr-1 leuB6 his65 argH46 thi-1 ara-13 gal-3 malA1</i> (λ^R) <i>xyl-7 mtl-2 tonA2 supE44?</i>	<i>E. coli</i> Genetic Stock Center, Yale, USA
CP79	As CP78, but <i>relA2</i>	<i>E. coli</i> Genetic Stock Center, Yale, USA
CP79 $\Delta spoT207$	CF1693 = MG1655 $\Delta relA251::kan$ $\Delta spoT207::cat$ used as donor to transduce $\Delta spoT207$ into CP79	This study
CP79 <i>spoT203</i>	CF3042 = CF930 = N99 <i>relA1 zib563::Tn10</i> (<i>spoT203</i>) used as donor to transduce <i>spoT203</i> into CP79	This study
CP79 $\Delta spoT207 \Delta relA251$	CF1652 = MG1655 $\Delta relA251::kan$ used as donor to transduce $\Delta relA251$ into CP79 $\Delta spoT207$	This study
CF1693 = MG1655	$\Delta relA251::kan \Delta spoT207::cat$	M. Cashel (Xiao <i>et al.</i> , 1991)
CF3042 = CF930 = N99	<i>relA1 zib563::Tn10</i> (<i>spoT203</i>)	M. Cashel (Xiao <i>et al.</i> , 1991)
CF1652 = MG1655	$\Delta relA251::kan$	M. Cashel (Xiao <i>et al.</i> , 1991)

graphy in 1.5 M KH₂PO₄, pH 3.4, was run at 5 °C until the solvent front was 16 cm from the origin. The radioactive spots were located by autoradiography on Kodak X-Omat film AR (Wright, 1996). The radioactive areas corresponding to ppGpp were cut off the plate, wetted with 0.25 ml water and counted in 5 ml cytoscent (ICN). The concentration of ppGpp in [pmol (OD₅₅₀ unit)⁻¹] was based on the specific radioactivity of the medium phosphate.

DNA manipulation. Chromosomal DNA from *E. coli* was isolated by the methods of Ausubel *et al.* (1989). Approximately 1 ng purified DNA was PCR amplified using a PCR Core Kit and *Taq* polymerase according to the manufacturer's instructions (Perkin Elmer Cetus). PCR was done by 30 cycles of 94 °C (1 min), 55 °C (2 min) and 72 °C (1.5 min). Primers for amplifying the entire *leuB* gene for cloning were based upon the nucleotide sequence of Kirino *et al.* (1994) (GenBank accession no. D17631) and included 5' TTTAAGCTTCAA-CGAAAACAACAAGGAAACCGTGTG (primer 1) and 5' TTTTCTAGAACAAATTTTCGTATAACGTCTTAGCC (primer 2). For screening revertants, PCR amplification of a 416 bp region encompassing the encoded Ser-286 → Leu lesion of *leuB* (nt 856–858) was accomplished by using 5' GATCC-ATCACAGTTTGACGTTCTGC (primer 3) and primer 2. Primers were synthesized with an Applied Biosystems (ABI) model 394 DNA synthesizer at The University of Montana Molecular Biology Facility.

PCR products containing the full-length *leuB* gene were excised from ethidium-bromide-stained agarose gels and then purified using GeneClean II according to the manufacturer's instructions (Bio101). The DNA was blunt-ended with Klenow fragment and then cloned into the *Sma*I restriction site of pUC19 (Yanisch-Perron *et al.*, 1985) by standard protocol (Sambrook *et al.*, 1989). *E. coli* DH5 α was then transformed with the resulting plasmids by the methods of Chung *et al.* (1985). Transformants were initially screened for recombinant DNA by blue/white screening on LB medium containing ampicillin (0.1 mg ml⁻¹), IPTG (Gibco BRL) and Blueo-Gal (Gibco BRL). Plasmid DNA from the white colonies was obtained by alkaline minipreps (Sambrook *et al.*, 1989), restriction-endonuclease-digested and then analysed for insert content on ethidium-bromide-stained agarose gels.

Nucleotide sequence analyses were done by the methods of Tracy & Mulcahy (1991) on an ABI automated nucleic acid sequencer (ABI; model 373A). Sequencing-grade templates were prepared by a Midi-Prep Kit (Qiagen) for *leuB*-containing plasmids or by GeneClean (Bio101) for the 416 bp PCR products from agarose gels. Computer analysis of the sequences was performed using PCGENE 6.8 software (Intelligenetics).

RESULTS

Growth rate determinations

Fig. 1 compares the exponential growth rates of different strains under the various culture conditions used in this investigation. The growth rates ranged from the lowest generation time of 100 min for strains CP78 and CP79 to the highest generation time of 652 min for CP79 in the presence of serine hydroxamate. In the latter case, the low rate of growth was followed for more than 10 h and did not deviate from linearity. The mean values of replicate determinations are summarized in Table 3.

Mutation rate determinations

leuB⁻ reversion rates of the two *spoT* strains grown with and without serine hydroxamate are shown in Table 2. In the absence of serine hydroxamate, the $\Delta spoT$ strain exhibited a reversion rate approximately threefold higher than its parent strain, CP79 (Tables 2 and 3). The reversion rate of CP79 $\Delta spoT207$ was only slightly stimulated by serine hydroxamate, in contrast to strain CP79 (Table 3). The reversion rate of the CP79 *spoT203* strain was approximately 10-fold higher than that of its parent strain, and was nearly halved by serine hydroxamate (Tables 2 and 3).

To find other conditions that might alter ppGpp levels and reversion rates in the *leuB* auxotrophs, various leucine derivatives were examined for their ability to

Fig. 1. Exponential growth curves for the strains and culture conditions used in determining the concentration of ppGpp under steady-state conditions. (a) ●, CP78; ▼, CP78 + 1.5 mM serine hydroxamate; ■, CP78 + 0.06 mM γ -glutamyl leucine; ○, CP79; ▽, CP79 + 1.5 mM serine hydroxamate. (b) ●, CP79 Δ spoT207; ▼, CP79 Δ spoT207 + 1.5 mM serine hydroxamate; ○, CP79 spoT203; ▽, CP79 spoT203 + 1.5 mM serine hydroxamate.

supply leucine at a low rate, thereby inducing a state of partial starvation and provoking the stringent response. Replacing leucine with the peptide γ -glutamyl leucine significantly lowered the growth rate of strain CP78 (Fig. 1). When grown in the presence of this peptide, the reversion rate of CP78 was about fourfold higher than in the presence of limiting leucine (Tables 2 and 3).

ppGpp levels

The steady-state levels of ppGpp were determined during exponential growth in the strains and under the conditions summarized in Fig. 1 and Table 3. Fig. 2 shows the mutation rates plotted against both the concentration of ppGpp and the generation time. The medium used for measuring ppGpp levels was essentially the same as that used in mutation rate experiments, except that the leucine concentration was necessarily in excess during exponential growth. Leucine was limiting (unless cell growth was inhibited) in mutation rate determinations to achieve a low cell density such that a significant fraction of the cultures would have no revertants. This was necessary to calculate rates by the 'zero' method (Luria & Delbrück, 1943). MES buffer was used in the ppGpp experiments because phosphate levels had to be lowered to minimally dilute the ^{32}P added. In separate experiments (not shown) it was found that mutation rates using medium with the MES-phosphate buffer were comparable to those in phosphate buffer.

Other investigators measuring ppGpp levels *in vivo* have labelled at least one (Friesen *et al.*, 1975) or two (Lagosky & Chang, 1980) generations with ^{32}P prior to sampling. In the experiments reported here, labelling with ^{32}P occurred for 4 h, which was equivalent to 0.4–2.5 generation times, depending upon the strain and the medium employed (Fig. 1). To ensure that the ppGpp pool was equilibrated with the P_i pool, samples were taken again at 4.5 h. In every case, the 4 and 4.5 h samples were indistinguishable, indicating that the small metabolite pools were turning over rapidly regardless of generation time. As the ppGpp pools were saturated, their concentration could be based on the specific radioactivity of the phosphate in the medium.

Revertant colony sizes

Colony counts on 20 plates from a typical 40-culture mutation rate experiment are shown in Table 4 (see Methods). Because growth was limited by threonine, the *leu*⁺ revertants had no selective advantage. Colony counts at 66 h after plating were used to calculate mutation rates. At least 70% of these proved to be true revertants, regardless of the strain in which they arose

Table 2. *leuB* reversion rates

The values given are the means of at least four independent mutation rate determinations. For experimental conditions and for the method of determining mutation rates, see Methods.

	CP79 Δ spoT207		CP79 spoT203		CP78
	Leucine-starved	Seryl-tRNA-starved	Leucine-starved	Seryl-tRNA-starved	Growth on γ -glutamyl leucine
$10^8 \times$ Total cell number	2.8 ± 0.76	0.31 ± 0.12	0.80 ± 0.14	1.85 ± 0.38	1.25 ± 0.18
P_0 (no. negative plates/total)	0.79 ± 0.03	0.96 ± 0.02	0.76 ± 0.04	0.69 ± 0.08	0.33 ± 0.07
$10^9 \times$ Mutation rate	0.60 ± 0.11	1.03 ± 0.09	2.5 ± 0.62	1.3 ± 0.32	6.4 ± 1.7

Table 3. Relationship of mutation rates to levels of ppGpp and generation time

Strain and condition*	$10^9 \times$ Mutation rate	ppGpp concn [pmol (OD unit) ⁻¹] [†]	Generation time (min) [‡]
CP78	1.5 ± 0.48 §	327 ± 30	100 ± 6
CP78 + SH	4.9 ± 0.37 §	479 ± 40	212 ± 39
CP78 + γ -Glu-Leu	6.4 ± 1.7	753 ± 81	630 ± 90
CP79	0.22 ± 0.08 §	234 ± 17	102 ± 8
CP79 + SH	4.4 ± 2.1 §	391 ± 29	652 ± 124
CP79 $\Delta spoT207$	0.60 ± 0.11	267 ± 77	156 ± 22
CP79 $\Delta spoT207$ + SH	1.0 ± 0.09	395 ± 81	371 ± 45
CP79 $spoT203$	2.5 ± 0.62	335 ± 54	207 ± 38
CP79 $spoT203$ + SH	1.3 ± 0.32	321 ± 60	494 ± 30

* SH, serine hydroxamate; γ -Glu-Leu, γ -glutamyl leucine.

[†] Means of four determinations $\pm$ SD; see Methods for details.

[‡] Means of four to eight determinations $\pm$ SD; see Methods for details.

§ Taken from Wright (1996).

Fig. 2. Reversion rate of *leuB* under different conditions (see Table 2 and Fig. 1) plotted against the generation time (▲) and against the ppGpp concn (●) during exponential growth. Bars indicate SD.

(see below). Very small colonies were rarely seen at that time. However, they began to appear after 90 h incubation and in general stopped appearing after about 138 h. The variance of the small colonies at 162 h was 4.8×10^4 , compared to the mean of 115, suggesting that these small colonies were the progeny of slow-growing clones that arose in the liquid cultures and thereafter grew slowly on the plates. In other words, they were not post-plating mutations. Nucleotide sequence analyses indicated that they were suppressor mutants.

Cloning and sequence analysis

The sequence of the *leuB* gene, which encodes the enzyme β -isopropyl malate dehydrogenase (EC 1.1.1.85), has been determined (Kirino *et al.*, 1994;

Table 4. *leu*⁺ revertants from threonine-starved cultures

CP78 cultures were grown in the presence of 0.15 mM leucine and limiting threonine (0.08 mM). Each culture was washed prior to plating on minimal agar lacking leucine (see Methods). Cell density at the time of plating was 1.3×10^8 ml⁻¹. Abbreviations: L, large colony size; S, small colony size.

Time (h):	42	66	90	114	138	162					
Plate no.	L	S	L	S	L	S	L	S	L	S	
1					160	228				228	
2					4	5				5	
3				1	104	104				104	
4					15	92				92	
5					31	211				211	
6					2	8				18	
7						3				3	
8	2		2		2	42	2	84	2	84	
9						640		640		~ 1000	
10				2		2		5		5	
11				1		6		150		150	
12			1	1	1	9	1	23	1	23	
13				1		3		7		9	
14			2	2	3	2	5	2	6	2	6
15						15		27		37	
16				1		12		13		13	
17			1	1	1	3	172	3	172	3	172
18				2		3		19		37	
19				1	1	24	1	60	1	63	
20				1	1	1	10	1	46	1	46

GenBank accession no. D17631). Sequence analysis of the cloned *leuB* genes from the *leuB*⁻ mutants CP78 and CP79 revealed complete nucleotide sequence identity to the published *leuB* gene except for C-to-T transitions at nucleotide 857. This point mutation would result in a

Table 5. Sequence of the *leuB*⁻ mutants and revertants at nt 856–858

Single base substitutions observed		
Sequence	Predicted amino acid 286	Description
TTG	Leucine	<i>leuB</i> ⁻ mutant
TCG	Serine	Wild-type <i>E. coli leuB</i>
GTG	Valine	Phenotypically wild type
ATG	Methionine	Phenotypically wild type
Predicted amino acid residue 286 in true revertants		
Strain	Amino acid (no. observed)	
CP78	Ser (7), Val (8)	
CP79	Ser (6), Val (1)	
CP79 $\Delta spoT207$	Ser (1), Val (6)	
CP79 <i>spoT203</i>	Ser (2), Val (3), Met (2)	

serine-to-leucine substitution at amino acid residue 286 of the LeuB protein. The sequence of the *leuB*⁻ mutant gene in the region of the mutation is shown in Table 5 and the possible single base substitutions in this codon and the corresponding amino acids are also summarized. Base substitutions encoding serine, valine and methionine were all observed among the revertants; the other possibilities (tryptophan or phenylalanine) were never observed, perhaps because they are too bulky to replace serine. Of the 36 true revertants, two had generation times significantly longer than wild-type cells. Revertants containing the Met-286 substitution were unique to *E. coli* strain CP79 *spoT203*. Of the 17 suppressors that were sequenced (i.e. an encoded Leu remained at amino acid residue 286), five had generation times comparable to wild type.

DISCUSSION

To study the relationship between mutation rates and the stringent response, two isogenic multiple auxotrophs were chosen, namely CP78 and CP79 (Table 1). These strains differ only in *relA* and have been widely used in investigations of the stringent response over the past 20 years. The *leuB* allele was chosen for intensive study since (a) the *leuB* gene has been sequenced (Kirino *et al.*, 1994), (b) revertant colonies appearing after 2 d were all of large, uniform size and (c) the reversion rate was conveniently low for using the 'zero' method of calculating mutation rates (Luria & Delbrück, 1943). This is preferable to the 'average' method (Lea & Coulson, 1949) which does not measure the number of initial mutational events and is subject to various artifacts (Stewart *et al.*, 1990). The validity of the 'zero' method depends only upon the survival of each revertant, which has been demonstrated in reconstruction experiments (Wright, 1996). Since the reversion rate depends upon the fraction of plates without colonies, the parental mutant may be grown in medium which

may or may not select for the revertants (Ryan, 1955). To obtain a significant fraction of 'zero' plates, the final cell concentration is adjusted by the concentration of a required amino acid, or of an inhibitor such as serine hydroxamate.

Using a series of *spoT* mutant alleles, an inverse linear relationship was found between steady-state rates of growth and ppGpp levels (Sarubbi *et al.*, 1988). The ppGpp concentrations and the reversion rates of *leuB*⁻ in the two CP79 *spoT* strains (Table 2) were higher than in CP79. This would be expected as a result of the blocking of ppGpp degradation by ppGpp synthase II. It is significant that serine hydroxamate did not affect ppGpp levels or mutation rates in these *spoT* transductants as much as it did in CP79, suggesting that the site of action of this inhibitor is ppGpp degradation. Growth on the γ -glutamyl leucine peptide had the largest effect on ppGpp levels and mutation rates in CP78 (Table 3). Use of this peptide as a substitute for leucine in *leu* auxotrophs is assumed to be comparable to the use of histidinol or formyl histidine as substitutes for histidine in *his* auxotrophs (Ames & Garry, 1959; Blasi & Bruni, 1981). Growth on either of these poor sources of histidine imposes partial starvation and provokes the stringent response; genes for the synthetic enzymes are derepressed, which could then result in increased rates of mutation. During exponential growth the most striking effects on ppGpp accumulation and reversion rates in strains CP78 and CP79 were seen when derepression and a low rate of cell division were both maintained due to the presence of 1.5 mM serine hydroxamate or γ -glutamyl leucine (Fig. 1). In *S. typhimurium* the addition of 2.0 mM serine hydroxamate inhibited growth and provoked an increase in ppGpp levels and *his* operon expression with a similar time course (Shand *et al.*, 1989).

Two general classes of *leu*⁺ revertants were observed: (a) true revertants, with base substitutions encoding serine (wild type), valine or methionine with generation times of about 100 min; and (b) suppressors, which retained the Leu-286 residue of the parental *leuB* mutant and usually had generation times in excess of 200 min. The distribution of these two classes of revertants suggests that they may arise by different mechanisms. As discussed above, the true revertants appeared with a low variance towards the end of growth when ppGpp levels showed a significant, transient increase from basal levels (Lazzarini *et al.*, 1971; Wright, 1996). Suppressor mutants originated throughout the growth phase of the liquid cultures and appeared with a high variance after 4–7 d incubation on the selective plates. These observations suggest that the mutations resulting in true reversions may have been influenced by the stringent response to a greater extent than the suppressor mutations.

Genes undergoing transcription are uniquely susceptible to mutations (Cordaro & Balbinder, 1967; Datta & Jinks-Robertson, 1995; Davis, 1989; Fitch, 1982; Herman & Dworkin, 1971; Lindahl & Nyberg, 1972,

1974; Savić & Kanazir, 1972; Siebenlist *et al.*, 1980; Singer & Kusmierck, 1982; see also Wright, 1997) and repair (Hanawalt & Mellan, 1993; Selby & Sancar, 1994). Cashel & Rudd (1987) believe that all effects of the stringent response can be attributed to the metabolism of ppGpp, and the extensive literature on this subject supports our conclusion that ppGpp stimulates transcription of the *leu* operon, which in turn affects reversion rates due to an increase in the concentration of single-stranded DNA. Mutation rates have now been correlated with ppGpp levels during exponential growth (Fig. 2) as well as during the transition period between the end of exponential growth and stationary phase (Wright, 1996). Studies in progress will examine the correlation between mutation rates and enzyme and mRNA levels of the *leu* operon. About 500 pmol ppGpp appears to be optimal to enhance reversion rates of the *leuB*⁻ allele (Fig. 1). It should be possible to determine whether this level of ppGpp is also optimal for the *de novo* synthesis of the enzymes encoded by the *leu* operon in an *in vitro* transcription-translation system. If so, this would further strengthen the correlation between the stringent response, transcription and mutation rates.

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