The Adaptive Response to Long-Term Nitrogen Starvation in *Escherichia coli* Requires the Breakdown of Allantoin

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**ABSTRACT** Bacteria initially respond to nutrient starvation by eliciting large-scale transcriptional changes. The accompanying changes in gene expression and metabolism allow the bacterial cells to effectively adapt to the nutrient-starved state. How the transcriptome subsequently changes as nutrient starvation ensues is not well understood. We used nitrogen (N) starvation as a model nutrient starvation condition to study the transcriptional changes in *Escherichia coli* experiencing long-term N starvation. The results reveal that the transcriptome of N-starved *E. coli* undergoes changes that are required to maximize chances of viability and to effectively recover growth when N starvation conditions become alleviated. We further reveal that, over time, N-starved *E. coli* cells rely on the degradation of allantoin for optimal growth recovery when N becomes replenished. This study provides insights into the temporally coordinated adaptive responses that occur in *E. coli* experiencing sustained N starvation.

**IMPORTANCE** Bacteria in their natural environments seldom encounter conditions that support continuous growth. Hence, many bacteria spend the majority of their time in states of little or no growth due to starvation of essential nutrients. To cope with prolonged periods of nutrient starvation, bacteria have evolved several strategies, primarily manifesting themselves through changes in how the information in their genes is accessed. How these coping strategies change over time under nutrient starvation is not well understood, and this knowledge is important not only to broaden our understanding of bacterial cell function but also to potentially find ways to manage harmful bacteria. This study provides insights into how nitrogen-starved *Escherichia coli* bacteria rely on different genes during long-term nitrogen starvation.

**KEYWORDS** *Escherichia coli*, nitrogen regulation, bacterial stress response, transcriptome, allantoin
growth over a period of nearly 2 days (5); Stapels et al. showed that nongrowing intracellular Salmonella organisms in host macrophages are transcriptionally active, and this activity is important to shape the host environment to benefit bacterial survival (6); similarly, nonreplicating Mycobacterium tuberculosis cells retained transcriptional activity in the lungs of mice (7). More recently, Gray et al. reported that Bacillus subtilis cells under oligotrophic conditions are transcriptionally active and replicate at an extremely low rate (8). Therefore, understanding the transcriptional landscape in growth-attenuated bacteria is important, as this knowledge could inform and inspire new antibacterial strategies, such as disrupting genetic networks and products that are activated during growth attenuation, which subsequently become essential for survival and/or optimal growth recovery.

In this study, we used nitrogen (N) starvation as a model condition to understand how the transcriptional landscape in nutrient starvation-induced growth-attenuated E. coli affects bacterial survival and the ability to recover growth when nutrients (i.e., N) become replenished. N is an integral nutrient to bacterial cell function, since N is an essential element of most macromolecules in the bacterial cell (proteins, metabolites, nucleic acids, and cell wall components). Therefore, an exponentially growing batch culture of E. coli rapidly attenuates growth upon N run-out (9–11). The initial adaptive response to N run-out, the N regulation (Ntr) stress response, results in the large-scale and multilevel reprogramming of the transcriptome, involving ~40% of all E. coli genes, to allow E. coli to adjust to N starvation (12–14). Since E. coli perceives N run-out as a decrease in the internal concentration of glutamine, the genes expressed as part of the Ntr response are predominantly involved in the transport and assimilation of nitrogenous compounds into glutamine or glutamate, yielding these catabolically or sparing the requirement for them in cellular processes (12, 13). Although our understanding of the initial adaptive transcriptional response to N starvation is well established, how the transcriptome changes during long-term N starvation is far from clear.

RESULTS AND DISCUSSION

Inhibition of transcription in N-starved E. coli compromises survival and growth recovery. To set up an experimental system to study how the transcriptome changes during long-term N starvation, we grew a batch culture of E. coli in a defined minimal growth medium in the presence of a limiting amount (3 mM) of ammonium chloride as the sole N source and excess (22 mM) glucose as the carbon (C) source. As shown in Fig. 1A (left and middle), under these conditions, when ammonium chloride becomes depleted (but sufficient glucose is still available to support growth), bacteria attenuate growth due to N starvation (12). From the onset of N starvation (referred to as N−), the number of cells in the population of growth-attenuated E. coli, as measured by the number of CFU in the population, increases at a low rate over the initial 24 h under N starvation (doubling time of ~39 h, which is ~80-fold less than that during exponential growth when N is abundant; referred to as N+ ) (Fig. 1A, right, orange area). This growth seems to occur despite the absence of any detectable ammonium chloride in the growth medium (Fig. 1A, left and middle). However, as N starvation ensues beyond 24 h (referred to as N-24), the population ceases growth before the number of viable cells gradually begins to decline after 48 h under N starvation (Fig. 1A, red area). Therefore, the first 24-h period following N−, which we refer to as the N-starved adaptive phase (NSAP), seems to be an important adaptive period during long-term N starvation. Transcriptional changes likely occur during NSAP, and we sought to determine how the inhibition of transcription during NSAP affects cell survival and growth recovery when N starvation stress is alleviated. To do this, we treated the bacteria with 150 μg/ml of the bacteriostatic antibiotic rifamycin (Rif) at ~1 h following N− for ~23 h. As previously shown by us, under these conditions, 150 μg/ml Rif prevents transcription elongation by the RNAP by trapping it at promoters (12). We initially compared the proportion of viable cells in the Rif-treated and untreated population of bacteria at N-24 (i.e., ~23 h following Rif treatment at N-1). As shown in Fig. 1B, at N-24, only ~50% of cells in the Rif-treated culture were viable compared to the level of the
FIG 1 Inhibition of transcription in N-starved E. coli compromises survival and growth recovery. (A, left) Graph showing a representative growth curve (measured by OD_{600} readings) of E. coli and concentration of ammonium chloride in the growth medium as a function of growth. The time points referred to as N+, N−, and N-24 in the text are indicated on the growth curve. (Middle) The bar graph shows the concentration of ammonium ions (NSAP) over time post-N. (Continued on next page)
untreated culture. Rif treatment also had a profound effect on the ability of bacteria from N-24 to resume growth compared to that of untreated bacteria. As shown in Fig. 1C and D, the lag time to growth recovery of the Rif-treated bacteria (following CFU correction to ensure an equal number of viable treated and untreated bacteria were used; based on data in Fig. 1B) from N-24 was delayed by ~4.8 h compared to that of untreated bacteria. We considered whether this difference in lag time between Rif-treated and untreated bacteria was due to the presence of a slower-growing subpopulation(s) of bacteria in the Rif-treated population that resumed growth slowly. To investigate this, we used a method called ScanLag (15), which allows the measurement of the appearance time of individual colonies on N-replete solid growth medium as a function of time following inoculation. We expected that if the Rif-treated population consisted of one or more subpopulations compared to the untreated population, then the appearance of individual colonies of bacteria treated with Rif would be more heterogenous than that of untreated bacteria. As shown in Fig. 1E, no heterogeneity in growth in the populations of Rif-treated and untreated bacteria from N-24 was detected. However, the ScanLag data substantiated the data shown in Fig. 1C and D and demonstrated that Rif-treated cells have an increased lag time to growth recovery compared to that of untreated cells. Once growth resumed, the doubling time of Rif-treated and untreated bacteria did not markedly differ (Fig. 1D). Overall, we conclude that the inhibition of transcription at the onset of NSAP has a detrimental effect on the ability of E. coli to adapt to long-term N starvation in terms of maximizing chances of viability and optimal growth recovery when N becomes available.

The transcriptome E. coli undergoes changes during the NSAP. To study the transcriptional changes that occur during the NSAP, we compared, as indicated in Fig. 2A, the transcriptomes of E. coli during exponential growth (N+), following initial N run-out (N−), and at N-24. We defined differentially expressed genes as those with expression levels changed ≥2-fold with a false discovery rate-adjusted P value of <0.05. As previously reported by us and others, the initial transcriptional response to N starvation involves differential expression of 1,787 genes primarily required for scavenging and catabolism of alternative N sources (13, 14, 16); thus, we did not investigate these genes further (phase A in Fig. 2A). However, during the NSAP, upon transition from N− to N-24 (phase B in Fig. 2A), 451 genes (~10% of E. coli genes) are differentially expressed, of which 105 genes (~2% of E. coli genes) that were not differentially expressed in phase A become differentially expressed in phase B (Fig. 2B). Functional categorization of the genes uniquely differentially expressed in phase B, here called NSAP genes, revealed that they are involved in diverse processes, including transcription, amino acid transport, and metabolism, as well as several genes of unknown function (Fig. 2C). Eleven (abgR, bbd, ileY, leuO, ompN, sibE, tauA, ycdD, yedL, yhdV, and yihO) of the upregulated and nine (evgA, glnA, gltI, hemH, ivy, pfbK, ybjX, yieP, and yjeL) of the downregulated NSAP genes were known to be regulated by either NtrC (glnA and gltI) or Nac (all other genes listed above) (16, 17), the master transcription regulators that activate the initial adaptive response to N starvation. However, the absence of either glnG (which encodes NtrC) or nac did not detectably affect the viability of bacteria following 24 h under N starvation or the ability of 24-h N-starved bacteria to recover growth when inoculated into fresh N-replete minimal growth media (Fig. 2D). Overall, we conclude that the transcriptome of E. coli undergoes changes

FIG 1 Legend (Continued)
chloride and glucose at N+; N−, and N-24, (Right) Graph showing viability, measured by counting CFU (CFU of E. coli over 5 days under N starvation). The inset table shows doubling time between different growth periods, and the NSAP is indicated. (B) Bar graph showing the proportion of viable cells, measured by number of CFU in the population of E. coli at N-1 and following 24 h under N starvation with (+Rif) and without (−Rif) Rif treatment at N-1. (C) Graph showing recovery growth in fresh N-replete medium as measured by OD600 readings. The time points and conditions from which bacteria were sampled for recovery growth experiments are indicated and discussed in the text. (D) Graph showing lag time and doubling time during growth recovery experiments from panel C. ***, P < 0.001. (E) ScanLag analysis of colony growth rate and appearance time after plating of 24-h N-starved bacteria on LB agar after Rif treatment and no Rif treatment at N-1. The various shades of the same color represent distinct biological replicates, where n = 3. In panels A to D, error bars represent SEM, where n = 3, and the schematics describe the different experiments for ease of understanding.
FIG 2 Transcriptome of E. coli undergoes changes during the NSAP. (A, left) Graph showing a representative growth curve (measured by OD600 readings) of E. coli and concentration of ammonium chloride in the growth medium as a function of growth. The points at which samples were taken for RNA-seq analysis (N/H11001, N/H11002, and N-24) and the phases, referred to as A and B in the text, are indicated. (Right) Graphs showing the log2 fold change in gene expression at N/H11002 compared to N/H11001 (phase A) and N-24 compared to N/H11002 (phase B). Genes in blue (phase A) or red (phase B; NSAP) represent significant differential expression above a log2 fold change of 1 and a false discovery rate-adjusted P value of <0.05. Genes in black (phase B) represent the NSAP genes, defined as having (Continued on next page)
during the NSAP, and this is consistent with the finding above that 150 μg/ml Rif treatment during the NSAP has a detrimental effect on the viability of the *E. coli* population following 24 h under N starvation and its ability to recover growth when N becomes replenished (Fig. 1B to D). Therefore, it seems that the adaptive transcriptional changes that occur during the NSAP happen subsequently to the initial adaptive changes to N starvation and do not depend on NtrC (and Nac), which coordinates the initial adaptive transcriptional response to N run-out.

**Four upregulated NSAP genes are important for optimal growth recovery from long-term N starvation.** We next investigated how the upregulated NSAP genes affected the viability and growth recovery of long-term N-starved *E. coli*. Of the 51 upregulated NSAP genes, 44 are available in the Keio single-gene knockout collection (18). Thus, we determined how the absence of any of the upregulated NSAP genes affected growth recovery following 24 under N starvation. To do this, we grew the library of upregulated NSAP gene mutant bacteria in minimal growth media containing excess (10 mM) ammonium chloride as the sole N source and measured cell density after 16 h of growth. The growth of the library of upregulated NSAP gene mutant strains was done in a plate reader incubator, which allowed continuous monitoring of the growth of each mutant strain simultaneously to enable better side-by-side comparison. The results shown in Fig. 3A indicate that the absence of any of the NSAP genes did not markedly affect growth under N-replete conditions. However, we cannot exclude the possibility that the relative dynamics of growth of the individual mutant strains differed from that of the wild-type (WT) strain over the 16-h period. We note that bacteria in which the tryptophan biosynthesis genes are deleted, that lack the γ subunit of formate dehydrogenase-N (*fdnI*), or that lack the ferric rhodotorulic acid outer membrane transporter (*thuE*) are unable to grow in our minimal growth media, most likely due to auxotrophy (19). We next reinoculated bacteria that grew under N-replete conditions into fresh minimal growth medium containing limiting, i.e., 3 mM, ammonium chloride and determined how the absence of the upregulated NSAP genes affected viability at N-24, i.e., following 24 h under N starvation. The results depicted in Fig. 3B show that the viabilities of the upregulated NSAP gene mutants were similar to that of the WT strain. When the 24-h N-starved mutant bacteria were inoculated into fresh N-replete minimal growth medium, the dynamics of growth recovery for the majority of the mutant strains were similar to those of the WT strain (Fig. 3C). However, Δ*allB*, Δ*leuO*, Δ*ynfP*, and Δ*ybcJ* mutant strains displayed noticeably compromised growth recovery properties compared to that of the WT strain (Fig. 3C). These genes encode an allantoinase (*allB*), a DNA-binding transcription dual regulator (*leuO*), a small, possibly fragmented, protein of unknown function (*ynfP*), and a putative RNA-binding protein (*ybcJ*) (19). As shown in Fig. 3D, although the doubling times of Δ*allB*, Δ*leuO*, Δ*ynfP*, and Δ*ybcJ* mutant strains were similar to that of the WT strain, their lag time to growth recovery was increased by ~1.5 to 4 h compared to that of the WT strain. Thus, it seems that the transcription of *allB*, *leuO*, *ynfP*, and *ybcJ* during the NSAP is required for optimal growth recovery from long-term N starvation. As the Δ*allB* mutant strain displayed the most pronounced compromised ability to recover growth compared to that of the WT strain (Fig. 3C), we decided to focus on the role of *allB* during the NSAP and recovery from long-term N starvation.

**Degradation of allantoin during the NSAP is required for optimal growth recovery from long-term N starvation.** To discount the possibility that the difference in lag time during growth recovery between the WT and Δ*allB* bacteria was not due to the presence of a subpopulation of slow-growing Δ*allB* bacteria, we measured the lag time during growth recovery and compared it to the lag time of the WT bacteria.
timedistributionoftheappearanceofindividualcoloniesduringgrowthrecoveryusing
ScanLag (15), as described for Fig. 1E. As shown in Fig. 4A, we observed the expected
lag time in growth recovery for the ΔallB bacteria, but no evidence of a slow-growing
subpopulation was seen. Further, we did not detect a lag in growth recovery of WT and
ΔallB bacteria sampled from N/H11002 when reinoculated into N-replete fresh growth
medium (Fig. 4B). This result underscores the view that allB has a specific role in
E. coli during the NSAP. To establish that the increased lag time in growth recovery of ΔallB
bacteria was directly due to the absence of allB, we complemented the ΔallB bacteria
with plasmid-borne allB. As shown in Fig. 4C and D, the lag time in growth recovery of ΔallB bacteria reverted to that of WT bacteria when allB was supplied via a plasmid. The
product of allB is allantoinase: a tetrameric enzyme that catalyzes the hydrolytic
conversion of (S)-(+) -allantoin to allantoate. A previous study (20) reported that substi-
tutions at invariant amino acid positions N94 and S317 (in both cases to D and V),
which are located proximal to the active center, rendered allantoinase inactive (Fig. 4E).
As shown in Fig. 4C and D, in the context of the complementation experiments,
FIG 4 Degradation of allantoin during the NSAP is required for optimal growth recovery from long-term N starvation. (A) ScanLag analysis of colony growth rate and appearance time after plating of 24-h N-starved WT and ΔallB bacteria on LB agar. Various shades of the same color represent (Continued on next page)
plasmid-borne allB-N94V and allB-S317V failed to fully decrease the lag time in growth recovery of ΔallB bacteria, as seen with the WT version of allB. In contrast, plasmid-borne allB-N94D and, to a greater extent, allB-S317D reverted the lag time in growth recovery of ΔallB bacteria, as seen with the WT version of allB. Although we are unable to explain why specifically substitution by D but not V at N94 or S317 renders allantoinase catalytically inactive under our experimental conditions, it seems that the degradation of allantoin occurs at some point during the NSAP in E. coli. This is conceivable because, as shown in Fig. 4F, the conversion of allantoate (the product of allantoinase) to (S)-ureidoglycine by allantoate amidohydrolase (AllC) and the subsequent conversion of (S)-ureidoglycine to (S)-ureidoglycolate by (S)-ureidoglycine aminohydrolase (AllE) produces ammonium, which may be needed for the maintenance of cellular functions required for optimal growth recovery from long-term N starvation. Intriguingly, compared to allB, transcripts corresponding to allC and allE were only moderately upregulated (~1.2- to 1.5-fold) in phases A and B in Fig. 2A and, thus, did not fall into the category of NSAP genes. However, we observed that 24-h N-starved ΔallC and ΔallE bacteria displayed an increased lag time to growth recovery similar to that of the ΔallB bacteria (Fig. 4G and H) when grown in fresh N-replete growth media. This suggests that the activity of the genes of the allantoin degradation pathway has a role in the adaptive response to long-term N starvation in E. coli. As allantoin is a product of the purine degradation pathway in N-starved E. coli (21), we considered whether the requirement for allB is a consequence of purine degradation in E. coli during the NSAP to sustain cellular functions via the allantoin degradation pathway (as shown in Fig. 4F). To indirectly determine whether purines are degraded in E. coli during the NSAP, we measured the integrity of total RNA as a surrogate for purine degradation in E. coli as a function of time in the NSAP by chip-based capillary electrophoresis (see Materials and Methods). As shown in Fig. 4I, we observed a gradual degradation of 16S and 23S rRNA as the NSAP ensued. This observation further underscores the view that the RNA landscape and turnover during the NSAP is plastic and consistent with the view that the transcriptome of E. coli undergoes changes during long-term N starvation. Collectively, the results support the view that allB contributes to optimal growth recovery from long-term N starvation by the degradation of allantoin during the NSAP in E. coli, which likely accumulates as a result of purine degradation.

Conclusions. N is central to all metabolic processes in the bacterial cell. As such, bacteria can utilize a wide range of N-containing compounds as sole sources of cellular N. Upon N run-out, E. coli cells activate the transcription of genes associated with N assimilatory metabolic pathways necessary for the utilization of N from the extracellular environment and catabolic biosynthetic pathways for intracellular assimilation of N. We have presented evidence that the growth of a population of N-starved E. coli does not completely cease upon N run-out and that E. coli cells continue to divide over a period of up to 24 h (the NSAP) following entry into N starvation. It seems that the NSAP represents an important adaptive phase in E. coli experiencing long-term N starvation to maximize chances of viability and optimize growth recovery should N become replenished. We have also presented evidence that the adaptive transcriptional response to N starvation in E. coli occurs in at least two distinct temporally coordinated phases, where the NtrC-dependent initial response to N starvation is followed by, to a

FIG 4 Legend (Continued)
large extent, an NtrC-independent transcriptional response. We have uncovered that an aspect of the adaptive transcriptional response, which occurs in the NSAP subsequent to the initial adaptive transcriptional response in *E. coli* under long-term N starvation, involves the degradation of allantoin. As ammonium is a by-product of the degradation of allantoin, we propose that allantoin degradation allows the assimilation of ammonium into glutamine and glutamate to maintain cellular processes during the NSAP to maximize chances of viability and optimize growth recovery when N becomes replenished. Previous studies have shown that allantoin can only be used as the sole N source by *E. coli* grown under anaerobic conditions (22, 23). Consistent with these results, we were unable to grow *allIB* and WT bacteria under our conditions when ammonium chloride (the sole N source used in our experiments) was replaced by allantoin in both the initial growth and recovery growth experiments (data not shown). Therefore, as the end product of allantoin degradation under aerobic conditions is 3-phosphoglycerate (in addition to the by-product, ammonium), which is used for energy production, we propose that the breakdown of allantoin could serve as some sort of metabolic threshold to allow optimal growth recovery of *E. coli* from long-term N starvation under aerobic conditions when N becomes replenished. Previous work by us (12, 14) and others (13) has also shown that genes associated with the degradation of pyrimidine (e.g., the rut operon) become upregulated at the onset of N starvation and remain upregulated throughout the NSAP (14). As allantoin is a product of the purine degradation pathway in N-starved *E. coli* (21), collectively, these observations suggest that the degradation of nucleotides is an essential component of the adaptive response to N starvation in *E. coli*. Although future work will focus on deciphering the metabolic and nucleotide landscape to unravel in depth the adaptive responses that define the NSAP in *E. coli*, this work has underscored the importance of studying the transcriptional changes that occur in long-term nutrient-starved bacteria to further our understanding of bacterial stress physiology.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids, growth conditions, and viability measurements.** WT and mutant *E. coli* BW25113 strains were used in this study (18). Bacteria were grown as described by Figueira et al. (24) in 10 mM NH₄Cl (for overnight cultures) or 3 mM NH₄Cl (for N starvation experiments) Gutnick minimal medium, consisting of 33.8 mM KH₂PO₄, 77.5 mM K₂HPO₄, 5.74 mM K₂SO₄, 0.41 mM MgSO₄, supplemented with Ho-LE trace elements and 0.4% (wt/vol) glucose, using NH₄Cl as the sole N source (16). Where relevant, rifampin was added at a final concentration of 150 μg/ml at N-1 or N₊ as stated, and 1-arabinose was added to complementation strains containing the pBAD18 plasmid at 0.2% (wt/vol) for induction of gene expression in growth cultures. Ampicillin was added at a final concentration of 100 μg/ml to all cultures containing derivatives of the pBAD18 plasmid. Measurement of growth was taken by measuring the optical density at 600 nm (OD₆₀₀) of bacterial cultures over time to generate growth curves. Ammonium and glucose concentrations in the media were determined using the Aquaquant ammonium quantification kit and glucose assay kit, respectively (both by Merck Millipore, UK), according to the manufacturer’s instructions. To determine population viability, the number of viable cells was calculated by conducting serial dilutions of cultures at designated time points, as shown in relevant figures, grown overnight on Luria-Bertani (LB) agar plates and measuring the number of CFU per milliliter. The pBAD18-*allB* plasmid was made by traditional restriction enzyme cloning to incorporate the ribosomal binding site of pET33b(+) with the *allB* gene to allow arabinose-inducible expression of the *allB* protein. The N94D, N94V, S317D, and S317V mutations in *allB* were done by standard site-directed mutagenesis using pBAD18-*allB* as the template.

**ScanLag.** Bacteria were grown in 3 mM Gutnick minimal medium to N-24, washed twice in 1 ml PBS, and diluted in PBS. One hundred microliters of diluted culture was spread on an LB agar plate and incubated at 33°C for 48 h with pictures of plates taken every 20 min. Analysis of colony growth rate and appearance time was modified from Levin-Reisman et al. (25) by Miles Priestman and is available at [https://github.com/mountainpenguin/NQBMatlab](https://github.com/mountainpenguin/NQBMatlab).

**RNA-seq.** The RNA sequencing (RNA-seq) data set was previously generated and analyzed by Switzer et al. (14). The analysis of differential gene expression during NSAP as well as the statistical analyses were also conducted in the same ways as those described previously. The sequencing data can be accessed under accession number E-MTAB-6491 in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress).

**Total RNA analysis.** Cultures were grown as described above and sampled at the following time points: at the onset of N starvation (N⁻) and 3 h (N-3), 6 h (N-6), 9 h (N-9), 12 h (N-12), 18 h (N-18), and 24 h (N-24) under N starvation. At least three biological replicates were taken at each time point. RNA was acquired and stabilized at specified time points using Qiagen RNA Protect reagent (76526; Qiagen). RNA extraction was carried out using the PureLink RNA minikit (12183025; Invitrogen) and DNase set (12185010; Invitrogen) per the manufacturer’s protocol. Electropherograms of total RNA integrity were...
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