Nitrogen Starvation Induces Persister Cell Formation in *Escherichia coli*

Daniel R. Brown^a

^aMRC Centre for Molecular Bacteriology and Infection, Imperial College London, London, United Kingdom

ABSTRACT To cope with fluctuations in their environment, bacteria have evolved multiple adaptive stress responses. One such response is the nitrogen regulation stress response, which allows bacteria, such as *Escherichia coli*, to cope with and overcome conditions of nitrogen limitation. This response is directed by the two-component system NtrBC, where NtrC acts as the major transcriptional regulator to activate the expression of genes to mount the response. Recently, my colleagues and I showed that NtrC directly regulates the expression of the *relA* gene, the major (p)ppGpp synthetase in *E. coli*, coupling the nitrogen regulation stress and stringent responses. As elevated levels of (p)ppGpp have been implicated in the formation of persister cells, here, I investigated whether nitrogen starvation promotes their formation and whether the NtrC-RelA regulatory cascade plays a role. The results reveal that nitrogen-starved *E. coli* synthesizes (p)ppGpp and forms a higher percentage of persister cells than nonstarved cells and that both NtrC and RelA are important for these processes. This study provides novel insights into how the formation of persisters can be promoted in response to a nutritional stress.

IMPORTANCE Bacteria often reside in environments where nutrient availability is scarce; therefore, they have evolved adaptive responses to rapidly cope with conditions of feast and famine. Understanding the mechanisms that underpin the regulation of how bacteria cope with this stress is a fundamentally important question in the wider context of understanding the biology of the bacterial cell and bacterial pathogenesis. Two major adaptive mechanisms to cope with starvation are the nitrogen regulation (ntr) stress and stringent responses. Here, I describe how these bacterial stress responses are coordinated under conditions of nitrogen starvation to promote the formation of antibiotic-tolerant persister cells by elevating levels of the secondary messenger (p)ppGpp.

KEYWORDS (p)ppGpp, *Escherichia coli*, nitrogen starvation, persister cell, transcription regulation

The survival and proliferation of bacteria require them to adapt to the fluctuating availability of macronutrients and micronutrients. To overcome these challenges, bacteria have evolved strategies in the form of adaptive stress responses to sustain growth on nonoptimal sources of nutrients, outcompete others for available resources, or enter a state of metabolic quiescence to survive until conditions improve. One such response is the nitrogen regulation (ntr) stress response, which allows *Escherichia coli* and related bacteria to cope with conditions of nitrogen starvation (1, 2). Nitrogen (N) is an essential element for life, as numerous cellular macromolecules contain nitrogen, including proteins, nucleic acids, and cell wall components. This adaptive response is coordinated by the two-component system NtrBC. NtrB is the sensor histidine kinase that senses and then transduces the nitrogen starvation signal upon which it phosphorylates and thereby activates the DNA binding transcription factor NtrC (3). NtrC is a member of a specialized family of transcriptional activators named the bacterial

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Address to correspondence to drbrown@imperial.ac.uk.

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enhancer binding proteins (bEBPs), which activate the alternative transcription machinery directed by the alternative sigma factor σ^{54} in response to diverse environmental stimuli (4, 5). The *ntr* response allows cells to rapidly sense and adapt to nitrogen limitation; it does this by actively scavenging for alternative nitrogen sources through the transcriptional activation of genes encoding nitrogen source transporters, catabolic enzymes, and amino acid biosynthetic operons (1). The response also induces the further expression of genes to survive N starvation through the action of the dual regulator Nac, the nitrogen accessory control protein (6). Recently, we showed that the least understood NtrC-regulated operon, *yeaGH*, which encodes a putative serine/threonine kinase and has a gene of unknown function, promotes the survival of *E. coli* when faced with sustained N starvation, appearing to act as a molecular brake, dampening the metabolic activity of N-starved *E. coli* (7, 8).

In a previous study, we sought to determine the NtrC regulon in N-starved *E. coli*. This study identified all of the above genes as being under the direct transcriptional control of NtrC (9). Interestingly, this genome-wide analysis also revealed a novel member of the NtrC regulon and uncovered a new branch to the *ntr* response. We discovered that NtrC binds upstream of, and activates, the σ^{54} -dependent transcription of the *relA* gene, which encodes the major (p)ppGpp synthetase in *E. coli* (9). Increased intracellular levels of the secondary messenger (p)ppGpp have pleiotropic effects upon major cellular processes, such as transcription, translation, and DNA replication, to reprogram the cell to cope with the change in the environment, in particular with regard to nutrient starvation (10). This action of (p)ppGpp is collectively known as the stringent response. Our finding illustrated an elegant mechanism in which nitrogen-starved *E. coli* uses the same regulatory mechanism to concurrently activate the transcription of genes to actively scavenge for alternative sources of nitrogen to survive while switching off genes required for exponential growth. The induction of (p)ppGpp has been previously implicated with the increase in levels of so-called persister cells (11–14), which are a small subpopulation of bacteria which can transiently tolerate high levels of antibiotics without being genetically resistant (11, 14). The formation of persisters is induced under a variety of conditions, including during infection (12). They have also been proposed as a reservoir for recurrent infection and are potentially a basis for the development of antibiotic resistance (15). Therefore, understanding the regulation that underpins their formation, maintenance, and resurgence is a key question which has yet to be fully answered. Previous work has shed light upon a multitude of conditions to understand how persisters form, such as different growth phases in rich medium, growth on defined carbon sources, and even the host environment (macrophages) (12, 16–19). In this case, I wanted to investigate whether nitrogen starvation induced the formation of persister cells and whether NtrC-dependent gene expression, in particular that of *relA*, plays a role.

RESULTS AND DISCUSSION

Nitrogen starvation induces an increase in ciprofloxacin-tolerant persisters.

The initial aim of this study was to understand whether *E. coli* subjected to N starvation form elevated levels of antibiotic-tolerant persisters in comparison with nonstressed bacteria. To accomplish this, I used the well-defined nitrogen starvation conditions that we previously established to study the nitrogen stress response (9, 20). To ensure that cells in the nonstressed condition (N+) were not sensing limitation to nitrogen and to ensure they were in a nonstressed state, they were grown in Gutnick minimal medium containing 10 mM NH_4Cl prior to exposure to antibiotics at early log phase. Nitrogen-starved cells (N–) were exposed to antibiotics 20 min post-runout of nitrogen following growth in Gutnick minimal medium containing 3 mM NH_4Cl . Growth rates of the cells are comparable when grown in either 10 mM or 3 mM NH_4Cl , as shown in Fig. 1A. The addition of antibiotics ($>10\times$ MIC; the ciprofloxacin MIC of all strains used in this study was determined to be 0.015 $\mu\text{g/ml}$) at either of these time points (N+ or N–) results in characteristic biphasic kill curves, with an initial fast killing of the susceptible population which then slows over time (Fig. 1B). When comparing the two conditions,

FIG 1 Nitrogen starvation promotes ciprofloxacin-tolerant persister formation. (A) Representative growth curve of NCM3722 wild-type *E. coli* in Gutnick minimal medium containing 3 mM or 10 mM NH₄Cl as the sole N source. Time points used for persister assays are highlighted (N+, nitrogen replete; N– nitrogen starved). (B) Persister assay results showing bacterial survival following N+ or N– challenged with 1 μg/ml ciprofloxacin for 24 h. (C) Persister assay results showing survival of bacteria at 24 h with respect to preantibiotic treatment. Error bars indicate standard deviation from the mean ($n = 6$). (D, right) Autoradiogram to show levels of (p)ppGpp from ³²P-labeled nucleotide extracts at N+ and N– in NCM3722 strains separated by PEI-cellulose thin layer chromatography. Control lanes 1 and 2 show levels of (p)ppGpp in (p)ppGpp-noninducing (–SHX) and (p)ppGpp-inducing (+SHX) conditions. (Left) Growth conditions and time points assayed for (p)ppGpp measurements.

there is an ~20-fold increase in the survival of the N-starved (N–) wild-type cells compared with nonstarved (N+) cells following 24 h of exposure to ciprofloxacin (Fig. 1C). Thus, this result indicates that there is a higher proportion of persisters in the bacterial population subjected to N-starved conditions than the nonstressed bacterial population.

In a previous study, we found that NtrC, the master regulator of the ntr response, regulated the transcription of *relA* in N-starved *E. coli* (9). To show that N starvation leads to the production of RelA-synthesized (p)ppGpp, here I measured (p)ppGpp and levels at nitrogen replete (N+) and nitrogen-starved (N–) conditions. As can clearly be seen in Fig. 1D, (p)ppGpp is produced by the wild-type strain following N starvation but not under N-replete conditions (Fig. 1D, lanes 3 and 4); this is also seen in the control lane (Fig. 1D, lanes 1 and 2) when bacteria are subjected to the serine hydroxamate (SHX) treatment, which is known to induce (p)ppGpp production (11). It is also clear that (p)ppGpp synthesis in N-starved *E. coli* is dependent upon the action of NtrC (encoded by *glnG*), as in the $\Delta glnG$ mutant, (p)ppGpp is not induced (Fig. 1D, lane 6). Further reinforcing these results, (p)ppGpp synthesis is restored in the N-starved complemented mutant expressing *glnG* from a plasmid (Fig. 1D, lane 8). The (p)ppGpp produced under N-starvation conditions appears to be mostly derived from the (p)ppGpp synthetase RelA, as in a $\Delta relA$ mutant, very little (p)ppGpp can be detected at either time point (Fig. 1D, lanes 9 and 10). This result reveals that (p)ppGpp synthesis in N-starved *E. coli* is regulated in an NtrC- and RelA-dependent manner.

NtrC-dependent gene expression promotes persister formation in N-starved *E. coli*. To understand whether persisters formed under N starvation require the action NtrC, next I determined the levels of persisters formed in its mutant, $\Delta glnG$, under the N-starvation growth conditions. Strikingly, despite seemingly similar persister levels to the wild-type under nonstressed conditions (N+), a mutant strain lacking NtrC ($\Delta glnG$)

survives much worse than the wild-type when challenged with ciprofloxacin following N starvation, with ~260-fold fewer persisters formed (comparing N[−], Fig. 1A and 2A; results are summarized in Fig. 2G). Importantly, this phenotype is restored when the *glnG* mutation is complemented via the expression of an inducible copy of *glnG* from a plasmid (comparing N[−], Fig. 1B and 2B; results are summarized in Fig. 2G). This indicates that under N starvation, NtrC-dependent gene expression is important for the formation of ciprofloxacin-tolerant persister cells.

To investigate whether the regulatory pathway that promoted persister formation under N starvation was via genes whose transcription is activated directly by NtrC (σ^{54} dependent) or by genes regulated by the NtrC-adaptor protein Nac (σ^{54} independent), I next determined the ability of a Δ *nac* mutant to form persisters under both N⁺ and N[−] conditions. Figure 2C and F show that following 24 h of exposure to ciprofloxacin, the N-starved Δ *nac* mutant forms similar levels of ciprofloxacin-tolerant persisters to the wild-type strain. This indicates that Nac-regulated genes do not impact persister formation; therefore, genes whose transcription is directly activated by NtrC and σ^{54} are the cause for this phenotype. The σ^{54} mutant does not grow in this minimal medium (see Fig. S1 in the supplemental material, which shows the growth of each of the strains used in this study under the experimental conditions), and the strict reliance on σ^{54} could not be tested; however, as NtrC can only activate transcription utilizing the σ^{54} -RNAP (4, 5), I am confident that σ^{54} -dependent transcription is involved in this regulatory process.

NtrC-activated *relA* is required under N starvation to promote persister formation. As in our previous study, I found that NtrC activates the expression of the *relA* gene in N-starved *E. coli*. Recall that RelA is the major (p)ppGpp synthase under these conditions (Fig. 1D) (9) and that (p)ppGpp has been implicated in formation of persister cells; therefore, it was logical to determine whether the *relA* mutant strain could form persisters following N starvation. The results in Fig. 2D clearly show that the Δ *relA* strain is impaired in persister formation in N-starved *E. coli*. As the RelA produced under these conditions is exclusively controlled by NtrC (9), this study implicates the NtrC-*relA* regulatory cascade, which results in ppGpp synthesis under N starvation (as seen in Fig. 1D), in persister formation.

Previous studies have indicated that (p)ppGpp plays a role in persister formation (11–14). It is also known that an increase in (p)ppGpp levels correlate with the increased stability of the general stress σ factor, σ^{38} , or RpoS and increased levels of RpoS and the gene expression of its regulon (21). RpoS has also been implicated in persister formation, with either mutants lacking *rpoS* or affecting RpoS stability producing both fewer or greater levels of persisters depending on the experimental setup (22–26). Although the gene expression program most associated with nitrogen stress is directed by the Ntr response and NtrC, σ^{54} , and Nac, RpoS-dependent gene expression may also play a role under these conditions. As can be seen in Fig. 2E, RpoS is clearly upregulated, and RpoS-dependent gene expression has been demonstrated to be active under our N-starvation conditions (8). Therefore, I decided to determine whether RpoS plays a role in the formation of N-starvation-induced persisters. However, as can be seen in Fig. 2G, the N-starved Δ *rpoS* mutant forms similar levels of persisters to the wild-type strain, ruling out its role in promoting the formation of persisters under these conditions.

Conclusions. Determining the mechanisms by which bacteria tolerate stress are fundamental in understanding how they live such adaptable lifestyles and manage to occupy such diverse niches. Despite extensive studies to understand what conditions induce the formation, maintenance, and reawakening of antibiotic-tolerant persister cells, the regulatory factors that underpin their formation in response to stressful environments are not fully understood. Previous studies have shown that an increase in (p)ppGpp levels lead to the formation of higher levels of persister cells (11, 13). Results presented here show that N-starved *E. coli* synthesize (p)ppGpp and form elevated levels of ciprofloxacin-tolerant persisters. The action of NtrC and RelA is key for both of these processes, as neither the Δ *glnG* nor the Δ *relA* mutants synthesize

FIG 2 NtrC and RelA are required for the formation of ciprofloxacin-tolerant persisters formed in response to nitrogen starvation. (A to D and F) Persister assay results showing bacterial survival following N+ or N- challenged with 1 μ g/ml ciprofloxacin for 24 h. Strain names are inset on each graph. (E) qPCR and Western blotting to show *rpoS* mRNA and σ^S protein levels at N+ and N-. (G) Persister assay results showing survival of bacteria at 24 h with respect to preantibiotic treatment. Error bars indicate standard deviation from the mean ($n = 3$ for mutants and 6 for wild type).

significant (p)ppGpp or generate persisters in response to N starvation. As under these conditions the transcription of *relA* is exclusively controlled by NtrC (9), the results presented here provide evidence that the NtrC/RelA regulatory cascade, which leads to (p)ppGpp synthesis under these conditions, promotes persister formation. However, it is interesting to note that RpoS, which has been implicated with the formation of persisters, is stabilized through the action of (p)ppGpp, is present under N starvation, and is not required for persisters to be formed under these conditions. Overall, these results display an apparent cross talk between regulatory mechanisms to survive a specific stress, N starvation, and those which allow bacteria to survive high doses of antibiotics. This study further underlines the important role that the alarmone (p)ppGpp plays in the stress tolerance of bacteria. This study also supports the hypothesis that (p)ppGpp is a key player in persister formation; however, the precise mechanism through which it does so remains elusive.

METHODS AND MATERIALS

Bacterial strains, plasmids, and growth conditions. All strains used in this study were derived from *E. coli* NCM3722, a derivative of *E. coli* K-12 strain MG1655. The NCM3722: Δ *glnG* and Δ *relA* strains were constructed as described previously (9, 20); briefly, the knockout *glnG* allele was transduced using the P1vir bacteriophage with JW3839 for *glnG* and JW2755 for *relA* from the Keio collection (27) serving as the donor strain and NCM3722 as the recipient strain. The kanamycin cassette was then cured from the strain using pCP20, leaving an unmarked knockout mutation. The NCM3722 Δ *rpoS* and Δ *nac* strains were constructed in the same way, with *E. coli* JW5437 and JW1967 (Keio collection), respectively, serving as the donor strains (27). A *glnG*-inducible complementing plasmid (pAPT-*glnG*-rbs31) was kindly provided by Wang et al. (28). Bacteria were grown in Gutnick medium (33.8 mM KH₂PO₄, 77.5 mM K₂HPO₄, 5.74 mM K₂SO₄, and 0.41 mM MgSO₄), supplemented with Ho-LE trace elements (29) and 0.4% glucose and containing either 10 mM NH₄Cl (for precultures and N⁺ persister assays) or 3 mM NH₄Cl (runout experiments and N⁻ persister assays) as the sole nitrogen source, at 37°C and 180 rpm. Ammonium concentrations in the medium were determined using the Aquaquant ammonium quantification kit (Merck Millipore, UK), according to the manufacturer's instructions.

Antibodies. For immunoblotting, the commercial mouse monoclonal antibody anti- σ^S (at a dilution of 1/500) (Neoclone, USA) was used as a primary antibody. The primary antibody was used in conjunction with the anti-mouse ECL horseradish peroxidase (HRP)-linked secondary antibody (at a dilution of 1/10,000) and ECL prime (GE Healthcare, UK).

(p)ppGpp measurements. (p)ppGpp levels were measured as described by Schneider et al. (30), with the following modifications. Overnight cultures were prepared in 5-ml potassium morpholinopropane sulfonate (MOPS) minimal medium containing 10 mM NH₄Cl as the sole nitrogen source. Following growth at 37°C with shaking at 180 rpm for 18 h, these precultures were used to inoculate 5-ml MOPS minimal medium containing 3 mM NH₄Cl as the sole nitrogen source at an optical density at 600 nm (OD₆₀₀) of 0.05 in 50-ml conical tubes. Bacteria were grown at 37°C with shaking at 180 rpm, and bacterial growth was monitored by OD₆₀₀ readings. At OD₆₀₀ of 0.1, 20 μ Ci/ml of H₃³²PO₄ was added as a phosphate source. At specified time points (N replete when OD₆₀₀ = 0.3 [N⁺], and 20 min post-N runout [N⁻]) 500- μ l samples were removed and added directly to 100 μ l ice cold 2 M formic acid, and samples were incubated on ice for 30 min prior to centrifugation at 17,000 $\times$ *g* for 5 min. To detect (p)ppGpp production, 10 μ l of the supernatant from each sample was spotted on a polyethylenimine cellulose F (dimensions, 20 by 20 cm; Merck Millipore) thin-layer chromatography (TLC) plate. The spots were then allowed to migrate to the top of the sheet (~70 min) in a TLC tank using 1.5 M KH₂PO₄ (pH 3.6) running buffer. The plate was then air dried before exposure onto a phosphor screen, following which it was viewed using a Typhoon phosphorimager (GE Healthcare). As a positive control, bacterial cultures were grown in the same way; however, 100 μ g/ml serine hydroxymate was added at an OD₆₀₀ of 0.45 to induce (p)ppGpp production and incubated for 10 min, and samples were recovered in the same way as described above.

Persister assays. Persister assays were completed as described in reference 12, with the following modifications. Bacteria were routinely grown as 50-ml cultures under either N⁺ (Gutnick minimal medium containing 10 mM NH₄Cl) or N⁻ (Gutnick minimal medium containing 3 mM NH₄Cl) conditions in 250-ml Erlenmeyer flasks, and growth was monitored via optical density measurements. Once bacteria reached the correct time point (N⁺, OD₆₀₀; N⁻, 20-min post-nitrogen runout, as defined previously [9]), 10-ml aliquots were removed, and bacteria were pelleted and resuspended in 10-ml Gutnick minimal medium, containing 10 mM NH₄Cl supplemented with an antibiotic at a concentration of at least 10 $\times$ MIC (1 μ g/ml ciprofloxacin or 100 μ g/ml ampicillin), in 50-ml conical tubes. Bacteria were then incubated for 24 h at 37°C with shaking at 180 rpm. Samples were taken for serial dilutions and counting of colony forming units (CFU) from the initial cultures (time point, 0 h) and 1, 4, and 24 h of antibiotic exposure. For CFU counting, bacteria were pelleted as 1-ml aliquots, and the supernatants were removed and resuspended in PBS prior to serial dilution, plating, and enumeration.

Determination of MICs. MICs of ciprofloxacin for each strain were determined using an M.I.C. Evaluator test (Oxoid, UK), as described by the manufacturer's instructions. Briefly, bacterial cultures were grown overnight in 5-ml cultures either in LB or Gutnick minimal medium containing 10 mM NH₄Cl. Bacterial suspensions of OD₆₀₀ of 1 were prepared and 100 μ l was spread on Iso-Sensitest agar plates.

An M.I.C. Evaluator strip impregnated with an increasing concentration gradient of ciprofloxacin was added to the plate and incubated at 37°C overnight. The MIC was determined as the lowest concentration of antibiotic that prevented growth.

qPCR. Quantitative real-time PCR (qPCR) was completed as described in reference 9. Briefly, total RNA samples were extracted from cells at specified time points, and RNA was stabilized with the Qiagen RNA protect reagent (Qiagen, USA) and extracted using the PureLink RNA minikit (Ambion Life Technologies, USA). Purified RNA was stored at -80°C in nuclease-free water. cDNA was amplified from 100 ng of RNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems, USA). qPCRs were completed in a final volume of 10 μl (1 μl cDNA, 5 μl TaqMan fast PCR master mix, 0.5 μl TaqMan probe [both Applied Biosystems, USA]). Amplification was performed on an Applied Biosystems 7500 fast real-time machine using the following conditions: 50°C for 5 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Real-time analysis was performed in triplicate on RNA from three independent cultures, and quantification of 16S expression served as an internal control. The relative expression ratios were calculated using the delta-delta method (PerkinElmer, USA). Statistical analysis of data were performed by one-way ANOVA, and a P value of ≤ 0.05 was considered to be a significant difference. Primer and probe mixtures were custom designed from Applied Biosystems (custom TaqMan gene expression assays, Life Technologies, USA), and sequences can be viewed in Table S1 in the supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00622-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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