Published in final edited form as:
Mol Microbiol. 2011 May ; 80(3): 695–714. doi:10.1111/j.1365-2958.2011.07602.x.

The complex logic of stringent response regulation in *Caulobacter crescentus*: starvation signaling in an oligotrophic environment

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Summary

Bacteria rapidly adapt to nutritional changes via the stringent response, which entails starvation-induced synthesis of the small-molecule, ppGpp, by RelA/SpoT homolog (Rsh) enzymes. Binding of ppGpp to RNA polymerase modulates the transcription of hundreds of genes and remodels the physiology of the cell. Studies of the stringent response have primarily focused on copiotrophic bacteria such as *E. coli*; little is known about how stringent signaling is regulated in species that live in consistently nutrient limited (*i.e*. oligotrophic) environments. Here we define the input logic and transcriptional output of the stringent response in the oligotroph, *Caulobacter crescentus*. The sole Rsh protein, $SpoT_{CC}$, binds to and is regulated by the ribosome, and exhibits AND-type control logic in which amino acid starvation is a necessary but insufficient signal for activation of ppGpp synthesis. While both glucose and ammonium starvation upregulate the synthesis of ppGpp, $SpoT_{CC}$ detects these starvation signals by two independent mechanisms. Although the logic of stringent response control in *C. crescentus* differs from *E. coli*, the global transcriptional effects of elevated ppGpp are similar, with the exception of 16S rRNA transcription, which is controlled independently of $spoT_{CC}$. This study highlights how the regulatory logic controlling the stringent response may be adapted to the nutritional niche of a bacterial species.

Keywords

Rsh; stringent response; IS3 insertion element; ribosome; L11; SpoT; Caulobacter; oligotroph; ppGpp

INTRODUCTION

Bacteria must coordinate growth rate with nutrient availability so as to balance survival under starvation conditions with reproduction in nutrient replete conditions. The stringent response is a bacterial signaling system that plays a critical role in this coordination of growth with nutrient availability (Potrykus & Cashel, 2008). This system is conserved in all bacterial species except obligate intracellular pathogens and obligate symbionts (Mittenhuber, 2001). In this response, **R**elA/**S**poT **h**omolog (Rsh) enzymes synthesize guanosine tetra- or penta-phosphate ((p)ppGpp) upon starvation. In gram-negative species, (p)ppGpp binds RNA polymerase (RNAP) and alters its activity at different promoters such that genes required for translation and cell building are downregulated while stress response

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genes are upregulated (Barker *et al*., 2001a, Barker *et al*., 2001b, Traxler *et al*., 2006, Traxler *et al*., 2008). (p)ppGpp also modulates the activity of certain DNA replication factors (Schreiber *et al*., 1995, Ferullo & Lovett, 2008, Lesley & Shapiro, 2008). Although the stringent response was initially defined in *E. coli* as the downregulation of stable RNA transcription in response to amino acid starvation (Cashel, 1969), several decades of research have demonstrated that (p)ppGpp is central in the vast network of bacterial sensory/signaling systems: this small molecule is rapidly synthesized upon multiple types of stress and alters transcription of large swathes of the genome (Potrykus & Cashel, 2008). We note that in the older literature the term “stringent response” refers specifically to the production of stable RNA after amino acid starvation. However, this term has come to encompass all aspects of cell physiology regulated by elevated ppGpp levels (Potrykus & Cashel, 2008). For the purposes of this study we use the term stringent response to refer to all effects of cytosolic ppGpp accumulation.

(p)ppGpp plays a central role in adaptation to changing environments in many bacterial species. For example, several mammalian pathogens use (p)ppGpp signaling to upregulate virulence genes upon host entry (Nakanishi *et al*., 2006, Dozot *et al*., 2006), to survive upon elimination from the host (Das *et al*., 2009), and to activate infection (Kim *et al*., 2005, Dalebroux *et al*., 2009, Edwards *et al*., 2009) or persistence (Dahl *et al*., 2003) factors. In *E. coli* the transition from the rich environment of the mammalian gut to the comparatively poor environment of the water table (Savageau, 1983) is mediated by (p)ppGpp signaling (Traxler *et al*., 2006). These species are all classified as copiotrophs, which experience an existence of feast and famine (Koch, 1971, Poindexter, 1981) and exhibit a “classic” stringent response whereby amino acid starvation induces the synthesis of (p)ppGpp. However, there are examples of species that exhibit a “non-classic” stringent response; the study of these cases provides important insight into the variable and conserved aspects of (p)ppGpp signaling, and highlights how the nutritional niche of a species can affect the structure of its molecular signaling systems.

Examples of species that do not produce ppGpp upon amino acid starvation include *Rhodobacter sphaeroides*, *Rhizobium meliloti* strain 41, *Rhizobium tropici*, *Azomonas agilis* and *Azobacter vinelandii* (Acosta & Lueking, 1987, Belitsky & Kari, 1982, Eccleston Jr. & Gray, 1973, Howorth & England, 1999). Both *R. meliloti* 41 and *R. sphaeroides* restrict rRNA transcription without production of ppGpp in amino acid starvation (Acosta & Lueking, 1987, Belitsky & Kari, 1982, Eccleston Jr. & Gray, 1973), but produce ppGpp in other types of starvation: *R. meliloti* 41 in carbon and ammonium starvation (Belitsky & Kari, 1982), and *R. sphaeroides*, a photosynthetic bacterium, upon downshift of light intensity (Eccleston Jr. & Gray, 1973). These species still downregulate rRNA synthesis during amino acid starvation and are thus “stringent” in the original sense of the word.

There are reports of “relaxed” species, including *Helicobacter pylori* and *Caulobacter crescentus*, which neither reduce rRNA transcription nor synthesize ppGpp in a few tested conditions of amino acid starvation (Scoarughi *et al*., 1999, Chiaverotti *et al*., 1981). Yet these species still synthesize ppGpp: *C. crescentus* in glucose (Lesley & Shapiro, 2008) and ammonium (Chiaverotti et al., 1981) starvation, and *H. pylori* in carbon and serum starvation and acid stress (Wells & Gaynor, 2006, Zhou *et al*., 2008). Both species also restrict rRNA transcription in conditions which activate ppGpp accumulation: carbon starvation in *H. pylori* (Wells & Gaynor, 2006) and nutrient downshift in *C. crescentus* (data not shown and (Amemiya, 1989)). To date, it has not been shown that ppGpp is responsible for rRNA transcriptional control in either species. Thus, while (p)ppGpp signaling is nearly ubiquitous in bacteria, it is clearly cued by different signals in different species; in some species other factors may be responsible for rRNA transcriptional control.

When studying diversity in the regulation of (p)ppGpp synthesis, it is necessary to consider the Rsh enzymes. The most well-studied of these are the paralogous enzymes $RelA_{EC}$ and $SpoT_{EC}$ of *E. coli*. $RelA_{EC}$ functions solely as a (p)ppGpp synthase (Aravind & Koonin, 1998); it binds directly to the ribosome and is activated by uncharged tRNA in the acceptor (A) site (Haseltine & Block, 1973). The C-terminal regulatory domains of $RelA_{EC}$ (Schreiber *et al*., 1991) and the L11 protein of the 50S ribosome (Friesen *et al*., 1974, Parker *et al*., 1976) function together to detect this uncharged tRNA signal. $SpoT_{EC}$, on the other hand, both synthesizes and hydrolyzes (p)ppGpp (Xiao *et al*., 1991) and is regulated by starvation for phosphate (Spira *et al*., 1995), carbon (Xiao *et al*., 1991), iron (Vinella *et al*., 2005), and lipids (Seyfzadeh *et al*., 1993) and by deprivation for multiple amino acids in combination. Unlike $RelA_{EC}$, starvation of single amino acids does not activate $SpoT_{EC}$ (Murray & Bremer, 1996). $SpoT_{EC}$ associates with the 50S subunit of the ribosome, but does not apparently associate with the translating ribosome (Jiang *et al*., 2007). It is not clear how $SpoT_{EC}$ is activated, except in the case of lipid starvation (Battesti & Bouveret, 2006).

In this work we examine the regulation of stringent signaling in *C. crescentus*, a bacterium that lives in nutrient poor (*i.e*. oligotrophic) freshwater environments, and exhibits a non-classical stringent response that is regulated by a single Rsh enzyme - annotated $SpoT_{CC}$. Our results confirm that *C. crescentus* synthesizes ppGpp in response to glucose and ammonium starvation, but not amino acid starvation. We show that $SpoT_{CC}$ associates with the ribosome, and exhibits AND-type signaling logic in which detection of an uncharged tRNA at the acceptor site is a necessary but insufficient signal for activation of ppGpp synthesis by $SpoT_{CC}$. Our work with $SpoT_{CC}$ and a *C. crescentus* ribosomal mutant provides evidence that glucose and ammonium starvation are detected via different mechanisms. A genome-scale transcriptional analysis delineates the global effects of increased cytosolic ppGpp on gene expression during carbon starvation. Quantification of 16S rRNA transcription by qRT-PCR shows that rRNA levels are restricted independently of $spoT_{CC}$, and only in select amino acid starvation conditions. This study defines regulatory features of the stringent response in an oligotrophic bacterium, and provides an example of how the signaling logic of a broadly conserved signaling system may be tailored to a particular environmental niche.

RESULTS

ppGpp synthesis in *Caulobacter crescentus* is regulated by glucose and ammonium, but not phosphate or amino acid starvation

To determine the types of starvation to which the stringent response of wild-type *C. cresecentus* strain NA1000 (Evinger & Agabian, 1977, Marks *et al*., 2010) is sensitive, we measured ppGpp accumulation by thin-layer chromatography (TLC) after selective removal of nutrients from glucose defined medium (M2G) supplemented with $KH_2{}^{32}PO_4$. It is known that *C. crescentus* synthesizes ppGpp under glucose (Lesley & Shapiro, 2008) and ammonium (Chiaverotti *et al*., 1981) starvation conditions. Our data confirm these results and show that $SpoT_{CC}$ does not respond to phosphate starvation (Figure 1A). Previous work has shown that starvation for arginine, proline, isoleucine, serine and lysine failed to induce ppGpp accumulation at short time points in *C. crescentus*. To more comprehensively examine the response of $SpoT_{CC}$ to amino acid starvation, we used strains that were auxotrophic for 17 amino acids – all except alanine, glutamine and asparagine. These strains have transposon insertions within, or deletions of, amino acid biosynthetic genes and thus require specific amino acids for growth. We measured ppGpp accumulation in these auxotrophs when starved for their respective required amino acid at short and long time points. Our results show that *C. crescentus* only accumulates very low levels of ppGpp in any of the tested amino acid starvation conditions (Figure 1B, S1).

In *E. coli*, $SpoT_{EC}$ does not synthesize ppGpp upon starvation for single amino acids, but rather upon depletion of multiple amino acids (Murray & Bremer, 1996). To test whether $SpoT_{CC}$ responds similarly to depletion of multiple amino acids for which it is prototrophic, we cultured wild-type *C. crescentus* with the 11 amino acids that do not inhibit growth of this species (Ferber & Ely, 1982) at 100 μg/ml in M2G with $KH_2{}^{32}PO_4$. We then washed all amino acids out of the medium and measured ppGpp accumulation. Our results show that simultaneous removal of multiple amino acids from the medium does not induce significant ppGpp accumulation (Figure 1B); thus $SpoT_{CC}$ differs from $SpoT_{EC}$ in its response to amino acid deprivation and phosphate starvation (Spira *et al*., 1995).

$SpoT_{CC}$ is physically associated with the ribosome

E. coli $RelA_{EC}$ associates with the ribosome (Ramagopal & Davis, 1974, Gentry & Cashel, 1995); this interaction is presumed to be required for regulation of $RelA_{EC}$ activity by uncharged tRNAs (Haseltine & Block, 1973). $SpoT_{EC}$ associates only with the 50S subunit (Jiang *et al*., 2007). *C. crescentus* $SpoT_{CC}$ differs from its *E. coli* Rsh homologs in that it is unresponsive to amino acid starvation (Figure 1B). We sought to test whether an Rsh protein that does not respond directly to amino acid starvation may still associate with the ribosome.

To assess ribosome association of $SpoT_{CC}$, we built a *C. crescentus* strain in which *spoT*$_{CC}$ is fused to an N-terminal HA tag (HA-*spoT*$_{CC}$). This tag does not interfere with $SpoT_{CC}$ activity or cause spurious ribosome association in a cytoplasmic control protein (Figure S2). Cell lysate from this strain was resolved on a sucrose gradient and ribosomal peaks were analyzed by Western blot with an α-HA antibody. Data from this experiment demonstrate that HA-$SpoT_{CC}$ is enriched in the 70S and polysome fractions of the lysate, while little or no HA-$SpoT_{CC}$ is present in the soluble fraction (Figure 2A). From this, we conclude that $SpoT_{CC}$ associates with the translating ribosome.

It is known that deletions of C-terminal regions of $RelA_{EC}$ and a P22L mutation in ribosomal protein L11 prevent $RelA_{EC}$ from detecting uncharged tRNA in the ribosomal A site (Jenvert & Schiavone, 2007, Schreiber *et al*., 1991). To test whether ribosome-association of $SpoT_{CC}$ is affected by similar mutations, we isolated *C. crescentus* ribosomes by sucrose cushion centrifugation (Cross, 1970, Spedding, 1996) and analyzed the ribosome pellet and soluble fractions by Western blot. HA-$SpoT_{CC}$ is present in the ribosome pellet fractions in both the C-terminal truncation of $SpoT_{CC}$, and in an L11 P22L ribosomal mutant (Figure 2B, Figure S3), demonstrating that these mutations do not affect ribosome association. The effects of these mutations on $SpoT_{CC}$ activity will be discussed below.

In vitro, $RelA_{EC}$ is thought to have a lower affinity for ribosomes during active ppGpp synthesis (Wendrich *et al*., 2002). To test whether ribosome association of $SpoT_{CC}$ is altered during ppGpp synthesis *in vivo*, we isolated ribosomes from unstarved and glucose-starved cells. $SpoT_{CC}$ is found only in the ribosome fraction, regardless of whether it is actively synthesizing ppGpp (Figure 2C).

Ribosome activity affects the regulation of cytosolic ppGpp levels by $SpoT_{CC}$

Given that amino acid starvation alone is not sufficient to induce robust ppGpp synthesis in *C. crescentus*, the functional role of $SpoT_{CC}$ ribosome association was not immediately evident. Antibiotics that inhibit different aspects of ribosome activity have been used to explore the mechanism of $RelA_{EC}$ regulation (Lund & Kjeldgaard, 1972, Sokawa & Sokawa, 1978). To test whether inhibition of the *C. crescentus* ribosome affects $SpoT_{CC}$ activity, we measured ppGpp decay during glucose starvation upon addition of the ribosome antibiotics tetracycline, chloramphenicol, streptomycin, spectinomycin and kanamycin. When added to cells pre-starved for glucose, tetracycline and chloramphenicol induce rapid

ppGpp decay; the other tested antibiotics do not induce decay (Figure 3A). The converse experiment yields consistent results: tetracycline and chloramphenicol prevent ppGpp accumulation upon glucose starvation, while streptomycin, spectinomycin and kanamycin do not (Figure 3B). Chloramphenicol inhibits peptidyltransferase activity and tetracycline blocks tRNAs from entering the A site: these drugs both bind to the 50S subunit (Gale *et al*., 1981). Chloramphenicol and tetracycline may prevent ppGpp accumulation either by inhibiting a ribosome function that is required for $SpoT_{CC}$ activity (see Discussion), or by some direct effect on $SpoT_{CC}$. However, $SpoT_{CC}$ is stable (Figure 3C) and co-purifies with the ribosome (Figure 3D) in the presence of tetracycline, ruling out antibiotic effects on $SpoT_{CC}$ stability or ribosome association.

As carbon starvation entails amino acid starvation (Ballesteros *et al*., 2001), uncharged tRNAs are expected to accumulate during glucose limitation and can function as a potential starvation signal. Our antibiotic data provide evidence that peptidyltransferase activity and binding of tRNA to the A site are necessary for $SpoT_{CC}$ activation. We therefore propose that, like $RelA_{EC}$, activation of $SpoT_{CC}$ requires an uncharged tRNA to occupy the A site. However, unlike $RelA_{EC}$, this signal is insufficient (Figure 1B, S1) and an additional signal induced by carbon or nitrogen starvation is required for full $SpoT_{CC}$ activation. This regulatory model is further tested below.

A two-tiered regulatory response of *C. crescentus* to ribosome inhibition and starvation

We observed that ppGpp levels did not completely decay to non-starvation levels when tetracyline and chloramphenicol were added to starved cultures (Figure 3A). This may be because the antibiotics directly, but incompletely, affect $SpoT_{CC}$ activity, or because they are interfering with only one of multiple signals sensed by $SpoT_{CC}$ during glucose starvation. Based on analogy to $RelA_{EC}$ regulation, we propose that tetracycline interferes with the signal for amino acid starvation. We therefore tested whether tetracycline-treated cells could still respond to relief of starvation by addition of glucose. We starved wild-type cells for glucose in the presence of $KH_2{}^{32}PO_4$ for two hours to induce ppGpp accumulation, then added tetracycline and measured ppGpp decay for 20 minutes. We then added glucose to these cells to relieve starvation (Figure 3E). We also measured ppGpp decay in a glucose-starved culture after the addition of glucose alone. These data show that when glucose is added to a starved culture, ppGpp levels quickly decay back to non-starvation levels. When tetracycline is added to a starved culture, ppGpp decays to a ppGpp/GTP ratio of ~0.3–0.5. Only when glucose is added to this tetracycline-treated culture does ppGpp return to non-starvation levels (Figure 3E). This two-tiered response implies that there are two signals controlling $SpoT_{CC}$ activity: tetracycline interferes with one signal while glucose removes both. We conducted the same experiment with ammonium-starved cells, and observed the same two-tiered response. These results imply that amino acid starvation is also a necessary (Figure 3F) but insufficient (Figure 1B, S1) signal for $SpoT_{CC}$ activation in ammonium starvation.

$SpoT_{CC}$ functions as a synergistic 'AND' logic gate

To genetically test whether glucose starvation in the absence of amino acid starvation is sufficient to activate $SpoT_{CC}$, we built strain Δ*fbp*s in which both genes for the gluconeogenic enzyme fructose 1–6 bisphosphatase have been deleted (Table 1). This strain is a glucose auxotroph that cannot grow with amino acids as the sole carbon source (data not shown), and which synthesizes ppGpp normally upon glucose starvation (Figure 4A). We cultured Δ*fbp*s in M2G defined medium with $KH_2{}^{32}PO_4$ and all 20 amino acids (100 μg/ml), then washed glucose from the medium and measured ppGpp accumulation. Our results show that glucose starvation during amino acid supplementation induces only a low level of ppGpp (Figure 4A), and is therefore not sufficient to induce a full ppGpp response in the

Δ*fbp*s strain. As a control, we show that wild-type cells starved for glucose in medium supplemented with amino acids do not accumulate detectable ppGpp (Figure 4A); these cells are able to synthesize glucose from the amino acids and are therefore not starving for any nutrient.

The converse experiment showed consistent results: glucose-starved Δ*fbp*s cells synthesize high levels of ppGpp, induce partial ppGpp decay upon addition of all 20 amino acids, and full decay upon addition of glucose (Figure 4B). These results provide evidence for a regulatory model in which $SpoT_{CC}$ functions as a synergistic 'AND' logic gate that requires both amino acid and glucose starvation signals for full activation. Individual starvation for either glucose or amino acids results only in very low levels of accumulated ppGpp (Figure 1B, 4A,B).

C-terminal truncations of $SpoT_{CC}$ decouple glucose and ammonium starvation responses

Rsh proteins have two conserved C-terminal domains, TGS and ACT, that are involved in detection of starvation signals (Battesti & Bouveret, 2006, Schreiber *et al*., 1991). To test the roles of these domains in starvation signal detection in $SpoT_{CC}$, we constructed strains in which the wild-type allele of $SpoT_{CC}$ was replaced with alleles carrying truncations within the C-terminus (Figure 5A).

These mutant strains were incubated for two hours in the presence of $KH_2{}^{32}PO_4$ in replete M2G defined medium, M2 without glucose, or M2G without ammonium, and then analyzed by TLC to assess ppGpp levels. All of the mutants have impaired ppGpp accumulation in glucose and ammonium starvation (Figure 5B). The ΔCTD mutant fails to accumulate ppGpp under any condition. Notably, while all of the mutants fail to accumulate significant ppGpp under glucose starvation conditions, the ACT domain mutants do accumulate ppGpp under ammonium starvation. The stability of each of these $SpoT_{CC}$ truncations was assessed by Western blot in strains with N-terminal HA tags on each. These data show that all of the mutants are stable except ΔACT, which is present at very low levels (Figure 5C). The relatively high ppGpp accumulation observed in ammonium starvation by the unstable ΔACT mutant implies that it is hyper-responsive under this starvation condition.

Ribosomal protein L11 is involved in transduction of ammonium starvation signals to $SpoT_{CC}$

In *E. coli*, a P22L mutation of L11 abrogates the ability of $RelA_{EC}$ to respond to amino acid starvation but has little effect on protein synthesis (Jenvert & Schiavone, 2007). To assess whether L11 is involved in the response of $SpoT_{CC}$ to starvation signals, we constructed an allelic replacement strain with a P22L mutation in L11 and tested its ability to accumulate ppGpp upon glucose and ammonium starvation. The data show that this mutant exhibits a defect in ppGpp accumulation under ammonium starvation but not glucose starvation (Figure 5D). This demonstrates that though the signals perceived by $RelA_{EC}$ and $SpoT_{CC}$ are different, there are conserved features of signal transduction through the ribosome. Moreover, the result that L11 P22L and SpoTΔACT alleles decouple the ammonium and glucose starvation responses provides evidence for independent mechanisms of carbon and nitrogen starvation signal detection by $SpoT_{CC}$.

Activated $SpoT_{CC}$ downregulates growth genes and upregulates stress and starvation genes

We sought to identify the genes that are regulated by *C. crescentus* $SpoT_{CC}$ upon carbon starvation. To quantify gene expression during the stringent response we grew wild-type strain NA1000 and NA1000 Δ*spoT*$_{CC}$ in M2G, washed glucose out of the medium and starved the cells for five minutes before extracting RNA and hybridizing labeled cDNA to

Affymetrix GeneChip, CauloHi1 (GEO microarray accession number GSE21206). The results from this experiment show large-scale downregulation of anabolic genes and upregulation of catabolic, sensory, chemotaxis and DNA recombination genes (Figure 6, Table S1). These results are generally consistent with the stringent transcriptional response described in other species, with the notable exception of amino acid biosynthetic genes, which are downregulated during carbon starvation by $spoT_{CC}$. This exception is likely due to our experimental protocol in which cells are starved for glucose, not amino acids. Several other genes of interest are regulated in carbon starvation by $spoT_{CC}$, including genes involved in cell division and nutrient granule metabolism (Table 2).

$SpoT_{CC}$ does not regulate rRNA transcription during glucose starvation

The first function assigned to $RelA_{EC}$ was that of a negative regulator of rRNA transcription during amino acid starvation (Stent & Brenner, 1961). To assess the role of $SpoT_{CC}$ in rRNA transcriptional control, we constructed a 16S rRNA transcriptional reporter plasmid that contains the 16SA promoter and the first 81 nucleotides of the 16SA leader followed by a segment of the *lacZ* gene from *E. coli*. The unstable transcript from this reporter can be quantified by qRT-PCR to yield a measure of 16S rRNA transcription (Figure 7A). Strains containing this reporter plasmid were grown in M2G medium and then starved by washing out glucose; RNA samples were collected from cells before and after starvation. For each sample, data from the 16S amplicon were normalized to data from an amplicon of the *ruvA* DNA helicase gene. *ruvA* was chosen as a normalization control because *ruvA* transcript levels did not change under starvation conditions as assessed using multiple unique probes on the Affymetrix microarray. The qRT-PCR data show that 16S transcription is quickly downregulated after glucose deprivation, but that this response is not dependent on $spoT_{CC}$ (Figure 7B). DksA is required for regulation of rRNA transcription in *E. coli* (Paul *et al*., 2004). We repeated the rRNA transcriptional assay in a Δ*dksA* strain: this mutation affects the basal level of 16S transcription but is not responsible for downregulation of transcription during glucose starvation (Figure 7B) in *C. crescentus*. These data provide evidence for a SpoT/DksA-independent mechanism of rRNA transcriptional control during starvation in *C. crescentus*.

Starvation for glutamate - but not phenylalanine - results in downregulation of rRNA transcription

While the data presented above show that ppGpp is not involved in stringent control of rRNA transcription (Figure 7B), we wished to test whether *C. crescentus* restricts rRNA synthesis upon amino acid starvation. It has been reported that net RNA synthesis in *C. crescentus* is not restricted upon the addition of chemical inhibitors of isoleucine, serine and lysine synthesis or utilization; however, controls demonstrating that these chemicals induced starvation were not described (Chiaverotti *et al*., 1981). With respect to *C. crescentus* growth, there are two classes of amino acids: nine which inhibit growth when added to minimal medium (Cys, His, Ile, Leu, Met, Phe, Ser, Thr, Val) and 11 which do not (Ferber & Ely, 1982). We hypothesized that the rRNA transcriptional response to starvation of amino acids in these two classes may be different. To study a representative from each class, we built phenylalanine (Δ*pheA*) and glutamate (Δ*gltB*) auxotrophs. These auxotrophic strains and a prototrophic strain were grown in M2G with phenylalanine or glutamate added. We then washed out the amino acids, starved the cells for two hours, and measured 16S rRNA transcription by qRT-PCR as described above. Our results show that glutamate deprivation results in a reduction of rRNA transcription only in the Δ*gltB* auxotroph, which experiences starvation (Figure 7C). Addition of phenylalanine, however, results in a reduction of 16S transcription in the prototroph and auxotroph. Starvation for phenylalanine in the auxotroph does not result in reduced rRNA transcription. The growth inhibition

observed in *C. crescentus* when phenylalanine is added to growth media (Ferber & Ely, 1982) may indirectly cause rRNA transcriptional repression.

ppGpp accumulation activates IS3 elements in the *C. crescentus* genome

We observed an increase in transcription of insertion sequence (IS) elements in our global transcriptional analysis (Figure 6, Table S1). This result was functionally confirmed in a strain with $SpoT_{CC}$H67A, which carries a point mutation that ablates ppGpp hydrolase activity (Hogg *et al*., 2004). Initial characterization of the $SpoT_{CC}$ H67A strain indicated slow growth, as expected for a strain unable to hydrolyze ppGpp. However, suppressors with wild-type growth rates appeared quickly, and we determined that they were no longer able to synthesize ppGpp (data not shown). Sequencing of the $spoT_{CC}$ locus in three independent suppressors revealed IS3 insertion at codon 115 in one and at codon 161 in the other two (Figure 5A). The transcriptional data and isolation of multiple IS3-mediated H67A suppressors provides evidence that increased cellular ppGpp, independent of starvation stress, is sufficient to activate IS3 insertion elements.

DISCUSSION

Prokaryotes inhabit a diverse range of ecological niches; the structure of environmental sensing and signaling mechanisms is correspondingly diverse. The stringent response has been most thoroughly studied in a few copiotrophic species including *E. coli* (Potrykus & Cashel, 2008), *B. subtilis* (Ochi *et al*., 1982), and *Legionella pneumophila* (Dalebroux *et al*., 2009, Edwards *et al*., 2009, Wells & Gaynor, 2006, Zhou *et al*., 2008). However, there is evidence that species living in different ecological niches have stringent response systems that are structured and regulated differently (Chiaverotti *et al*., 1981, Acosta & Lueking, 1987, Belitsky & Kari, 1982, Howorth & England, 1999, Scoarughi *et al*., 1999, Wells & Gaynor, 2006, Zhou *et al*., 2008). In this work we illuminate several regulatory features of the stringent response in the model oligotroph *C. crescentus*.

Amino acid starvation is not a sufficient signal to induce ppGpp accumulation in the oligotroph *C. crescentus*

For *E. coli*, amino acids are a constant and important nutrient source in the intestine. In the transition from intestine to the environment outside its commensal host, amino acid concentration scales with total nutrient availability (Savageau, 1983). Thus measuring amino acid levels is a convenient proxy for *E. coli* to assess environmental nutrient status. In contrast, *C. crescentus* lives in oligotrophic environments where amino acid concentrations are consistently low (Poindexter, 1981, Thomas, 1997) and may have little correlation with total nutrient availability. *C. crescentus* is able to respond to low cytoplasmic concentrations of amino acids by upregulating expression of amino acid biosynthetic genes (Tarleton *et al*., 1994), but, in contrast to *E. coli* and other copiotrophs, it does not activate the stringent response upon amino acid starvation alone.

$SpoT_{CC}$ is bound to the ribosome

The *E. coli* bifunctional ppGpp hydrolase/synthetase $SpoT_{EC}$ binds to the 50S ribosomal subunit, but not the translating ribosome (Jiang *et al*., 2007). Bifunctional Rsh proteins that are regulated solely by amino acid starvation and bind the ribosome have been previously identified (Avarbock *et al*., 2000, Avarbock *et al*., 1999, Martinez-Costa *et al*., 1998). Our data are the first to demonstrate association of a bifunctional Rsh protein that does not respond solely to amino acid starvation with the translating ribosome (Figure 2A). Deletion of the C-terminal domain of $SpoT_{CC}$ does not prevent ribosome association (Figure 2B), indicating that either the N-terminus alone mediates association, or that both halves of the protein independently associate with the ribosome. In the case of *E. coli* $RelA_{EC}$, it is not

known if the C-terminal domains bind to the ribosome, only that they receive a signal through the ribosome (Schreiber *et al*., 1991, Gropp *et al*., 2001). It is noteworthy that on the ribosomal side, association with $RelA_{EC}$ is mediated by L10 (Howard *et al*., 1976) but signal transduction is mediated by L11 (Friesen *et al*., 1974, Schreiber *et al*., 1991): thus binding and signal transduction are spatially separated.

An L11 P22L ribosome mutation perturbs $SpoT_{CC}$ regulation (Figure 5D), but does not abrogate ribosome association (Figure 2B). This mirrors the observation in *E. coli* that L11, though important for signaling, does not bind directly to $RelA_{EC}$ (Yang & Ishiguro, 2001). $SpoT_{CC}$ does not appear to modulate its association with the ribosome during glucose starvation (Figure 2C), although this experiment cannot rule out small changes in $SpoT_{CC}$-ribosome affinity. Antibiotics that bind to the 50S but not the 30S subunit interfere with $SpoT_{CC}$ activity (Figure 3A,B). The L11 protein and the amino-acylation portion of the A-site tRNA are near each other on the 50S subunit (Voorhees *et al*., 2010). We therefore propose that $SpoT_{CC}$ binds near these signals (Figure 8B).

Combinatorial signal integration by $SpoT_{CC}$: Evidence for 'AND' logic

The ribosome-association result reported herein was initially surprising given the observation that $SpoT_{CC}$ does not respond to amino acid starvation in the same way as $RelA_{EC}$ or $SpoT_{EC}$ (Figure 1, S1). However, our functional data demonstrate a role for ribosomal protein L11 in $SpoT_{CC}$ regulation (Figure 5D) and pharmacological data show that certain ribosome functions are required for ppGpp accumulation during glucose starvation (Figure 3). Together, these data provide support for a model in which the ribosome plays an important role in $SpoT_{CC}$ regulation.

Chloramphenicol and tetracycline both appear to interfere with the signal of the uncharged tRNA at the A site of the ribosome, which is required for $RelA_{EC}$ activation (Sokawa & Sokawa, 1978, Lund & Kjeldgaard, 1972, Gale *et al*., 1981, Haseltine & Block, 1973). Both chloramphenicol and tetracyline bind the 50S subunit of the ribosome (Gale *et al*., 1981), where $RelA_{EC}$ has been shown to bind (Howard *et al*., 1976). These same drugs also interfere with $SpoT_{CC}$ function (Figure 3). While it is possible that chloramphenicol and tetracycline inhibit Rsh protein activity by affecting binding to the ribosome (Gale *et al*., 1981), our data show no effect of chloramphenicol (data not shown) or tetracycline on $SpoT_{CC}$ association with the ribosome (Figure 3D) or stability (Figure 3C). The fact that these drugs both prevent ppGpp accumulation (Figure 3B) and induce ppGpp decay in pre-starved cultures (Figure 3A,E,F) also supports the argument that they do not inhibit the $SpoT_{CC}$ enzyme but rather remove the signal required for its activation toward synthesis.

Three antibiotics did not interfere with ppGpp accumulation or maintenance (Figure 3A,B): these all bind to the 30S subunit. Streptomycin causes mistranslation and inhibits initiation (Gale *et al*., 1981). Kanamycin binds to the decoding region of the A site and prevents discrimination between cognate and non-cognate tRNAs (Magnet & Blanchard, 2005). Spectinomycin binds to the head domain and blocks translocation (Borovinskaya *et al*., 2007). Our pharmacological data therefore suggest that an active peptidyltransferase site and binding of tRNA to the acceptor site are required for $SpoT_{CC}$ activation, while translational accuracy, initiation, and translocation are not. In *E. coli*, ppGpp synthesis by $RelA_{EC}$ is inhibited by chloramphenicol, tetracycline and spectinomycin but not by kanamycin and streptomycin (Cortay & Cozzone, 1983), indicating that tRNA binding, peptidyltransferase and translocation are essential for $RelA_{EC}$ activity, but initiation and fidelity are not. Experiments with spectinomycin show that $RelA_{EC}$ and $SpoT_{CC}$ are different with respect to translocation. This and the data with the mutated L11 protein (Figure 5C) show that detection of amino acid starvation at the ribosome occurs via subtly different mechanisms in

$SpoT_{CC}$ and $RelA_{EC}$. It is not clear why $SpoT_{CC}$ should be insensitive to inhibition of translocation.

Both glucose and ammonium starvation lead to accumulation of uncharged tRNAs and an increase in ppGpp (Figure 1A). Our genetic studies using amino acid auxotrophs demonstrate that amino acid starvation alone is not sufficient to induce ppGpp accumulation (Figure 1B, S1). Using a glucose auxotroph, we show that glucose starvation in the presence of amino acids is also not sufficient to induce full accumulation of ppGpp (Figure 4A). In ppGpp decay assays there is a two-tiered response to relief of these two types of starvation. When glucose or ammonium starved cells are treated so as to solely remove the amino acid starvation signal - by adding tetracycline (Figure 3E,F) or by adding amino acids (Figure 4B) – ppGpp levels are partially reduced but level off at a ppGpp/GTP ratio of ~0.3 – 0.5. When these cells are subsequently treated with glucose or ammonium, full ppGpp decay is induced (Figure 3E,F, 4B).

Our data support a model in which $SpoT_{CC}$ has regulatory properties of a synergistic 'AND' logic gate in which the presence of one activating signal results in low levels of ppGpp accumulation (Figure 1B, S1, 3, 4A). Only when both amino acid AND glucose OR ammonium starvation signals are present do high levels of ppGpp accumulate (Figure 1A, 4A, 8A). In glucose starvation it is possible that a glycolytic intermediate could be a molecular signal for $SpoT_{CC}$ activity, as may be the case in *E. coli* (Schneider & Gourse, 2003). The Δ*fbp*s strain would have abnormal levels of some glycolytic intermediates during glucose starvation and amino acid supplementation due to the block in gluconeogenesis, and this could prevent full ppGpp accumulation in this condition (Figure 4A,B). However, we think this an unlikely explanation of the results in Figure 4 because the analogous experiments with tetracycline show similar levels of ppGpp accumulation (Figure 3A, 3E, 3F).

The 'AND' gate signaling logic we propose for the *C. crescentus* stringent response entails a stricter definition of starvation than is observed in *E. coli*, which has 'OR' signaling logic in its stringent response (Figure 8A). This higher threshold for stringent response activation is likely an adaptation to the near constant starvation conditions encountered by *C. crescentus* in its oligotrophic niche. A regulatory structure in which multiple starvation cues are required to fully trigger the stringent response is likely better suited to oligtrophic niches where organisms must utilize their substantial metabolic capacity to convert nutrients and continue growth whenever possible.

Evidence for independent mechanisms of $SpoT_{CC}$ regulation by glucose and ammonium starvation signals

Rsh proteins have two conserved C-terminal domains: TGS and ACT, which are important for regulation. The TGS domain of $SpoT_{EC}$ binds to holo-acyl carrier protein during lipid starvation and induces synthesis of ppGpp (Battesti & Bouveret, 2006). The ACT domain and a region N-terminal to it are important in sensing amino acid starvation via ribosomal protein L11 in $RelA_{EC}$ (Schreiber *et al*., 1991, Durfee *et al*., 2008). We constructed strains in which the entire $SpoT_{CC}$ C-terminus or the ACT domain alone were deleted and tested them for ppGpp accumulation upon starvation. Our data show that $SpoT_{CC}$ with a missing ACT domain responds to ammonium but not glucose starvation. Thus signals perceived by $SpoT_{CC}$ are specific to the type of starvation and the ACT domain is not required for responding to ammonium starvation.

Further evidence for independent mechanisms of $SpoT_{CC}$ regulation by glucose and ammonium comes from our experiments with a ribosomal mutant. The L11 P22L mutant strain of *C. crescentus* was unable to accumulate ppGpp at a wild-type rate in ammonium

starvation but showed no defect upon glucose starvation. This indicates that detection of an ammonium starvation signal by $SpoT_{CC}$ involves ribosomal protein L11 and that glucose starvation signals are either transduced differently through L11 or not transduced through this protein at all.

$SpoT_{CC}$ regulates large-scale transcriptional changes during carbon starvation

Experiments assessing global transcriptional change during the stringent response have been conducted in a number of species (Brockmann-Gretza & Kalinowski, 2006, Eymann *et al*., 2002, Gaynor *et al*., 2005, Kazmierczak *et al*., 2009, Nascimento *et al*., 2008, Traxler *et al*., 2008, Durfee *et al*., 2008). In these experiments ppGpp synthesis was induced by amino acid starvation. In general, many anabolic genes were downregulated while stress response genes and certain amino acid biosynthetic genes were upregulated. Work in *E. coli*, however, shows that the regulation of amino acid biosynthetic genes is variable and complex (Durfee *et al*., 2008). In *E. coli* grown under glucose-lactose diauxie, most anabolic genes are downregulated and stress response genes are upregulated. However, histidine and arginine biosynthetic genes were transiently upregulated (Chang *et al*., 2002), though this may not be dependent on ppGpp (Traxler *et al*., 2006). Overall, the general picture from microarray analyses of the stringent response is that anabolic genes are downregulated and stress response genes are upregulated. Amino acid biosynthetic genes are sometimes an exception to this trend, but this result is partially confounded by the fact that the tested cells were in most cases starved for amino acids.

The transcriptional effects of elevated ppGpp in glucose starvation in *C. crescentus* are similar to those reported in other species. We observe downregulation of genes encoding translation machinery, energy metabolism and anabolic metabolism (Figure 6, Table S1). In addition, almost all of the amino acid biosynthetic genes were downregulated; this is not consistently seen in other microarray experiments, but is consistent with adaptation to total growth arrest and nutrient deprivation. The one gene that is involved in amino acid biosynthesis that is upregulated in our data set encodes homoserine O-acetyltransferase (Figure 6, Table S1), which is required for biosynthesis of methionine as well as S-adenosyl-L-methionine, a universal methyl-donor molecule (Karp *et al*., 1999, Romero *et al*., 2001).

The majority of genes in the microarray data set involved in catabolic, or degradative processes, are upregulated. Thus $SpoT_{CC}$ activates the breakdown of cellular components during starvation, presumably for energy. We also observe that genes that function in motility, chemotaxis and environmental sensing are largely upregulated by $SpoT_{CC}$, implying that the *C. crescentus* stringent response increases the capacity of the cell to respond to a wider range of nutrient sources. Such a response would be adaptive in an oligotrophic environment (Poindexter, 1981), and is not seen in *E. coli* in the amino-acid-starvation strigent response, where flagellar and chemotaxis genes are downregulated (Durfee *et al*., 2008, Lemke *et al*., 2009).

It has been predicted that oligotrophs should degrade storage polymers during starvation, and that cessation of growth should augment polymers of non-limiting nutrients (Poindexter, 1981). The PHB degradation enzyme Poly(3-hydroxyalkanoate) depolymerase is upregulated by $spoT_{CC}$ during carbon starvation, providing evidence that PHB is being used as a carbon source (Table 2). Additionally, $SpoT_{CC}$ downregulates the enzyme exopolyphosphatase, which degrades polyphosphate, while upregulating polyphosphate kinase (Table 2). Thus, $SpoT_{CC}$ regulates the ability of *C. crescentus* to increase its polyphosphate stores during stalled growth.

ccrM, a gene involved in cell division control, was downregulated by *spoT* (Table 2). CcrM methylates DNA, thereby controlling the transcription of genes with methylation-sensitive

promoters. Normally *ccrM* is expressed just before cell division: its activity represses trancription of *ctrA* - a regulator responsible for expression of genes required for swarmer cell development and septation (Reisenauer & Shapiro, 2002) – and activates expression of DnaA, which initiates chromosome replication (Collier *et al*., 2007). Downregulation of *ccrM* during starvation should cause CtrA levels to remain high and DnaA levels to remain low, perhaps permitting completion of the ongoing cell division but repression of the next round of DNA replication (Lesley & Shapiro, 2008). The regulation of other genes involved in cell division hints at a complex integration of cell division and starvation signals (Table 2).

Rsh signaling diversity and an undetermined mechanism of rRNA regulation

Our data show that amino acid starvation is only conditionally linked to ppGpp synthesis (Figure 1B, 4A,B) and inconsistently linked to rRNA transcriptional control (Figure 7C). Moreover, we demonstrate that $SpoT_{CC}$ (and hence, ppGpp) is not required to downregulate 16SA rRNA transcription upon carbon starvation (Figure 7B). There are two rRNA operons in *C. crescentus* and we have only examined transcription of one of them, the A operon. We draw no conclusions about the regulation of the B operon, but it is clear that a *spoT/dksA* - independent mechanism regulates the A operon in carbon starvation. As described in the introduction, there are other examples of species in which rRNA transcriptional restriction occurs in the absence of ppGpp accumulation (Acosta & Lueking, 1987, Belitsky & Kari, 1982). Our study is the first to show ppGpp-independent downregulation of rRNA transcription in a condition that still induces ppGpp accumulation in the wild-type (Figure 7B). In *E. coli* the concentration of the initiating NTP (iNTP) controls rRNA transcription during outgrowth, and ppGpp controls it during nutrient shifts (Murray *et al*., 2003). It is possible that in *C. crescentus* the iNTP controls rRNA transcription under all conditions. The iNTP for the ribosomal A operon is GTP (Amemiya, 1989). We have data showing that GTP levels upon carbon starvation fall to similar extents in wild-type and *spoT* null strains, thus it is possible that iNTP levels alone could control rRNA transcription in *C. crescentus* (Figure S4). It is notable that in *E. coli* subjected to amino acid starvation, levels of the iNTPs fall in the wild-type strain but actually rise in the *relA* null strain (Edlin & Neuhard, 1967). Further study will be required to determine the mechanism of rRNA control in *C. crescentus*.

Activation of IS elements by the stringent response

Activation of the stringent response in *C. crescentus* results in increased transcription of IS transposases and DNA recombination enzymes (Figure 6, Table S1). The observation that strains carrying a hydrolase-deficient $SpoT_{CC}$ grow slowly initially and quickly acquire suppressors with IS3 elements interrupting the *spoT* locus provides a confirmation of our transcriptome data. It is likely that IS3 elements mobilized to many locations in the genome of the SpoT H67A strain; however, we only recovered strains with elements that interrupted the *spoT* locus, thereby relieving the growth inhibition by elevated ppGpp levels. These elements are a member of the IS3 family - the most prevalent family in bacteria (Mahillon *et al*., 1999). Our study shows that accumulation of ppGpp activates IS elements, independently of starvation. It is notable that a $RelA_{EC}$ null strain was isolated and shown to have an IS2 element within the RelA gene, disrupting its activity (Metzger *et al*., 1989). IS element activation may therefore be a conserved feature of ppGpp accumulation.

Concluding remarks

This work describes a stringent response signaling system in which a nearly universal bacterial signaling molecule, ppGpp, is used to effect transcriptional changes and growth arrest in a manner similar to that observed in *E. coli* and other species. *C. crescentus* is apparently adapted to its oligotrophic niche through divergence of its Rsh protein, $SpoT_{CC}$,

which has a high threshold for activation, requiring multiple starvation signals before it induces growth arrest via the stringent response.

MATERIALS AND METHODS

Bacterial strains and growth conditions

All strains used in this study are listed in Table 1. Experimental strains were derived from *Caulobacter crescentus* strain NA1000 (CB15N) (Evinger & Agabian, 1977, Marks e*t al*., 2010). NA1000 strains were grown in M2G minimal medium (6.1mM Na_2HPO_4, 3.9mM KH_2PO_4, 9.3mM NH_4Cl, 500μM $MgSO_4$, 500μM $CaCl_2$, 1X $FeSO_4$/chelate (Sigma #F10518), 0.2% glucose) at 30°C. All auxotrophic strains except the glutamate auxotroph were grown in M2G supplemented with 100 μg/ml of the amino acid for which the strain was auxotrophic plus 200 μg/ml of alanine to relieve growth inhibition (Ferber & Ely, 1982). The glutamate auxotroph was grown in M2G supplemented with 500 μg/ml of glutamate. The concentration of kanamycin used for plasmid selection in *C. crescentus* was 5 μg/ml in liquid media and 25 μg/ml on solid media. *E. coli* strains TOP10 (Invitrogen, Carlsbad, CA) and Mach1 (Invitrogen) were used for cloning. *E. coli* strains were grown in Terrific Broth supplemented with 50 μg/ml kanamycin at 30°C.

Construction of strains

Strains in which genes were deleted, truncated, or fused to epitopes were constructed by homologous recombination using a two-step kanamycin selection/ *sacB* counterselection protocol with the suicide plasmid pNPTS138, as illustrated (Hinz *et al*., 2003, Fiebig *et al*., 2010). For gene deletions, ≈500 bp fragments upstream and downstream of the deletion target were PCR amplified, joined by triple ligation or gene stitching (Higuchi *et al*., 1988) and then cloned into pNPTS138. The Δ$spoT_{CC}$ strain encodes the first 29 codons and the last 24 codons of gene number *CCNA_01622*. The Δ*dksA* strain encodes the first 8 codons and the last 17 codons of gene number *CCNA_02663*. The Δ*pheA* strain encodes the first ten and last 17 codons of gene number *CCNA_03028*. The Δ*gltB* strain encodes the first 35 and last 30 codons of gene number *CCNA_03722*. The Δ*fbp*s strain encodes the first three codons of *CCNA_01448* and the last 15 codons of *CCNA_01449*. The *spoT*Δ$ACT_{668–719}$ strain encodes codons 1–667 and 720–744 of $spoT_{CC}$. To create the SpoT domain truncation strains, ≈500 bp fragments surrounding the region to be mutagenized were PCR amplified and cloned into TOPO Blunt (Invitrogen); site-directed mutagenesis was used to change the appropriate codon to a stop codon. The mutagenized gene fragments were then cloned into pNPTS138 and used to replace the wild-type chromosomal allele using the two-step recombination protocol cited above. In the *spoT*ΔCTD strain Y366 (TAC) was mutated to stop (TAG). In the *spoT*ΔACT strain A671 (GCC) was mutated to stop (TAG).

To create N-terminal HA epitope fusions, mutagenic gene-stitching PCR (Higuchi *et al*., 1988) was used to introduce an HA tag into a DNA fragment containing the upstream and 5’ region of the target gene. This fusion allele was cloned into pNPTS138 and used to replace the wild-type chromosomal allele as above. The amino acid sequence of the beginning of the HA-SpoT protein is: MYPYDVPDYA followed by amino acids 2–744 of the wild-type protein. The p16S*lac* reporter plasmid was constructed by fusing the 16SA rRNA promoter region to a piece of *E. coli lacZ* using gene-stitching PCR, and cloning the resulting fusion into pMT585 (Thanbichler *et al*., 2007) using the *Asc*I and *Nhe*I cut sites. The amplified region of the 16SA promoter starts 570 bp upstream of the transcription initiation site (Amemiya, 1989) and ends 80 bp after the initiation site. This chromosomal fragment was amplified using the following primers: 5’-atatggcgcgccaaacagctgatcgccaag-3’ and 5’-tatcggcctcaggaagtttctagcgaagcgtctgg-3’. The fragment of *lacZ* was amplified from pPR9TT (Santos *et al*., 2001) using the following primers: 5’-gcttcgctagaaacttcctgaggccgatactgtc-3’

and 5'-atatgctagccattaaagcgagtggcaaca-3'. All plasmids were introduced into *C. crescentus* by tri-parental conjugation using *E. coli* strain MT607 carrying the pRK600 helper plasmid (Finan *et al*., 1986).

Measurement of cellular ppGpp levels

ppGpp was measured as described (Cashel, 1969) with protocol modifications outlined below. Cells were grown in M2G, then washed in either M2G-labeling (M2G with 12.2 mM NaCl and 3.9 mM KCl instead of Na_2HPO_4 and KH_2PO_4), M2-labeling (M2G-labeling without glucose) or M2G-N-labeling (M2G-labeling without NH_4Cl) twice and then resuspended in the appropriate labeling medium. 100μCi/ml of $KH_2{}^{32}PO_4$ (PerkinElmer, Waltham, MA) was added and the cells were incubated at 30°C for 2 hours. In experiments with 2 hour time points, an equal volume of 2M formic acid was then added, and the formic acid extracts were placed on ice for at least 15 minutes. For shorter time points, cells were labeled in the appropriate labeling medium for 2 hours, spun for 1 minute in a microfuge at 12K rpm, the labeling media was pulled off, the cells were resuspended in 1 ml of the starvation or recovery medium, spun again, the washing medium was pulled off and then cells were resuspended in the appropriate starvation-labeling media with 20μCi/ml of $KH_2{}^{32}PO_4$. Aliquots were then taken and formic acid-extracted at time points after resuspension in the starvation media. The extracts were spun in a microfuge for three minutes and the cleared lysate was spotted on PEI-cellulose TLC sheets (EMD Chemicals) and developed in 1.5M KH_2PO_4 pH 3.4. TLC plates were imaged on a Typhoon Phosphoscanner and analyzed with QuantityOne software. The glutamate auxotroph strain FC1250 was cultured as described above, and starved in M2G-labeling media with $KH_2{}^{32}PO_4$, the other auxotrophs were starved in M2G-labeling media with $KH_2{}^{32}PO_4$ and 200 μg/ml of alanine. The Δ*fbp*s strain was starved in M2-labeling media with 100 μg/ml of each amino acid added. The concentrations of antibiotics used in the ppGpp assays was as follows: chloramphenicol, 50 μg/ml; tetracycline, 50μg/ml; streptomycin, 250μg/ml; kanamycin, 250 μg/ml; spectinomycin, 1250 μg/ml: in each case this is 50X the concentration of antibiotic normally used for selection.

Purification of ribosomes/polysomes by sucrose gradient centrifugation

800 ml of culture at OD_{660}=0.2–0.3 was pelleted and resuspended in 3ml of buffer TM (20 mM Tris pH7.5, 15 mM $MgCl_2$, 1mg/ml PMSF and EDTA-free Protease Inhibitor Cocktail (Roche, Indianapolis, IN)). 200 μl of 10 mg/ml lysozyme was added and the sample lysed by two successive freeze-thaw rounds in ethanol-dry ice / 10°C water bath. 60 μl of 10% deoxycholate and 40 μl of Rnase-free DnaseI (NEB, Ipswich, MA) was added and the sample was incubated on ice for 15 minutes, and then spun at 16,000 rpm for 15 minutes. The cleared lysate was removed and 50 OD_{260} was layered on top of a gradient of 10–40% sucrose in Buffer E (10 mM Tris, 10 mM $MgCl_2$, 100 mM NH_4Cl, 3mM β-mercaptoethanol) with 0.2 mg/ml PMSF and EDTA-free Protease Inhibitor Cocktail. The sucrose gradient was spun in a Beckman L8-M Ultracentrifuge at 27,000 rpm for 5 hours at 7°C. The gradient was manually fractionated by siphon into a UV-transparent 96-well plate and the OD_{260} of each fraction was read in a BioTek Synergy plate reader (BioTek, Winooski, VT). The fractions comprising the 70S and polysome peaks were combined and brought up to 30ml with Buffer E + 0.2 mg/ml PMSF, and then spun at 30,000 rpm for 12 hours. The supernatant was decanted and the pellets were resuspended in Buffer E. The soluble fractions from the gradient were combined and concentrated in an Amicon Ultra centrifugal filter device (Amicon, Billerica, MA). The concentrated ribosome and soluble fractions were then normalized to OD_{260} (1/5 of the OD_{260} of the ribosome fractions was used for the soluble fraction, so that background levels were comparable), separated by SDS-PAGE and immunoblotted to detect HA-SpoT.

Isolation of ribosomes by sucrose cushion centrifugation

To assay ribosome association of the various SpoT mutant proteins, we purified ribosomes using the sucrose cushion centrifugation method (Cross, 1970, Spedding, 1996). 150 ml of culture in M2G or M2 at OD_{660}=0.2 was pelleted, lysed and cleared as described above. 0.5 ml of lysate was layered on top of a sucrose cushion (1M sucrose, 50mM Tris pH 7.6, 15mM $MgCl_2$, 6mM BME, and 100mM NH_4Cl) and spun in a Beckman Optima TLX Ultracentrifuge at 70,000 rpm for one hour. The top layer was removed and concentrated to a volume of 200 µl in an Amicon Ultra centrifugal filter device (Amicon). The sucrose cushion was decanted and the high-density ribosome pellet was resuspended in 200 µl of Buffer E. The soluble fractions and ribosome pellet fractions were then separated by SDS-PAGE and immunoblotted todetect HA-SpoT.

Detection of SpoT by immunoblot

Whole cells or samples from biochemical fractionation were boiled in SDS loading dye and proteins were separated on 12% SDS-PAGE gels. Proteins were transferred to a Immobilon polyvinylidene fluoride membrane (Millipore) by wet transfer. The membrane was then blocked in 5% milk for at least 30 minutes and then cut in half, when appropriate, so the FixJ loading control protein and the HA-SpoT proteins could be probed separately. HA-tagged proteins were hybridized by incubating for at least 40 minutes in a 1:5,000 dilution of purified monoclonal mouse anti-HA antibodies (Sigma-Aldrich), washing three times and then incubating for at least 40 minutes in a 1:2,500 dilution of goat anti-mouse antibodies conjugated to HRP (Thermo Scientific). FixJ was hybridized by incubating the membrane in 5% milk with a 1:2,000 dilution of polyclonal rabbit α-FixJ antibody for 40 minutes, washing twice, and then incubating with a 1:10,000 dilution of HRP-cojugated goat anti-rabbit antibodies (Thermo Scientific) for 40 minutes. All membranes were then washed five times, and the secondary antibodies were detected with SuperSignal West Femto Substrate (Thermo Scientific). Blots were exposed to film for 10 seconds to 5 minutes.

Transcriptional analysis of the *C. crescentus* stringent response by DNA microarray

Three independent replicate cultures each of wild-type strain NA1000 and NA1000Δ*spoT*$_{CC}$ were grown to OD_{660}=0.2–0.3 in M2 with 0.2% glucose (M2G), washed once in glucose-free M2 medium, resuspended in 5ml of glucose-free M2, and rolled at 30°C for an additional 5 minutes to fully activate the stringent response. Cells were then pelleted and flash-frozen in liquid nitrogen. RNA was extracted using Trizol as previously described (Boutte *et al*., 2008) and RNA integrity assessed using an Agilent Bioanalyzer (Agilent, Santa Clara, CA). For each of the six RNA samples, 10 µg was processed to produce single-strand cDNA; RNA was removed by addition of 1N NaOH. cDNA was column-purified, fragmented using DNAseI (GE Life Sciences, Piscataway, NJ), and end labelled using GeneChip labelling reagent (Affymetrix, P/N 900542). Labelled cDNA was hybridized to GeneChip CauloHi1 (McGrath *et al*., 2007) according to GeneChip Expression analysis technical manual (Affymetrix, Santa Clara, CA). Hybridization proceeded for 16 hours at 50°C. Arrays were washed using protocol PRO-GE-W52-V3 and stained on a GeneChipFluidics Station (Affymetrix) according to the Genechip Expression analysis technical manual. The arrays were scanned using the Affymetrix Gene Chip Scanner 3000 7G and CEL intensity files were generated by GCOS (Gene Chip Operating Software) v. 1.4. Stringent response microarray data are deposited in the GEO database (http://www.ncbi.nlm.nih.gov/geo) under series accession GSE21206.

Data were analyzed by first calculating the mean expression for the wild-type and Δ*spoT* replicates and then calculating the wild-type / Δ*spoT* ratio. Only probes on the coding strand with at least a three-fold difference in expression were considered for further analysis (Table S1). In some cases there were multiple probes within a gene or its promoter region: the data

for these probes were averaged. The standard deviation for the wild-type and Δ*spoT* replicates was calculated, and genes for which the standard deviation was greater than the mean for either sample set were discarded. Functional assignments for genes were made using BioCyc (Karp et al., 1999, Romero et al., 2001).

RNA isolation and qRT-PCR analysis

Strains for qRT-PCR analysis were grown in M2G + 5 μg/ml kanamycin plus appropriate amino acid supplements for the auxotrophic mutants. Cells for starvation samples were washed and resuspened in starvation media and rolled at 30°C for 15 minutes to one hour for glucose starvation and for two hours for amino acid starvation. 1.5ml of cells at OD_{660}=0.2 were pelleted and resuspended in 1ml Trizol (Invitrogen), flash frozen in liquid nitrogen and stored at −80°C until purification. RNA was extracted according to the Trizol (Ambion, Austin, TX) protocol, the aqueous layer was extracted again with acid phenol-chloroform (Ambion) in phase-lock tubes (5 Prime). The aqueous layer from this extraction was decanted and precipitated with isopropanol. The RNA was then treated with TurboDNase (Ambion) for 4 hours at 37°C. Dnase Inactivating Reagent (Ambion) was used to remove DNase. 0.375 μg of RNA from each sample was used in a 15μl reverse transcription reaction with the iScript cDNA synthesis kit (BioRad) according to protocol. 4μl of the RT reactions was used in each 10μl qRT-PCR reaction. qRT-PCR was performed according to protocol using SsoFast EvaGreen Supermix (BioRad). The following primers were used to quantify 16S rRNA transcription from the pP16Slac plasmid: 5’-cgaaagggagttgcatcg-3’ and 5’cgtgcatctgccagtttg-3’. RuvA (gene number *CCNA_03345*) was used as an endogenous control, with the following primers: 5’-cgagtgaggaagccgtagag-3’ and 5’-gaccctgttgcacatcgag-3’. A relative starting quantity (SQ) of transcript was calculated by the Biorad CFX Manager software from a standard curve. The SQ value for the 16S amplicon was divided by the SQ value from the *ruvA* amplicon for each sample. The resulting ratio was the value recorded as “Relative transcript level” for each sample.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

C.B. was supported by National Institutes of Health (NIH) Training Grant 5T32GM007183-34. S.C. acknowledges support for this project from the Arnold and Mabel Beckman Foundation (BYI), and the Mallinckrodt Foundation. We thank Ling Chan for advice and guidance with many of the experiments, and the Ira Wool Lab for the use of centrifuges. We also thank Melissa Marks for critical feedback and Emmanuelle Bouveret (LISM, CNRS-Marseille) and Aretha Fiebig for critical readings of the manuscript. Patrick Viollier (Université de Genève) generously provided a *C. crescentus* transposon insertion collection from which we isolated several amino acid auxotrophs.

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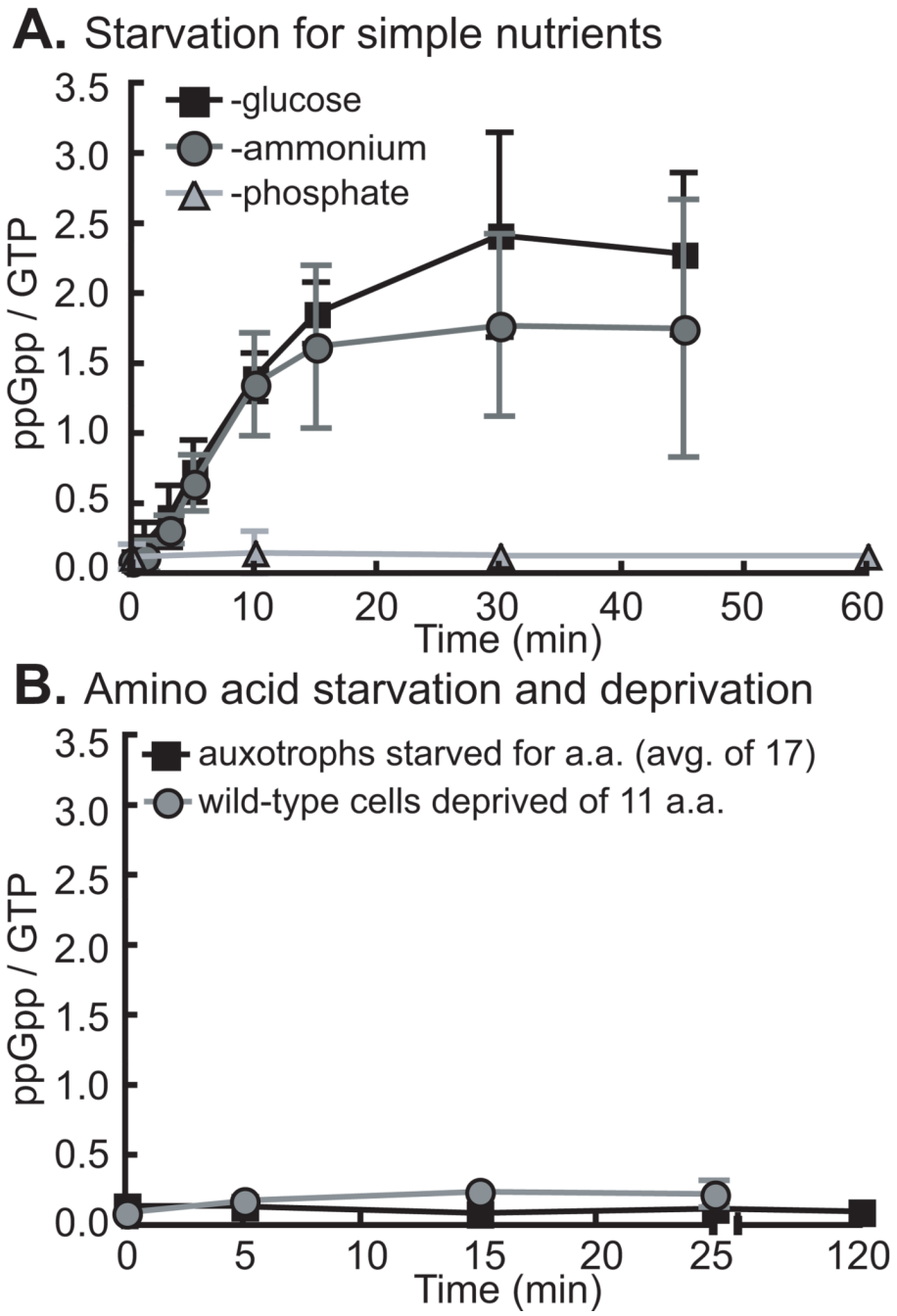

Figure 1. SpoT$_{CC}$ responds to glucose and ammonium, but not amino acid or phosphate starvation

A) ppGpp accumulation in wild-type cells in glucose, ammonium and phosphate starvation. For glucose and ammonium starvation, n=4; for phosphate starvation, n=2. B) ppGpp production in a series of auxotrophic strains starved for their respective amino acids at 5 minute and 120 minute time points. Data for separate auxotrophic strains starved individually for 17 different amino acids are similar and have been averaged (black line). ppGpp starvation curves for individual amino acid auxotrophs are presented in Figure S1. Also, presented, ppGpp accumulation in wild-type cells cultured in the presence of all 11

non-inhibitory amino acids and then simultaneously deprived of these amino acids (gray line; N=2). Error bars refer to standard deviation for all experiments.

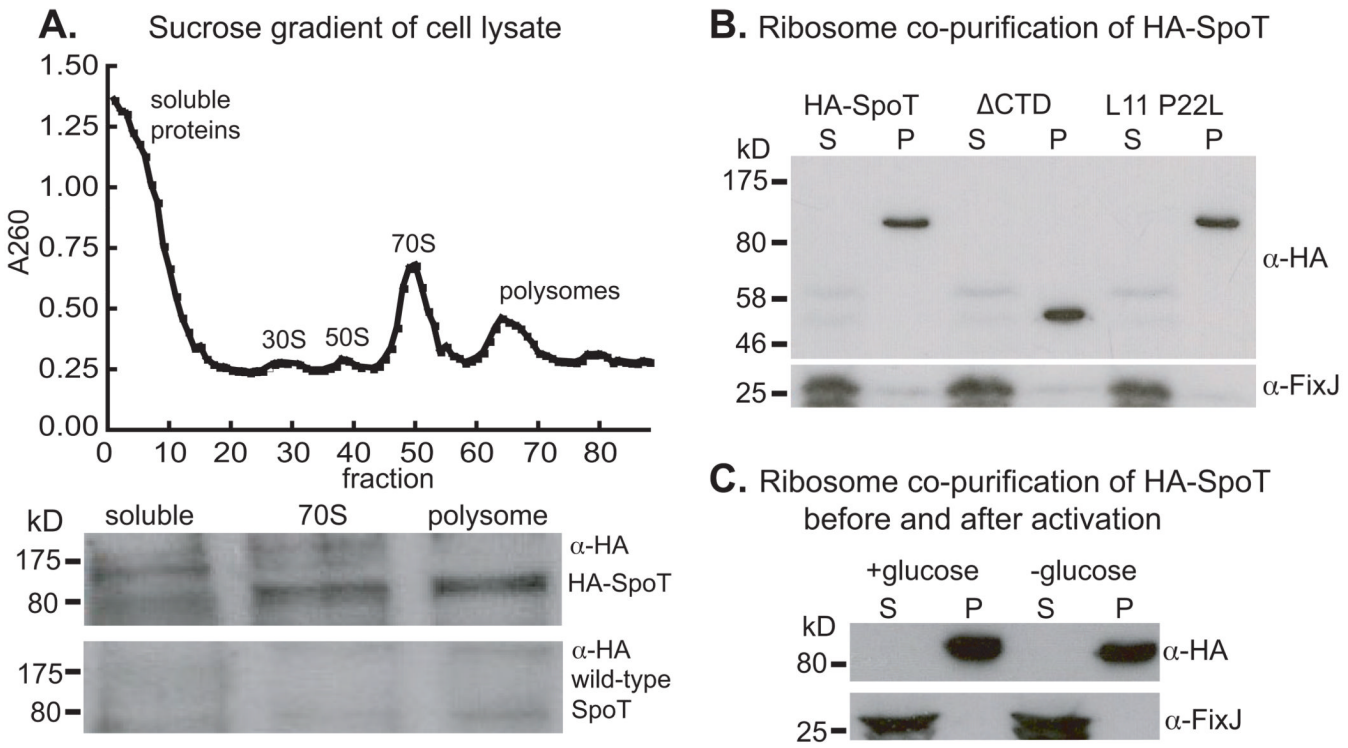

Figure 2. SpoT$_{CC}$ associates with the ribosome

A) sucrose-gradient polysome profile (top) and western blot (bottom) of pooled fractions from the soluble, 70S and polysome peaks. The top blot strip is from the HA-SpoT strain, the bottom strip is from the wild-type strain, which serves as a negative control. B) Western blots of supernatant (S) and pellet (P) fractions from cell lysates spun through a 1M sucrose cushion to separate the ribosomes from other cell constituents (See Figure S2). HA-SpoT co-purifies with the ribosome when the C-terminal domains are removed (ΔCTD) and in the L11 P22L ribosomal mutant. C) HA-SpoT co-purifies with the ribosome in M2G and after 15 minutes of glucose starvation in M2. FixJ is presented as a non-ribosome-associated control.

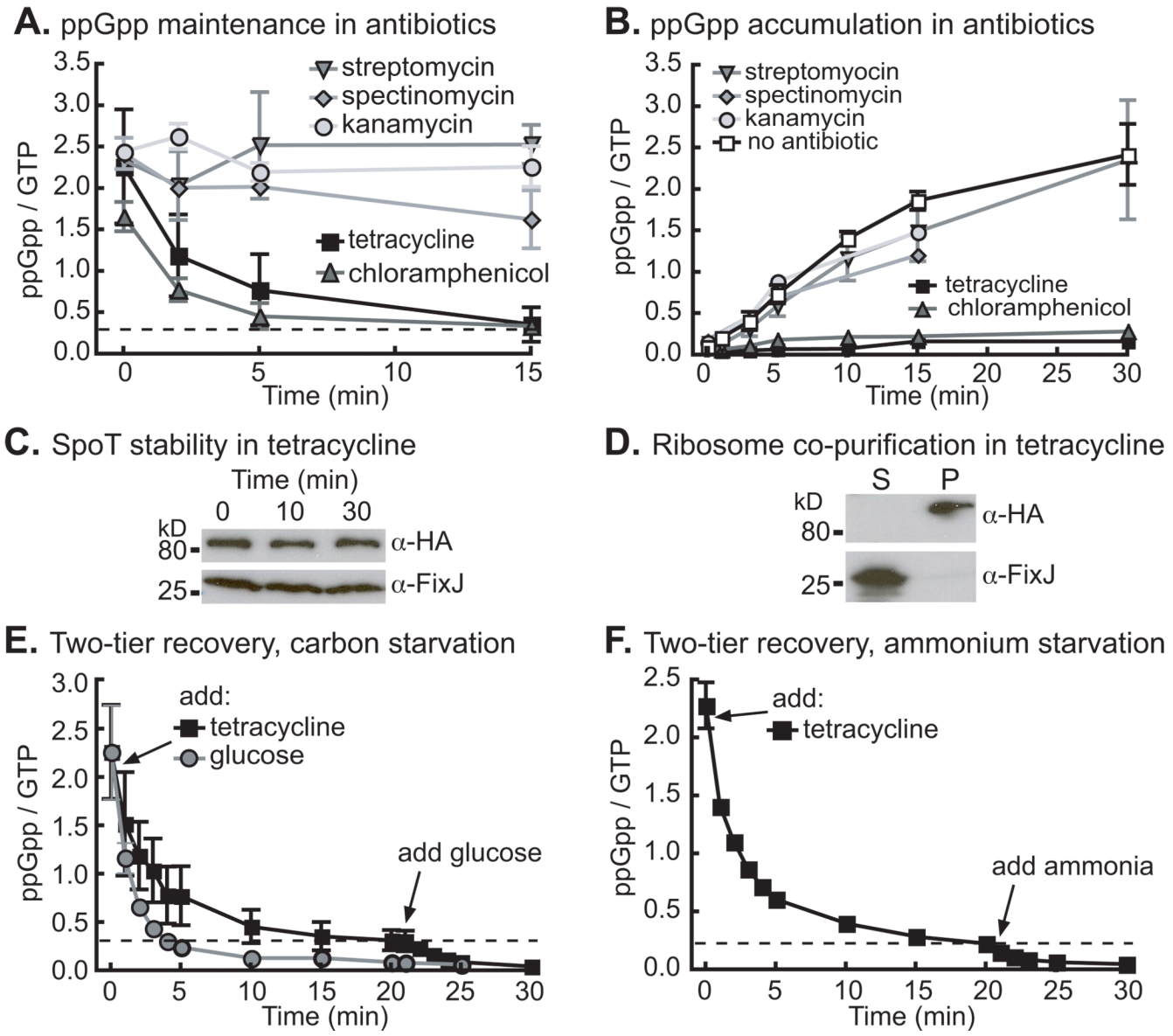

Figure 3. SpoT$_{CC}$ response to starvation is abrogated by certain ribosome poisons

A) ppGpp decay in wild-type cells starved for glucose for two hours and then treated with chloramphenicol (50 μg/ml), tetracycline (50 μg/ml), kanamycin (250 μg/ml), spectinomycin (1250 μg/ml) or streptomycin (50 μg/ml). B) ppGpp accumulation in wild-type cells grown in M2G, and then washed and resuspended in M2 without glucose and with an antibiotic at the same concentration as in A. C) Western blot of HA-SpoT culture aliquots taken before (0) and after 50 μg/ml tetracycline was added to the culture. D) Western blots of supernatant (S) and pellet (P) fraction from an HA-SpoT culture that was treated with 50 μg/ml tetracycline for 15 minutes before being lysed and separated through a sucrose cushion. FixJ is a non-ribosome-associated control. E) ppGpp decay in wild-type cells starved for glucose for two hours and then treated with 50 μg/ml tetracycline or 0.2% glucose for 20 minutes: the tetracycline-treated cells were then treated with 0.2% glucose and analyzed for ppGpp content for an additional 10 minutes. F) ppGpp decay in wild-type cells starved for ammonium for two hours, treated with 50 μg/ml tetracycline for 20

minutes, then treated with 9.3 mM NH_4Cl and analyzed for ppGpp content for an additional 10 minutes. N=2 for each experiment, error bars refer to the standard deviation.

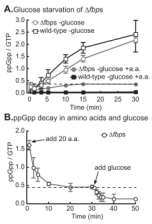

Figure 4. Glucose starvation in the presence of amino acids does not induce significant ppGpp accumulation

A) ppGpp accumulation in Δ*fbp*s (glucose auxotroph) and wild-type cells starved for glucose with all 20 amino acids supplemented. Cells were grown in M2G with all 20 amino acids supplemented at 100 μg/ml each. Cells were then washed and resuspended in media with all 20 amino acids but no glucose, and ppGpp measured at times after glucose removal. The data for both strains starved for glucose without amino acid supplementation is included for comparison. B) ppGpp decay in Δ*fbp*s cells starved for glucose for two hours and then treated with addition of all 20 amino acids, after 30 minutes 0.2% glucose was added and further time points were taken. N=2 for each experiment, error bars refer to the standard deviation.

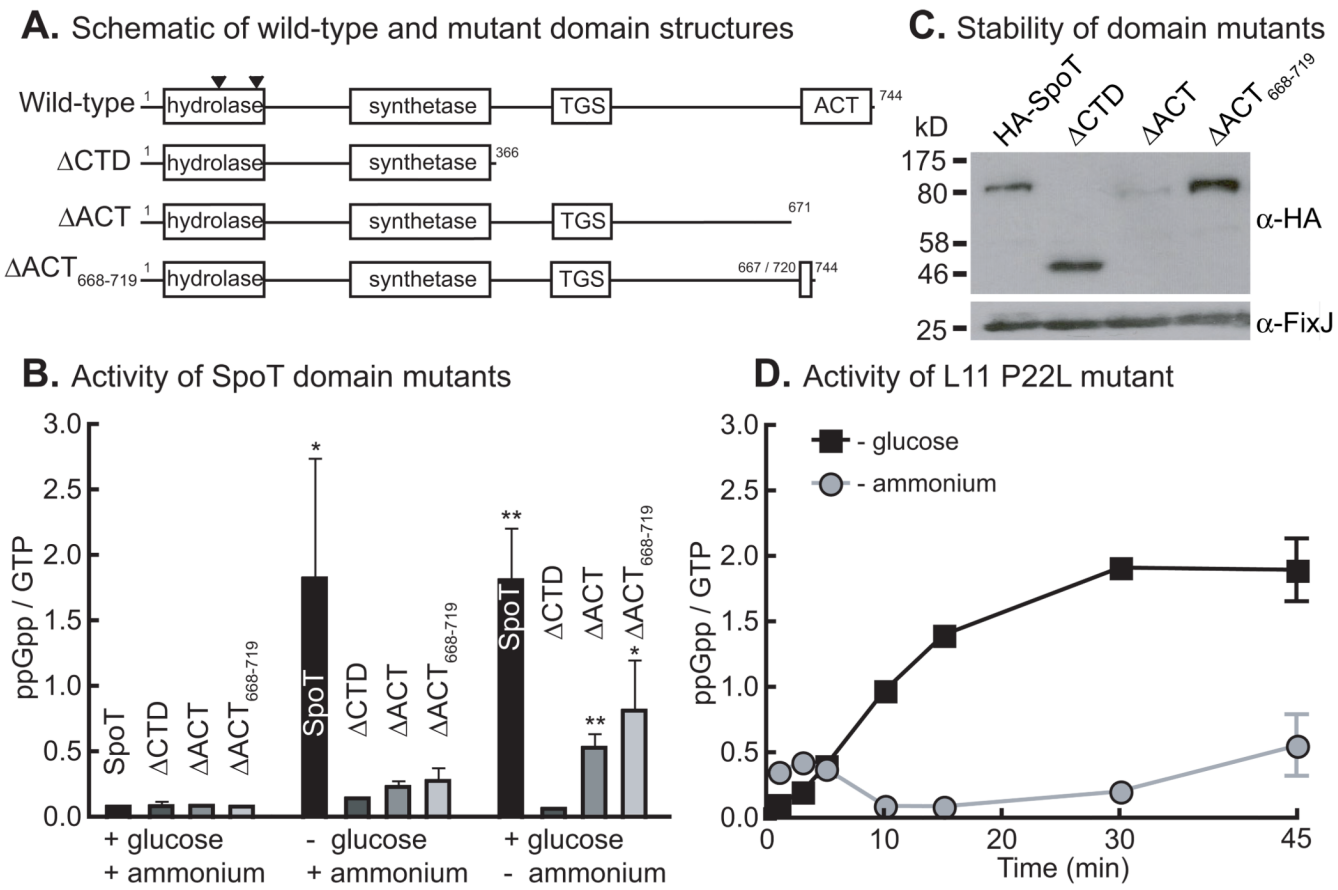

Figure 5. Glucose and ammonium starvation are sensed by different mechanisms

A) Schematic showing domain structure of the full length SpoT protein and domain-deletion alleles. Locations of IS insertion elements found in hydrolase suppressor mutants are indicated by black triangles. (See last section of Results). B) *In vivo* ppGpp accumulation by the wild-type and mutant SpoT proteins in M2G, or after 2 hours of glucose or ammonium starvation. The stars indicate statistically significant difference, by student's t-test, from the ΔCTD strain for each condition where *= $p<0.01$ and **=$p<0.001$. N=3. C) Western blot showing the stability of each domain-deletion protein in cells. N-terminal HA-tagged versions of each mutant were used. FixJ was used as a loading control. D) ppGpp accumulation in an L11 P22L mutant starved for glucose or ammonium. N=2. Error bars refer to standard deviation for all experiments

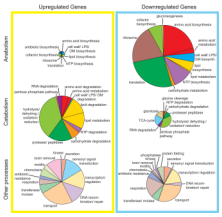

Figure 6. Microarray analysis of genes regulated by *C. crescentus* SpoT$_{CC}$

The genes included in this figure are protein-coding open reading frames that were regulated at least 3-fold in the wild-type versus Δ*spoT*$_{CC}$ Affymetrix data set. The size of each pie is proportional to the number of genes in that category. In total, 379 genes were upregulated at least 3-fold (17-anabolic, 120-catabolic, 126-other processes, 8-cell cycle/nutrient granule, 108-unknown function), and 382 genes were downregulated at least 3-fold by *spoT* (166-anabolic, 44-catabolic, 94-other processes, 6-cell cycle/nutrient granule, 71-unknown function) (see Table S1). Genes of unknown function and cell cycle/nutrient granule genes in Table 2 are not included in the pie charts

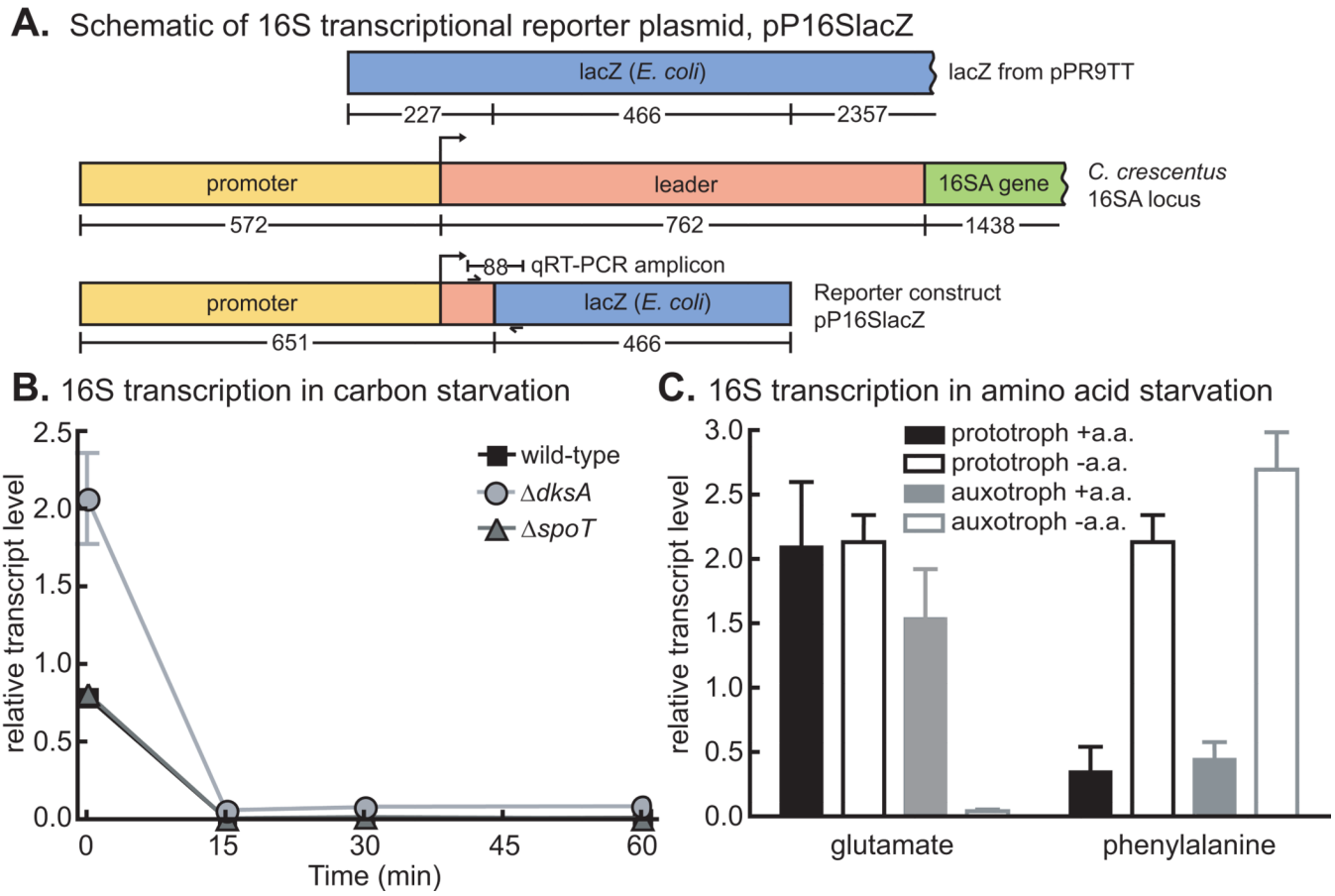

Figure 7. rRNA transcriptional control

A) Schematic of 16S transcriptional reporter plasmid, which produces an unstable transcript from the 16S A rRNA promoter. Numbers below genes represent the number of nucleotides. Location of qRT-PCR amplicon primers are indicated by small arrows. B) 16S rRNA promoter activity in wild-type, Δ*spoT* and Δ*dksA* strains before (0 minutes) and during an hour of glucose starvation. N=3. C) 16S rRNA promoter activity in the prototrophic strain (NA1000 pxyl::pP16Slacz) and the auxotrophic strains (NA1000 Δ*gltB* pxyl::pP16Slacz and NA1000 Δ*pheA* pxyl::pP16Slacz) in M2G with and without added glutamate or phenylalanine. The cells were grown up in M2G + glu or M2G + phe + ala, and then washed and resuspended in M2G or M2G + ala and grown in those conditions for two hours before the starvation samples were taken. All data were normalized to the signal from the *ruvA* amplicon on the same biological sample (N=3). Error bars refer to standard deviation for all experiments.

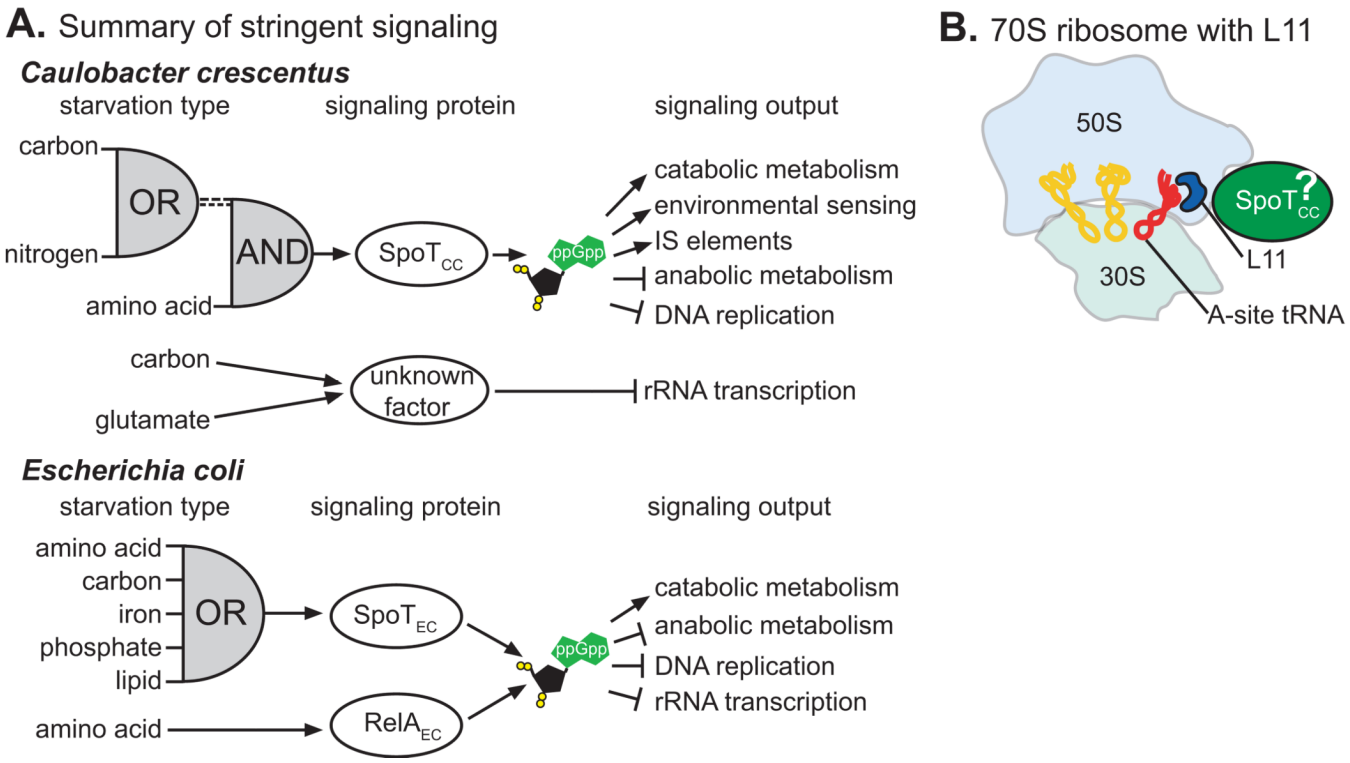

Figure 8. Summary of the regulatory logic of the stringent response in a copiotrophic and an oligotrophic species, and a ribosome model

A) Comparison of the signaling proteins, signaling logic, and signaling output of the stringent response in the oligotroph, *C. crescentus*, and the copiotroph, *E. coli*. The dashed lines from the OR gate of *C. crescentus* represent the different signals that activate the stringent response during carbon and nitrogen starvation. B) Cartoon of the bacterial 70S ribosome. SpoT$_{CC}$ is in green. SpoT$_{CC}$ is presumed to be near the 70S A site and ribosomal protein L11 based on regulatory data presented herein.

Table 1

Strains

FC #	Genotype	CCNA#, Gene altered	Reference
20	*Caulobacter crescentus* NA1000		(Evinger & Agabian, 1977)
769	NA1000 Δ*spoT*	CCNA_01622	This work
1220	NA1000 Δ*pheA*	CCNA_03028	This work
1145	NA1000 *CCNA0559*::hMu	CCNA_00559	(Huitema *et al*, 2006)
1165	NA1000 l*ysA*::mar414	CCNA_02296	(Huitema *et al*, 2006)
1166	NA1000 *serA*::mar414	CCNA_03322	(Huitema *et al*, 2006)
1164	NA1000 *CCNA1419*::mar414	CCNA_01419	(Huitema *et al*, 2006)
1250	NA1000 Δ*gltB*	CCNA_03722	This work
1183	NA1000 *cysE*::hMu	CCNA_02734	(Huitema *et al*, 2006)
1167	NA1000 *asp*::mar414	CCNA_01603	(Huitema *et al*, 2006)
329	NA1000 *thrB*::mar414	CCNA_03475	(Huitema *et al*, 2006)
316	NA1000 *proC*::hMu	CCNA_00528	(Huitema *et al*, 2006)
1163	NA1000 *argB*::hMu	CCNA_00285	(Huitema *et al*, 2006)
1144	NA1000 *ilvB*::tn5	CCNA_02185	(Huitema *et al*, 2006)
314	NA1000 *leuC*::hMu	CCNA_00196	(Huitema *et al*, 2006)
325	NA1000 *trpE*::mar414	CCNA_01972	(Huitema *et al*, 2006)
323	NA1000 *hisD*::mar414	CCNA_02431	(Huitema *et al*, 2006)
943	NA1000 HA-SpoT	CCNA_01622	This work
1105	NA1000 HA-SpoTΔCTD	CCNA_01622	This work
1283	NA1000 HA-SpoT L11 P22L	CCNA_01622,CCNA_00678	This work
1095	NA1000 SpoTΔCTD	CCNA_01622	This work
1010	NA1000 SpoTΔACT	CCNA_01622	This work
772	NA1000 SpoTΔACT(668–719)	CCNA_01622	This work
1019	NA1000 SpoTΔTGS	CCNA_01622	This work
1068	NA1000 HA-SpoTΔACT	CCNA_01622	This work
1155	NA1000 HA-SpoTΔACT(668–719)	CCNA_01622	This work
1083	NA1000 HA-SpoTΔTGS	CCNA_01622	This work
1334	NA1000 L11 P22L	CCNA_00678	This work
1257	NA1000 xylX::pP16Slacz		This work
1258	NA1000 Δ*spoT* xylX::pP16Slacz	CCNA_01622	This work
1304	NA1000 Δ*dksA* xylX::pP16Slacz	CCNA_02663	This work
1259	NA1000 Δ*pheA* xylX::pP16Slacz	CCNA_03028	This work
1261	NA1000 Δ*gltB* xylX::pP16Slacz	CCNA_03722	This work
1397	NA1000 Δ*fbp* Δ*glpX, i.e.*, Δ*fbp*s	CCNA_01448, CCNA_01449	This work

Table 2

Regulated cell cycle and nutrient granule genes

Upregulated genes				
Function	**Gene #**	**Gene name**	**WT/ΔspoT**	**References**
cell division	CCNA_02635	Cell division protein ftsW	4.23	(Mercer & Weiss, 2002)
PHB degradation	CCNA_00250	Poly(3-hydroxyalkanoate) depolymerase	3.04	(Karp *et al*., 1999, Romero *et al*., 2001)
PHB metabolism	CCNA_00544	Acetyl-CoA acetyltransferase	4.32	(Karp *et al*., 1999, Romero *et al*., 2001)
PHB metabolism	CCNA_00545	acetoacetyl-CoA reductase	3.45	(Karp *et al*., 1999, Romero *et al*., 2001)
PHB metabolism	CCNA_01444	Poly(3-hydroxyalkanoate) polymerase	3.03	(Karp *et al*., 1999, Romero *et al*., 2001)
PHB metabolism	CCNA_03293	Enoyl-CoA hydratase	5.28	(Karp *et al*., 1999, Romero *et al*., 2001)
polyphosphate synthesis	CCNA_03529	Polyphosphate kinase	7.86	(Karp *et al*., 1999, Romero *et al*., 2001)
replication inhibition	CCNA_01451	Cold shock protein cspD	3.78	(Yamanaka *et al*., 2001)

Downregulated genes				
Function	**Gene #**	**Gene name**	**WT/ΔspoT**	**References**
cell division	CCNA_03792	Intracellular septation protein	0.33	(Siomoin *et al*., 1996)
cell division inhibition	CCNA_02427	Septum formation protein Maf	0.26	(Butler *et al*., 1993)
DNA methylation/ cell cycle	CCNA_00382	modification methylase CcrMI	0.16	(Collier *et al*., 2007, Reisenauer & Shapiro, 2002)
polyphosphatase	CCNA_01780	Exopolyphosphatase	0.06	(Karp *et al*., 1999, Romero *et al*., 2001)
replication	CCNA_01596	DNA helicase II	0.04	(Lesley & Shapiro, 2008)
replication	CCNA_03701	Integration host factor beta-subunit	0.19	(Lesley & Shapiro, 2008)