Strain variation in ppGpp concentration and RpoS levels in laboratory strains of *Escherichia coli* K-12

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Laboratory strains and natural isolates of *Escherichia coli* differ in their level of stress resistance due to strain variation in the level of the sigma factor σ^S (or RpoS), the transcriptional master controller of the general stress response. We found that the high level of RpoS in one laboratory strain (MC4100) was partially dependent on an elevated basal level of ppGpp, an alarmone responding to stress and starvation. The elevated ppGpp was caused by two mutations in *spoT*, a gene associated with ppGpp synthesis and degradation. The nature of the *spoT* allele influenced the level of ppGpp in both MC4100 and another commonly used K-12 strain, MG1655.

Introduction of the *spoT* mutation into MG1655 also resulted in an increased level of RpoS, but the amount of RpoS was lower in MG1655 than in MC4100 with either the wild-type or mutant *spoT* allele. In both MC4100 and MG1655, high ppGpp concentration increased RpoS levels, which in turn reduced growth with poor carbon sources like acetate. The growth inhibition resulting from elevated ppGpp was relieved by *rpoS* mutations. The extent of the growth inhibition by ppGpp, as well as the magnitude of the relief by *rpoS* mutations, differed between MG1655 and MC4100. These results together suggest that *spoT* mutations represent one of several polymorphisms influencing the strain variation of RpoS levels. Stress resistance was higher in strains with the *spoT* mutation, which is consistent with the conclusion that microevolution affecting either or both ppGpp and RpoS can reset the balance between self-protection and nutritional capability, the SPANC balance, in individual strains of *E. coli*.

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INTRODUCTION

Large numbers of influential studies in bacterial physiology, genetics and gene regulation have used laboratory strains of *Escherichia coli* K-12, but evidence is accumulating that stocks kept in various laboratories are not comparable. The MG1655 strain used in genomic studies differs in several respects between laboratories (Soupene *et al.*, 2003). Another commonly used sequenced strain, W3110, also differs in important respects in various stocks (Jishage & Ishihama, 1997). There are also numerous physiological and regulatory differences between MG1655 and another commonly used K-12 lineage, MC4100 (King *et al.*, 2004). Stored archival cultures of bacteria can be heterogeneous (Sutton *et al.*, 2000), which is also likely to contribute to strain variation in stocks of K-12 strains. We are interested in the molecular basis of strain variation and in this study identify some of the factors important not only in strain differences but also in the settings for global gene regulation in bacteria.

One identified contributor to strain variation is the conditional advantage of the sigma factor RpoS (σ^S) in

different environments. RpoS is responsible for the general stress response of *E. coli* (Weber *et al.*, 2005) and strains with high levels of RpoS are more resistant to stress but grow more slowly under a variety of conditions (Ferenci, 2005). The balance between self-protection and nutritional capability, the SPANC balance, is frequently reset by mutation, resulting in many different combinations of stress resistance and nutritional capability in the species (King *et al.*, 2004). Growth of bacteria on poor carbon sources or low levels of nutrients selects for reduced or abolished RpoS levels, whereas stressful environments require RpoS (King *et al.*, 2006). Laboratory strains contain one of several different alleles of *rpoS* (Atlung *et al.*, 2002). Here we investigate some of the factors that determine RpoS levels and its variation in strains of *E. coli* K-12.

The role of RpoS in *E. coli* is complementary to that of the alarmone ppGpp, whose concentration also responds to stress and shifts transcription away from vegetative growth towards stress resistance (Ferenci, 2005; Gentry *et al.*, 1993; Jishage *et al.*, 2002; Typas *et al.*, 2007). ppGpp and RpoS co-operate both mechanistically and strategically, and expression of RpoS-controlled genes is partly dependent on ppGpp (Kvint *et al.*, 2000; Nystrom, 2004). The level of

Abbreviation: SPANC, self-protection and nutritional capability.

ppGpp is also important because it helps to control the amount of RpoS in the cell, as ppGpp increases the cellular concentration of RpoS by several-fold during nutritional stress or upon entry into the stationary phase. The absence of ppGpp impairs or severely delays the accumulation of RpoS (Gentry *et al.*, 1993). ppGpp also positively affects the efficiency of *rpoS* translation in stationary phase under stress conditions as well as *rpoS* basal expression under conditions of optimal growth (Brown *et al.*, 2002; Hirsch & Elliott, 2002). The response to phosphate starvation additionally involves stabilization of RpoS protein mediated by ppGpp (Bougourd & Gottesman, 2007). At several levels then, ppGpp is intertwined with *rpoS* regulation and function; here we show ppGpp is also subject to strain variation.

ppGpp directly and indirectly affects many important bacterial characteristics, from stress responses to pathogenesis to biofilm formation (Braeken *et al.*, 2006; Cashel *et al.*, 1996; Magnusson *et al.*, 2005; Nakanishi *et al.*, 2006). ppGpp accumulates in bacteria undergoing nutritional stress, such as amino acid, carbon or phosphate limitation (Bougourd & Gottesman, 2007; Cashel & Gallant, 1969; Lazzarini *et al.*, 1971; Spira *et al.*, 1995). Accumulation of ppGpp triggers the 'stringent response', in which a radical decrease in ribosome synthesis results in a general inhibition of protein synthesis and growth arrest (Cashel *et al.*, 1996). The strategic overall role of ppGpp is the allocation of transcriptional resources so they are appropriate to the level of stress and growth rate of the bacteria. This, as with RpoS, necessarily involves a trade-off in the expression of genes related to stress resistance and vegetative growth. The presence of ppGpp may be beneficial in stressful environments but may be an impediment to vegetative growth in intermediate-stress situations. It is therefore interesting to analyse the evolutionary selection pressures imposed on the genes involved in ppGpp synthesis or degradation and how these shape bacterial behaviour.

ppGpp levels are controlled by two proteins, encoded by the genes *relA* and *spoT* (Cashel *et al.*, 1996). Synthesis of ppGpp under intracellular amino acid starvation is dependent on *relA*. The RelA protein senses the presence of uncharged tRNAs at ribosomes, which triggers the RelA-dependent synthesis of ppGpp. *relA* mutants display a relaxed phenotype in response to amino acid limitation, characterized by a further decrease in ppGpp concentration and the continuous production of stable RNA (Cashel *et al.*, 1996). SpoT is a bifunctional cytosolic enzyme capable of both ppGpp synthesis and degradation. Under nutritional stresses other than amino acid limitation, accumulation of ppGpp is largely *relA*-independent, as the rise in ppGpp concentration is mainly due to inhibition of SpoT hydrolytic activity (Murray & Bremer, 1996).

Because ppGpp is needed to obtain high levels of *rpoS* expression, it was expected that ppGpp would also affect

the equilibrium between metabolic capacity essential during vegetative growth and stress resistance, the so-called SPANC balance (Ferenci, 2005). ppGpp may have a direct effect on bacterial nutritional ability, as it binds to RNA polymerase and affects the expression of many genes either positively or negatively (Cashel *et al.*, 1996; Magnusson *et al.*, 2005; Reddy *et al.*, 1995). Additionally, ppGpp may affect cellular properties indirectly through RpoS (Cashel *et al.*, 1996); we tested this prediction in this study. We also showed that ppGpp levels are remarkably strain-specific and that *spoT* is particularly important in microevolution, fixing the basal level of ppGpp in response to selective pressures on stress resistance and nutrition. Finally, we demonstrate that strain variation in ppGpp contributes to the difference in the level of RpoS in *E. coli* and discuss the consequences of polymorphisms at the core of bacterial regulation.

METHODS

Strains and media. The strains used in this study are described in Table 1. Bacteria were cultured in T-salts, a Tris-buffered minimal medium supplemented with different concentrations of glucose and KH_2PO_4 (Spira *et al.*, 1995), and minimal medium A (MMA; as described by Miller, 1972). For growth on acetate and L-agar plates (Miller, 1972), overnight cultures were harvested, washed twice and resuspended in MMA to a density of 2×10^8 cells ml^{-1} . Aliquots of suspensions (5 μl) were spotted on MMA-acetate or L-agar plates and incubated at 37 °C.

Sequence analysis. For the sequencing of the *spoT* ORF, DNA was amplified by PCR using primers *spoTF1* (5'-CAGTATCATGCC-AGTCATTTCTTC-3') and *spoTR2* (5'-GGTAGTACTGGTTTCGCCGTGCTC-3'). Sequencing analysis of both DNA strands was performed with primers *spoTF1*, *spoTF2* (5'-AAAAGCGTCGCCGAGCTGGTAGAGG-3'), *spoTF3* (5'-TGATCGGCCCGCACGGTGTGCCGG-3'), *spoTF5* (5'-TGATCGGCCCGCACGGTGTGCCGG-3'), *spoTR1* (5'-TGCACCATCGCCATAATCATCTTGC-3'), *spoTR2* and *spoTR3* (5'-CTTGATTTCGGTGATGAACCTCTG-3') at the Australian Genome Research Facility. Sequences were assembled with the help of the Staden Package (<http://staden.sourceforge.net/>).

Transductional transfer of *spoT* alleles and *rpoS*::Km. The different *spoT* alleles were transferred by P1 transduction (Miller, 1972). Strains CF12269, CF12271 and CF12273 served as lysate donors for the *spoT1655*, *spoT202* and *spoT203* alleles, respectively, and MC4100TF served as the recipient strain. The proximity of the *spoT* locus to *zib563*::Tn10 (80 % co-transduction) was used to select the transductants for tetracycline resistance; these transductants were later tested for *spoT*-dependent phenotypes, such as ppGpp level, iodine staining and growth on acetate. For the transduction of the *spoT4100* allele into MG1655, the *zib563*::Tn10 marker was first transduced into MC4100 and then a lysate of MC4100 *zib563*::Tn10 was used to transduce MG1655. The *rpoS*::Km mutation was transduced into other strains using strain ZK1000 as a donor.

ppGpp assay. ppGpp was extracted from cells growing at 37 °C in T-salts minimal medium containing 100 μCi (3.7 MBq) $\text{KH}_2^{32}\text{PO}_4$ ml^{-1} . To measure basal levels of ppGpp, cells growing exponentially in T-salts were resuspended in T-salts supplemented with 0.2 % glucose and 0.2 mM $\text{KH}_2^{32}\text{PO}_4$ at OD₆₀₀ 0.1. Samples were harvested after 90, 100, 110 or 120 min. ppGpp was extracted from amino acid-starved cells

Table 1. *E. coli* strains used in this study

Strain	Relevant genotype	Reference/source
CF12269	N99 <i>relA1 zib563::Tn10 spoT⁺</i> (<i>spoT1655</i>)	M. Cashel
CF12271	N99 <i>relA1 zib563::Tn10 spoT202</i>	M. Cashel
CF12273	N99 <i>relA1 zib563::Tn10 spoT203</i>	M. Cashel
CF1693	MG1655 Δ <i>relA::Km</i>	M. Cashel
MC4100	F [−] <i>araD139 (argF-lac)U169 rpsL150 deoC1 relA1 thiA ptsF25 flbB5301 rbsR</i>	Lab collection
MC4100BS	MC4100 from B. Spira lab	Lab collection
MC4100TF	MC4100 from T. Ferenci lab	Lab collection
MC4100TF <i>relA</i>	MC4100TF Δ <i>relA::Km</i> (transduction from CF1693)	This study
MC4100TF <i>rpoS</i>	MC4100TF <i>rpoS::Km</i> (transduction from ZK1000)	This study
MC4100TF <i>spoT1655</i>	MC4100TF <i>spoT1655 zib563::Tn10</i> (transduction from CF12269)	This study
MC4100TF <i>spoT202</i>	MC4100TF <i>spoT202 zib563::Tn10</i> (transduction from CF12271)	This study
MC4100TF <i>spoT202 rpoS</i>	MC4100TF <i>spoT202 rpoS::Km zib563::Tn10</i> (transduction from CF12271 and ZK1000)	This study
MC4100TF <i>spoT203</i>	MC4100TF <i>spoT203 zib563::Tn10</i> (transduction from CF12273)	This study
MC4100TF <i>spoT203 rpoS</i>	MC4100TF <i>spoT203 rpoS::Km zib563::Tn10</i> (transduction from CF12273 and ZK1000)	This study
MG1655	Wild-type <i>E. coli</i> K-12	Lab collection
MG1655 <i>relA</i>	MG1655 Δ <i>relA::Km</i> (transduction from CF1693)	This study
MG1655 <i>rpoS</i>	MG1655 <i>rpoS::Km</i> (transduction from ZK1000)	This study
MG1655 <i>spoT4100BS</i>	MG1655 <i>spoT4100BS (spoT from MC4100BS) zib563::Tn10</i>	This study
MG1655 <i>spoT4100BS rpoS</i>	MG1655 <i>spoT4100BS zib563::Tn10 rpoS::Tn10</i>	This study
MG1655 <i>spoT4100TF</i>	MG1655 <i>spoT4100TF (spoT from MC4100TF) zib563::Tn10</i>	This study
MG1655 <i>spoT4100TF rpoS</i>	MG1655 <i>spoT4100TF zib563::Tn10 rpoS::Tn10</i>	This study
ZK1000	ZK126 <i>rpoS::Km</i>	Bohannon <i>et al.</i> (1991)

using the same method except that amino acid starvation was triggered by adding 1 mg serine hydroxamate ml^{−1} to the cultures.

The labelled samples were mixed immediately with 0.5 vols of cold formic acid and stored overnight at −20 °C. The extracts were centrifuged for 5 min at 10 000 r.p.m. to precipitate cell debris; 3–5 µl was applied to PEI-cellulose TLC plates. The labelled nucleotides were resolved by one-dimensional TLC using 1.5 M KH₂PO₄ as solvent. The amounts of ppGpp on the chromatograms were estimated by measuring the radioactivity of the spots in a Phosphor-Imager (Molecular Dynamics) and calculating the level of ppGpp relative to that of GTP + ppGpp. The densitometric analysis was performed with the help of the Image J software (available at <http://rsb.info.nih.gov/ij/>).

Determination of growth rate. The growth rate of K-12 strains was measured in cultures growing in unlimited T-salts minimal medium. Overnight cultures were diluted to OD₆₀₀ 0.02 and grown at 37 °C for 4 h. Samples were taken every hour and OD₆₀₀ was determined. The growth rate (μ , h^{−1}) was calculated using the formula $\mu = \ln(N/N_0)/t$, where N_0 is the initial and N is the final cell concentration, and t is the time course of the growth curve.

Steady-state growth conditions in chemostats. T-salts supplemented with 0.02 % glucose and 1.0 mM KH₂PO₄ was used to set up an 80 ml chemostat culture as described by Notley-McRobb *et al.* (2002). The dilution rate was set to 0.1 h^{−1}. Daily samples were taken to monitor the *rpoS* status of members of the population. The *rpoS* status was determined by iodine staining (see below).

Detection of *rpoS* status by iodine staining. The RpoS level was qualitatively assessed by staining glycogen with an iodine solution as described by Hengge-Aronis & Fischer (1992). Patches of bacteria or

diluted chemostat samples were grown overnight on L-agar plates, stored at 4 °C for 24 h and then flooded with iodine. The intensity of the brown colour varies according to glycogen concentration in the cell and indirectly measures the level of RpoS (King *et al.*, 2004; Spira & Ferenci, 2008). *rpoS⁺* strains stain brown to dark brown.

Stress resistance assays. Bacteria from overnight cultures grown in L-broth were washed twice in 0.9 % (w/v) NaCl and diluted in 0.9 % NaCl to a density of 4×10^3 cells ml^{−1}. For oxidative stress, freshly diluted H₂O₂ (final concentration 1.5 mM) was added to 1 ml culture and held at room temperature. Samples (100 µl) were taken at 5, 10, 15 and 20 min, spread on L-agar plates and incubated overnight at 37 °C. For osmolarity, suspensions of 4×10^3 cells ml^{−1} were incubated in 1.5 M NaCl at room temperature. Samples (100 µl) were withdrawn at 1, 3 and 4 h and plated onto L-agar. For cold stress, bacteria resuspended in 0.9 % NaCl were held at 4 °C and survival was assessed at 2, 4, 6 and 8 days.

Quantification of RpoS. Bacteria from overnight cultures were resuspended in L-broth and grown at 37 °C up to OD₆₀₀ 2.0, then 2×10^9 cells were centrifuged, resuspended in 200 µl application buffer (0.5 M Tris/HCl, 2 % SDS, 5 % 2-mercaptoethanol, 10 % v/v, glycerol and 0.01 % bromophenol blue) and boiled for 5 min. Proteins were resolved in a 12.5 % denaturing polyacrylamide gel and transferred to a nitrocellulose membrane (GE HealthCare) by capillary action. Following blocking with 5 % skim milk, the membrane was incubated with 2000-fold diluted monoclonal anti-RpoS antibodies (Neoclone) and 20 000-fold diluted peroxidase-conjugated anti-mouse IgG (Pierce). The Super Signal West Pico kit (Pierce) was used to detect the RpoS bands as recommended by the manufacturer. The membrane was exposed to X-ray films for various periods of time and the signal intensities were scanned and computed using the Image J software.

RESULTS

Level of ppGpp and nature of the *relA*/*spoT* genes in a high-RpoS strain

The MC4100 strain from the Ferenci lab (subsequently called MC4100TF) maintains higher levels of RpoS than other K-12 strains, including MG1655 (King *et al.*, 2004). As ppGpp is an important factor in controlling RpoS synthesis and function (Gentry *et al.*, 1993; Kvint *et al.*, 2000), the level of ppGpp in MC4100 was compared to that of MG1655. As shown in Table 2, the basal level of ppGpp was twofold greater in MC4100TF than in MG1655 under steady-state growth conditions at similar growth rates. The higher ppGpp in MC4100 was unexpected, given the known genotypes (*relA*⁺ in MG1655 and *relA1* in MC4100) and the wild-type *spoT* genes in both. Amino acid starvation induced by an amino acid analogue resulted in a relaxed response in MC4100TF consistent with the presence of the *relA1* defect (data not shown), which was closer to the expected result. To test if undetected genetic differences were present, both the *relA* and *spoT* genes associated with ppGpp synthesis were sequenced in MC4100TF. The *relA1* insertion mutation in MC4100 was indeed found in MC4100TF (data not shown). Surprisingly, the *spoT* sequence in MC4100TF differed from that in MG1655 and included a substitution (H255Y)

and a two-amino acid insertion between residues 82 and 83 (+QD) of the SpoT protein. The gene with this pair of changes is subsequently called *spoT4100*.

To test if ppGpp differences occurred as a result of the MC4100TF *spoT* mutation, the *spoT4100* gene of MC4100TF was transferred into MG1655 by co-transduction with a linked marker. Reciprocally, the wild-type *spoT* gene of MG1655 was transferred to MC4100TF. The identity of the *spoT* allele in all transductants in Table 2 was confirmed by DNA sequencing. As shown in Table 2, the effect of the *spoT4100* gene on MG1655 was to increase the basal level of ppGpp. Consistent with this, introduction of the wild-type *spoT* from MG1655 into MC4100TF reduced the ppGpp level to that found in MG1655. Hence the *spoT4100* allele is likely to be responsible for the elevated basal level of ppGpp in MC4100TF. However, the *spoT4100* gene does not result in a rise in ppGpp to the extent found with the previously isolated *spoT202* and *spoT203* mutations (Sarubbi *et al.*, 1988) when transferred to the same background (Table 2).

A different MC4100 stock maintained in another laboratory, MC4100BS, was also sequenced for *spoT* and contained the same pair of changes as MC4100TF. The *spoT4100* from MC4100BS also elevated ppGpp in the MG1655 background to similar basal levels as found in the MC4100TF transductant. The mutational differences in *spoT* and ppGpp between MG1655 and MC4100 indicate the variability of these features of *E. coli* laboratory strains.

Table 2. ppGpp basal levels in different genetic backgrounds

ppGpp was extracted from exponential-phase cells growing in T-salts. Values represent the radioactive spots of ppGpp/GTP+ppGpp and are the mean ($\pm$ SD) of at least three independent experiments.

Strain/genotype	ppGpp	Growth rate*
MC4100BS	0.27 ($\pm$ 0.02)	0.67
MC4100TF		
MC4100TF	0.31 ($\pm$ 0.03)	0.53
<i>rpoS</i>	0.28 ($\pm$ 0.02)	0.65
Δ <i>relA</i>	0.29 ($\pm$ 0.03)	0.54
<i>spoT202</i>	0.42 ($\pm$ 0.01)	0.31
<i>spoT202 rpoS</i>	0.4 ($\pm$ 0.02)	0.45
<i>spoT203</i>	0.39 ($\pm$ 0.02)	0.68†
<i>spoT203 rpoS</i>	0.43 ($\pm$ 0.01)	0.46
<i>spoT1655</i>	0.12 ($\pm$ 0.01)	0.62
MG1655		
MG1655	0.15 ($\pm$ 0.01)	0.49
<i>rpoS</i>	0.15 ($\pm$ 0.02)	0.53
Δ <i>relA</i>	0.15 ($\pm$ 0.02)	0.53
<i>spoT4100</i>	0.33 ($\pm$ 0.01)	0.45
<i>spoT4100 rpoS</i>	0.3 ($\pm$ 0.0)	0.44
<i>spoT4100BS</i>	0.27 ($\pm$ 0.01)	0.44
<i>spoT4100BS rpoS</i>	0.37 ($\pm$ 0.01)	0.44

*Values represent the growth rates (μ , h⁻¹) of exponentially growing cells in T-salts minimal medium.

†Growth rate is unusually high due to the appearance of fast-growing mutants (see Fig. 2).

Differences in RpoS levels between MG1655 and MC4100 lineages

The effect of different endogenous ppGpp concentrations on the level of RpoS protein were tested. As shown in Fig. 1, using anti-RpoS antibodies and three to six replicate immunoblots of each strain, different levels of RpoS were found both as a function of strain background and as a result of different *spoT* alleles. The difference between MG1655 and MC4100TF (Fig. 1) is fully consistent with an earlier study (King *et al.*, 2004). More surprisingly, in *spoT1655* strains, RpoS levels were also lower in MG1655 than in MC4100TF. Since both MC4100TF transduced with the *spoT1655* allele and MG1655 showed similar ppGpp levels (Table 2), it is necessary to invoke other differences in RpoS regulation to explain the protein levels. The increased RpoS levels found in *spoT4100* strains relative to *spoT1655* strains were consistently higher in either MC4100TF or MG1655 backgrounds. Likewise, ppGpp was approximately twofold higher in the *spoT4100* strain. With the *spoT202/203* mutations and even higher ppGpp, RpoS did not increase substantially above the *spoT4100* level, suggesting saturation of *rpoS* regulation by ppGpp. The trends in protein amount in Fig. 1 between *spoT1655* and *spoT* mutants are consistent with the effect of ppGpp levels (Table 2) on *rpoS* expression (Gentry *et al.*, 1993).

Fig. 1. Quantification of RpoS protein. Bacteria were harvested from early stationary phase cultures of strains with various *spoT* alleles; samples were lysed and proteins were resolved by SDS-PAGE. The blots were incubated with anti-RpoS monoclonal antibodies. The bands were scanned and quantified. Values represent the mean $\pm$ SEM of at least three independent experiments. A mutant lacking RpoS is also shown.

A more unexpected result was that the RpoS level in MC4100BS was considerably lower than that in MC4100TF. Given that the two MC4100 stocks have the same ppGpp level and the same *spoT* allele, and that MC4100TF and MC4100BS also have the same *rpoS* sequence (unpublished results), it is necessary to postulate

that there is also a strain difference in another of the numerous extragenic inputs into the control of RpoS synthesis or degradation (Hengge-Aronis, 2002). These MC4100 stocks had a common ancestor over 30 years ago (Casadaban, 1976), but have been in separate laboratories for more than 25 years. Independent propagation or storage conditions have led to strain variation in RpoS of one or both strains due to mutations outside *spoT*. Sequencing of the MC4100TF chromosome that is currently under way may help to resolve the additional causes of high RpoS in this strain.

Effect of ppGpp and *spoT* differences on growth rates, nutrition and the SPANC balance

The influence of *spoT* alleles on RpoS levels suggested that ppGpp would also affect downstream RpoS-dependent phenotypes. A simple test of RpoS function is through testing for glycogen accumulation, which is RpoS-dependent and proportional to its protein level (Spira & Ferenci, 2008). As shown in Fig. 2(c), staining with iodine showed that glycogen accumulation is very much in parallel with the independently determined RpoS estimations. The strong staining in the high-ppGpp, high-RpoS strains (MC4100TF *spoT*202, MC4100TF *spoT*203) contrasts with the poor staining in MG1655 and MC4100BS. It is also obvious that there is an increase in staining in *spoT*4100 compared with *spoT*1655. These data are entirely consistent with those in Fig. 1.

Fig. 2. Growth of bacterial patches on different carbon sources. Washed cultures (5 μ l) containing equal amounts of cells were grown on minimal agar plates containing 0.4% acetate as the sole carbon source (a); the faster-growing colonies in some of the slow-growing patches are *rpoS* mutants selected after 2 days on plates (King *et al.*, 2004). Bacterial patches were grown on L-agar as described above and growth was recorded after 12 h at 37 $^{\circ}$ C (b); these patches were stained with iodine to detect the level of glycogen in the cell (c). The intensity of staining is a function of glycogen content and indirectly of RpoS protein concentration (Spira & Ferenci, 2008).

The level of RpoS determines the nutritional competence of *E. coli* cultured on poor carbon sources; *E. coli* displays weak growth on substrates such as acetate when RpoS levels are high, as in MC4100TF (King *et al.*, 2004). The effect of different *spoT* alleles was tested on the growth on acetate (Fig. 2a). The low-ppGpp, low-RpoS strain, MG1655, grows much better on acetate than MC4100TF. Elevation of ppGpp in MG1655 slows growth somewhat, but not as much as in MC4100TF. The slower growth on acetate is corrected by an *rpoS* null mutation, and indeed *rpoS* mutants are seen as faster-growing colonies within the MC4100TF patch (King *et al.*, 2004). An *rpoS* mutation does not affect ppGpp levels (Table 1), so the growth reduction that is seen on acetate is more likely to result from the indirect influence of ppGpp on RpoS levels. Consistent with this notion, artificially increasing ppGpp levels through the *spoT4100* allele or the characterized *spoT202* or *spoT203* alleles (Sarubbi *et al.*, 1988) also reduced growth on acetate. The growth defect could be relieved by introduction of *rpoS* mutations into these strains (Fig. 2).

The difference in growth rates with the set of strains in Table 2 was evident, even with good carbon sources such as glucose. Furthermore, growth rate differences can be seen even in rich Luria–Bertani medium (Fig. 2b). The slower growth of high-ppGpp strains was at least partially due to RpoS, as *rpoS* mutants were able to grow faster even with rich nutrients. Even with the very high-ppGpp strains containing the *spoT202/203* alleles, an *rpoS* mutation relieves most of the growth inhibition. Hence, selection for improved growth rates with any carbon source can be a potential cause of polymorphisms affecting RpoS and ppGpp.

Effect of ppGpp differences on stress resistance

The *spoT* alleles affected the nutritional side of the SPANC balance, so twofold variation in basal ppGpp levels, such as between MC4100TF and MC4100TF *spoT1655*, should also be reflected in differences in the general stress responses of the bacteria. The effects of H₂O₂, high osmolarity and cold stress were tested on these strains (Fig. 3). In each stress condition, the strain with the *spoT4100* allele performed better. Particularly interesting is the disparity upon cold storage. Given the advantage of the *spoT4100* allele in stressful situations (as may occur in prolonged laboratory storage at 4 °C), the difference in stress survival may explain the selection pressure for mutations in *spoT* towards higher ppGpp levels in the MC4100 lineage or any *E. coli* strain stored for long periods in a stressful environment. The above results suggest that an increase in basal ppGpp levels can improve resistance and tilt the SPANC balance away from effective nutrition; lower ppGpp improves growth on different carbon sources, but reduces stress survival.

Fig. 3. Effect of the *spoT* background on stress resistance. Cultures of strains MC4100TF (■), MC4100TF *spoT1655* (▲) and MC4100BS (●) were challenged for resistance to 1.5 mM H₂O₂, high osmolarity (1.5 M NaCl) and cold (4 °C). Viable bacteria were determined from plate counts.

Connection between variations in ppGpp levels and the enrichment of *rpoS* mutations in *E. coli* populations

Previous results indicated that the high level of RpoS in strains such as MC4100TF is associated with a measurably greater incidence of *rpoS* mutations in nutrient-limited populations than that which is found with low-RpoS strains like MG1655 (King *et al.*, 2004). To see if the elevated ppGpp resulting from *spoT4100* influenced the selection pressure for *rpoS* mutations under nutrient limitation, the kinetics of spread of *rpoS* mutations was followed in chemostat cultures limited by glucose, with all cultures growing at the same rate ($\mu=0.1\text{ h}^{-1}$). The rate of enrichment of *rpoS* mutations was measured by determining the decrease in RpoS⁺ cells in strains containing different *spoT* alleles as shown in Fig. 4. The populations containing *spoT* alleles resulting in higher ppGpp levels (*spoT4100*, *spoT202*) started to accumulate significant numbers of *rpoS* mutations within 2 days of continuous culture. The *spoT202* mutant with the highest ppGpp is particularly prone to RpoS change; more than 50% of the population were RpoS⁺ after 24 h of culture. When the wild-type *spoT* gene (MC4100TF *spoT1655*), *rpoS* mutations still proliferated in the culture, but more slowly. MG1655 did not accumulate mutations in *rpoS* under these conditions. Introduction of *spoT4100* into MG1655 increased the selective pressure on *rpoS*, resulting in a measurable rate of accumulation of *rpoS* mutations. This assay measures the benefit of the loss of *rpoS* (King *et al.*, 2006), so it can be concluded that the nature of *spoT* influences the burden imposed by RpoS under nutrient limitation. Interestingly, pairs of strains containing the same *spoT* allele (MG1655 and MC4100TF *spoT1655*; or

Fig. 4. Loss of RpoS in bacteria with different ppGpp backgrounds during steady-state growth. Cultures of strains MC4100TF (◇), MC4100TF *spoT1655* (▲), MC4100TF *spoT202* (●), MG1655 (□) and MG1655 *spoT4100* (■) were grown separately for 4 days in glucose-limited chemostats at a 0.1 h⁻¹ dilution rate. Samples were harvested daily, diluted and plated on L-agar. Changes in *rpoS* status were followed by iodine staining. The proportion of RpoS⁺ cells is shown; the value for each strain is representative of at least two experiments.

MG1655 *spoT4100* and MC4100TF) had similar ppGpp levels (Table 2) but exhibited different rates of *rpoS* mutant accumulation. Hence, differences between the MC4100 and MG1655 backgrounds besides ppGpp need to be invoked to explain the extent of *rpoS* changes under glucose limitation.

DISCUSSION

The *spoT* gene is central to the ppGpp-dependent response of bacteria to changes in growth rate and nutrient starvation (Cashel *et al.*, 1996) so it is sobering to note that it is subject to polymorphism, as the sequence variation in laboratory strains of *E. coli* suggests. It is probably no historical accident that the *spoT* variations were first noted in some laboratory lineages that differed from other K-12 strains and exhibited a 'spotless' phenotype (Laffler & Gallant, 1974). Distinct mutations in *spoT* were also found in *E. coli* after thousands of generations of laboratory growth on glucose (Cooper *et al.*, 2003), suggesting that *spoT* is subject to selection under repeat-batch culture conditions as well.

The structure–function relationships in the bi-functional SpoT protein are only partly understood (Gentry & Cashel, 1996), so the *spoT* mutations found in this study can only be tentatively interpreted. The insertion at amino acid residues 82/84 probably compromises the ppGppase activity of the protein. Interestingly, one of the high-ppGpp mutations in *spoT* (*spoT202*) is nearby, affecting residue 78 (T78I, M. Cashel, personal communication). The *spoT203* mutation affecting residue 140 (R140C, M.

Cashel, personal communication) and the H255Y substitution in the *spoT4100* strain are more likely to affect synthesis in a positive way. Further protein–function studies are needed to fully resolve these questions, as current speculations are based on the structure of a hybrid protein related to SpoT (Hogg *et al.*, 2004).

In many respects, *spoT4100* resembles the previously described *spoT1* mutation that had elevated basal levels of ppGpp (Lagosky & Chang, 1980; Sarubbi *et al.*, 1989). Strain variation in *spoT* has already been associated with the presence of a *spoT1* allele in a subset of K-12 lineages (Laffler & Gallant, 1974). Indeed, sequencing of the archival *spoT1* gene revealed the same double mutation as in MC4100 (M. Cashel, personal communication), so it is possible that an undocumented *spoT1* mutation was incorporated into the MC4100 strain during its complex construction (Casadaban, 1976). A full genealogical analysis of the studied strains was not undertaken, however, as it is clear from the strain variation, even between the two MC4100 stocks (the TF and BS variants), that frequent changes are common in stored cultures. It is also unclear whether *spoT1*-like mutations are more prevalent in *relA1* mutants.

The environmental influence on ppGpp regulation is affected by the same dichotomy already observed and discussed for RpoS (Ferenci, 2005), namely the different needs of the cell in response to nutrient limitation and stress resistance. As described for the MC4100TF strain and its *spoT1655* derivative, or MG1655 and its *spoT4100* derivative, the nature of the *spoT* allele that is present in *E. coli* simultaneously influences the level of RpoS, stress resistance and nutritional capabilities of *E. coli*. The benefit of high basal ppGpp levels is seen in the greater stress resistance that is conferred (Fig. 3), whereas the benefit of low ppGpp is evident from increased growth, particularly under nutrient limitation or with poor nutrients (Fig. 2a, b). Depending on the environment, the antagonistic pleiotropy between resistance and nutrition can change the cell's SPANC balance. Given that external stresses and nutritional constraints are common in natural settings, it is not surprising that mutations occur and are constantly selected in shifting environments. Indeed, the variation in *spoT* resembles the polymorphisms in *rpoS*, which are, if anything, even more extensive (Atlung *et al.*, 2002; Ferenci, 2005).

In the past, the relationship between growth rate and ppGpp levels was discussed mainly in the context of high ppGpp resulting in a reduction in stable RNA synthesis and thereby reduced translational capacity (Sarubbi *et al.*, 1988). After the effect of ppGpp on RpoS was discovered (Gentry *et al.*, 1993), it was suggested that some of the ppGpp effect could be indirect and could be acting through elevated expression of the alternative sigma factor (Cashel *et al.*, 1996). Indeed, as shown in this study, the elimination of RpoS results in faster growth on a range of substrates. Even on rich media, the inhibitory effect of artificially

elevated ppGpp can be largely overcome by *rpoS* mutations. Hence, the growth rate effects of high ppGpp are more complex than simple control of translational processes and they probably involve competition between sigma factors as well. Adding to the complexity, both ppGpp and RpoS levels differ between strains, so the overall effect of elevated ppGpp is also likely to be strain-specific.

In summary, the physiological and regulatory roles of RpoS and ppGpp are interlinked at several levels, including strain variation. Increased ppGpp generally has a positive effect on RpoS levels, but differences in RpoS between the MC4100 and MG1655 backgrounds demonstrate that other factors besides ppGpp need to be invoked to explain the extent of *rpoS* changes. Previous results have pointed to considerable polymorphisms in *rpoS* between *E. coli* strains (Ferenci, 2003); these new results suggest that at least one of the genes involved in ppGpp synthesis and degradation is also subject to the same kind of selective pressures as is *rpoS*. In this respect, *spoT* and *rpoS* are involved in the dynamic balancing of SPANC within a bacterium in response to the nature of the immediate environment and demand for nutrients. These findings are significant for molecular studies of bacteria, because it is now known that two of the global systems that control the allocation of transcriptional resources are actually strain-specific, since ppGpp and RpoS levels are potentially non-uniform in *E. coli* under identical growth conditions. This has major consequences for global studies of the pattern of expression of hundreds of genes controlled directly or indirectly (by competition) at the level of RNA polymerase. Equally importantly, many of the numerous traits controlled by ppGpp (Braeken *et al.*, 2006; Cashel *et al.*, 1996; Magnusson *et al.*, 2005; Nakanishi *et al.*, 2006) are also likely to be subject to strain variation.

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