Transcriptional occlusion caused by overlapping promoters

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RpoS (σ^{38}) is required for cell survival under stress conditions, but it can inhibit growth if produced inappropriately and, consequently, its production and activity are elaborately regulated. Crl, a transcriptional activator that does not bind DNA, enhances RpoS activity by stimulating the interaction between RpoS and the core polymerase. The *crl* gene has two overlapping promoters, a housekeeping, RpoD-(σ^{70}) dependent promoter, and an RpoN (σ^{54}) promoter that is strongly up-regulated under nitrogen limitation. However, transcription from the RpoN promoter prevents transcription from the RpoD promoter, and the RpoN-dependent transcript lacks a ribosome-binding site. Thus, activation of the RpoN promoter produces a long noncoding RNA that silences *crl* gene expression simply by being made. This elegant and economical mechanism, which allows a near-instantaneous reduction in Crl synthesis without the need for transacting regulatory factors, restrains the activity of RpoS to allow faster growth under nitrogen-limiting conditions.

transcriptional repression | lncRNA

In *Escherichia coli*, RNA polymerase consists of a core, and a σ factor to provide specificity for promoter recognition. RpoD (σ^{70}) is the housekeeping σ factor and is essential for growth (1, 2). Six other σ factors compete for core polymerase with RpoD and regulate specific genes (3). One such factor is RpoS (σ^{38}), the general stress response σ factor (4, 5); another is RpoN (σ^{54}), the nitrogen-stress σ factor (6).

The RpoS regulon provides defense against harsh environmental conditions, and cells that lack RpoS are highly susceptible to stress (7, 8). However, high levels of RpoS can inhibit growth (9). For example, mutants lacking RpoS grow faster on poor carbon sources like succinate (9, 10) and mutants with attenuated RpoS activity have a distinct fitness advantage known as growth advantage in stationary phase (GASP; ref. 11). Bacteria must achieve a fine balance between self-preservation and nutritional competence (SPANC; ref. 12) and for this reason, RpoS is polymorphic in wild and domesticated laboratory strains (9, 13). Not surprisingly, given the functional duality of the protein, RpoS is tightly regulated at all levels: transcription, translation, protein stability, and its activity (5). The regulatory mechanism described here regulates RpoS at yet an additional level by controlling the synthesis of an RpoS activator, Crl.

RpoS has the lowest affinity of all σ factors for core polymerase (14). One of the ways that RpoS can overcome its lack of affinity for the core polymerase is by its interaction with its activator, the prosigma factor Crl. Crl is a transcriptional activator that does not bind DNA (15), rather it biases the competition between sigma factors by stimulating the interaction between RpoS and core polymerase (16–19). Thus, Crl plays an important role in global gene regulation by enhancing RpoS activity (18, 19).

Our efforts to understand how *Escherichia coli* controls the activity of RpoS when protein synthesis is restricted by nitrogen limitation led to the discovery that the *crl* gene produces less protein despite the fact that it is transcribed at a substantially increased rate under these conditions. Unraveling this paradoxical result led to the elucidation of a completely unexpected and unique mechanism of transcriptional regulation. This mechanism has as its basis two overlapping promoters and a long noncoding RNA (lncRNA). Expression of the shorter transcript blocks expression of the longer, but the former cannot be translated because it lacks a ribosome-binding site (*rbs*). This simple, but economical, mechanism allows a near instantaneous reduction in Crl synthesis without the need to produce any transacting regulatory factor.

Results

The Role of Crl Under Nitrogen-Stress Conditions. In rapidly growing cells, RpoS is made but it is directed by the adaptor protein SprE (RssB) to ClpXP where, in ATP-dependent fashion, it is unfolded and rapidly degraded (15, 20, 21). Under carbon starvation conditions, ATP levels drop, proteolysis ceases, and RpoS levels increase 20-fold (22, 23). Under nitrogen-starvation conditions, ATP levels do not drop, proteolysis continues, and RpoS levels increase only twofold (22). Nonetheless, transcription from the *dps* promoter, which strongly depends on RpoS, increases ~30-fold under both conditions (Fig. 1*A*).

Because the effects of Crl on RpoS activity are greatest when RpoS levels are low (16), we wondered whether Crl might be especially important under nitrogen-starvation conditions. To test this possibility, we monitored transcription of the RpoS-dependent gene *dps* in the presence and absence of Crl. As shown in Fig. 1*A*, there is a significant reduction in *dps* transcription in a *crl* null strain under nitrogen-starvation, but not carbon-starvation, conditions. Thus, Crl plays a significant role specifically under nitrogen-starvation conditions. It should be noted that other effectors of σ factor competition,

Significance

Under nitrogen-limiting conditions, the master regulator RpoS protects against stress, but slowed growth is the price cells pay for this protection. Under these conditions, transcription of the gene for the RpoS activator Crl increases. Strikingly, we show that this increase in transcription actually decreases Crl protein levels, thus decreasing RpoS activity and increasing growth rate. This unique regulatory mechanism has as its basis two overlapping promoters. Transcription from the promoter used under nitrogen-limitation prevents transcription of the functional *crl* mRNA producing instead a long noncoding RNA that has no function. Under conditions where protein synthesis is compromised, this simple, but economical mechanism causes a near-instantaneous reduction in Crl levels and RpoS activity without the need to synthesize a regulatory molecule.

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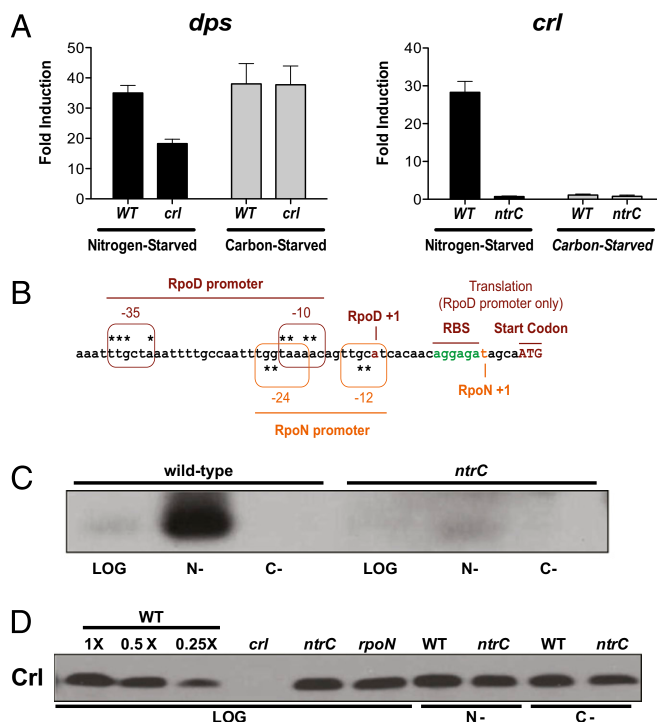

Fig. 1. RpoS activity and *crl* expression under nitrogen-starvation conditions. (*A*) Induction of *dps* (*Left*) and *crl* (*Right*) transcription measured under carbon-starvation and nitrogen-starvation conditions relative to nonstarved cells. Error bars indicate the SEM. (*B*) The *crl* promoter sequence with both RpoD and RpoN promoters are highlighted. The TSSs are indicated with a +1, and the *rbs* is indicated in green; asterisks mark the consensus sequence for each of the promoters. (*C*) Northern blot analysis of *crl* mRNA in wild-type and *ntrC* mutant strains. (*D*) Western analysis of Crl levels in carbon-starved (C-) or nitrogen-starved (N-) and nonstarved cells (LOG).

such as Rsd and 6S RNA, also contribute to *dps* expression under starvation conditions (Fig. S1; ref. 24).

crl Gene Expression Under Nitrogen-Starvation Conditions. The *crl* gene has two functionally overlapping promoters, one that is RpoD-dependent and another that depends on RpoN (Fig. 1*B*). RpoN-containing polymerase always requires a transcriptional activator, a role played by nitrogen regulatory protein C (NtrC) under nitrogen-stress conditions. Because Crl has an RpoN-dependent promoter, and as it played an active role at the *dps* promoter under nitrogen starvation, we hypothesized that it may be up-regulated in an RpoN/NtrC-dependent manner. Indeed, there was more than a 20-fold increase in *crl* transcript abundance under nitrogen starvation as observed by quantitative real-time PCR (qRT-PCR) (Fig. 1*A*), a result confirmed by Northern blot hybridization (Fig. 1*C*), and this increase was abolished by removal of NtrC.

We expected that a 20-fold increase in mRNA would yield a substantial increase in Crl protein levels. Strikingly, we did not observe any change in Crl protein levels either in log phase in wild-type, *rpoN*, or *ntrC* mutant cells or in starved wild-type or *ntrC* mutant cells (Fig. 1*D*). Because Crl is a stable protein (Fig. S2), it would appear that the synthesis of Crl is not increased under these conditions.

crl Gene Expression Under Nitrogen-Limiting Conditions. Cells starved for nitrogen stop growing quickly, thus limiting meaningful physiological experimentation. To probe the paradoxical disconnect between *crl* transcription and translation in more detail, we introduced a constant level of nitrogen stress by growing cells at a steady state in media containing the poor nitrogen source arginine. We denote this nitrogen-stress medium as M63 (Arg^{+}) in contrast to the typical M63 medium that contains the preferred nitrogen source ammonia. Arginine is catabolized via the *ast* pathway, and expression of these genes is induced by nitrogen stress in RpoN/NtrC-dependent fashion (25, 26).

Under steady-state growth conditions in M63 (Arg^{+}) media, *dps* transcription increased to a similar level (~30-fold) as observed under nitrogen starvation. This increase completely depended on RpoS, and it was significantly lowered in strains lacking Crl (Fig. 2*A*). Transcription of *crl* was also increased, albeit more modestly, threefold (Fig. 2*A*), than observed under nitrogen starvation. However, under nitrogen-limiting conditions, we could measure Crl protein levels under steady-state growth conditions, a more meaningful measure of Crl protein levels than was possible with starved, nongrowing cells. Surprisingly, although *crl* transcript levels are increased threefold, we find that Crl protein levels are actually decreased threefold (Fig. 2*B*). Note that under these conditions, increased transcription of *crl* also is accompanied by decreased levels of Crl protein in strain MG1655, demonstrating that this surprising result is not strain specific (Fig. S3).

The Role of the RpoN-Dependent _crl_ Transcript. As mentioned earlier, *crl* has two overlapping promoters (Fig. 1*B*): One is RpoD dependent, and the other is RpoN dependent. RpoN is known to bind to the *crl* promoter element (27, 28), and the transcription start sites (TSS) have been determined for both promoters. The RpoD-dependent TSS is upstream from a conserved ribosome-binding site (*rbs*; ref. 29), and the mRNA is translated efficiently as evidenced by the amount of Crl present in nonstressed cells (Figs. 1*D* and 2*B*). However, the RpoN-dependent TSS is further downstream and lacks an *rbs* (30). Transcripts that begin with the initiation codon (leaderless mRNA) are still translated, but the RpoN-dependent *crl* transcript has 5 bp upstream of the ATG, and such transcripts are known to be very poorly translated (31). Thus, it appears that the RpoN-dependent transcript was selected to be translated poorly, if at all, and this missing *rbs* likely

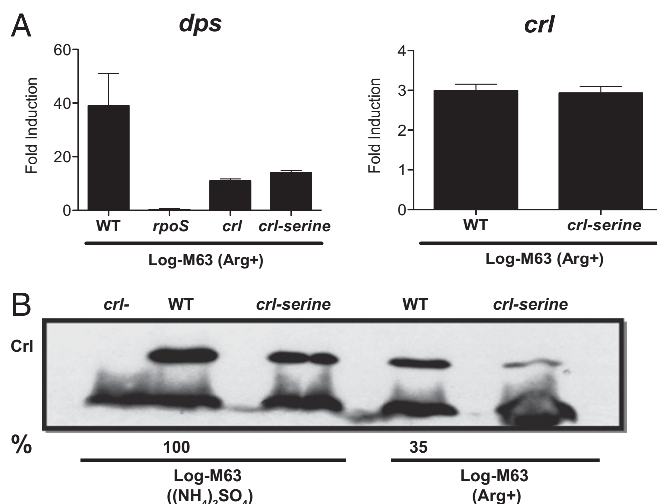

Fig. 2. RpoS activity and *crl* expression under nitrogen-limiting, M63 (Arg+), conditions. (*A*) Induction of *dps* (*Left*) and *crl* (*Right*) transcription measured under nitrogen-limiting conditions relative to nonstarved cells. Error bars represent the SEM. (*B*) Western blot analysis of Crl and Crl-serine levels in nitrogen-limited and nonlimited cells. Shown is a representative of four independent experiments. Relative differences in Crl levels, as calculated by ImageJ, are shown as percentage of WT. The average difference in Crl levels between the two conditions in the WT strain is threefold.

explains the disconnect between *crl* mRNA and protein levels under nitrogen stress conditions. What is the purpose of the RpoN-dependent *crl* transcript if not to make protein?

We have considered the possibility that the *crl* transcript, or some part of it, functions as a regulatory RNA. Northern blot hybridizations do not detect the small differences in the RpoD- and RpoN-dependent transcripts, and they provide no evidence for RNA processing (Fig. 1*C*). More revealing are studies with a *crl* mutant in which the three cysteine codons are changed to serine (Crl-serine). This mutant gene is transcribed normally (Fig. 2*A*), but the mutant protein is inactive and somewhat unstable (Fig. 2*B*). Strains carrying this *crl-serine* mutant gene behave like mutants carrying a *crl* deletion; although the *crl* transcript is made at normal levels, RpoS activity is not enhanced (Fig. 2*A*), suggesting that the transcript has no functional relevance.

We propose instead that the RpoN polymerase has a negative regulatory role at the *crl* promoter functioning in a manner analogous to a traditional repressor at an RpoD-dependent promoter (Fig. 3). To test this possibility, we cloned the *crl* gene with its promoters and upstream elements into a low copy plasmid, *pZS*11* (32). When this plasmid is introduced into a *crl* deletion strain, we see a fivefold increase in the *crl* transcript under nitrogen-limiting conditions (Fig. 4*A*) and a threefold decrease in Crl protein levels (Fig. 4*B*). Using site-directed mutagenesis, we replaced the conserved GC sequence in the -12 element of the RpoN promoter with AT (GC-12AT).

Although the GC-12AT change does not completely abolish the increased *crl* transcription seen under nitrogen-limiting conditions, it clearly reduces it (Fig. 4*A*). However, this decrease in transcription allows a significant increase in Crl protein levels returning them to levels similar to that seen in nonstressed cells (Fig. 4*B*). These results argue strongly that the RpoN polymerase itself is

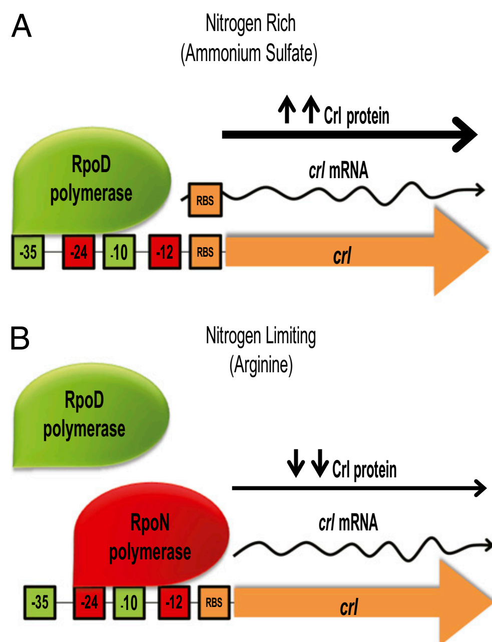

Fig. 3. Transcriptional occlusion at *crl*. (*A*) Under nitrogen-rich conditions, the transcript made by the RpoD polymerase (green) is translated well, resulting in high Crl levels. (*B*) Under nitrogen-limiting conditions the RpoN polymerase (red) acts as a repressor occluding the RpoD polymerase and makes a shorter *crl* transcript that lacks an *rbs*, thus inhibiting Crl synthesis and reducing Crl levels.

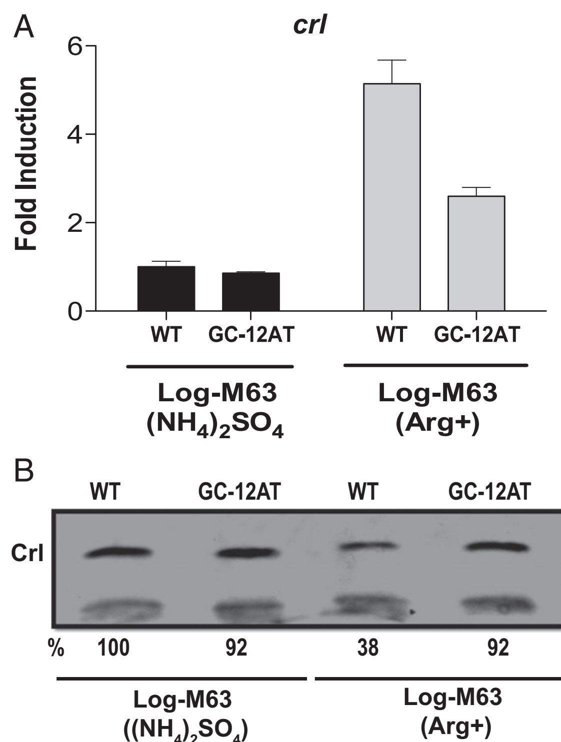

Fig. 4. The effect of the RpoN promoter mutation GC-12AT on *crl* expression. (*A*) *crl* transcription was measured in exponential phase *crl* null strains carrying plasmid *pZS*11crl-1000* with and without the GC-12AT promoter mutation. Data are presented as relative induction over cells carrying the wild-type *crl* plasmid grown in complete minimal media. Error bars represent the SEM. (*B*) Western blot analysis of Crl levels in nitrogen-limited and nonlimited cells. Shown is a representative of four independent experiments. Differences in Crl levels, as calculated by ImageJ, are shown as percentage of WT.

responsible for both the increase in *crl* transcription and the decrease in Crl protein levels. We suggest that the reduced occupancy of the RpoN promoter caused by the GC-12AT mutations relieves repression of the RpoD promoter, allowing for increased transcription of an mRNA with a functional *rbs*. The net result is an increase of Crl protein levels.

The Physiological Role of the RpoN Promoter. The Jekyll and Hyde nature of RpoS provides a convenient way for us to test the physiological relevance of the RpoN-dependent *crl* promoter. RpoS is beneficial to cells growing under nitrogen-stress conditions because it protects them from a variety of different stresses. Cells lacking RpoS are far more sensitive to high temperature than are their wild-type counterparts (Fig. S4). However, this protection comes at a price. As shown in Fig. 5*A*, wild-type strains grow poorly in M63 (Arg^+) media and under these conditions, removing RpoS improves growth dramatically.

Strains lacking Crl or producing the inactive Crl-serine mutant protein grow better than wild type and, as expected, the effect of removing Crl is not additive with the removal of RpoS. Fig. 5*B* shows that introducing the low-copy *crl* plasmid into strain lacking Crl slows growth. However, the same plasmid carrying the RpoN GC-12AT promoter mutation, which allows production of more Crl protein, slows growth even more: The generation time is more than 100 min longer. Under nitrogen-limiting conditions, growth is inversely proportional to RpoS activity and the higher the levels of Crl, the greater the RpoS activity. When active, the RpoN promoter decreases Crl levels, thus decreasing RpoS activity and increasing growth rate.

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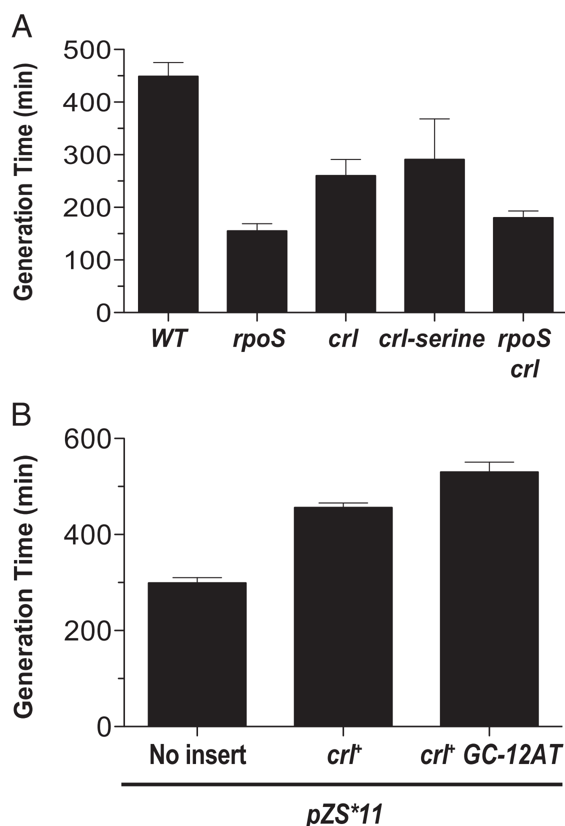

Fig. 5. Generation times under nitrogen-limiting, M63 (Arg+), conditions. Generation time for MC4100 and *rpoS::kan* mutant in M63 minimal media supplemented with 0.4% glucose was determined to be ∼150 min. (*A*) Wild-type strains carrying the indicated mutations. (*B*) *crl* null strains carrying the indicated plasmids. Error bars represent the SEM.

Discussion

We have discovered a unique mechanism that regulates Crl, a regulator of RpoS, allowing precise control of the activity of this general stress response σ factor, and thus growth rate, under nitrogen-stress conditions. Our data indicate that the RpoN-dependent *crl* transcript, which lacks an *rbs*, is a lncRNA. This lncRNA has no transacting function. Rather, the RpoN polymerase that makes this lncRNA serves as a transcriptional repressor to occlude the housekeeping promoter and prevent production of the translatable *crl* mRNA.

A regulatory mechanism that produces an lncRNA might at first seem wasteful. However, protein synthesis requires far more energy input than does RNA synthesis (33). Moreover, in nitrogen-limited cells, where amino acids are limiting, the toll taken on cellular resources by protein synthesis becomes even greater (34–36). The transcriptional occlusion mechanism operating at *crl* is ideally suited for operation under these conditions. First, it is economical because it does not require the synthesis of a repressor protein. Second, it reduces Crl synthesis in near-instantaneous fashion, even faster than a transacting sRNA could act. As soon as the RpoN polymerase is activated, synthesis of the productive *crl* mRNA is repressed, thus further conserving valuable cellular resources.

There are many examples of regulatory, nonprotein coding RNAs in bacteria (37–40). Most bacterial regulatory RNAs are small RNAs (sRNAs) 50–150 bp in length. These sRNAs often regulate translation by base pairing with mRNAs. There are longer noncoding regulatory RNAs, such as RNAIII in several Gram-positive bacteria, which are similar in size to the *crl* lncRNA (∼400 bp; refs. 36 and 38). However, what makes the *crl* lncRNA special and different from all of these prokaryotic regulatory RNAs is functionality. Unlike these RNAs, the *crl* lncRNA has no transacting function.

In mammalian cells, there are many regulatory lncRNAs. Most of these eukaryotic lncRNAs function *in trans* to silence gene expression directly. However, for at least one of these lncRNAs, *Airn*, the gene silencing mechanism is different (41). Like the RpoN-dependent prokaryotic *crl* lncRNA, the mammalian *Airn* lncRNA is nonfunctional; it is the act of making these lncRNAs, not the lncRNA themselves, that is responsible for gene silencing.

The transcriptional occlusion mechanism described has as its basis overlapping promoters. At *crl*, the overlapping promoters produce similarly sized mRNAs, but only one of the resulting transcripts has an *rbs* and can be translated. There are many overlapping promoters in bacteria, and in most cases, the functional significance of this overlap is not yet known (42, 43). No other case, where one of the overlapping promoters would produce an *mRNA* without an *rbs*, has been reported. However, there may well be examples where one of the mRNAs is translated more efficiently than the other or where only one of the mRNAs is sensitive to a transacting sRNA. We suggest that the regulatory potential of promoter overlap may be largely unappreciated.

Experimental Procedures

Media and Growth Conditions. All strains and plasmids are listed in Table S1. Standard microbial techniques were used for strain constructions. Rich media (LB) and M63 liquid medium and agar were supplemented with appropriate antibiotics as needed (44). M63 media with 0.2% arginine and 0.4% glucose was used with arginine as a sole nitrogen source. For sudden starvation, a single colony was grown overnight in 5 mL of M63 minimal media supplemented with thiamine (final concentration 100 μg/mL), $MgSO_4$ (1 mM), and glucose (0.4%). Overnight cultures were diluted 1:100 in M63 media and grown at 37 °C to early log-phase OD_{600} ∼0.3 (3 h). Cells were collected, washed three times with media lacking the appropriate carbon or nitrogen source, and finally resuspended in M63 media lacking in either carbon or nitrogen source. They were then incubated on a rotor for 1 h at 37 °C. For generation time experiments, overnight cultures in M63 media supplemented with glucose were diluted 1:100 in fresh M63 (Arg^+) growth media. Cultures were grown at 37 °C. Aliquots were removed at 10-, 14-, 16-, and 20-h time interval and plated on LB plates to determine CFUs. For reference, the generation time for MC4100 in M63 media with glucose is approximately 150 min.

Plasmid Construction. For construction of *pZS*11crl-1000* (low copy 3–5 copies per cell), colony PCR was performed on MC4100 cells with primers that amplify the *crl* ORF and 1,000 bases upstream of the Crl ATG, so that the plasmid construct had native *crl* promoter, activator, and ribosomal binding site present. The primers pZS-XbaI-crl-1000upstream and pZS-KpnI-Crl-c-terminal were used for cloning (Table S2). The *crl* construct was put in reverse orientation so that expression was only driven by the *crl* promoter and not by the *tet* promoter. After digestion with KpnI and XbaI, the resulting PCR product was ligated into the KpnI and XbaI sites of *pZS*11* (32). Constructs were sequenced by Genewiz with primer Crl-200F.

Site-Directed Mutagenesis. Plasmids were mutagenized by using the Gene Tailor Site-Directed Mutagenesis system (Invitrogen) according to the manufacturers' instructions. The GC-12AT was introduced into the plasmid *pZS*11crl-1000* by using primers Crl(+)gc-AT and Crl(-)gc-AT. Mutagenized plasmids were transformed into DH5α. Plasmids were extracted by using Qiagen plasmid kit as per manufacturers' instructions and sequenced by Genewiz using primer Crl-200F.

λ Red-Mediated Recombination for Strain Construction. Recombination using a single-stranded oligonucleotide was carried out as described (45). To construct the $crl_{C28S\ C37S\ C41S}$ allele (MJM372), the oligo (MJM335ss) was used. RFLP analyses were carried out for loss of an *Hpy*CH4V site at codon 28.

qRT-PCR. The qRT-PCR assay was carried out as described (46). Five microliters of the 1×, 0.1×, or 0.01× concentrations of cDNA for standards and 5 μL of 0.1× for all other samples were added to MicroAmp Optical 96-well reaction plates. SYBR Green PCR master mix supplied by Applied Biosystems was used

in each reaction. Samples were run on an ABI Prism 7900 HT real-time PCR system, and data was analyzed (standard curve method) by using SDS software (v.2.1, Applied Biosystems). The *ompA* transcript was used as an internal control to adjust for differing amounts of input cDNA. All reactions were run in triplicates, and each experiment was repeated at least three times.

We examined the expression of a number of genes in the RpoS regulon that are induced under nitrogen-starvation or nitrogen-limiting conditions, including *bolA*, *tktB*, *osmY*, *poxB*, and *dps*. We show results with *dps* because its induction was most strongly dependent on RpoS (Fig. 2*A*).

Gel Electrophoresis and Analyses. Western and Northern blot analyses were performed as described (22, 47).

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