New Insights into the Role of Sigma Factor RpoS as Revealed in *Escherichia coli* Strains Lacking the Phosphoenolpyruvate:Carbohydrate Phosphotransferase System

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Key Words

Phosphoenolpyruvate:carbohydrate phosphotransferase system • RpoS • SpoT • Gpp • ppGpp • Carbon limitation • Adaptive evolution

Abstract

It has been demonstrated that about 10% of the *Escherichia coli* genes are under direct or indirect control of RpoS. Therefore, Weber et al. [2005] proposed that this sigma subunit should be considered a second vegetative sigma factor under non-optimal growth conditions. In this report we demonstrate that in the phosphoenolpyruvate:carbohydrate phosphotransferase system-deficient (PTS⁻) derivatives, PB11 and PB12 of strain JM101 that permanently grow slowly on glucose, the inactivation of *rpoS* resulted in decreased growth rates of 50 and 10%, respectively. Real-time PCR (RT-PCR) analysis confirmed the important role of this sigma factor in the PTS⁻ strains and allowed the identification of 19 genes including almost all the glycolytic genes, not previously reported, to be at least partially dependent on RpoS. The transcription level of *gpp*, *spoT*, *ppa* and *ndk* whose products are involved in ppGpp metabolism was upregulated in strain PB12 as compared to the parental strains PB11 and JM101. In the PTS⁻ strains, at least three of these genes (*gpp*,

spoT and *ppa*) were mainly or partially regulated by RpoS which is known to require ppGpp for activation, while only *gpp* was highly RpoS-dependent in the parental PTS⁺ strain JM101. The role of RpoS in the transcription of these genes was analyzed and evidence that the expression of this group of genes could be regulated by a common factor in addition to RpoS was discussed.

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Introduction

In *Escherichia coli*, the primary response to glucose limitation ($\mu = 0.1 \cdot \text{h}^{-1}$), known as hunger or scavenging response, is the activation of a group of genes that allow high affinity uptake of different carbohydrates as well as other carbon sources such as acetate [Hengge-Aronis, 1996, 2002; Notley-McRobb and Ferenci, 1996, 2001]. These nutrient-specific systems are part of the CRP regulon for the use of alternative carbon sources [Gosset et al., 2004]. The *E. coli* genome encodes several sigma factors that enable the use of alternative promoter sequences, some of which can transcribe the same gene in different metabolic and stress conditions. The *rpoS* gene codes for the sigma factor S or RpoS which has been involved in the

transcription of several genes expressed in the stationary phase and on glucose-limited growth conditions. It has been reported that while the cellular sigma S concentration is the highest in early stationary phase cells, exponentially growing cells in minimal medium maintain low levels of this sigma factor [Lange and Hengge-Aronis, 1994; Notley-McRobb and Ferenci, 1996; Hengge-Aronis, 2002]. Interestingly, recent genome-wide expression analysis by Weber et al. [2005] indicates that up to 10% of the *E. coli* genes are under direct or indirect control of RpoS. Therefore, these authors have proposed that this sigma subunit should be considered a second vegetative sigma factor with major impact not only on stress tolerance but on the entire cell physiology under non-optimal growth conditions.

RpoS-dependent promoters require ppGpp to induce gene transcription even in the presence of high levels of sigma S [Kvint et al., 2000]. Under favorable growth conditions a steady-state level of pppGpp and ppGpp is maintained in the cytosol mainly by the activity of three enzymes: RelA, SpoT and Gpp. RelA and SpoT synthesize pppGpp while Gpp transforms this metabolite into ppGpp [Lengeler et al., 1999; Chatterji and Ojha, 2001]. In a *relA-spoT* double mutant, where no ppGpp is produced, the cellular content of sigma S is greatly decreased indicating that the transcription of the *rpoS* gene depends on the presence of ppGpp [Lange et al., 1995]. It is known that ppGpp alarmone levels are increased following the depletion of amino acids (triggering the stringent response), upon starvation of carbon or nitrogen, as well as in cells growing slowly in a chemostat [Notley-McRobb and Ferenci, 1996; Lengeler et al., 1999; Teich et al., 1999; Hengge-Aronis, 2002].

Many regulons whose transcription is controlled by alternative sigma factors, such as sigma S, are poorly induced in cells lacking the ppGpp alarmone. In fact, the absence of this alarmone prevents cell growth in minimal medium [Cashel et al., 1996]. An in vitro transcription/competition assay with reconstituted RNA polymerase showed that addition of ppGpp reduces the ability of wild-type sigma 70 to compete with sigma S for its binding to the RNA polymerase core. Similarly, an in vivo competition assay demonstrated that the ability of sigma S to compete with sigma 70 is diminished in cells lacking ppGpp. Consistently, the fraction of sigma S bound to the RNA polymerase core is drastically reduced in ppGpp-deficient cells. Thus, stringent and carbon limitation responses apparently encompass a mechanism that alters the relative competitiveness of sigma factors for the RNA polymerase core [Cashel et al., 1996; Maeda et al., 2000;

Baker et al., 2001a, b; Chatterji and Ojha, 2001; Hengge-Aronis, 2002; Jishage et al., 2002]. In addition, it has been reported that ppGpp acts on transcription initiation, inhibiting the formation of the open promoter complex and thereby transcript elongation. This failure in gene transcription may increase the availability of RNAP holoenzyme, favoring in turn the binding of RpoS versus RpoD to the RNA polymerase core. As a consequence of this, the RpoS-dependent transcription is activated. Hence, an increase in ppGpp alarmone favors the transcription of sigma S-dependent promoters [Wagner, 2002; Artsimovitch et al., 2004].

In *E. coli*, glucose transport and phosphorylation are mainly dependent on the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) [Postma et al., 1996; Saier, 2002]. In a strain lacking PTS, such as PB11, the cells grow very slowly ($\mu = 0.1 \cdot \text{h}^{-1}$) in minimal medium on glucose as the sole carbon source. This is explained by the fact that they have lost most of their glucose transport capacity and therefore, they permanently sense very low levels of intracellular glucose or glucose-6-phosphate even in the presence of a high glucose concentration in the medium. As a result of this type of growth under non-optimal conditions, these cells induce the synthesis of galactose. This molecule in turn inactivates the galactose repressor causing the upregulation of *galP*, the gene coding for the GalP permease responsible for glucose uptake in this strain [Death and Ferenci, 1994; Notley and Ferenci, 1996; Hua et al., 2004; Flores S et al., 2002, 2005; Flores N et al., 2005; Weber et al., 2005]. It has been reported that in a strain lacking the *crr* gene such as PB11, the translation of the *rpoS* mRNA is no longer repressed by the IIA^{Glc} component of PTS, allowing higher levels of RpoS protein even during the exponential growth phase [Ueguchi et al., 2001; Flores N et al., 2005]. Therefore, it is expected that this strain, growing in non-optimal nutrient conditions, may increase the synthesis of ppGpp as a starvation-stress signal since RpoS, in conjunction with ppGpp, is responsible for its own synthesis and high concentrations of this alarmone are required for RpoS-RNA polymerase transcription [Lange et al., 1995; Lengeler et al., 1999; Kvint et al., 2000; Baker et al., 2001a, b; Hengge-Aronis, 2002]. In agreement with these considerations, it has been previously reported that the transcription of *rpoS*, *poxB*, *talA* and *tktB*, members of the RpoS modulon, is upregulated in PB11, as compared to the wild-type strain JM101 when growing on glucose as the only carbon source [Weber et al., 2005; Flores N et al., 2005].

Strains PB12 and PB13 were derived from PB11 in an adaptive evolution fermentation experiment. It has been published that PB12 carries at least two mutations that allow higher rates of glucose transport and growth [Flores N, 1995, Flores N et al., 1996]. Moreover, it has been reported that in the PTS⁻ strain PB12, a fraction of the phosphoenolpyruvate that is not consumed for glucose transport, could be redirected into the synthesis of aromatic compounds [Flores N et al., 1996; Gosset et al., 1996; Báez et al., 2001, 2004]. Due to this biotechnological potential, we have been interested in characterizing these and other PTS⁻ strains [Flores N et al., 2004; de Anda et al., 2006]. In PB12, which grows faster on glucose than PB11 ($\mu = 0.4$ vs. $0.1 \cdot \text{h}^{-1}$), the carbon flux distribution is modified at various portions of the central carbon metabolism as compared to the parental strains. It has also been shown that *glk*, *pgi*, *lpd* as well as many other genes coding for TCA enzymes are upregulated in PB12 as compared to the parental strains [Flores N, et al., 2005].

In the present contribution, we provide and discuss evidence showing that PB12, as well as PB13, carry a point mutation in the *rpoS* gene that, in principle, should generate a truncated RpoS polypeptide. However, these strains also carry the *supE* gene that may suppress this mutation, at least partially. This last proposition is supported by the fact that in PB12 the transcription levels of many genes that are members of the RpoS modulon are still upregulated but at different and lower levels of those found in PB11. Furthermore, knockout inactivation of *rpoS* in PB11 and PB12 by insertion of a Tc^r cassette differentially decreases the transcription of previously reported *rpoS*-dependent genes and many others, thus indicating an important role of this sigma subunit in both PTS⁻ strains. Finally, the transcription level of a group of genes involved in ppGpp synthesis and degradation are 3- to 5-fold upregulated in PB12 and PB13 as compared to JM101 and PB11 strains. The role of RpoS in the transcription of these genes is discussed and evidence that the expression of this group of genes may be regulated by a common factor in addition to RpoS, is presented.

Results and Discussion

Detection of a Point Mutation in the rpoS Gene in Strains PB12 and PB13

It has been reported that *E. coli* strains can adapt their metabolism to higher growth rates on specific carbon sources as a result of specific mutations [Raghunathan and Palsson, 2003]. During the process utilized for the

selection of rapidly growing strains PB12 and PB13 from the PTS⁻ PB11 strain, at least two non-cotransducible mutations were selected that allowed PB12 to consume glucose in the absence of PTS at a higher rate than PB11 [Flores N, 1995; Flores N et al., 1996].

The nucleotide sequences of several genes of PB12 and PB13 have been previously determined and only a single point mutation was detected in the *arcB* gene of PB12 [Flores N et al., 2005]. A second mutation in these strains located at the *rpoS* gene was identified and characterized in the present contribution. It has been reported that the appearance of mutations at the *rpoS* gene is a frequent event when *E. coli* grows slowly ($\mu = 0.1 \cdot \text{h}^{-1}$) in glucose-limited controlled conditions [Notley-McRobb et al., 2002; Ferenci, 2003; King et al., 2006]. The mutation found in PB12 occurred at position 98 and changed the coding triplet TCG to the stop codon TAG. As a result of this point mutation a truncated 32-amino acid residue polypeptide should be produced instead of the original 228-residue wild-type protein. However, it is known that the parental strain JM101 carries a *supE* mutation that suppresses stop codon amber mutations [Bolívar et al., 1977]. Therefore, the translation of *rpoS* mRNA might produce functional RpoS for the transcription of RpoS-dependent genes. Evidence will be presented showing that the *rpoS* gene is at least partially functional since the transcription of RpoS-dependent genes is still present in PB12 (and PB13, data not shown) as shown in the next section. Finally, inactivation of *rpoS* by insertion of a Tc^r cassette in PB12 and in the parental PB11 generated strains PB12 *rpoS*::Tc^r and PB11 *rpoS*::Tc^r, respectively. Inactivation of *rpoS* by this method clearly demonstrates, as will be presented below, that there is sufficient RpoS activity in PB12 because the transcription of many RpoS-controlled genes was higher in the parental strains than in the *rpoS*::Tc^r derivative mutants. Nevertheless, from these results it cannot be concluded that the point mutation in PB12 is completely suppressed. It can be speculated that this point mutation was selected to reduce the amount of active RpoS, especially if it was not completely suppressed, thus allowing or facilitating the preferential transcription of certain RpoS-dependent genes by RpoD. This in turn could allow a faster growth on glucose to the cell carrying this mutation. Furthermore, as will be presented below, an additional mutation could have been selected in PB12 or in a precursor strain that modified, probably reduced, the amount of ppGpp in the cell. This second event could also have contributed to further reducing the transcription capacity of RpoS, favoring growth increase as well. It is important to point out that

no mutation was detected in the *rpoD* gene of PB11 and PB12 (data not shown), excluding the possibility of an altered sigma 70 subunit competing with RpoS for the transcription of certain genes.

Transcription Levels of RpoS-Controlled Genes

The transcription levels of 67 genes, some of which are known to contain RpoS-dependent promoters, were determined by RT-PCR in the strains JM101, PB11, PB12 and their *rpoS*::Tc^r derivatives (table 1). The expression levels of most of the tested genes in the *rpoS*::Tc^r derivative of the parental wild-type strain JM101 were not significantly modified as compared to the wild-type strain when cells were growing logarithmically in minimal medium on glucose. Nevertheless, some interesting differences among these strains were observed. Some genes known to be highly RpoS-dependent such as *osmY*, *pfkA*, *talA*, *tktB* were downregulated in JM101 *rpoS*::Tc^r [Weber et al., 2005]. Moreover, genes not previously reported as part of the RpoS modulon, like *fbaA*, *gpp*, *ppa* and *rpoS*, were also downregulated. There was a slight upregulation in the transcription of *gapC-1* and *gapC-2* as well as *aceEF* and *lpd* (table 1). If this upregulation is significant, being within the resolution limits of the RT-PCR technique, it is in agreement with results previously published by Rahman et al. [2006] which demonstrated that the transcription of these last three genes is upregulated in an *E. coli* B *rpoS*⁻ strain when the cells are growing logarithmically on Luria broth (LB).

It has previously been reported that when PB12 and the parental strain PB11 are grown in minimal medium with glucose (2 g/l) as the only carbon source, there is an upregulation of certain genes (*poxB*, *rpoS*, *talA* and *tktB*) that are part of the RpoS modulon, as compared to the wild-type JM101 [Flores N et al., 2005]. Results in table 1 show that in PB11 there was a higher level of transcription of most of the highly RpoS-dependent genes [Weber et al., 2005], including *rpoS*, in comparison with PB12. These results indicate that PB11 is under severe stress when growing on glucose, as it senses very low internal levels of glucose or glucose-6-phosphate, which resulted in permanent non-optimal growing conditions [Flores N et al., 2005]. Under these conditions, RpoS-dependent genes are expressed [Weber et al., 2005]. As expected, in the PB11 *rpoS*::Tc^r derivative strain the transcription levels of most of these genes were significantly decreased. For some highly RpoS-dependent genes, such as *talA*, *tktB*, *galE*, *gadA*, *adhP*, *fbaB*, *katE* and *osmY*, the transcription levels were decreased more than 10-fold, while in others (*pfkA*, *pfkB*, *pykA* and *poxB*) these levels were decreased

about 2- to 3-fold. There are 19 genes, *aceBA*, *aceEF*, *ackA*, *acnB*, *acs*, *eno*, *fbaA*, *gapA*, *gmk*, *gpmA*, *gpp*, *pgi*, *ppa*, *pykF*, *sfcA*, *spoT*, and *tpiA*, that have not been classified as part of the RpoS modulon whose transcription levels were also decreased in the PB11 *rpoS*::Tc^r derivative. Interestingly, many of these genes are involved in central carbon metabolism. In fact, transcription levels of all the glycolytic genes with the exception of *lpd* and *pgk* were at least 2-fold downregulated in PB11 *rpoS*::Tc^r strain as compared to PB11, showing the important role of RpoS in this strain when growing on glucose under these conditions. Transcription levels of the genes involved in the glyoxalate shunt and the *acs* gene, highly upregulated in PB11, were still, but not as highly, upregulated in the PB11 *rpoS*::Tc^r derivative. Finally, the transcription levels of the genes involved in the TCA cycle did not change significantly in this strain as compared to PB11, with the exception of *acnB* that was 2-fold downregulated and the *sdhAB* genes that were 2-fold upregulated (fig. 1; table 1). In the case of PB12, *rpoS* inactivation in the derivative strain PB12 *rpoS*::Tc^r also differentially decreased the transcription of many genes as compared to PB12. The transcription of *poxB*, *talA*, *tktB*, *gpp*, *katE* and *osmY* was downregulated more than 10-fold. Likewise, the transcription levels of most glycolytic genes (*pgi*, *pfkA*, *pfkB*, *fbaA*, *tpiA*, *pykA*, *pykF* and *aceEF*) were decreased approximately 2-fold although not all glycolytic genes were downregulated in this strain. Interestingly, the transcription levels of the genes involved in the glyoxalate shunt as well as the *acs* gene were also downregulated. The higher transcription level of many RpoS-dependent genes in PB12 indicates an active role of this sigma factor in this strain. Finally, the transcription levels of most of the TCA genes was not significantly modified in PB12 *rpoS*::Tc^r, as compared to PB12, with the exception of *acnB* whose transcription was 2-fold downregulated and the *sdhAB* genes that were increased approximately 2-fold (fig. 2; table 1). This last result is in agreement not only with the fact that the transcription of the TCA genes are already upregulated in this strain because PB12 carried a mutation in the *arcB* gene [Flores N et al., 2005], but also with the report that the transcription of *sdh* genes is upregulated in *rpoS*⁻ mutants [Weber et al., 2005].

It is clear that RpoS inactivation exerted various and different effects on the strains analyzed in this report. While *rpoS* inactivation did not modify the specific growth rate (μ) in the PTS⁺ strain JM101 *rpoS*⁻ as compared to JM101, the μ of both PTS⁻ PB11 *rpoS*::Tc^r and PB12 *rpoS*::Tc^r were diminished by 50 and 10%, respectively, as compared to their parental *rpoS*⁺ strains PB11

Table 1. Relative transcription levels determined by RT-PCR of several groups of genes from strains JM101, PB11, PB12 and their *rpoS*::Tc^r derivatives

Gene	<i>rpoS</i>	JM <i>rpoS</i> ⁻	PB11	11 <i>rpoS</i> ⁻	PB12	12 <i>rpoS</i> ⁻
<i>aceA</i>	*	1.0 ± 0.1	11.9 ± 1.2	9.1 ± 2.8	1.9 ± 0.2	0.8 ± 0.1
<i>aceB</i>	*	1.0 ± 0.0	15.6 ± 2.5	11.0 ± 2.1	3.7 ± 0.3	0.8 ± 0.0
<i>aceE</i>	*	1.4 ± 0.1	0.4 ± 0.0	0.2 ± 0.0	1.4 ± 0.0	0.8 ± 0.0
<i>aceF</i>	*	1.5 ± 0.1	0.6 ± 0.0	0.4 ± 0.1	1.2 ± 0.0	0.7 ± 0.0
<i>ackA</i>	*	1.4 ± 0.0	0.4 ± 0.1	0.2 ± 0.0	0.8 ± 0.0	0.41 ± 0.0
<i>acnA</i>	+	1.7 ± 0.1	5.2 ± 0.6	0.7 ± 0.1	5.7 ± 0.2	3.5 ± 0.3
<i>acnB</i>	*	0.8 ± 0.1	1.7 ± 0.4	0.2 ± 0.0	1.7 ± 0.4	0.7 ± 0.0
<i>acs</i>	*	0.8 ± 0.0	5.6 ± 0.7	4.1 ± 0.1	8.0 ± 0.1	3.4 ± 0.1
<i>adhP</i>	+	1.2 ± 0.0	9.0 ± 3.0	0.7 ± 0.0	3.3 ± 0.2	0.9 ± 0.1
<i>arcA</i>	+	1.0 ± 0.0	1.2 ± 0.0	0.7 ± 0.0	1.3 ± 0.1	1.1 ± 0.0
<i>eno</i>	*	0.9 ± 0.0	0.5 ± 0.1	0.2 ± 0.0	0.5 ± 0.1	0.4 ± 0.0
<i>fbaA</i>	*	0.3 ± 0.0	0.9 ± 0.0	0.3 ± 0.0	1.1 ± 0.0	0.4 ± 0.1
<i>fbaB</i>	+	1.0 ± 0.1	10.6 ± 1.1	0.9 ± 0.0	1.1 ± 0.0	0.9 ± 0.0
<i>fbp</i>		1.0 ± 0.2	2.4 ± 0.7	1.9 ± 0.4	2.8 ± 0.1	2.2 ± 0.1
<i>fumA</i>		1.0 ± 0.2	3.3 ± 0.7	2.3 ± 0.5	3.6 ± 0.7	3.8 ± 0.1
<i>fumB</i>		1.2 ± 0.2	0.3 ± 0.0	1.2 ± 0.0	0.4 ± 0.0	0.4 ± 0.1
<i>fumC</i>	+	0.7 ± 0.2	4.3 ± 1.0	2.9 ± 0.2	3.5 ± 1.5	2.4 ± 0.3
<i>gadA</i>	+	1.4 ± 0.0	18.0 ± 4.0	1.0 ± 0.0	1.0 ± 0.2	0.8 ± 0.0
<i>galE</i>	+	1.0 ± 0.1	38.8 ± 6.8	1.4 ± 0.1	46.6 ± 6.5	25.0 ± 2.1
<i>gapA</i>	*	0.9 ± 0.1	0.4 ± 0.1	0.1 ± 0.0	1.7 ± 0.2	1.4 ± 0.2
<i>gapC-1</i>	+	1.7 ± 0.1	6.7 ± 0.1	0.9 ± 0.1	3.1 ± 0.1	1.0 ± 0.2
<i>gapC-2</i>	+	1.8 ± 0.0	3.9 ± 0.2	0.7 ± 0.1	3.2 ± 0.0	0.9 ± 0.1
<i>gdhA</i>	+	1.1 ± 0.1	0.5 ± 0.0	0.4 ± 0.0	3.5 ± 0.2	3.0 ± 0.0
<i>glcB</i>	+	1.0 ± 0.0	11.7 ± 1.4	2.5 ± 0.2	3.7 ± 0.4	1.8 ± 0.2
<i>glk</i>		1.0 ± 0.1	1.0 ± 0.0	0.7 ± 0.1	2.2 ± 0.1	1.7 ± 0.1
<i>gltA</i>		0.9 ± 0.0	0.8 ± 0.1	1.3 ± 0.2	1.3 ± 0.2	1.2 ± 0.2
<i>gmK</i>	*	1.4 ± 0.1	0.9 ± 0.1	0.3 ± 0.0	2.0 ± 0.2	0.4 ± 0.0
<i>gpmA</i>	*	0.8 ± 0.1	0.9 ± 0.0	0.6 ± 0.1	1.8 ± 0.0	1.7 ± 0.3
<i>gpmB</i>		0.9 ± 0.2	1.5 ± 0.2	1.6 ± 0.2	2.7 ± 0.4	1.9 ± 0.1
<i>gpp</i>	*	0.0 ± 0.0	1.4 ± 1.2	0.0 ± 0.0	5.2 ± 0.3	0.1 ± 0.0
<i>icdA</i>		1.1 ± 0.1	1.0 ± 0.1	1.2 ± 0.0	1.9 ± 0.0	2.4 ± 0.0
<i>katE</i>	+	1.4 ± 0.1	24.2 ± 8.2	2.2 ± 0.1	7.0 ± 0.0	0.8 ± 0.1
<i>lpd</i>		1.4 ± 0.1	1.1 ± 0.1	1.3 ± 0.3	2.0 ± 0.1	1.7 ± 0.1
<i>maeB</i>		0.9 ± 0.0	1.2 ± 0.2	1.6 ± 0.0	2.5 ± 0.0	1.7 ± 0.0
<i>malP</i>	+	1.3 ± 0.3	5.9 ± 0.4	0.2 ± 0.0	1.0 ± 0.0	0.7 ± 0.1
<i>mdh</i>		1.3 ± 0.2	1.2 ± 0.1	1.4 ± 0.3	1.8 ± 0.5	1.4 ± 0.1
<i>ndk</i>		0.8 ± 0.1	0.4 ± 0.0	0.5 ± 0.0	3.2 ± 0.1	3.3 ± 0.1
<i>osmY</i>	+	0.6 ± 0.0	16.0 ± 5.2	1.4 ± 0.1	6.7 ± 0.3	0.7 ± 0.1
<i>pckA</i>		1.0 ± 0.2	0.8 ± 0.1	0.9 ± 0.2	0.8 ± 0.0	1.3 ± 0.1
<i>pfkA</i>	+	0.4 ± 0.0	0.3 ± 0.0	0.0 ± 0.0	0.5 ± 0.0	0.2 ± 0.0
<i>pfkB</i>	+	1.2 ± 0.2	1.2 ± 0.2	0.4 ± 0.0	1.9 ± 0.2	0.9 ± 0.1
<i>pgi</i>	*	0.8 ± 0.1	1.0 ± 0.1	0.4 ± 0.1	6.6 ± 0.3	3.7 ± 0.1
<i>poxB</i>	+	0.7 ± 0.1	4.2 ± 0.3	1.5 ± 0.1	5.7 ± 0.5	0.5 ± 0.0
<i>ppa</i>	*	0.6 ± 0.0	1.0 ± 0.0	0.5 ± 0.1	6.1 ± 1.4	3.8 ± 0.1
<i>ppc</i>		1.2 ± 0.0	0.6 ± 0.1	0.8 ± 0.1	0.8 ± 0.0	0.5 ± 0.1
<i>ppsA</i>		0.6 ± 0.1	3.7 ± 0.2	3.3 ± 0.2	2.4 ± 0.1	1.1 ± 0.2
<i>pta</i>		1.8 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	1.5 ± 0.3	0.6 ± 0.2
<i>ptsG</i>		1.0 ± 0.0	1.3 ± 0.0	1.5 ± 0.2	1.3 ± 0.6	2.3 ± 0.1
<i>pykA</i>	+	1.0 ± 0.2	0.4 ± 0.0	0.1 ± 0.0	1.0 ± 0.0	0.5 ± 0.1
<i>pykF</i>	*	0.8 ± 0.0	0.8 ± 0.0	0.2 ± 0.0	0.9 ± 0.0	0.5 ± 0.1
<i>recG</i>		4.9 ± 0.2	0.8 ± 0.1	1.0 ± 0.2	6.6 ± 1.0	3.9 ± 0.4
<i>relA</i>		1.1 ± 0.1	1.3 ± 0.0	0.9 ± 0.1	1.6 ± 0.2	1.1 ± 0.0
<i>rhlB</i>		1.4 ± 0.1	0.9 ± 0.0	0.6 ± 0.0	1.9 ± 0.1	1.3 ± 0.0
<i>rpoD</i>		0.7 ± 0.2	0.8 ± 0.2	0.7 ± 0.2	0.9 ± 0.2	1.1 ± 0.1

Table 1 (continued)

Gene	<i>rpoS</i>	JMrpoS ⁻	PB11	11rpoS ⁻	PB12	12rpoS ⁻
<i>rpoS</i>	+	0.1 ± 0.0	2.9 ± 0.1	0.1 ± 0.0	2.2 ± 0.2	0.2 ± 0.0
<i>rpoZ</i>		1.3 ± 0.0	0.9 ± 0.1	0.7 ± 0.0	0.8 ± 0.2	1.2 ± 0.4
<i>sdhA</i>	–	1.0 ± 0.1	0.9 ± 0.0	3.7 ± 0.2	2.0 ± 0.3	4.0 ± 0.0
<i>sdhB</i>	–	0.9 ± 0.0	0.8 ± 0.0	2.2 ± 0.0	1.7 ± 0.2	2.4 ± 0.3
<i>sfcA</i>	*	1.8 ± 0.2	1.9 ± 0.3	1.1 ± 0.1	1.7 ± 0.1	1.0 ± 0.0
<i>spoT</i>	*	1.1 ± 0.1	1.2 ± 0.2	0.5 ± 0.1	4.7 ± 0.2	0.7 ± 0.1
<i>sucA</i>		1.3 ± 0.0	1.4 ± 0.1	1.2 ± 0.2	1.7 ± 0.1	1.0 ± 0.0
<i>sucC</i>		1.4 ± 0.1	0.9 ± 0.1	1.3 ± 0.1	1.6 ± 0.0	1.5 ± 0.0
<i>talA</i>	+	0.3 ± 0.0	10.6 ± 1.9	1.1 ± 0.3	5.1 ± 0.7	0.2 ± 0.0
<i>talB</i>		0.8 ± 0.0	1.1 ± 0.1	0.7 ± 0.0	1.6 ± 0.0	1.4 ± 0.2
<i>tktA</i>		0.7 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	2.4 ± 0.1	2.1 ± 0.3
<i>tktB</i>	+	0.4 ± 0.0	11.6 ± 0.2	0.3 ± 0.0	5.7 ± 0.1	0.7 ± 0.0
<i>tpiA</i>	*	0.2 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	1.8 ± 0.0	0.3 ± 0.0

This table shows the relative transcription levels of the genes involved in different pathways and processes. For each gene in all strains, the transcription level of the gene for the wild-type strain was used as the control to normalize the data using the wild-type RT-PCR value for that gene as one. Therefore, data in this table and in figures 1–3 are reported as relative expression levels, compared to the expression level of JM101. The results are the averages of four independent measurements of the RT-PCR expression values for each gene. Two of the values were obtained from one fermentation procedure and the two others from an in-

dependent fermentation grown under identical conditions for each strain. The RT-PCR expression values obtained for each gene differ between them in most of the genes by less than 30% (see Experimental Procedures for a more detailed explanation). The values for some of these genes have been published previously and are depicted for comparative purposes.

RpoS regulated reported genes [Weber et al., 2005, Byocyc <http://www.ecocyc.org/>] are shown with +/- signs. Genes marked with an asterisk are genes reported in this work as regulated by RpoS.

and PB12 (table 2). These values indicate the different relative roles of RpoS in these strains. In agreement with the previous results, the transcription of many genes was more RpoS-dependent in the absence of PTS. As shown in table 3, the transcription levels of 18 genes (group 1) was downregulated in the PTS⁻ *rpoS*::Tc^r strains, whilst they were unmodified in the wild-type PTS⁺ *rpoS*⁻ derivative. The inactivation of *rpoS* decreased the transcription levels of another group of 8 genes in both genetic backgrounds, PTS⁻ and PTS⁺ (table 3, group 2). Interestingly, inactivation of *rpoS* in JM101 resulted in the upregulation of a group of 5 genes that were downregulated in the PTS⁻ strains (table 3, group 3). Finally, the *sdhAB* genes were upregulated in PB12 *rpoS*⁻ and PB11 *rpoS*⁻ as compared to their parental strains PB11 and PB12 (table 3, group 4). As mentioned these results give evidence of the differential roles of RpoS in the two strains and also confirm that the promoters of these genes share both, sigma 70 and sigma S recognition sites.

A possible explanation for the diverse roles of RpoS in the transcription of various genes in the two genetic back-

Table 2. Specific growth rate determinations (μ = h⁻¹) for the JM101 parental strain, the PB11 and PB12 PTS⁻ derivatives and their *rpoS*::Tc^r derivatives

Strain	Glc	Glc-Ac
JM101	0.71	0.68
JM101 <i>rpoS</i> ::Tc ^r	0.71	0.68
PB11	0.12	0.26
PB11 <i>rpoS</i> ::Tc ^r	0.06	0.2
PB12	0.42	0.48
PB12 <i>rpoS</i> ::Tc ^r	0.38	0.42

The specific growth rate determinations are the results of the averages of three independent growth experiments. The cells were grown on glucose or glucose plus acetate (Ac) as carbon sources.

grounds might be that in PTS⁻ backgrounds the cells sensed a permanent condition of glucose or glucose-6-phosphate internal limitation which resulted in decreased growth rates and thereby increased levels of ppGpp. Un-

Fig. 1. Transcription values of central metabolic genes in PB11 and PB11 *rpoS*::Tc^r. Central metabolic routes showing key metabolites and the genes involved in their transformation. RT-PCR values are shown close to the gene in parentheses for PB11 *rpoS*::Tc^r and underlined in parentheses for PB11; see table 1 for the complete set of RT-PCR values. The abbreviations are: GLC = glucose; G6P = glucose-6-phosphate; F6P = fructose-6-phosphate; F1,6P = fructose-1,6-phosphate; DHAP = dihydroxyacetone-phosphate; G3P = glyceraldehyde-3-phosphate; G1,3P = glyceraldehyde-1,3-phosphate; 3PG = 3-phosphoglycerate; 2PG = 2-phosphoglycerate; PEP = phosphoenolpyruvate; PYR = pyruvate; AC-CoA = acetyl-CoA; Ac-P = acetyl phosphate; A-AMP = acetyl-AMP; CIT = citrate; ICT = isocitrate; Gox = glyoxylate; α-KG = α-ketoglutarate; SUC-CoA = succinyl-coenzyme A; SUC = succinate; FUM = fumarate; MAL = malate; OAA = oxaloacetate; 6PGNL = 6-phosphogluconolactone; 6PGNT = 6-phosphogluconate; RU5P = ribulose-5-phosphate; R5P = ribose-5-phosphate; X5P = xylulose-5-phosphate; S7P = sedoheptulose-7-phosphate; E4P = erythrose-4-phosphate; KDPGNT = 2-keto-3-deoxy-6-phosphogluconate; DAHP = 3-deoxy-D-arabino-heptulosonate-7-phosphate; mez = malic enzymes.

der these circumstances, the genes commonly transcribed by sigma 70 in non-stress conditions (genes in group 1) were preferentially transcribed by RpoS. The transcription of the second group of genes was highly RpoS-dependent in both PTS⁻ and PTS⁺ strains, since the inactivation of *rpoS* clearly decreased their transcription. In the third group, there was apparently an opposite role of RpoS in the different backgrounds. For these genes, RpoS

had a repressor-like role in the PTS⁺ background, while it apparently had an activator role in the PTS⁻ strains. Finally, in the case of *sdhAB* genes, the inactivation of RpoS increased their transcription levels indicating a possible repressor role for RpoS. It is interesting that repressor-like roles for RpoS have been reported in an *E. coli* B *rpoS*⁻ derivative growing logarithmically for the TCA, *aceEF* and *lpd* genes [Rahman et al., 2006].

Fig. 2. Transcription values of central metabolic genes in PB12 and PB12 *rpoS*⁻Tc^r strains. This figure and table 1 present the relative transcription values of central metabolic genes in PB12 and PB12 *rpoS*⁻Tc^r strains. RT-PCR values are shown close to the gene in parentheses for PB12 *rpoS*⁻Tc^r and underlined in parentheses for PB12. See table 1 for the complete set of RT-PCR values. Symbols as in the legend to figure 1.

Transcription and Regulation of the Genes Involved in the Synthesis of pppGpp and ppGpp

As has been mentioned, many *E. coli* genes, whose transcription is controlled by alternative sigma factors such as RpoS, are poorly induced in cells lacking the alarmone ppGpp because this metabolite is required to increase the RpoS-binding capacity to the RNA polymerase core. It is known that RelA and SpoT are involved in the

synthesis of pppGpp and that the enzyme Gpp trans-forms this metabolite into ppGpp. Furthermore, SpoT is also involved in the degradation of this alarmone into GDP and PPi, and Ppa transforms PPi into Pi. Finally, Ndk converts GDP into GTP, while SpoT utilizes this metabolite to produce pppGpp [Lengeler et al., 1999, fig. 3]. It is remarkable that the transcription levels of *spoT*, *gpp*, *ppa* and *ndk* that encode these related enzymes were 3- to

Table 3. Transcription dependency on RpoS

Gene	JM101 <i>rpoS</i> ⁻	PB11 <i>rpoS</i> ⁻	PB12 <i>rpoS</i> ⁻
Group 1: Genes displaying transcription downregulation by <i>rpoS</i> inactivation only on PTS ⁻ strains			
<i>aceBA</i>	=	▼	▼
<i>ackA</i>	=	▼	▼
<i>acnB</i>	=	▼	▼
<i>acs</i>	=	▼	▼
<i>adhP</i>	=	▼	▼
<i>arcA</i>	=	▼	▼
<i>eno</i>	=	▼	▼
<i>fbaB</i>	=	▼	▼
<i>gadA</i>	=	▼	▼
<i>galE</i>	=	▼	▼
<i>katE</i>	=	▼	▼
<i>malP</i>	=	▼	▼
<i>pfkB</i>	=	▼	▼
<i>pgi</i>	=	▼	▼
<i>poxB</i>	=	▼	▼
<i>pykA</i>	=	▼	▼
<i>pykF</i>	=	▼	▼
<i>spoT</i>	=	▼	▼
Group 2: Genes displaying transcription downregulation by <i>rpoS</i> inactivation on PTS ⁻ strains and wild-type derivatives			
<i>fbaA</i>	▼	▼	▼
<i>gpp</i>	▼	▼	▼
<i>osmY</i>	▼	▼	▼
<i>pfkA</i>	▼	▼	▼
<i>ppa</i>	▼	▼	▼
<i>rpoS</i>	▼	▼	▼
<i>talA</i>	▼	▼	▼
<i>tktB</i>	▼	▼	▼
<i>tpiA</i>	▼	▼	▼
Group 3: Genes displaying an opposite transcription effect of <i>rpoS</i> inactivation. Upregulation in JM101 and downregulation in PTS ⁻ derivatives			
<i>aceEF</i>	▲	▼	▼
<i>ackA</i>	▲	▼	▼
<i>acnA</i>	▲	▼	▼
<i>gapC-1</i>	▲	▼	▼
<i>gapC-2</i>	▲	▼	▼
<i>sfcA</i>	▲	▼	▼
Group 4: Genes displaying upregulation by <i>rpoS</i> inactivation in PTS ⁻ mutants			
<i>sdhA</i>	=	▲	▲
<i>sdhB</i>	=	▲	▲
▼ Indicates that the transcription level is downregulated; = indicates no significant change in the transcription level, and ▲ indicates transcription upregulation.			

5-fold upregulated in both PB12 and PB13, as compared to PB11 and JM101 (fig. 3; table 1). Interestingly, the transcription level of the *relA* gene was not significantly modified. These results are in agreement with the proposition that during carbon starvation an alternative RelA-independent pathway for the synthesis of ppGpp is activated [Lengeler et al., 1999]. The upregulation of all these genes located in different transcription units at different positions of the *E. coli* chromosome [Gentry et al., 1993; GenBank accession number NC000913], could be the result of a mutation inactivating a common regulatory molecule which in turn allowed a higher transcription of these genes. A higher transcription level of *spoT* and *gpp* could in turn modify the levels of SpoT and Gpp. The high level transcription of RpoS-dependent genes in PTS⁻ strains could be important not only for maintaining the transcription of certain genes under nutrient-limited growth conditions, but also for the selection of strains capable of reaching a higher μ on glucose as is the case for PB12 and PB13. In agreement with this proposition, *rpoS* inactivation in PB11 *rpoS*::Tc^r strain clearly decreased its specific growth rate by 50% as compared to PB11, while the PB12 *rpoS*::Tc^r strain decreased its μ by only 10% as compared to PB12. On the contrary, the μ of the wild-type strain was not affected by the presence of the *rpoS*::Tc^r mutation (table 2). These results are also in accordance with the 3- and 2-fold transcription upregulation of the *rpoS* gene in PB11 and PB12, respectively, indicating that under these non-optimal nutrient-limited growing conditions, the role of RpoS was important in both PTS⁻ strains, although in PB11 it was more relevant.

There are different explanations for the isolation and preservation of a mutation upregulating the *spoT* and *gpp* genes. In a first scenario, the mutation that caused upregulation of these genes could be responsible for generating higher levels of ppGpp in PB12 or in a precursor strain. This mutation could have been selected and maintained because it allowed the transcription of RpoS-dependent genes in this PTS⁻ strain, in which the level of RpoS could be lower because the *rpoS*⁻ point mutation was not completely suppressed. Alternatively, the mutation upregulating *spoT* and *gpp* could have been selected to increase the transcription of RpoS-dependent genes in the PB11 background and this in turn could have increased the μ of this strain. In this second scenario, the *rpoS* point mutation was later selected in PB12 or a precursor strain and this could have diminished the levels of *rpoS* transcription because this mutation was not completely suppressed. In a third scenario, the upregulation of *spoT* and *gpp* could have decreased the levels of ppGpp in PB12 as compared

Fig. 3. Relative transcription expression values of genes involved in ppGpp metabolism. This figure and table 1 present the comparative expression of genes whose products are involved in ppGpp metabolism. The transcription values of *relA*, *gpp*, *spoT*, *ppa* and *ndk* are presented. The figure also shows, in the left upper part, the role of the enzymes coded by these genes in ppGpp metabolism.

Fig. 4. Nucleotide sequences of the putative 41-bp regulatory boxes located at the 5' upstream regions of the *gpp*, *ppa*, *rhlB* and *gmk-spoT* transcription units. The numbers indicate the distances in base pairs to the ATG start codons of the depicted genes.

to PB11, in order to reduce the transcription role of RpoS in PB12, or in a precursor strain. In this scenario, the appearance of the *rpoS* point mutation could have lowered the amount of active RpoS even further. This last scenario is in agreement with the fact that PB12 may sense less nutritional stress conditions than PB11, a condition in which less ppGpp is expected to be present. This fact could have given PB12 an altered transcription capability where sigma 70 could play a more important role but still maintains RpoS transcription of some important genes, allowing this strain a faster growth on glucose than PB11. However, since PB12 did not arise directly from PB11 and at least two mutations that appeared in an unknown order gave rise to PB12, it was difficult to propose a simple hypothesis to explain how the levels of ppGpp were modified and selected giving rise to PB12 which certainly grows faster than PB11 but still is RpoS-dependent to some extent. In any case, a mutation involved in ppGpp metabolism was selected that allowed the upregulation of *spoT* and *gpp* genes in PB12 or in a precursor strain.

We cannot rule out that this mutation was also responsible for the upregulation of other genes in PB12 (such as *glk* and *pgi*). However, it is interesting to point out that three of these four genes *spoT*, *gpp*, and *ppa* share a 41-bp DNA box located at the 5' terminus region outside their transcriptional units (fig. 4) that apparently is not present in any other region in the bacterial chromosome, except for the *rhlB* gene whose expression was also upregulated (table 1). This box, if functional, could play a role in the recognition of a regulatory protein involved in the transcriptional upregulation of these genes. Preliminary evidence shown in figure 5 indicates that this box could play a role in the expression of these genes. A 586-bp DNA fragment, carrying the wild-type 5' region including the 41-bp box and the first 20 nucleotides of the *gmk-spoT* operon, was cloned in front of a reporter *lacZ* gene. When this DNA fragment was integrated into the chromosome of the wild-type JM101 and PB11 strains, white colonies were obtained when the cells were plated in the reported medium (see Experimental Procedures). On the other hand, when the same fragment was integrated into the PB12 chromosome, blue colonies were obtained (appear as dark colonies in fig. 5). These results indicate that the proposed regulatory protein thought to be inactive in PB12, and therefore allowing the upregulation of *gmk-spoT* and other genes in this strain, was active in JM101 and PB11 and did not allow the upregulation of either *gmk-spoT* (as indicated by the RT-PCR results) or the reporter *gmk-lacZ* fusion in these two parental strains. A second DNA fragment, carrying only the first 297 bp

from the 5' region including 20 bp of the *gmk* structural gene, was also used to construct a different *gmk-lacZ* fusion. This new construction did not include the putative 41-bp box, or the additional 248 bp upstream from the putative regulatory DNA region (fig. 5). When this new construction was integrated into the chromosome of JM101, PB11 and PB12, all derivative strains gave rise to blue colonies. These results indicate that the 289-bp DNA region including the identified regulatory 41-bp box, modulated the transcription of the *gmk-spoT* operon. A possible explanation might be that a putative protein could bind to this DNA region with a regulatory effect on the downstream genes. When this DNA region, which carried the reporter *gmk-lacZ* fusion gene, was absent in JM101 and PB11 derivative strains, the putative regulatory protein was unable to bind to DNA and this enabled the transcription upregulation of these genes as indicated by the appearance of blue colonies of these derivative strains. As expected, in the case of PB12, both types of DNA fragments gave rise to blue colonies due to the absence of the putative regulatory protein that should be inactive in PB12, permitting the upregulation of *gmk-spoT* and perhaps *ppa* and *gpp*. As expected in all the blue colonies, β -galactosidase activity was detected (between 2000 and 4000 units) while in the white colonies no activity was found (data not shown). These experiments, while not conclusive, indicate a possible role of this 41-bp putative DNA box in the regulation of these genes. Although this putative regulatory site is located far upstream from its corresponding regulatory target, it is known that in *E. coli* long-range interactions of DNA regions are feasible by the formation of DNA-looping repressor structures by specific proteins and nucleoprotein complexes that may include H-Ns or Fis. The formation of this kind of DNA structure can block the access of the RNA polymerase to the promoter region and thus regulate gene expression [for review, see Semsey et al., 2005; Fried and Hudson, 1996].

Finally, It is important to note that the inactivation of *rpoS* in both strains PB11 *rpoS*::Tc^r and PB12 *rpoS*::Tc^r clearly decreased the transcription level of *gpp* and reduced in half the transcription levels of *spoT* and *ppa*, indicating that the transcription of the first gene was highly dependent whereas *spoT* and *ppa* transcriptions were at least partially dependent on RpoS. On the other hand, the transcription of *ndk* and *relA* was not affected by the *rpoS* mutation in the PTS⁻ strains. Interestingly, the transcription level of *gpp* was also clearly reduced in JM101 *rpoS*::Tc^r, while the transcription levels of *spoT* as well as *ndk* and *relA* were not affected in this PTS⁺ strain (fig. 3; table 1).

Fig. 5. Construction of the *gmk-lacZ* transcriptional chromosomal fusions. For the construction of the chromosomal translation *gmk-lacZ* fusions two DNA regions were employed. The first corresponded to the first 586 bp upstream of the *gmk* structural gene. This fragment included: the 297-bp *ligB-gmk* intergenic region containing the first 20 bp of the *gmk* structural gene, and part of the *ligB* gene where the 41-bp putative box is located. The second DNA fragment used in the *gmk-lacZ* translation fusion only included the 297-bp DNA intergenic fragment carrying the first 20 bp of the *gmk* structural gene (**A**). These amplified DNA frag-

ments were cloned into plasmid pRS415, including the β -lactamase gene (*bla*) with the *T14* terminator, allowing the construction of the two different *gmk-lacZ* fusions (**B**). These fusions were transferred into phage λ RS45 (**C**). Phage derivatives containing the two different *gmk-lacZ* fusions were utilized for the infection of strains JM101, PB11 and PB12. Blue and white phenotypes (that appear as dark and pale grays) were screened for the different derivative strains carrying the 586- and 297-bp *gmk-lacZ* fusions (**D**). For a more detailed explanation see the Experimental Procedures and Discussion sections in the text.

Growing Capacities of the *PTS*⁻ Strains and Their *rpoS*⁻ Derivatives

As has been mentioned, the μ on glucose of the *rpoS*::Tc^r derivatives of strains PB11 and PB12 was decreased by 50 and 10%, respectively, as compared to the parental strains, while the μ of the JM101 *rpoS*::Tc^r derivative was

not affected when compared to the wild-type strain (table 2). These results indicate the different and important roles of RpoS in the *PTS*⁻ strains, when growing in this carbohydrate as the unique carbon source. Interestingly, the gluconeogenic capacities of PB11 when growing on glucose are still present in the PB11 *rpoS*::Tc^r derivative

strain. The transcription of the glyoxalate shunt genes *aceBA* and *glcB*, as well as *acs* involved in acetate conversion into AcCoA is still upregulated, although not as highly as in PB11 (fig. 1; table 1). These results explain why this strain is still capable of growing more than 100% faster on glucose complemented with acetate as compared to the growth with only glucose as the sole carbon source given its capacity of co-utilizing both carbon sources as occurred with the parental strain PB11 (table 2). In the case of the PB12 *rpoS*::Tc^r, the transcription of *aceBA* and *glcB* involved in the glyoxalate shunt was clearly diminished to the levels of the wild-type strain, while the transcription of *acs* was still 3-fold upregulated. When glucose and acetate are both present as carbon sources this strain increased its μ about 10% in a similar level to that of PB12 which increased its μ from 0.42 to 0.48 (table 2) [Flores N et al., 2005; Flores S et al., 2005].

Concluding Remarks

The role of the RpoS sigma factor has recently been reevaluated. Weber et al. [2005] demonstrated that RpoS is involved in the transcription of more than 10% of *E. coli* K-12 genes, and Rahman et al. [2006] have shown that in *E. coli* B this sigma factor plays an important role in the transcription of many genes of its central metabolic pathway, not only in carbon starvation conditions but also in logarithmic growing cultures. These results indicate that several central carbon metabolism genes are both RpoS- and RpoD-dependent.

We studied the effect of *rpoS* inactivation in the JM101 strain and its PTS⁻ derivatives. When wild-type strain JM101 was grown logarithmically on glucose, the inactivation of *rpoS* did not substantially modify the transcription of most of the carbon central metabolism genes. These results indicate that most of the genes in PTS⁺ strains, growing logarithmically in minimal medium on glucose, are mainly sigma 70-dependent, as expected.

However, in the case of PTS⁻ derivatives of JM101, which grow permanently under non-optimal nutrient conditions on glucose, there is an important RpoS dependency in the transcription of many genes. The specific growth rate of the PB11 *rpoS*::Tc^r strain decreased 50% as compared to PB11 by the inactivation of *rpoS*, showing that important genes involved in these growing conditions are at least partially RpoS-dependent. This conclusion is supported by the fact that the transcription levels of many genes are downregulated in PB11 *rpoS*::Tc^r as compared to the parental PB11 strain, including most

glycolytic genes. 19 of these genes have not been previously reported as part of this modulon in *E. coli* K12 [Weber et al., 2005]. These results clearly demonstrate the important role of RpoS in this PTS⁻ *E. coli* strain.

During the adaptive evolution fermentation process for the selection of PB12 and PB13 from PB11, as strains capable of faster growth on glucose, several mutations occurred that favored this purpose [Flores N et al., 1996]. In this report we identified one of these changes, corresponding to a point mutation that generated a stop codon in the *rpoS* gene in both PB12 and PB13 strains. However, since these strains carry the *supE* gene that suppresses stop codon amber mutations, the *rpoS* point mutation should be at least partially suppressed in PB12. In accordance with this proposition, the transcription of highly RpoS-dependent genes in PB12 is still upregulated but not as high as PB11. A possible selective force behind the appearance of this type of mutation at the *rpoS* gene could be the appearance of a strain with less RpoS activity in which the point mutation was not completely suppressed. Less RpoS protein could in turn allow this strain a faster growth on glucose because certain RpoS-dependent genes could then be transcribed by RpoD. Interestingly, in strains PB12 and PB13, but not in the parental JM101 and PB11, the transcription levels of several genes such as *gpp*, *spoT*, *ppa* and *ndk* related to ppGpp metabolism are increased 3- to 5-fold. It is possible that the enhanced transcription rate of this group of genes was selected, in PB12 or in a precursor strain during the adaptive evolution fermentation experiment, to allow a faster growth on glucose by modifying, probably decreasing, the ppGpp concentration. This should allow a strain already carrying a *rpoS*⁻ point mutation, which could be only partially suppressed, an even more important role than RpoD over RpoS in the transcription of key genes, reflecting in this manner less stressed conditions but at the same time permitting the partial transcription by RpoS of certain genes required for the selection and/or the maintenance of a faster growth in a still non-optimal nutrient condition. Among these genes, a likely candidate could be *pgi* whose transcription is upregulated 6-fold in PB12 as compared to PB11. When *rpoS* was inactivated in PB12, generating strain PB12 *rpoS*::Tc^r, the transcription levels of several genes, known to be transcribed by RpoS were decreased as was the case of *pgi*. Therefore, it is clear that the translation of the *rpoS* mRNA in PB12 produced enough functional RpoS to transcribe certain genes. However, the inactivation of *rpoS* in strain PB12 *rpoS*::Tc^r decreased its growth rate by only 10% as compared to PB12. These results indicate that PB12 still senses non-optimal nutrient

growing conditions, although to a less significant degree than PB11 when glucose is utilized as the only carbon source. Consequently, the level of ppGpp in PB12 should be lower than in PB11. Nevertheless, the upregulation of many RpoS-dependent genes would not be possible without the presence of enough ppGpp to enable the utilization of RpoS for their transcription.

Interestingly, the transcription of *gpp*, *spoT* and *ppa* was also dependent on RpoS, since their expressions clearly diminished in strains PB11 *rpoS::Tc^r* and PB12 *rpoS::Tc^r*. On the other hand, the transcription of *relA* and *ndk* was found to be independent of RpoS. These data provide clear evidence, not previously reported, for the different roles of RpoS in these two mechanisms that are involved in the synthesis of ppGpp. One of the mechanisms is related to RelA through the stringent response and the other through carbon starvation where the transcription of *spoT* and *gpp* is dependent on RpoS, at least under non-optimal carbon-limited growth conditions in these PTS⁻ strains. Our results are in agreement with and extend those reported by Weber et al. [2005], where 10% of the genes of the bacterial genome in the PTS⁺ wild-type strain were reported to be under RpoS control in different non-optimal growth conditions. Apparently, in the PTS⁻ strains non-optimal growing conditions are permanent when growing on glucose, especially in PB11. As mentioned, the inactivation of *rpoS* in these PTS⁻ strains allowed the identification of an additional group of 19 genes, not previously reported whose transcription is at least partially controlled directly or indirectly by RpoS.

Finally, the isolation of a mutation that simultaneously upregulated the transcription of the *spoT*, *gpp*, *ppa* and *ndk* genes, required in the synthesis and degradation of ppGpp, could not be directly obtained from the parental JM101 PTS⁺ strain as wild-type strains have several redundant mechanisms to deal with carbon utilization in logarithmic and starvation conditions. The isolation of this mutation became feasible in a PTS⁻ strain (PB12), permanently growing under carbon-limited non-optimal conditions on glucose, most probably because this situation changed the levels of ppGpp allowing a transcription modification specificity of many genes permitting a more relevant role for RpoD, although still maintaining transcription by RpoS of important genes in this strain. This is a clear example of the importance of the utilization and characterization of strains with mutations in general regulators, such as PTS and RpoS, for the purpose of identifying and characterizing other important regulatory elements in the network, such as the one described here, which is involved in the regulation of the

expression of genes responsible for the synthesis and degradation of the alarmone ppGpp that is required for sigma S functionality. We cannot rule out that the upregulation of other genes in PB12 (such as *glk* and *pgi*) could depend on the same regulatory element responsible for the upregulation of *spoT*, *gpp* and *ppa*. However, it is interesting that these three genes share a putative DNA box sequence present in *E. coli* only at the 5' upstream regions of these genes localized at different chromosomal positions with only one exception, the *rhlB* gene, which encodes a putative ATP-dependent RNA helicase. In this report, preliminary data are presented in favor of a possible role of this DNA box in the expression of these genes. So far, a possible regulatory protein involved in the expression of this group of genes has not been reported. Attempts will be made towards its isolation, characterization and to understand in more detail its possible role in the genetic network under different metabolic conditions.

Experimental Procedures

Bacterial Strains, Construction of rpoS⁻ Derivatives, Media and Growth Conditions

E. coli strains PB12 and PB13 (JM101 $\Delta(ptsH, ptsI, crr)::kan$, Glc⁺) were obtained from PB11 (JM101 $\Delta(ptsH, ptsI, crr)::kan$), a PTS⁻ mutant derivative of *E. coli* JM101 (F' *traD36 proA⁺ proB⁺ lacI^q $\Delta(lacZ)$ Z15/supE thi $\Delta(lac-proAB)$) [Bolívar et al., 1977; Flores N, 1995]. *rpoS⁻* derivatives of these strains were obtained by P1 transduction using strain RH90 *rpoS::Tc^r* as donor of *rpoS::Tc^r*. Duplicate cultures for RNA isolation, acetate and glucose measurements by HPLC and specific growth rate (μ) determinations were grown on 1 liter fermentors on M9 medium with 2 g/l of glucose or 2 g/l of glucose plus 12 mM of acetate, at 37°C, 600 rpm controlling pH at 7 with NaOH and air flow rate of 1 vvm. Bacterial growth monitoring started at an OD₆₀₀ of 0.05 and cells were collected when growing in the log phase at an OD₆₀₀ of 1. *E. coli* strain DH5 α [Simons et al., 1987] was used for the construction of *gmk-lacZ* fusions.*

RNA Extraction and cDNA Synthesis

Total RNA extraction was performed using hot phenol equilibrated with water. After extractions, RNA was precipitated with 3 M sodium acetate/ethanol and centrifuged 20,000 g at 4°C. Supernatant was discarded and the RNA resuspended in water. RNA was treated with DNase kit (DNA-free™, Ambion) and its concentration measured by densitometry in agarose gels and by 260/280 nm ratio absorbance. cDNA was synthesized using RevertAid™ H First Strand cDNA Synthesis kit (Fermentas Inc.) and a mixture of specific DNA primers. The list with the sequences of the primers of some of the specific genes utilized in this work has been published in Flores N et al. [2005]. The non-published sequences are listed in table 4. cDNA was used as template for RT-PCR assays. Reproducibility of this procedure was determined by

performing two separate cDNA synthesis experiments from the RNA extracted for each strain. Similar results were obtained for the transcription of all measured genes.

Real-Time PCR

RT-PCR was performed with the ABI Prism 7000 Sequence Detection System (Perkin-Elmer/Applied Biosystems) using the SYBR Green PCR Master Mix (Perkin-Elmer/Applied Biosystems). Amplification conditions were 10 min at 95°C, and a two-step cycle at 95°C for 15 s and 60°C for 60 s for a total of 40 cycles. Primers for specific amplification were designed using the Primer Express software (PE/Applied Biosystems) and have been published [Flores N et al., 2005]. The only primers not published are shown in table 4. The size of all amplimers was 101 bp. The final primer concentration, in a total volume of 15 µl was 0.2 µM. Five nanograms of target cDNA for each gene utilized in this work was added to the reaction mixture. All experiments were performed in triplicate for each gene of each strain, obtaining very similar values (differences of less than 0.3 SD). A non-template control reaction mixture was included for each gene. The quantification technique used to analyze data was the $2^{-\Delta\Delta CT}$ method described by Livak and Shmittgen [2001], and the results were plotted.

Data were normalized using the *ihfB* gene as an internal control (housekeeping gene). The same expression level of this gene was detected in all the strains in the conditions tested. For each analyzed gene, the transcription level of the wild-type gene was considered as one and was used as the control to normalize the data. Therefore, data are reported as relative expression levels, compared to the expression level of JM101. The results presented in table 1 and in figures 1–3 are the averages of four independent measurements of the RT-PCR expression values for each gene. Half of the values were obtained from two different cDNAs generated from one fermentation procedure and the other two from another identical fermentation. RT-PCR expression values obtained for each gene differ by less than 30%.

Construction of *gmk-lacZ* Chromosomal Fusions

Chromosomal *gmk* regulatory region-*lacZ* fusions were constructed as described by Simons et al. [1987]. Briefly, a chromosomal region of 586 bp (including 289 bp, the possible regulatory 41-bp box, the intergenic *ligB-gmk* region and the first 20 bp of the 5'-*gmk* structural gene) and a second chromosomal region of 297 bp (including the intergenic *ligB-gmk* region excluding the possible regulatory 41-bp box, but including the first 20 bp of the *gmk* structural gene) were amplified (fig. 4, 5). *EcoRI* restriction sites were incorporated in the two forward (Fw) primers (Fw1 5' CCCC GAATTC ATTTT GCGTACACCCGATGGG 3', Fw2 5' CCCC GAATTC ATCGCCATCCATACTTTTCATCATCAC3'), and a *BamHI* site was incorporated in the reverse (Rv) primer (5' CCCC GGATCCTAAAGCGTGCCTTGAGCCAT 3'; fig. 5). Each amplified product was digested and ligated into *EcoRI/BamHI* digested plasmid pRS415. Recombinant molecules were used to transform *E. coli* strain DH5α. Transformant cells were plated on LB with ampicillin (200 µg/ml). Alkaline minipreps of several colonies were performed and plasmids digested with *EcoRI/BamHI* to verify the size of the ligated chromosomal DNA region. The two different translational fusions of the *gmk* regulatory region and its first seven codons with the reporter *lacZ* (*gmk-lacZ*) fusions thus obtained were transferred to λRS45 phage using the following technique: 1-ml aliquots of overnight culture LB sup-

Table 4. Nucleotide sequences of primers not previously published and used for RT-PCR assays

Gene	Primer name	Primer sequence
<i>kgm</i>	gmka	5' ATCCAGCCTGATTGAGGCTTT 3'
	gmkb	5' CGTGGACTTCACCAGGACG 3'
<i>gpp</i>	gppa	5' AATTGCTGGCGACGCTACTG 3'
	gppb	5' GCAGAGTTGTTCTGCGAGCC 3'
<i>katE</i>	katEa	5' CCAAAGCGGATTCCTCTCA 3'
	katEb	5' ATCACGCACGGTATCAGCAG 3'
<i>malP</i>	malPa	5' CTGGCGGCTCTGTTGGATAA 3'
	malPb	5' TGACGGAATTTTCGCATCATC 3'
<i>osmY</i>	osmYa	5' AAAGTTGAAACCACCGACGG 3'
	osmYb	5' ACACCATCTACCGCTTTGGC 3'
<i>ppa</i>	ppaa	5' AATTGCTGGCGACGCTACTG 3'
	ppab	5' GCAGAGTTGTTCTGCGAGCC 3'
<i>ptsG</i>	ptsGa	5' AAAAGCGACAGGTACCAGCG 3'
	ptsGb	5' CGCGCAGACGGGTAATACAT 3'
<i>rhlB</i>	rhlBa	5' ATCAGCCGCGTGCCTTAAT 3'
	rhlBb	5' ACCCAGCTTCAGGCCAGTAG 3'
<i>rpoD</i>	rpoDa	5' GATTCTGCGACCACCGAAAG 3'
	rpoDb	5' TCGATACCGAAACGCATACG 3'
<i>spoT</i>	spoTa	5' ATCCGATCTCTTCCCGGATG 3'
	spoTb	5' GCACTGCATAAGCGAAGTCG 3'

The sequences of primers of the remaining genes have been reported [Flores N et al., 2005].

plemented with 0.2% maltose and 10 mM MgCl₂ of both DH5α/pRS552 *gmk-lacZ* fusions were washed with buffer λ (0.61 g/l Trizma base pH 7.5, 0.123 g/l MgSO₄·7H₂O, 0.0278 g/l anhydrous CaCl₂, 0.46 g/l NaCl) and infected with 100 µl of λRS45 phage. 3 ml of TAB soft agar were added to infected cells that were plated and incubated on at 37°C. The λRS45 phage containing *gmk-lacZ* fusions was harvested and a 10⁻³ dilution of phage suspension was used overnight to infect LB cultures of JM101, PB11 and PB12 strains grown in TB agar supplemented with maltose, NaCl and MgCl₂. After infection, cells were washed and plated in TB agar supplemented with X-Gal and ampicillin (200 µg/ml). White or blue phenotypes were screened in LB with X-Gal for JM101, PB11 and PB12 derivative strains carrying 586- and 297-bp regulatory region *gmk-lacZ* fusions.

Chromosomal DNA was extracted from JM101, PB11, PB12 and derivative strains carrying the 586 and 297 *gmk-lacZ* fusions to verify the presence of the fusions by amplification using the adequate set of flanking primers. The size of the fragments obtained indicated the correct size of the amplified products for the different *gmk-lacZ* fusions. DNA sequencing of amplified products indicated the correct sequences of the amplified products (see below).

Nucleotide Sequence Determination and Analysis of *rpoS* and Other DNA Regions

200 bp located at the 5' end and 100 bp at the 3' end of the *rpoS* structural gene and their coding regions were amplified by PCR using a set of primers designed with the Clone Manager Program.

Chromosomal DNA (100 ng) from JM101, PB11, PB12 and PB13 strains was used as template for amplification. Also DNA from strains JM101, PB11 and PB12 carrying 586 and 297 bp *gmk-lacZ* fusions integrated at the chromosome was amplified by PCR with the Elongase enzyme mix, in accordance with the supplier's recommendations (Invitrogen, Inc.). PCR products were analyzed for expected size and purified using a PCR purification kit (Marpigen, BioScience Inc.). Nucleotide sequences (data not shown) were determined from PCR templates by the Taq FS Dye Terminator Cycle Fluorescence-Based Sequencing method, with an Applied Biosystems Model 377-18 sequencer.

Computer Analysis of Putative Regulatory Regions

The 400 bp upstream regions of the operons carrying the genes whose products are involved in ppGpp metabolism in *E. coli* (*relA*, *gpp*, *spoT*, *ppa* and *ndk*), were used to search for significant over-represented sequence motifs; their comparative expressions are depicted in figure 4. For this purpose, the public domain motif Multiple EM for the Motif Elicitation (MEME) program was utilized [Bailey and Elkan, 1994]. The common motif found by

this analysis and its distance to the ATG start codon of the first gene of the operons is shown. The specificity of this motif was evaluated by search in all upstream regions of the *E. coli* genome using the MEME counterpart Motif Alignment and Search tool MAST [Bailey and Gribskov, 1998]. No new sequences were found in this search. The *E. coli* genome sequences used in the analysis were obtained from GenBank (AC number NC_000913).

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