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Synthetic growth phenotypes of *E.coli* lacking ppGpp and transketolase A (*tktA*) are due to ppGpp-mediated transcriptional regulation of *tktB*

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Summary

Many physiological adjustments to nutrient changes involve ppGpp. Recent attempts to deduce ppGpp regulatory effects using proteomics or gene profiling can rigorously identify proteins or transcripts, but the functional significance is often unclear. Using a random screen for synthetic lethals we found a ppGpp-dependent functional pathway that operates through transketolase-B (TktB), and which is "buffered" in wildtype strain by the presence of an isozyme, transketolase-A (TktA). Transketolase activity is required in cells to make erythrose-4-phosphate, a precursor of aromatic amino acids and vitamins. By studying *tktB*-dependent nutritional requirements as well as measuring activities using P*talA-tktB′-lacZ* transcriptional reporter fusion we show positive transcriptional regulation of the *talA-tktB* operon by ppGpp. Our results show the existence of RpoS-dependent and independent modes of positive regulation by ppGpp. Both routes of activation are magnified by elevating ppGpp levels with a *spoT* mutation (*spoT*-R39A) defective in hydrolase but not synthetase activity or with the stringent suppressor mutations *rpoB*-A532Δ or *rpoB*-T563P in the absence of ppGpp.

Keywords

RpoS; transketolase; synthetic lethal; *spoT*; *rpoB*; and stringent

Introduction

Bacteria have evolved with complex protective global responses to stress. In enteric bacteria, the accumulation of guanosine 5′-diphosphate, 3′-diphosphate and/or guanosine 5′-triphosphate, 3′-diphosphate, collectively referred to as the (p)ppGpp nucleotides (Cashel and Gallant, 1969) is a common response to different sources of nutritional stress. Here we shall use ppGpp as an abbreviation for pppGpp and ppGpp. There are many examples of regulation that involve ppGpp as a function of normal growth and during stress. Phenotypes associated with a complete deficiency of ppGpp ($ppGpp^0$) include multiple amino acid requirements, filamentation, nucleoid partitioning defects, agglutination changes, adhesion and motility defects arising from the absence of fimbriae and flagella respectively and decreased virulence in pathogenic bacteria (Xiao *et al*., 1991; Cashel *et al*., 1996; Magnusson *et al*., 2005, 2007; Breaken *et al*., 2006).

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Random genetic approaches to define ppGpp function have not been frequently used. Instead, mutants of the *relA* and *spoT* genes were encountered in different strains of *E. coli*, mapped, isogenic strains were constructed and phenotypes characterized. Selections based on these phenotypes identified mutations in *relB* and *relC* but not the genes involved in the wide range of functions just mentioned (Cashel *et al*., 1996). One way to identify cellular functions mediated by ppGpp is to isolate spontaneous extragenic suppressors of defects in ppGpp^0 cells. Many such mutations have been isolated that reverse the multiple amino acid auxotrophic phenotype of ppGpp^0 cells to allow growth on minimal glucose media. So far, such mutations occur exclusively in *rpoB*, *rpoC* and *rpoD* RNA polymerase subunit genes and have been termed "stringent" mutations (Murphy and Cashel, 2003; Zhou and Jin, 1998).

We used a synthetic lethal approach to look for genes involved in ppGpp-dependent functions. Two mutations are synthetic lethal if either in isolation is viable but together cause inviability. Two separate non-lethal mutations that confer a growth defect more severe than either single mutation can be called synthetic growth inhibition (Ooi *et al*., 2006; Phizicky and Fields, 1995). The interpretation is that synthetic growth inhibition reflects an important genetic interaction, whereas synthetic lethality reflects an essential genetic interaction. Such interactions reveal genes that function in parallel pathways and "buffer" each other biologically or function within the same pathway but independently contribute to the strength of the signal in the pathway. In general, synthetic lethal screens help uncover pathway(s) that are conditionally essential or significantly influence growth.

Here a genetic screen is used to search for pathways that show ppGpp-mediated regulation. We isolated an insertion in *tktA* that gives synthetic growth defects in a ppGpp^0 strain. This led to the identification of *tktB* as a transcriptional target of ppGpp and evidence that activation of *tktB* transcription by ppGpp occurs both through the modulation of RpoS levels and independent of RpoS. The physiological relevance of the two modes of regulation is assessed.

Results

A screen for synthetic-lethal mutations in a ppGpp^0 host identifies an insertion in *tktA*

The rationale behind the screen is that an unstable plasmid replicon carrying a gene required for growth would be retained through selection during growth conditions that favor plasmid loss through segregation (Phizicky and Fields, 1995). Low copy number plasmid pHR14 is a temperature-sensitive pSC101-replicon with functional *spoT* and *lacI*^{q} genes. Replication of pHR14 is stable at 30°C significantly restricted at 38°C and completely abolished at 42^0. Growth at 38°C without selection causes plasmid loss and dilution of cellular LacI levels. In the Δ*lacI* (*lacZ*$^+$)Δ *relA251* Δ*spoT207* strain CF11722 carrying plasmid pHR14, plasmid loss results in an increase in β-galactosidase expression and appearance of blue colonies in plates containing the chromogenic substrate X-gal. Mutations that limit plasmid loss will give rise to white or pale blue colonies. Among many other possibilities, mutations that render *spoT* gene functions essential for growth are expected to select against plasmid loss. A similar approach has been used to identify a synthetic lethal mutation in *ftsEX* mutant (Reddy M, 2007)

CF11722 with pHR14 was subjected to Tn5 transposon mutagenesis and dilutions were plated on LB X-gal plates with trimethoprim to obtain about 200 well separated single colonies on each plate. A pale blue colony was identified after screening 5,000 blue colonies. The transposon insertion in this clone impaired growth when moved into the ppGpp^0 strain CF10237 (by P1vir transduction) but not in the wildtype strain CF1648 (See Fig. 1 panel B). Thus, growth inhibition is dependent on ppGpp deficiency and the growth phenotype from the transposon insertion is a severe growth impairment rather than lethality. Sequencing the transposon-chromosome junction localized the insertion to the distal half of the *tktA* open reading frame and it was designated as *tktA*::Tp.

In *E. coli*, *tktA* and *tktB* genes encode redundant transketolases that catalyze synthesis of a key metabolic intermediate, D-erythrose-4-phosphate. As shown in Fig. 2, lack of transketolase activity would result in the failure to synthesize erythrose-4-phosphate, a precursor required for biosynthesis of aromatic amino acids, aromatic vitamins like para-amino benzoic acid and pyridoxine (PN), a precursor of pyridoxal phosphate (Fraenkel, 1987;Pittard, 1996; Wallace and Pittard, 1969; Zhao and Winkler, 1994).

A Δ*tktB*::kan mutation confers synthetic growth defects in the *tktA* mutant similar to that observed from ppGpp deficiency

We examined LB growth in the presence of *tktA* and *tktB* mutations singly and in combination. We chose two *tktA* alleles, *tktA*::Tn10, an undefined insertion in *tktA* (Iida *et al*., 1993) and the Δ*tktA::kan* deletion-insertion allele from the Keio collection (National Bioresource project, Japan) as well as two Δ*tktB::kan* alleles (Iida *et al*., 1993 and Keio collection). The *tktA* mutants were slightly slower growing on LB while the *tktB* mutants showed no growth defect. However when combined, the *tktA tktB* double mutant shows growth inhibition in LB comparable to that observed in the *tktA* ppGpp^0 strains (Fig. 1B; Table 1A, last column).

Comparison of growth requirements between *tktA* ppGpp^0 mutant and the *tktA tktB* double mutant

Transketolase mutants require aromatic amino acids and pyridoxine (Fraenkel, 1987; Iida *et al*., 1993; Zhao and Winkler, 1994). The growth requirements of the *tktA tktB* double mutants are due to the lack of erythrose-4-phosphate. This compound is generated from glyceraldehyde-3-phosphate and fructose-6-posphate by transketolase or from sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate by transaldolase. However, the syntheses of the latter two substrates require transketolase. Therefore, erythrose-4-phosphate is not synthesized in a *tktA tktB* double mutant (Fig. 2).

Growth on minimal glucose casaminoacids plates with or without tryptophan and pyridoxine is shown in Fig. 1, panels C & D. The growth requirements of *tktA* ppGpp^0 strain differs from that seen in *tktA tktB* mutant; the former does not show an absolute requirement for pyridoxine; when each of the aromatic amino acids is additionally omitted, both strains fail to show growth (Table 1A). A requirement for pyridoxine as well as aromatic amino acids has been reported in a *tktA tktB* double mutant (Zhou and Winkler, 1994). Since phenylalanine requirement is also observed in ppGpp^0 strain (Xiao *et al*., 1991), it will not be considered further as a synthetic phenotype of the ppGpp^0 *tktA* mutant. The pyridoxine requirement seen in the *tktA tktB* double mutant is not observed in ppGpp^0 strains with the *tktA*::Tn10 or the *tktA*::Tp alleles (Table 1A). This difference could be due to a trace of transketolase activity in the ppGpp^0 *tktA* strain as compared to the complete absence of activity in the *tktA tktB* mutant (see below). We assume that a small amount of pyridoxine is sufficient to support growth due to the catalytic use of vitamins as opposed to stoichiometric consumption of amino acids. Unlike ppGpp^0 *tktA* mutant the ppGpp^0 *tktB* mutant strain has growth phenotypes identical to the ppGpp^0 parental strain (data not shown).

Supplementing LB medium with aromatic amino acids and/or pyridoxine did not improve growth while the addition of 0.2% glucose partially improved growth (data not shown). Colony sizes are equivalent on LB glucose and in minimal glucose with all 20 amino acids and PN when incubated for the same amount of time (data not shown). It is notable that the *tktA tktB* mutant does not appear to require para-amino benzoic acid and related vitamins under our growth and media conditions. We do not have a good explanation for this phenotype based on our current understanding of the metabolic pathways.

The ppGpp^0 – *tktA* synthetic phenotypes arise from low transketolase activity

The *tktA* gene is located at the 66.3 min region of the genome with 6 ORFs (*cmtB* to *yggC*) downstream of *tktA* and oriented in the same direction (Fig. 3A). Therefore *tktA* could be the first gene of an operon and the synthetic phenotypes of an insertion in *tktA* might be due to polar effects. In order to ensure that the loss of transketolase activity caused the observed phenotype, we looked for phenotypic rescue by ectopic expression of the transketolase B isozyme (74% amino acid identity with *tktA*) from an IPTG-inducible promoter in the ppGpp^0 *tktA* mutant. Table 1B shows that IPTG-induced expression of a minimal *tktB* gene from plasmid pHR30 completely reverses the synthetic growth defect of *tktA*-ppGpp^0 strains while the plasmid vector had no influence on the synthetic growth phenotypes. This verifies that the phenotypes conferred by *tktA* insertions are a consequence of lowered transketolase catalytic activity. This result implies that ppGpp deficiency could lower transketolase B activity.

The growth phenotypes of the ppGpp^0 *tktA* mutant strain are alleviated by functional SpoT

There are two genes for ppGpp synthesis in *E.coli*, namely *relA* and *spoT* (Cashel *et al*., 1996). A *relA tktA* (*spoT* $^+$) strain does not exhibit growth impairment on LB, but shows a partial tyrosine requirement (Table 2, rows 1–3). Tyrosine and tryptophan auxotrophy is observed when the entire *spoT* ORF is deleted (*spoT*212) in the *relA tktA* background (Table 2 row 4). We conclude that functional SpoT is sufficient to alleviate synthetic growth phenotypes, especially the growth defect on LB. The converse experiment of deleting *spoT* in a *tktA relA*$^+$ strain could not be performed since such a construct is inviable because excess ppGpp inhibits growth (Xiao *et al*., 1993). However, as described below, RelA-mediated ppGpp synthesis also contributes to the synthetic growth phenotypes.

The growth phenotypes in ppGpp^0 *tktA* mutant strain reflect the overall ppGpp biosynthetic capacity of the cell

There are at least two known functions for SpoT protein, namely, ppGpp synthesis and hydrolysis (Xiao *et al*., 1991). We wanted to understand the SpoT function required to alleviate synthetic growth phenotypes. It is even possible that this function is *spoT*-dependent but ppGpp-independent, because a number of proteins have been identified that interact with SpoT. We were unable to test this by providing a weak enough source of ppGpp to allow survival of a Δ*relA* Δ*spoT* strain (Table S2, row 3). Examples of proteins that interact with SpoT are acyl-carrier protein (Battesti and Bouveret, 2007); CgtA (Wout *et al*., 2004; Jiang *et al*., 2007) and numerous small and large subunit ribosomal proteins (Butland *et al*., 2005). The balance of SpoT hydrolase and synthetase activities respond to variety of environmental signals (Cashel *et al*., 1996; Murray and Bremer, 1996) but little is known of the mechanisms coupling SpoT responses to these signals except in the case of fatty acid synthesis (Battesti and Bouveret, 2007).

We constructed a pair of single amino acid substitution alleles of SpoT designed to eliminate either the hydrolase or the synthetase activity of SpoT but otherwise minimally altering the protein. To do this, we exploited predictions from mutants and structures solved for Rel$_{Seq}$, the SpoT homolog from *Streptococcus equisimilis* (Mechold *et.al*., 2002; Hogg *et al*., 2004). The residues chosen to be altered in each of the two catalytic centers were SpoT-R39 to limit hydrolase activity (H^-S^+) and SpoT-E319 to limit synthetase (H^+S^-) activity. The residues were selected because their homologs in Rel*Seq* were deduced to display maximum movement during structural changes in their catalytic pocket when ligands bind the opposing catalytic center (Hogg *et al*., 2004). Growth tests in a *relA* mutant to characterize *spoT*-R39A and *spoT*-E319Q alleles are described in supplementary information (Table S2). The hydrolase mutation (H^-S^+) slows growth in LB and in minimal media (Table S2) consistent with higher basal levels of ppGpp during growth. The E319Q synthetase mutation in this host entirely

eliminates ppGpp synthesis because the mutant fails to grow in minimal media when the *relA256* in-frame ORF deletion is present (Xiao *et al*., 1993).

Substituting the synthetase mutant (H^+S^-) allele for a complete s*poT* deletion confers growth requirements in a *tktA relA256* background. If this strain is made $RelA^+$, growth is normal on LB and aromatic amino acid requirements are not seen; RelA becomes the source of ppGpp (Table 2, rows 6 & 7). Introduction of the *spoT*-R39A allele eliminates the growth requirements of the parental *tktA* $ppGpp^0$ strain (Table 2, row 5) and is not viable in a $relA^+$ background (data not shown). Apparently, transketolase B activity can be down-regulated by a single residue change in the synthetase catalytic center of the 702-residue SpoT protein. This suggests a key role for ppGpp synthetase function and eliminates other putative regulatory functions of SpoT protein that are unaltered in the E319Q allele. The simplest interpretation of the results is that ppGpp regulates transketolase B activity in the *tktA relA256* mutant. The extent of *tktB* activation reflects the cellular capacity to synthesize ppGpp either from RelA or SpoT.

Independent and synergistic roles of ppGpp: DksA and RpoS modulate *tktB*-dependent growth requirements

Finding that ppGpp is required for *tktB* function leads to the need to assess the roles for DksA and RpoS, two proteins whose regulatory functions are coordinated with that of ppGpp in many instances. DksA, a multi-copy suppressor of DnaK (Kang and Craig, 1990), functions at the level of transcription initiation in vitro as a co-factor of ppGpp to mediate both positive and negative regulatory effects on gene expression (Perederina *et al*. 2004; Paul *et al*., 2004a, 2004b & 2005). Studying RpoS is relevant because during entry into stationary phase the accumulation of this stationary phase sigma factor is delayed in the absence of ppGpp or *dksA*; during exponential growth RpoS levels increase upon gratuitous induction of ppGpp (Gentry *et al*., 1993; Brown *et al*., 2002). A requirement for ppGpp exists not only at the level of accumulation of RpoS but also for RpoS-dependent gene expression (Kvint and Nystrom, 2000).

Deleting *dksA* confers several amino acid requirements but these do not include tryptophan or tyrosine (Brown *et al*., 2002). The same *dksA* allele when combined with *tktA* reduces, but does not eliminate growth in the absence of tyrptophan or tyrosine (Table 3, row 2). Apparently the absence of one co-factor (DksA) only partially mimics the absence of the other (ppGpp). The results could be interpreted as independent regulation of *tktB* expression by *dksA* or potentiation of ppGpp-mediated regulation by *dksA*. The latter is supported by the observation that absence of DksA and ppGpp give phenotypes only as severe as those seen in the absence of ppGpp (Table 3, rows 3 & 4).

In an otherwise wildtype host, deleting *rpoS* does not result in amino acid or vitamin requirements, upon further inactivation of *tktA*, growth impairment is slight with all amino acids and PN present, similar to a *tktA* mutant (Table 1, rows 1 & 2; Table 3, rows 5 & 6). However, the *rpoS tktA* double mutant, unlike each single mutant, shows partial requirements for tryptophan, tyrosine (Table 1, row 2; Table 3, rows 5 & 6) and phenylalanine (data not shown). Adding a *relA* deletion (*rpoS tktA relA*) gives strong growth requirements for PN and amino acids (Table 3, rows 7). A similar phenotype is observed in the *rpoS tktA* $ppGpp^0$ strain, making these strains phenotypically identical to the *tktA tktB* mutant (Table 1A, row 4; Table 3, rows 7,9). We confirmed that growth requirements in mutant strains arise from reduced levels of TktB by rescuing growth through ectopic *tktB* expression using plasmid pHR30 (Table 3, rows 6–10). The results indicate independent regulatory roles for ppGpp and *rpoS* in *tktB* transcription (see discusson).

As mentioned previously, DksA over-expression using multicopy plasmid can suppress some $ppGpp^0$ phenotypes. Table 4, rows 3 & 4 show that DksA over-expression in the $ppGpp^0$

tktA mutant restores prototrophy for tryptophan and tyrosine and that the suppression requires RpoS. The pyridoxine requirement is overcome by DksA over-expression in a ppGpp^0 *tktA rpoS* mutant (Table 4, compare rows 2 & 4). Therefore, over-expression of DksA can suppress pyridoxine and amino acid requirement in the presence of RpoS and only the pyridoxine requirement in the absence of RpoS.

Suppression of auxotrophic requirements by RNA polymerase mutations

About 60 spontaneous mutant alleles have been isolated that restore the growth of ppGpp^0 strain on minimal glucose and mapped to *rpoB, rpoC* and *rpoD* genes. Some of these have been studied extensively in vitro (Cashel *et al*., 1996; Murphy and Cashel, 2003; Zhou & Jin, 1998; Barker *et al*., 2001). We chose for this study two well known *rpoB* alleles that confer rifampicin resistance, *rpoB*-T563P and *rpoB*-A532Δ (alias *rpoB*3370 and *rpoB*3449 respectively) which mimic ppGpp regulatory behavior *in vivo* and *in vitro* (Zhou & Jin, 1998). We first asked if the alleles change RpoS expression pattern in the absence of ppGpp. Figure 4 is an immunoblot using anti-RpoS antibody in ppGpp^0 strains with or without the *rpoB*3449 and *rpoB*3370 alleles. The presence of the suppressor mutations elevates RpoS protein levels 20-fold over the levels observed in ppGpp^0 cells in the log phase of growth. The RpoS level in the *rpoB* mutant strains are about 5-fold higher than in the wildtype strain in log phase (data not shown).

Table 4 shows that both *rpoB* alleles completely suppress the growth requirements associated with low transketolase activity in the *tktA* ppGpp^0 host (rows 5 & 8). This is consistent with their ability to induce RpoS accumulation. However, when *rpoS* is deleted, growth in the absence of PN and tryptophan or tyrosine persists although the suppression is considerably weakened (Table 4, rows 6 & 9). Therefore, the suppression activity of the *rpoB* alleles is not entirely RpoS-dependent. We thought it was also important to ask if suppression of growth requirements by the *rpoB* alleles is entirely through the activation of *tktB* or has an alternate explanation (say, the activation of a cryptic transketolase gene). Table 4, rows 7 & 10 show that suppression requires *tktB*; these results indicate the *rpoB* alleles can increase TktB activity independent of RpoS and ppGpp (see below).

RpoS and ppGpp activate *tktB*-lacZ transcriptional fusions

The nutritional requirements of the mutant strains indicate regulation of *tktB* expression by ppGpp, RpoS, DksA and the stringent *rpoB* mutations. To find out if transcription can account for regulation of growth requirements, reporter activity of *tktB*-lacZ operon fusions were measured during growth in LB using a *tktA*$^+$ strain.

Previous studies have identified two closely spaced promoters upstream of *talA* (P1 and P2 in figure 3B), and one within the *talA* ORF just upstream of *tktB* (Lacour and Landini, 2004; Jung *et al*., 2005). We constructed three transcriptional fusions (Fig. 3B) to look at activity of the promoters. Fusion A measures transcription from P1 P2 promoters upstream of *talA*, fusion B from the promoter reported in the *talA* ORF with fusion joints identical to the one described in Jung *et al*., 2005. Fusion C detects transcription from the entire region upstream of *tktB* (Fig. 3B) and extends 238 nucleotides upstream of *talA*.

Table S3 is a survey of reporter activities using the three fusions in wildtype strains for exponential and stationary growth phases. Fusion B is marginally active, whereas Fusions A and C have measurable activities during exponential growth, which are induced 7- and 9- fold respectively in stationary phase. Under our conditions *talA* and *tktB* genes seem to comprise an operon. We chose fusion C for the studies reported below.

The reporter activity from fusion C is shown in Fig. 5, measuring activities in log, early stationary and stationary phase for different strains. Transcription is lowered 5–6 fold during all phases of growth in the ppGpp^0 strain (panels A & C) whereas the absence of RpoS lowers the *tktB* activity increasingly during growth from log to stationary phase (7-to 16- fold). In ppGpp^0 strain the absence of RpoS lowers activity at least 3- fold further in all growth phases (compared to *rpoS* mutant) and the activity is virtually absent in the log phase cells (~0.6 Miller units). The results are consistent with an independent regulation of *tktB* expression by ppGpp and RpoS (see discussion).

Stringent *rpoB* alleles increase *tktB*-lacZ expression

The presence of the "stringent" *rpoB* suppressor mutations T563P and A532Δ (in the ppGpp^0 background) results in a large increase in *tktB* expression (32-fold in *rpoB*A532Δand 42-fold in *rpoB*T563P) during exponential growth. When these strains go into stationary phase only a modest additional increase in expression occurs (Fig. 5 panels G and I). For both *rpoB* mutants the increase in *tktB* expression is largely RpoS-mediated (panels H and J). However, in the absence of RpoS, they have a 20-fold higher activity in log phase compared to isogenic ppGpp^0 strain (compare panel D with H & J).

Hydrolase-deficient *spoT*-R39A allele increases *tktB*-lacZ expression

The activation of *tktB* transcription by the hydrolase-deficient *spoT*-R39A allele is consistent with a positive regulatory role for ppGpp. The *tktB* expression pattern seen when *spoT* synthetase is not balanced by hydrolase, is strikingly similar to that observed in the *rpoB* mutant strains: a large increase in exponential phase and a moderate increase thereafter. An 8- fold or 44-fold increase in expression is observed in exponential phase when compared to wildtype or ppGpp^0 strains (Fig. 5 compare panels A, C & E). The RpoS-independent *tktB* expression in the *spoT* mutant is once again similar to that seen in the *rpoB* mutant strains (compare panels F, H & J in Fig. 5) underscoring a possibility of similar regulatory mechanisms (see discussion).

Positive regulation of *tktB*-lacZ expression by DksA

A *dksA* deletion reduces *tktB* transcriptional activity roughly by half during all phases of growth, compared to wild-type strain; in a ppGpp^0 strain the same deletion has no further effect (data not shown). DksA over-expression does not significantly alter *tktB* transcription in the presence of ppGpp, but restores expression close to wildtype levels in a ppGpp^0 strain. This positive effect of DksA on *tktB* transcription in the ppGpp^0 strain is primarily RpoS-dependent but the small RpoS- independent effect is also noted (Fig. 6B). These results are consistent with the growth phenotypes observed during DksA over-expression in the absence of ppGpp and in the presence or absence of RpoS (Table 4, rows 1 to 4).

Discussion

This work shows that genetic screening for synthetic lethals can be applied to define ppGpp-dependent functions. We explain the synthetic growth defects arising from the inactivation of *tktA* in a ppGpp^0 host strain as due to inactivation of a *tktB*-dependent redundant pathway. We show that ppGpp regulates *tktB* transcription through the modulation of RpoS activity and by RpoS-independent positive stringent regulation as well. The contribution of each pathway may depend on growth conditions.

Transketolase activity and growth in LB

It is unclear how transketolase activity affects growth in LB agar or broth. Supplementing LB with glucose, aromatic amino acids and pryridoxine only marginally improves growth (data not shown). Unlike in LB, in minimal glucose media containing all the supplements, growth

of the double mutant is only slightly slower than that of an isogenic wildtype strain (Table 1A). Presence of inhibitors in LB has been suggested previously (Zhou and Winkler, 1994). Alternatively, the unique need for transketolase activity could be specific for growth on carbohydrate-poor complex peptide digest medium like LB and related to its metabolic function at the intersection of gluconeogenesis and pentose phosphate shunt. The synthetic growth defect arising from *tktA* and ppGpp deficiency or the absence of both transketolase isozymes can be completely reversed by ectopic expression of TktB (Table 1B), indicating that growth defects are due to general transketolase deficiency rather than from a specific function of TktA. It is important to consider this possibility because a genetic selection for mutants with reduced chromosomal negative supercoiling has uncovered a role for TktA and DksA along with H-NS, Fis and SeqA/Pgm in the maintenance of chromosomal superhelicity (Hardy and Cozzarelli, 2005).

Positive regulation of *tktB* transcription by ppGpp: the RpoS-dependent and -independent modes of regulation

The assay for transketolase-B function based on growth requirements indicates that the cellular transketolase-B activity is almost entirely dependent on the presence of ppGpp and RpoS because growth properties of *tktA tktB* mutant is identical to that of *tktA rpoS* ppGpp^0 mutant. The *tktB*-lacZ fusion results show that regulation by ppGpp and RpoS is almost entirely at the level of transcription. The data also indicates that there are at least two routes for activation of *tktB* transcription; one requires ppGpp and RpoS while the other is transcriptional activation by ppGpp independent of RpoS.

The transcriptional fusion C in *tktB* shows a 16-fold drop in expression in stationary phase in the *rpoS* mutant, consistent with a microarray study that reported 14-fold RpoS-dependent increase in *tktB* transcripts in stationary phase (Weber *et al*., 2005). Previous studies have established that ppGpp^0 strains phenocopy RpoS deficiency and gratuitous induction or increase in ppGpp basal levels leads to increased RpoS protein levels (Gentry *et al.*, 1993; Brown *et al.*, 2002). Also, ppGpp facilitates transcription by pre-existing RpoS (Kvint *et al*., 2000) and its absence diminishes the ability of RpoS to compete against RpoD for the core (Jishage *et al*., 2002). There is growing evidence from many other studies that lead to the proposal that ppGpp helps alternative sigma factors to compete for RNAP core (Magnusson *et al*., 2005). Therefore, down-regulation of *tktB* expression observed in ppGpp^0 strain can result from a combination of lowered RpoS protein levels and diminished RpoS function. It is possible that low RpoS levels could be a reason why *tktB* was not identified in a transcriptional profile of stringent response (Durfee *et al*., 2008), since the response was elicited in early exponential phase cultures using serine hydroxymate which inhibits protein synthesis. The induction by elevated ppGpp alone in the absence of RpoS was probably below detection levels.

The partial growth defects observed in *rpoS tktA* mutant indicate low level TktB activity (Table 3). Additional inactivation of *relA* or the absence of ppGpp eliminates residual activity, because growth requirements of *rpoS tktA relA*, *rpoS tktA* ppGpp^0 and *tktA tktB* mutants are identical (Table 3). Similarly, the low lacZ reporter activity in the *rpoS* mutant is lowered further to barely detectable levels in the absence of ppGpp (Fig. 5). These results are consistent with a role for ppGpp that is independent RpoS in the regulation of *tktB* expression and suggest a mechanism for feedback regulation in the aromatic amino acid biosynthetic pathway (Fig. 2). Aromatic amino acid starvation activates RelA, leading to ppGpp synthesis, activation of *tktB* transcription and aromatic amino acid biosynthesis. This leads to disappearance of the signal.

Based on existing models, the transcriptional activation mediated by ppGpp we see could be due to either direct effect of ppGpp and DksA at the promoter (Paul et.al., 2005) or due to an indirect effect resulting from increased availability of free RNAP as a consequence of inhibition

of rRNA transcription (Zhou and Jin, 1998; Barker *et al*., 2001) that facilitates competition by alternative sigma factors for the RNAP core (Jishage *et al*., 2002; Magnusson *et al*., 2005; Szalewska-Palasz *et al*., 2007; Costanzo *et al*., 2008).

Two transcription start sites were localized upstream of *talA* and used to deduce promoters P1 and P2 based on a -10 consensus for RpoS dependent promoters (Lacour and Landini, 2004). This microarray study found RpoS-dependence for *talA* but in contrast to our results and the other microarray study cited earlier (Weber *et al*., 2005) *tktB* transcripts were not found to be RpoS-dependent. Interestingly the P1 promoter has a sequence tgctatgcttttt followed by the +1 transcription start site, that is, an extended −10 (underlined) together with AT rich discriminator. This could fuse RpoS-dependence with activation by ppGpp. It is also possible that the two promoters respond differentially, one to RpoS and another to ppGpp perhaps through sigma-70 or an alternative sigma factor.

RpoS-mediated positive and negative regulation of *tktB* and *tktA* respectively was reported; it was suggested that isozymes TktA and TktB may play their main roles in exponential and stationary phase respectively (Jung *et al*., 2005). However, using an identical construction (fusion B, Fig. 3) we are unable to observe activity (Table S2) or observe RpoS regulation (data not shown); the reason for the disparity is not apparent.

Overexpression of DksA and activation of tktB transcription

Suppression by over-expression of DksA is a feature found for many $ppGpp^0$ phenotypes including the restoration of RpoS induction (Brown *et al*., 2002 ; Magnusson *et al*., 2007). Consistent with an effect mediated through RpoS, restoration of *tktB* expression by DksA over-expression requires functional RpoS (Fig. 6A & B). However a small positive effect can be observed in the absence of RpoS and ppGpp; this is supported by the suppression of vitamin requirement observed in growth assays (Fig. 6B; Table 4). The ability of DksA to substitute for a complete absence of ppGpp argues DksA and ppGpp are not co-factors (like cAMP and CRP).

Stringent *rpoB* mutations functionally mimic constitutive high levels of ppGpp

The *spoT*-R39A mutation is deduced to change the ppGpp hydrolase-synthetase balance in favor of synthesis and increase ppGpp basal levels (Table S2). The *spoT*-R39A mutant and the stringent *rpoB* mutant strains used in this study show essentially identical *tktB* transcriptional regulation and TktB activity in vivo as deduced from lacZ fusion data and growth requirements of these strains (Tables 2 & 4; Fig. 5). The phenotypic similarities between the strains persist in the absence of RpoS. The results suggest that an increase in intracellular ppGpp level and presumably polymerase conformation changes can have similar functional consequences for positive transcriptional regulation mediated by ppGpp. A previous study with the same polymerase mutants showed they mimic the negative transcriptional regulation mediated by ppGpp (Zhou and Jin, 1998). The *rpoB* mutants increase RpoS protein levels (Fig. 4) and the same is observed for the *spoT*-R39A hydrolase mutant (data not shown). Together, the results provide further evidence for the passive regulatory model and extend it for ppGpp-mediated RpoS regulation. The *spoT*-R39A and stringent *rpoB* mutants soften the wildtype “stair step” like increase in *tktB* expression with growth phase. If high ppGpp levels slow the metabolic turnover of RpoS during growth in LB like it does during phosphate starvation through *iraP* (Bougdour and Gottesman, 2007) similar effects might be expected.

This study shows that *tktB* is subject to RpoS-mediated regulation through ppGpp, excess DksA in the absence of ppGpp, and “stringent” *rpoB* mutations. In addition, transcription activation is also seen independent of RpoS (Fig. 7): (i) under conditions that increase cellular ppGpp levels; (ii) by “stringent” *rpoB* mutations in the absence of ppGpp and (iii) DksA over-

expression in the absence of ppGpp. It is possible that in each case transcriptional activation is mediated through a similar conformational change in RNAP. In vitro transcription studies are warranted to find out if the effects are direct or involve additional factors.

Experimental procedures

Media and growth conditions

Table S1 list the *E.coli* strain derivatives of MG1655 and plasmids used in this study. Cultures were grown in LB broth in rotary shaker flasks at 37°C. The media used are described by Miller (1972) but modified as follows: LB contains 0.5% NaCl and M9 glucose minimal contains 15 μM thiamine and may be supplemented with either 0.4% casiamino acids, all 20 amino acids (each at 40 μg/ml), 19 amino acids lacking either phenylalanine, tryptophan or tyrosine and pyridoxine (10 μM). Final concentration of antibiotics was: ampicillin (100 μg/ml in LB and 50 μg/ml in minimal media), tetracycline (20 μg/ml), kanamycin (30 μg/ml) and trimethoprim (100 μg/ml). Quantitative estimates of growth at 37°C are based on colony diameters: ± is 0.5 mm or less; + is 1–1.5 mm; + + is 2–2.5 mm; + + + is 3 mm or more. In general, colony sizes are scored on LB plates and on minimal media after incubating for 24 hrs and after 48 hrs respectively.

Bacterial strains, plasmids and Genetic procedures

Plasmid pMA2 is a temperature-sensitive, pSC101 replicon (obtained from the cloning vector collection at NIG, Mishima, Japan). pMA2 was digested with *Sph*I and re-circularized to obtain plasmid pHR13, and was used to clone the *spoT* gene from plasmid pHX41 (Gentry *et al*., 1996) as a 2.1 kb *Sph*I fragment yielding pHR14. Plasmid pJK537 has been described in Kang & Craig, 1990. Plasmid pHR30 was constructed using pQE80L (Qiagen vector) for IPTG-inducible TktB expression. A 2041 bp fragment encompassing the entire *tktB* ORF was PCR-amplified from MG1655 genome using *Pfu*-polymerase and primers 5′-atgaattccagccacggagt-3′ (upstream) and 5′-ttggatccggcaatcacc atca-3′ (downstream). *Eco*RI and *BamH*I sites in the primers (underlined) were used to clone the fragment in the *Eco*RI/*BamH*I sites of pQE80L.

Phage P1(vir) transductions were performed by standard procedures (Miller, 1972). When constructing strains with the *tktA rpoS*, ppGpp^0 *tktA*, and ppGpp^0 *rpoS tktA* genotypes the last allele to be introduced is *tktA* and transductants were selected and maintained on LB plates containing 0.2% glucose which improved the growth of these strains. Plasmid DNA was extracted using Qiagen kits, transformations and recombinant DNA procedures were performed generally as described in Sambrook *et al*., 1989. Recombineering was performed as described in Yu *et al*., 2000, using the linear DNA transformation protocol to engineer the *spoT*- synthetase and hydrolase mutations.

Construction of transcriptional-lacZ fusions

The *talA-tktB*prime;::lacZ fusion (fusion-C) was constructed using a 1331 bp fragment retrieved from the chromosome of strain MG1655. This fragment has 238 bp of non-coding region upstream of *talA*, the entire *talA* structural gene, the *talA- tktB* intergenic region and 124 nucleotides of *tktB* coding sequence; it was recombined into a linear pBR322 derived fragment using a protocol described in Court *et al*., 2002. The primers for generating the linear fragment for recombination with the chromosomal sequence (italics in the primer sequence) were 5′-cagggtaataatgtgcgccacgttgtgggcaggggaattcgcgt ttcggtgatgacggtg-3′ (bottom strand) and 5′-gggcatggctgatattgccgaagtgctgtggaacggatccga aagggcctcgtgatacgc-3′ (top strand). The chromosomal fragment was then subcloned into the operon fusion vector pRS415 using restriction enzyme *Eco*RI and *BamH*I (underlined in the primers) and recombined first into λRS45 and then into the λ*att* site on the chromosome as described in Simons *et al*., 1987. The

talA′::lacZ fusion (fusion A) was constructed using a 319 bp fragment containing the same 5′ end point as fusion C and 81 bp of the *talA* coding sequence and obtained by PCR using primers 5′-atgaattcccctgccca caacg-3′ (top strand) and 5′-atggatccgatgataatggcgaatggactc -3′. The '*talA-tktB*'::lacZ fusion (fusion B) contains a 344 bp fragment with 200 bp of the 3′-coding sequence of *talA*, 19 bp of intergenic sequence and 124 bp of *tktB* coding sequence and obtained using the primers 5′-atgaattcctgcaggaaaaagtttcgcc -3′ (top strand) and 5′-atggatcctcgttccacagcac ttc-3′ (bottom strand). These fusions were also transferred to the λ*att* site as described for fusion C.

Transposon mutagenesis and sequencing of chromosomal transposon-insertion sites

Random mutagenesis was carried out using the EZ-Tn5™ < DHF R-1> transposon kit from Epicentre (Madison, WI). The protocol for sequencing insertions is published in Epicentre forum (Ducey and Dyer, 2002).

β-galactosidase assays

Cultures were grown after a 1:500 dilution of an overnight culture and the lacZ fusions were assayed for β-galactosidase activity as described in Miller, 1972 with activities reported in Miller units.

Gel electrophoresis and Western blotting for RpoS

Lysates were prepared after precipitation with cold trichloroacetic acid using final concentrations of 5% and 10% for exponential and stationary phase cells, respectively. After centrifugation, pellets were washed once with 0.5 ml of ice-cold 80% acetone, air dried and resuspended in SDS-gel loading buffer. Equal quantities of proteins were separated on precast SDS-12% PAGE acrylamide gels (Invitrogen) and transferred to PVDF membranes. The membranes were incubated with anti-RpoS antibody (Neoclone, Madison, WI) at 1:1000 dilution and the blots developed using horseradish peroxide-conjugated goat anti-rabbit antibody by the enhanced chemiluminescence protocol (GE health sciences).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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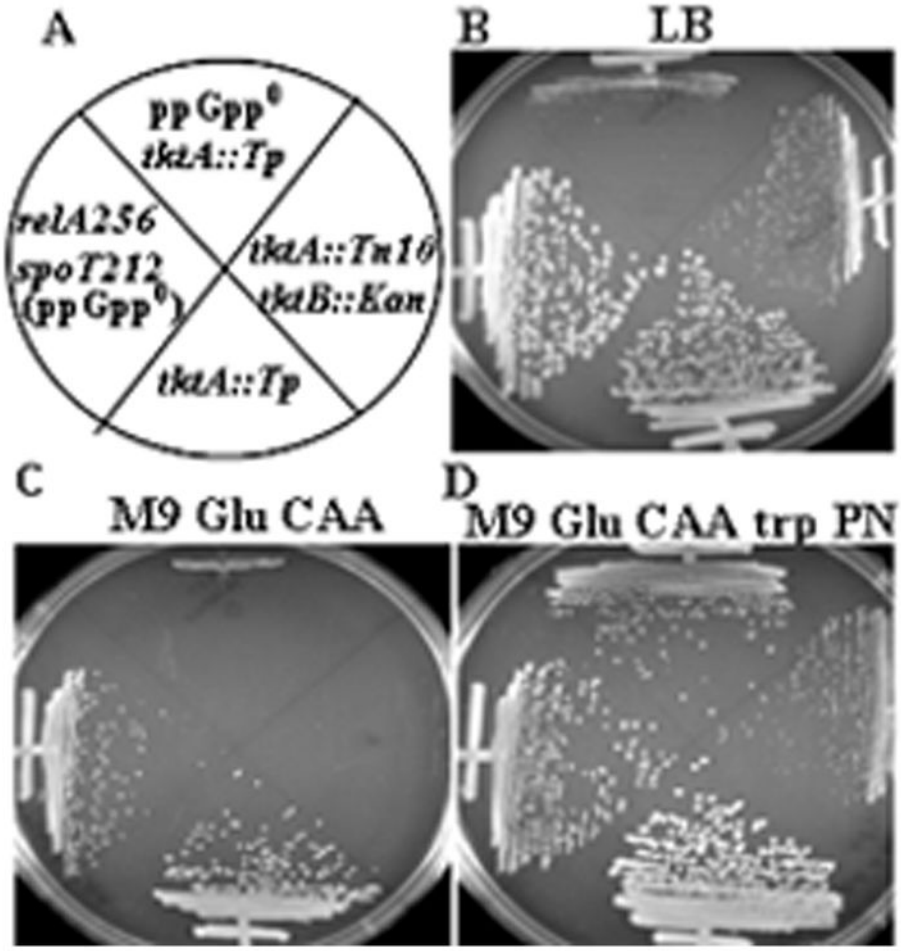

Figure 1.

Growth properties of transketolase mutants. A. Strains on plate: *tktA*::Tp (CF13942), ppGpp^0 i.e., *relA*256 *spoT*212 (CF10237), ppGpp^0 *tktA*::Tp (CF13926) and *tktA tktB* (CF13927); B. LB agar after 18 hours; C. Minimal media with glucose and casamino acids after 36 hrs; and D. Minimal media with glucose casamino acids, tryptophan and pyridoxine after 36 hrs. All incubations were at 37°C.

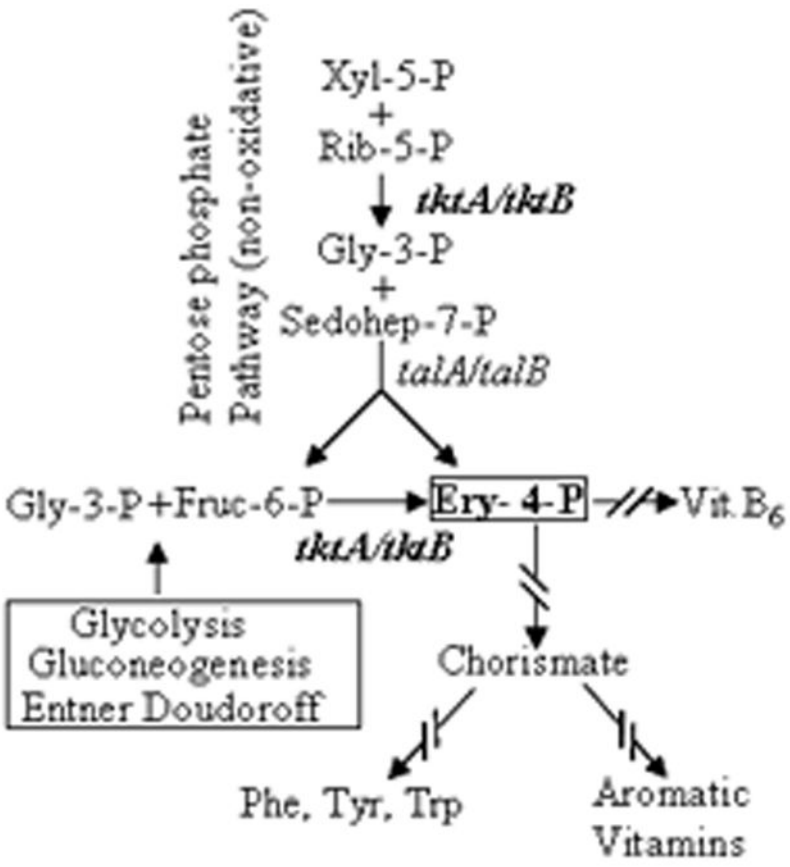

Figure 2.

Pathways for biosynthesis of the intermediary metabolite D-erythrose-4-phosphate and amino acids and vitamins derived from it. The enzymes involved at each step are indicated by gene names that encode them; broken arrows represent multiple steps in the pathway; abbreviations, Xly-5-P : D-xylulose-5-phosphate; Rib-5-P : D-ribose-5-phosphate; Gly-3-P : D-Glyceraldehyde-3-phosphate; Sedohep-7-P : Sedoheptulose-7-phosphate; Fruc-6-P : D-fructose-6-phosphate and Ery-4-P : D-erythrose-4-phosphate.

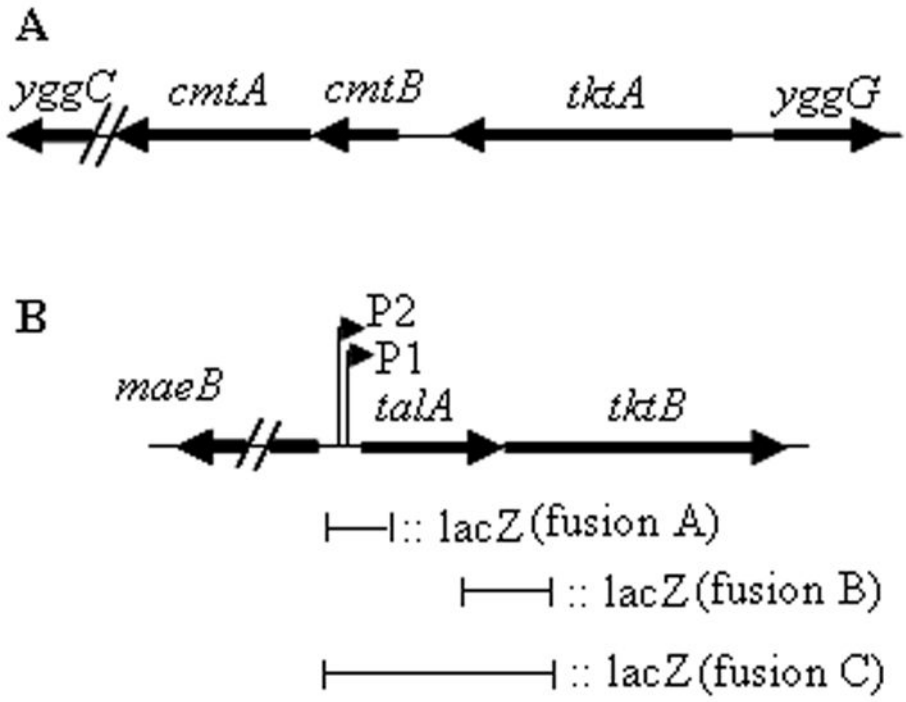

Figure 3.

Schematic representation of the genomic neighborhoods of *tktA* and *tktB* genes and the DNA segments in each lacZ operon fusion. A. *tktA* and proximal ORF's; B. *tktB* and proximal ORF's. Open-reading frames are represented by thick filled arrows; P1 and P2 refer to promoters characterized in the intergenic region (Lancour and Landini, 2004); Fusions A, B and C refer to the transcriptional fusions described in materials and methods. Horizontal bracketed lines refer to DNA segments present in each fusion; fusions A and C have identical start points upstream of the *talA* coding sequence and fusions B and C have identical end points within *tktB*; the contents of each fusion are described in materials and methods.

Figure 4.

Stringent *rpoB* suppressor mutations increase RpoS protein levels of exponentially growing cells. Extracts were made from LB grown cells taken at different stages of growth. Extracts from cells equivalent to 0.1 A_{600} were used for immunoblotting with anti-RpoS antibody. Lanes, 1, 4 & 7 have extracts from $ppGpp^0$ strains CF14276; lanes 2, 5 & 8 from the *rpoB*T563P derivative CF14278 and lanes 3, 6 & 9 from the *rpoB*A532Δ derivative CF14277. Log, early stationary phase and stationary phase correspond to A_{600} values of 0.6–0.8; 2–2.2; and 3.5–3.6, respectively.

		WT (S.D)[a]	ppGpp0	R39A	A532Δ	T563P
rpoS+	log	31 (2.94)	6 (0.28)	267 (21.4)	195 (6.94)	253 (7.93)
	Early Stat	71 (5.26)	18 (1.70)	563 (22.4)	260 (7.36)	308 (8.17)
	Stat	282 (22.8)	43 (4.54)	877 (42.2)	508 (16.3)	595 (31.8)
Δ*rpoS*::Kan	log	4 (0.53)	1 (0.13)	21 (2.05)	18 (1.87)	22 (1.56)
	Early Stat	6 (0.31)	2 (0.40)	26 (1.24)	23 (2.06)	35 (3.27)
	Stat	17 (1.38)	7 (0.30)	36 (1.69)	28 (1.89)	40 (2.04)

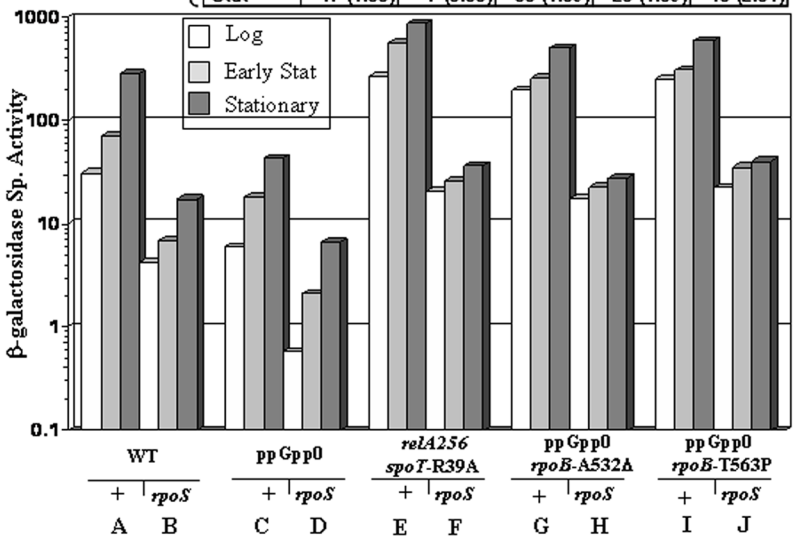

Figure 5.

Regulation of *tktB* transcription – the role of ppGpp, RpoS and stringent *rpoB* mutations. *tktB* transcription was monitored during growth in LB with the *talA-tktB'*::lacZ fusion C. Strains used are, CF14213 and CF14241 (columns A & B); CF14214 and CF14242 (columns C & D)); CF15008 and CF15023 (columns E & F); CF14277 and CF14281 (columns G & H); CF14278 and CF14280 (columns I & J); For each culture activity was measured in log phase (A_{600} 0.5–0.6), early stationary phase (A_{600} 1.5–2) and stationary phase (A_{600} 2.5–3.5). β-galactosidase specific activities are the mean of three independent experiments expressed in Miller units. In the data table, but not the bar graph, values are rounded to the nearest whole number. a – standard deviation (S.D).

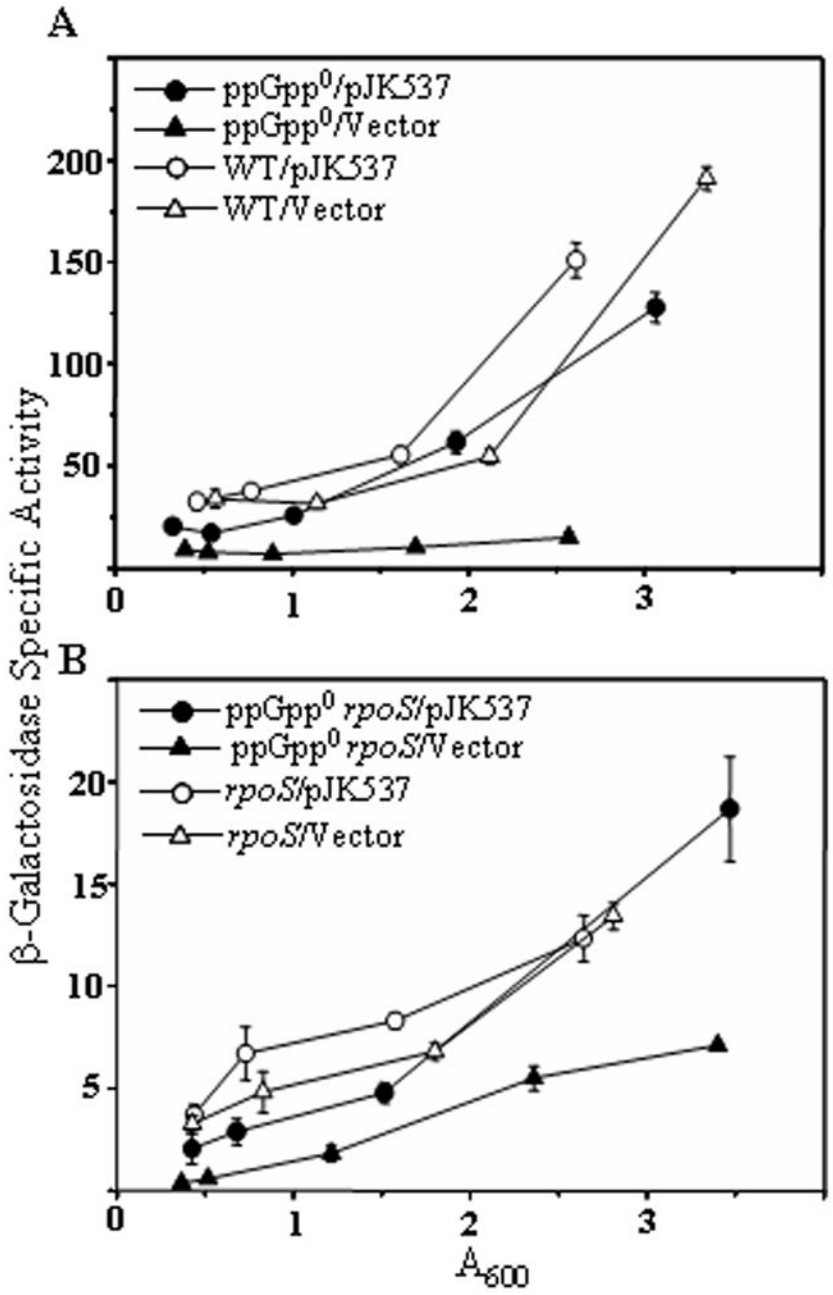

Figure 6.

Effect of over-production of *dksA* on *talA-tktB'*::lacZ expression. β-galactosidase specific activities are plotted against A_{600} during growth in LB in the presence of plasmid pJK537 or the vector control pBR322 in wild-type (CF14213) or $ppGpp^0$ strains (CF14314) (A); the *rpoS* mutant derivatives of wildtype (CF14241) or $ppGpp^0$ strains (CF14242)(B); the activities plotted are mean of three independent experiments expressed in Miller units.

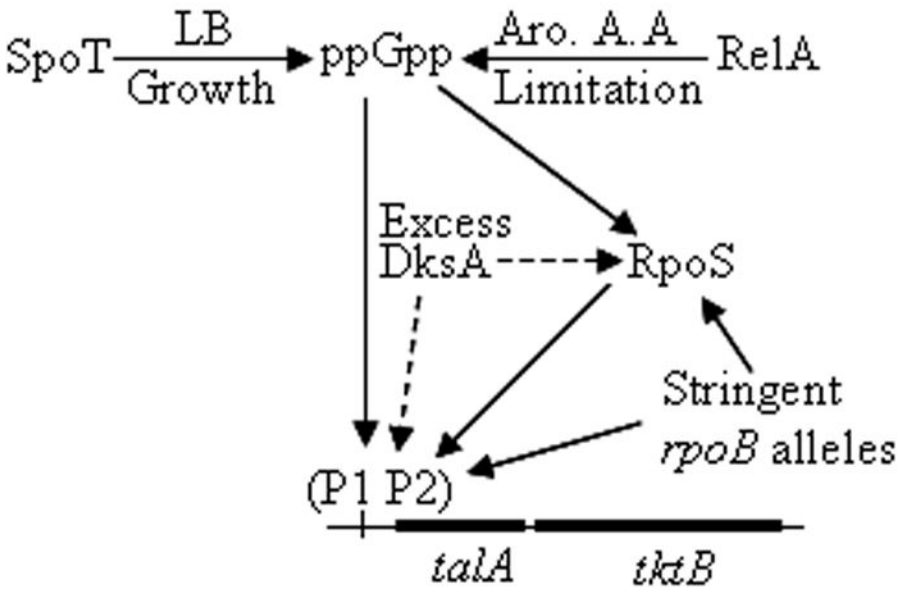

Figure 7.

A model for the transcriptional regulation observed in this study for the *talA-tktB* operon. Two known promoters P1 and P2 upstream of *talA* are represented by a vertical line. Horizonal bars indicate the *talA-tktB* operon. Excess DksA refers to over-expression of the protein using plasmid pJK537. Continuous line arrows refer to activation signals; broken line arrows refer to activation signals observed only in the absence of ppGpp.

Table 1

Growth deficiency and requirements seen in *tktA* mutants in the absence of ppGpp arise from lowered transketolase-B activity.

	Relevant Genotypes	M9 Glucose minimal with (48 hr)					LB (24 hr)
		20 AA +PN	20 AA	20 AA −phe	20 AA −trp	20 AA −tyr	
A							
1	Wild type	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
2	*tktA*	+ +	+ +	+ +	+ +	+ +	+ +
3	*tktB*	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
4	*tktA tktB*	+ +	−	−	−	−	±
5	ppGpp^0	+ +	+ +	−	+ +	+ +	+ + +
6	ppGpp^0 *tktA*::Tn10	+ +	+	−	−	−	±
7	ppGpp^0 *tktA*::Tp	+ +	+	−	−	−	±
B							
1	*tktA tktB*/pHR30	+ +	+ +	+ +	+ +	+ +	+ +
2	*tktA tktB*/pHR30 + IPTG	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
3	*tktA tktB*/vector	+ +	−	−	−	−	±
4	*tktA tktB*/vector + IPTG	+ +	−	−	−	−	±
5	ppGpp^0 *tktA*/pHR30	+ +	+	−	+	±	+
6	ppGpp^0 *tktA*/pHR30+IPTG	+ +	+ +	−	+ +	+ +	+ + +
7	ppGpp^0 *tktA*/vector	+ +	+	−	−	−	±
8	ppGpp^0 *tktA*/vector +IPTG	+ +	+	−	−	−	±

Growth estimates are based on single colony size as described in the methods, with IPTG used at 0.25 mM where indicated. For plasmid bearing strains, ampicillin was used at final concentration of 100μg/ml in LB and 50μg/ml in minimal media. Strains in panel A rows 1 to 7 are: CF1648, CF13912, CF13969, CF13927, CF10237, CF13913, and CF13926; panel B CF13927 (rows 1 – 4) and CF13913 (rows 5 – 8). pHR30 is a plasmid for inducible expression of *tktB*.

Table 2

Growth phenotypes correlate with the loss of cellular ppGpp synthesis.

Relevant Genotype	M9 glucose minimal (48 hr)			LB (24 hr)
	20 AA	20 AA − trp	20 AA − yr	
1 Wildtype	+ + +	+ + +	+ + +	+ + +
2 Δ*relA*	+ + +	+ + +	+ + +	+ + +
3 Δ*relA tktA*	+ +	+ +	+	+ +
4 Δ*relA* Δ*spoT tktA*	+	−	−	±
5 Δ*relA spoT*-R39A *tktA*	+ +	+ +	+ +	+
6 *spoT*-E319Q *tktA*	+ +	+ +	+ +	+ +
7 Δ*relA spoT*-E319Q *tktA*	+	−	−	±

Growth estimates are as in Table 1. Strains in rows 1 to 7 are: CF1648, CF15010, CF15036, CF15007, CF15025, CF15039 and CF15005. Mutant alleles: *spoT*-R39A is hydrolase defective and synthetase-proficient, *spoT*-E319Q is synthetase defective and hydrolase-proficient.

Table 3

Independent and synergistic effects of ppGpp, DksA and RpoS in the modulation of *tktB*-dependent growth requirements.

	Relevant Genotypes	M9 glucose minimal (48 hr)			
		20 AA +PN	**20 AA**	**20 AA −trp**	**20 AA−tyr**
1	*dksA*	+ +	+ +	+ +	+ +
2	*dksA tktA*	+ +	+ +	+	+
3	ppGpp^0 *tktA*	+ +	+	−	−
4	*dksA tktA* ppGpp^0	+ +	+	−	−
5	*rpoS*	+ + +	+ + +	+ + +	+ + +
6	*rpoS tktA*	+ +	+ +	+	±
7	*rpoS tktA relA*	+ +	−	−	−
8	*rpoS tktA*/pHR30 + IPTG	+ + +	+ + +	+ + +	+ + +
9	*rpoS tktA* ppGpp^0	+ +	−	−	−
10	*rpoS tktA* ppGpp^0/pHR30 + IPTG	+ +	+ +	+ +	+ +

Growth estimates are as in Table 1. Strains in rows 1 to 10 are: CF14309, CF14955, CF14966, CF15240, CF14241, CF14965, CF15282, CF14965 with pHR30, CF14967, and CF14967 with pHR30. Wherever indicated, IPTG is present at 0.25 mM. For plasmid bearing strains ampicillin was used at 50μg/ml final concentration.

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Table 4

Suppression of growth requirements by multicopy DksA and the stringent *rpoB* mutations

	Relevant genotype	M9 glucose minimal with (48 hr)			
		20 AA+PN	**20 AA**	**20 AA −trp**	**20 AA −tyr**
1	ppGpp^0 *tktA*/pBR322	+ +	+	−	−
2	ppGpp^0 *tktA* Δ*rpoS*	+ +	−	−	−
3	ppGpp^0 *tktA*/pJK537	+ +	+ +	+	+
4	ppGpp^0 *tktA* Δ*rpoS*/pJK537	+ +	+	−	−
5	ppGpp^0 *tktA rpoB*-A532Δ	+ +	+ +	+ +	+ +
6	ppGpp^0 *tktA rpoB*-A532Δ Δ*rpoS*	+ +	+	+	±
7	ppGpp^0 *tktA rpoB*-A532Δ *tktB*	+	−	−	−
8	ppGpp^0 *tktA rpoB-T563P*	+ +	+ +	+ +	+ +
9	ppGpp^0 *tktA rpoBT-563P* Δ*rpoS*	+ +	+	+	±
10	ppGpp^0 *tktA rpoBT-563P tktB*	+	−	−	−

Growth estimates are as in Table 1. Strains in rows 1 to 10 are: CF15041, CF14967, CF14946, CF14953, CF14998, CF15000, CF14959, CF14999, CF15001, and CF14960. For plasmid bearing strains, ampicillin was used at 50μg/ml final concentration.