A novel regulatory factor affecting the transcription of methionine biosynthesis genes in Escherichia coli experiencing sustained nitrogen starvation

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Abstract

The initial adaptive transcriptional response to nitrogen (N) starvation in Escherichia coli involves large-scale alterations to the transcriptome mediated by the transcriptional activator, NtrC. One of these NtrC-activated genes is yeaG, which encodes a conserved bacterial kinase. Although it is known that YeaG is required for optimal survival under sustained N starvation, the molecular basis by which YeaG benefits N starved E. coli remains elusive. By combining transcriptomics with targeted metabolomics analyses, we demonstrate that the methionine biosynthesis pathway becomes transcriptionally dysregulated in ΔyeaG bacteria experiencing sustained N starvation. It appears the ability of MetJ, the master transcriptional repressor of methionine biosynthesis genes, to effectively repress transcription of genes under its control is compromised in ΔyeaG bacteria under sustained N starvation, resulting in transcriptional derepression of MetJ-regulated genes. Although the aberrant biosynthesis does not appear to be a contributing factor for the compromised viability of ΔyeaG bacteria experiencing sustained N starvation, this study identifies YeaG as a novel regulatory factor in E. coli affecting the transcription of methionine biosynthesis genes under sustained N starvation.

INTRODUCTION

Conditions that sustain constant bacterial growth are seldom found in nature. Environments where growth is limited by availability of nutrients are common, for example, soil, water, or even host environments such as macrophages can lack essential nutrients to support growth. As such, many bacteria spend most of their time in states of little or no growth due to starvation. The starved and growth attenuated state is now widely considered as an important physiological condition in bacterial pathogenesis and survival. Nitrogen (N) is an essential element of most macromolecules (proteins, nucleic acids and cell wall components) within a bacterial cell. Many bacterial pathogens appear to experience N limitation in host environments such as the urinary tract (e.g. uropathogenic Escherichia coli [1]) or macrophages (e.g. Salmonella Typhimurium [2]), and respond by activating specific adaptive processes. Emerging evidence also indicates that bacterial N metabolism and N stress responses have a role in the development of dysbiosis [3]. Further, we recently reported that the specific adaptive response to N starvation in E. coli is directly coupled to the production of guanosine pentaphosphate, (p)ppGpp: the pleiotropic stress signalling nucleotide and effector of the stringent response [4, 5]. Thus, the adaptive response to N starvation clearly plays an integral and fundamental role in bacterial physiology and pathogenesis.

The initial adaptive response to N starvation is mediated by the transcriptional activator, NtrC, and involves the expression of genes largely associated with activation of transport systems, catabolic and biosynthetic operons for the scavenging of alternative N sources, transcriptional reprogramming and managing cellular resources until growth conditions improve. One of the most highly expressed operons during the initial adaptive response to N starvation consists of yeaG and yeaH. Both genes are conserved across several
bacterial species, especially Enterobacteriaceae, including plant and animal pathogenic bacteria (e.g. Shigella, Salmonella, Erwinia and Pseudomonas species) [5–7]. They are also induced in response to diverse stresses (including low pH [8], high osmolarity [9], stationary phase [8] and low sulphur [10]), which suggests a wider importance of yeaG and yeaH and pathways affected by them in bacterial adaptive processes. Although the product of yeaH has no sequence or structural homology to any known protein, YeaG is a Hank’s type kinase [11, 12].

In earlier work, we revealed that the viability of ΔyeaG E. coli becomes significantly compromised only after 24 h under N starvation and thus proposed a role for YeaG in the adaptive response to sustained N starvation [6]. Further, we showed that the intracellular concentration of RpoS (σ^38), the major bacterial sigma (σ) factor required for the transcription of general stress response genes, was decreased by more than twofold in ΔyeaG bacteria compared to the wild-type [6], and consequently suggested that ΔyeaG bacteria might be compromised to effectively adapt their transcriptional programme to survive a sustained period of time under N starvation. In the present study, we compare the transcriptomes of wild-type and ΔyeaG bacteria to assess how YeaG affects the transcriptional response to N starvation. The results reveal that YeaG plays a role in the adaptive response to initial and sustained nitrogen starvation, and identifies YeaG as a novel regulatory factor in E. coli affecting the transcription of methionine biosynthesis genes under sustained N starvation.

METHODS

Bacterial strains, plasmids, growth conditions and viability measurements

Strains and plasmids used in this study were derived from Escherichia coli K-12 and are listed in Table S1 (available in the online version of this article). The NtrC-FLAG allele was transduced into the BW25113 strains (wild-type and ΔyeaG) using P1 phage transduction from the NtrC-FLAG NCM3722 strain as described in [5]. The metJ-FLAG strains were constructed using the λ Red recombinase method to introduce an in-frame fusion encoding three repeats of the FLAG epitope [3×(DYKDDDDK)] followed by a kanamycin resistance cassette, amplified from the pDOC-F plasmid [13], to the 3’ end of metJ. The ΔmetJ strain was also constructed using the λ Red recombinase method to introduce an in-frame fusion encoding a kanamycin resistance cassette amplified from the pDOC-K plasmid [13] in place of the metJ gene in the wild-type BW25113 strain. Bacteria were grown in Gnutnick minimal medium (33.8 mM KH₂PO₄, 77.5 mM K₂HPO₄, 5.74 mM K₂SO₄, 0.41 mM MgSO₄), supplemented with Ho-LE trace elements [14] and 0.4 % (w/v) glucose according to Figuereira et al. [6], where the sole source of nitrogen is NH₄Cl; overnight cultures were grown in medium containing 10 mM NH₄Cl and N-limiting growth curves were carried out in medium containing 3 mM NH₄Cl. To measure the biological activity of MetJ-FLAG, the metJ-FLAG strains were grown in Luria–Bertani (LB) medium supplemented with 100 µg ml⁻¹ methionine to induce repression of the methionine biosynthesis pathway as described in [15]. Samples were taken during exponential growth for analysis of metF transcript levels as described below by quantitative reverse transcription PCR. All growth curves were generated by measuring the optical density (OD₆₀₀ nm) of the bacterial cultures over time. The number of viable cells was determined by measuring c.f.u. ml⁻¹ from serial dilutions on LB agar plates at the designated time points shown in Fig. 1.

Antisera and immunoblotting

Immunoblotting was completed according to standard laboratory protocols [16]. To produce antisera against YeaG, His-tagged YeaG (expressed from the arabinose-inducible plasmid: pBAD18-yeaG in MC1061) was purified using nickel affinity purification. Eurogentec produced the antisem against YeaG through immunization of rabbits with the purified YeaG protein. This protein was also used to affinity-purify the antibodies from the sera following standard laboratory protocols [16]. The YeaG antibody was used at 1:2500 dilution. The following commercial primary antibodies were used: mouse monoclonal anti-RNA polymerase (RNAP) α subunit 4RA2 at 1:10,000 dilution (Biolegend), and anti-FLAG M2 at 1:1000 dilution (Sigma, F3165). Both secondary antibodies were used at 1:10,000 dilution, either rabbit anti-mouse IgG H and L horseradish peroxidase (HRP)-linked secondary antibody (Abcam, ab97046) or Amersham ECL Rabbit IgG, HRP-linked whole antibody (from donkey) (GE Healthcare, NA934). ECL Prime Western blotting detection reagent was used for all the immunoblotting experiments done in this study (GE Healthcare, RPN2232). All blots were analysed on the ChemiDoc MP imaging system and bands quantified using Image Lab software version 5.2.1.

RNA sequencing (RNA-seq)

Cultures were grown as in [6] and sampled at the following time points: N+, when the OD₆₀₀ nm≈0.3; N–, 20 min following N ran-out; and N-24, where bacteria were subjected to 24 h of N starvation. Two biological replicates were taken for N+ and N−, and three for N-24. Cultures were mixed with 1:19 phenol:ethanol solution at a ratio of 9:1 cultures:solution and then pelleted. Cell pellets were sent to Vertis Biotechnologie AG for downstream processing exactly as previously described by [17]. The RNA extracted for RNA-seq was examined by capillary electrophoresis before rRNA depletion, cDNA synthesis and next-generation sequencing. The data analyses were performed with CLC Genomics Workbench 7 using standard parameters. RNA-seq reads were mapped to the E. coli K-12 MG1655 (U00096.3) genome using Burrows–Wheeler Aligner. Reads that mapped uniquely were used for further analysis. Total read counts were used to normalize the data, the number of reads mapping to each gene was calculated, and a matrix of read counts was generated. The matrix was analysed using the DESeq2 BioConductor package for differential gene
expression analysis. Genes with ≤10 reads mapped to them were excluded from analysis. All statistical analyses were performed in R version 3.4.2. The data have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/ arrayexpress) under accession number E-MTAB-6481.

Quantitative reverse transcription PCR (qRT-PCR)
RNA was acquired and stabilized at specified time points using Qiagen RNA Protect reagent (Qiagen, 76526). RNA extraction was carried out using the PureLink RNA mini kit (Invitrogen, 12183025) and DNase Set (Invitrogen, 12185010). The A260/280 and A260/230 ratios of the extracted RNA for qRT-PCR was analysed and deemed suitable for downstream processing. The High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, 4368814) was used to convert 100 ng of purified RNA to cDNA. 400 ng µl⁻¹ of cDNA was used per qRT-PCR reaction containing: 10 µl PowerUp SYBR Green Master Mix (Applied Biosystems, A25742), 1 µl forward primer (5 µM), 1 µl reverse primer (5 µM) and 1 µl template cDNA in a 20 µl reaction volume. Amplifications were performed on the StepOnePlus Real-Time PCR system (Applied Biosystems, 4376600) using the following cycle: 95 °C (10 min) followed by 40 cycles of 95 °C (15 s), 59 °C (15 s), 68 °C (45 s), and a melt curve of 95 °C (15 s), 60 °C (1 min), 95 °C (15 s). Sequences of all primers used in this study are listed in Table S2. All real-time analysis was performed in triplicate and compared against 16S rRNA expression as an internal control.

Metabolomics
Metabolite analysis was carried out using liquid chromatography mass spectrometry (LC-MS)-based metabolomics. Briefly, cultures of wild-type and ΔyeaG strains were grown in 100 ml of Gutnick minimal medium in triplicate as described above for the RNA-seq experiment. The cultures were then spun down rapidly at 3000 g for 5 min, the excess of the supernatant was discarded, and the remaining 10 ml of culture was passed through a 0.22 µm pore size filter (mixed cellulose ester membrane; Millipore #G5WP02500). The metabolites were quenched by submerging the filter containing the bacteria directly into 1 ml solution of acetonitrile: methanol: water (ACN: MeOH:H₂O; 2:2:1 v/v/v) that was pre-chilled at −40 °C on dry ice. The whole mixture was transferred into a 2 ml screw cap polypropylene tube containing around 400 µl of acid-washed beads (150–212 µm; Sigma #G1145) and ribolysed for 40 s at 6.5 speed. Following lysis, the tubes were centrifuged at 12 000 r.p.m. for 10 min and supernatants containing soluble metabolites were passed into a Spin-X 0.22 µm cellulose acetate centrifuge tube filter (Costar; #8160) and stored at −80 °C. Prior to LC-MS, metabolite samples were mixed with acidified ACN (0.2 % acetic acid) at a 1:1 ratio, spun down at top speed for 5 min and transferred into LC-MS vials. LC-MS was performed in an Agilent 1200 LC system coupled with an Agilent Accurate Mass 6230 TOF apparatus using the method as described in [18]. Briefly, the LC system was equipped with a solvent degasser, a binary pump, temperature-controlled auto-sampler and temperature-controlled column compartment with a Cogent Diamond Hydride Type C silica column (150×2.1 mm; dead volume 315 µl) that was employed for liquid chromatography. The mobile phase method used consisted of 0 min 85 % B; 0–2 min 85 % B; 2–3 min to 80 % B; 3–5 min 80 % B; 5–6 min to 75 % B; 6–7 min 75 % B; 7–8 min to 70 % B; 8–9 min 70 % B; 9–10 min to 50 % B; 10–11 min 50 % B;
null–11.1 min to 20 % B; 11.1–14 min hold 20 % B, where solvent A consists of deionized water with 0.2 % acetic acid and solvent B consists in acetonitrile with 0.2 % acetic acid. The flow rate on Agilent 1200 LC was 0.4 ml min$^{-1}$. On the Agilent Accurate Mass 6230 TOF apparatus, dynamic mass axis calibration was achieved by continuous infusion of a reference mass solution using an isocratic pump connected to a multimode ionization source, operated in the positive-ion and negative-ion mode. ESI capillary and fragmentor voltages were set at 3500 and 100 V, respectively. The nebulizer pressure was set at 40 p.s.i. and the nitrogen drying gas flow rate was set at 101 min$^{-1}$. The drying gas temperature was maintained at 250°C. The MS acquisition rate was 1.5 spectra s$^{-1}$ and m/z data ranging from 50 to 1200 were stored. This instrument routinely enabled accurate mass spectral measurements with an error of less than 5 parts-per-million (p.p.m.), mass resolution ranging from 10 000 to 25 000 over the m/z range of 121–955 atomic mass units, and a 100 000-fold dynamic range with picomolar sensitivity. Data were collected in the centroid mode in the 4 GHz (extended dynamic range) mode. Metabolite standard samples of O-succinylhomoserine ([M+H]+/m/z=220.0822, RT=7.77) methionine ([M+H]+/m/z=150.5609, RT=8.28), S-adenosylmethionine (SAM) ([M+H]+/m/z=400.1497, RT=17.1), S-adenosylhomocysteine (SAH) ([M+H]+/m/z=386.3493, RT=13.36) and N-formyl-l-methionine ([M+H]+/m/z=178.0543, RT=1.33) were dissolved at a range of concentrations in the same matrix solution as the bacterial samples (ACN:MeOH:H$_2$O: 2:2:1 v/v/v) and were run alongside the experimental samples for quantification purposes. Metabolite abundances were performed using Agilent’s Mass Hunter suite and Mass Profinder software (Fig. S1). A standard curve of the ion count abundances of each standard metabolite versus its concentration was used for the quantification of the same metabolite in the experimental samples. Metabolite abundances were normalized with residual protein of each sample, which was estimated using a BCA protein assay kit (Pierce; 23225).

Chromatin immunoprecipitation (ChIP)

Cultures were grown in 3 mM NH$_4$Cl Gutnick minimal medium as above; 25 ml was sampled at N+ , N− and N-24, and processed exactly as previously described by [5]. Either anti-FLAG (M2) or anti-β (WP002) antibodies were used for the MetJ-FLAG ChIP and RNAP ChIP, respectively. Primers used for rate limiting semi-quantitative PCR are listed in Table S2.

Statistical analysis

Unless otherwise specified, all data show the mean average of at least three independent experiments, where variation shown is the SEM. Statistical significance was determined using Student’s t-test or one-way ANOVA where a probability (P) value of <0.05 was considered statistically significant (* P<0.05, ** P<0.01, *** P<0.001, NS – not significant).

RESULTS AND DISCUSSION

The initial 24 h of N starvation represents a physiologically important period during which YeaG-mediated adaptation to sustained N starvation occurs

To decipher the molecular basis by which YeaG contributes to adaptation to sustained N starvation, we began our study by measuring the intracellular levels of YeaG in whole-cell extracts from cells experiencing N starvation over 5 days. Results revealed that YeaG levels peaked within the first 24 h following entry into N starvation, but subsequently diminish (Fig. 1a). Although YeaG per se might be subject to regulation, this observation suggests that the YeaG-mediated adaptive response to N starvation occurs, at least under our conditions, within the first 24 h upon entry into N starvation. Further, since the viability of ΔyeaG bacteria gradually decreased only after 24 h under N starvation (N-24) (resulting in ~22 % of the mutant population surviving after 5 days under N starvation compared to the wild-type; Fig. 1b), the results imply that the initial 24 h of N starvation represents a physiologically important period during which YeaG-mediated adaptation to sustained N starvation happens.

The NtrC-mediated transcriptional response to N starvation is dynamic

We next compared the transcriptomes of wild-type and ΔyeaG bacteria during exponential growth under N replete conditions (N+), following the initial response to N starvation (20 min following N run-out; N−) and after experiencing 24 h of N starvation (N-24) to understand how YeaG contributes to adaptation to sustained N starvation. We defined differentially expressed genes as those with expression levels changed ≥ twofold with a false-discovery-rate-adjusted P-value <0.05. The initial transcriptional response to N starvation involves the activation of genes required for scavenging of alternative N sources by NtrC [4, 5]. We therefore compared how the transcript levels of NtrC-activated genes (as defined in [5]) differed in wild-type and ΔyeaG bacteria at N− (compared to N+) and N-24 (compared to N− and N+). In wild-type bacteria, as expected, the transcript levels of the majority of NtrC-activated genes increased in response to N starvation at N− (Fig. 2a). Following sustained N starvation at N-24, the transcript levels of NtrC-activated genes become overall reduced (Fig. 2b), but remain elevated in comparison to N+ (Fig. 2c). We also note that the rut operon, which encodes proteins associated with pyrimidine degradation, becomes further upregulated during sustained N starvation (Fig. 2b, c). Consistent with this observation, NtrC was still present following 24 h of N starvation in both wild-type and ΔyeaG bacteria (Fig. 2d). Interestingly, we note that the intracellular level of NtrC at N-24 was ~ twofold higher in ΔyeaG bacteria compared to wild-type bacteria at N+, N− and N-24. Since the transcript levels of glnG, the gene encoding for NtrC do not significantly differ in wild-type and mutant samples, we consider it possible that NtrC is
Fig. 2. The NtrC-mediated transcriptional response to N starvation is dynamic. (a–c) Log$_2$ fold change in mean expression of the 46 genes within the NtrC regulon [as described in (5)] for wild-type bacteria from N+ to N− (a), N− to N-24 (b) and N+ to N-24 (c). (d) Representative immunoblot of whole-cell extracts of bacterial cells sampled at N+, N− and N-24 in wild-type (left) and ΔyeaG (right) bacteria, probed with anti-FLAG antibody (to detect NtrC) and anti-RNA polymerase α subunit antibody (loading control). Fold change (FC) between NtrC levels in wild-type and ΔyeaG bacteria was calculated by obtaining the ratio in band intensities between the band corresponding to NtrC and the RNAP α subunit (loading control), and dividing the values obtained for ΔyeaG samples by the values obtained for the wild-type samples from the corresponding time point. The bands corresponding to NtrC and α subunit shown are from the same blot. (e) Heat maps showing the log$_2$ fold change in expression levels of the NtrC-regulated genes in wild-type (top) and ΔyeaG (middle) bacteria at N− relative to N+, N-24 relative to N−, and N-24 relative to N+, and in ΔyeaG relative to wild-type bacteria at N−,
subject to post-transcriptional regulation. We next considered whether the expression of the NtrC-activated genes becomes dysregulated in the absence of YeaG. As shown in the heat maps of genes differentially expressed in wild-type and ΔyeaG bacteria (Fig. 2e), the absence of YeaG does not substantially change the expression profile of NtrC-activated genes at N− or N-24. Overall, it seems that the NtrC-mediated transcriptional response to N starvation is dynamic and possibly subject to temporal regulation. It further seems that the expression of the NtrC-activated genes does not substantially differ between wild-type and ΔyeaG bacteria in the initial response to N starvation (N−) and following sustained N starvation (N-24).

ΔyeaG bacteria experiencing sustained N starvation have subtle dysregulation of the σ38 regulon

In our previous work [6], we used qRT-PCR to establish that the transcript levels of two toxin/antitoxin (TA) modules: magR/magA and dinJ/yafQ, were upregulated (by ~four and ~threefold, respectively) in ΔyeaG bacteria compared to wild-type bacteria following sustained N starvation (at N-24). The magR/magA TA module directly represses the transcription of σ38 [19, 20]. Similarly, DinJ, the toxin component of the dinJ/yafQ TA module, has been implicated in reducing σ38 levels through directly repressing the transcription of cspE, which in turn positively affects σ38 translation through stabilization of rpoS mRNA [21]. Consistent with our previous observations, analysis of the relative transcript levels of magR, magA, dinJ and yafQ in the transcriptome of ΔyeaG bacteria at N-24 was upregulated compared to wild-type bacteria; conversely, transcript levels of cspD and cspE were lower (Fig. S2a). However, none of these (except for cspD) differences were statistically significant based on the cut-off criteria of the RNA-seq data analysis (see above). Nevertheless, and consistent with previous observations [6], the protein levels of σ38 were reduced by 1.9 and ~fourfold in ΔyeaG bacteria compared to wild-type bacteria at N− and N-24, respectively (Fig. S2b). Therefore, we considered whether dysregulated expression of the σ38 regulon (as defined by [8]) could be a reason for the compromised ability of the ΔyeaG bacteria to survive sustained N starvation. As seen with the NtrC-activated genes (Fig. 2a–c), in wild-type bacteria, the transcript levels of σ38-dependent genes were upregulated at N−. Following sustained N starvation, at N-24, the transcript levels of these genes become overall reduced but remain higher still than at N+ (Fig. S2c). This observation further underscores the view that the transcriptome of E. coli experiencing sustained N starvation is dynamic. As shown in Fig. 3a, none of the σ38-dependent genes were differentially expressed in ΔyeaG bacteria at N+ (except yehH; see later) and N−. However, a small subset (9/113, ~8%) of the σ38-dependent genes were differentially expressed at N-24 (Fig. 3c). Of these nine genes, the biological roles of six (euhH, yciG, yebf, ygbA, yjdc and ymgA) are unknown; whereas the remaining three (amyA, narU and yfcF) have diverse functions (amylase, nitrate/nitrite transporter and glutathione S-transferase, respectively). Overall, although we cannot exclude a role for these nine σ38-dependent genes in adaptation to sustained N starvation, it seems that, despite σ38 levels being ~fourfold lower, the σ38 regulon is only subtly dysregulated in ΔyeaG bacteria.

A role for YeaG in regulating motility-associated gene expression during the initial adaptive response to N starvation

We generated volcano plots to compare the complete transcriptomes of wild-type and ΔyeaG bacteria at N+, N− and N-24 (Fig. 4a–c). A total of 21 and 199 genes were differentially expressed in ΔyeaG bacteria compared to the wild-type at N− and N-24, respectively (Fig. 4b, c, Tables S3 and S4). As above (Fig. 3a), only yehH appears differentially expressed at N+ in ΔyeaG bacteria compared to the wild-type (Fig. 4a). Further, analysis of the transcript levels of yehH by qRT-PCR at N+, N− and N-24 showed that yehH transcripts were increased by ~fivefold in ΔyeaG bacteria compared to wild-type bacteria at N+ but expressed to identical levels at N− and N-24 (Fig. S3, and also see Fig. 4b, c and Tables S3 and S4). We cannot conclusively explain why yehH transcript levels are increased at N+ but not at N− or N-24 in ΔyeaG bacteria, however, we are confident that the properties of ΔyeaG bacteria described below are solely due to the absence of yeaG (and unlikely due to the dysregulation of yehH) as the properties of mutant bacteria described can be complemented (see Fig. S5 later). Categorization of differentially expressed genes using Clusters of Orthologous Groups (COG) analysis [22] revealed that the majority of upregulated genes at N− in ΔyeaG bacteria were associated with the biosynthesis of flagella and motility (flag, flhD, motA, motB, tap, tar, tsr, fiml and flgK) (Fig. 4d and Table S3). Spinning of E. coli cells is indicative of the presence of functional flagella when tethered to a glass surface. Therefore, we examined samples of ΔyeaG and wild-type bacteria from N− by phase-contrast microscopy. As shown in Movie S1 (MP4, 71.47 Kb in the supplementary material), we failed to detect any spinning cells in the wild-type sample. In contrast, the ΔyeaG sample contained several spinning and motile cells (Movie S2 – MP4, 71.95 Kb in the supplementary material). Interestingly, however, in Movie S2 we note that ~2% of the ΔyeaG cells were spinning or motile, while most cells resembled wild-type bacteria, suggesting that the upregulation of motility genes in the ΔyeaG population at N− could occur in a heterogeneous manner. We further note that the motility-associated genes
A role for YeaG in regulating methionine biosynthesis genes in E. coli experiencing sustained N starvation

Since the viability of the ΔyeaG population begins to decline after 24 h under N starvation, we next focused on differences in the transcriptomes of wild-type and ΔyeaG bacteria at N-24. COG analysis of differentially expressed genes at N-24 revealed that the majority of upregulated genes were either uncharacterized or associated with amino acid transport and metabolism, and of the latter group, genes involved in the biosynthesis of methionine were most highly upregulated (Fig. 4c, e and Table S4). Conversely, genes associated with coenzyme transport and metabolism, and particularly NAD+ biosynthesis, were most highly downregulated (Fig. 4c, e and Table S4). Interestingly, aspartate serves as the precursor for both NAD+ and methionine biosynthesis, thus the data suggests that aspartate becomes preferentially directed towards methionine biosynthesis in ΔyeaG cells at N-24.

In E. coli, the transcription of methionine biosynthesis genes is under negative regulation by MetJ. In wild-type bacteria, when cells become nitrogen starved (i.e. when they transition from N+ to N-), the transcript levels of genes within the MetJ regulon decreased. This response occurs in ΔyeaG bacteria to a similar extent (Fig. 5a). However, during sustained nitrogen starvation (i.e. when the cells transition from N- to N-24), a subset of these genes become upregulated in both wild-type and mutant bacteria (Fig. 5b), consistent with the view that the transcriptome of E. coli experiencing N starvation is dynamic. Strikingly, in ΔyeaG bacteria, the magnitude by which this upregulation occurred was substantially higher compared to the wild-type (Fig. 5b, c, and Table S4). Quantification of transcript levels of the most dysregulated genes in ΔyeaG bacteria (artJ, metA, metB, metF, metR, mmuP, nadA, nadB, pnuC, and yhdV) by qRT-PCR in wild-type, ΔyeaG bacteria and ΔyeaGH bacteria as in ΔyeaG bacteria at N-24 (Fig. S6), underscoring previous observations that YeaG and YeaH functionally interact [6].

Aberrant activation of the methionine biosynthesis pathway after 24 h in N starved ΔyeaG bacteria

As synthesis of one methionine molecule in E. coli requires seven molecules of ATP [23], we thus considered whether the aberrant synthesis of methionine and associated metabolites of the pathway in ΔyeaG bacteria compromises the viability of the mutant population after 24 h under N starvation. We used liquid chromatography-electrospray ionization mass spectrometry metabolomics to carry out a targeted analysis of selected intermediates [O-succinylhomoserine, methionine, S-adenosylmethionine,...]
with the methionine biosynthesis pathway in wild-type and ΔyeaG bacteria at N+, N− and N-24. The intracellular levels of the measured metabolites did not significantly differ between the wild-type and ΔyeaG bacteria at N+ and N− (Fig. 6). However, at N−, the intracellular levels of the measured metabolites were overall lower than at N+ (Fig. 6). Consistent with the differences in the transcription of methionine biosynthesis genes between wild-type and ΔyeaG bacteria at N-24 (Figs 4c and 5, Table S4), the intracellular levels of O-succinylhomoserine, methionine, SAM and SAH were higher in ΔyeaG cells than in the wild-type at N-24 (Fig. 6). We note that levels of N-formylmethionine, the initiating amino acid for most proteins, are similar in wild-type and ΔyeaG at N-24, suggesting that methionine is routed towards the SAM cycle in ΔyeaG bacteria. Overall, the results unambiguously confirm that the methionine biosynthesis pathway is active and aberrantly upregulated in ΔyeaG bacteria following 24 h of N starvation.

Transcriptional derepression leads to activation of the methionine biosynthesis pathway in 24 h N starved ΔyeaG bacteria

Most of the methionine biosynthesis genes that become dysregulated at N-24 in ΔyeaG bacteria are negatively regulated by the transcription repressor, MetJ, along with its cofactor, SAM, which increases the affinity of MetJ to bind cognate sites by 100–1000-fold [24]. Although intracellular SAM levels are ~threefold higher and metJ transcript levels are ~eightfold higher in ΔyeaG cells compared to wild-type cells at N-24 (Fig. 6 and Table S4 respectively), somewhat paradoxically, it seems that MetJ activity becomes compromised in mutant bacteria during sustained N starvation. We thus considered whether either the stability or the affinity of MetJ to bind its cognate binding sites (called met boxes) might be altered in ΔyeaG bacteria under sustained N starvation. To investigate whether the stability of MetJ is affected during the initial 24 h under N starvation in ΔyeaG bacteria, we introduced an in-frame fusion encoding three repeats of the

Fig. 4. A role for YeaG in regulating motility-associated gene expression during the initial adaptive response to N starvation. (a–c) Volcano plots revealing expression levels of all genes at N+, N− and N-24 (for (a), (b) and (c) respectively) in ΔyeaG bacteria as a log2 fold change from wild-type, where red indicates differentially expressed genes changed ≥twofold with a false-discovery-rate-adjusted P-value <0.05. The identities of genes that are most differentially expressed and relevant to the study are provided and shown in red. (d) Graph categorizing all differentially expressed genes at N− based on their COG annotation. (e) As in (d) but for N-24.
FLAG epitope at the 3' end of MetJ in wild-type and ΔyeaG bacteria. Initially, we confirmed that the presence of the FLAG epitope did not adversely affect the activity of the MetJ protein by comparing the relative levels of metF transcripts (a gene which contains five met boxes and thus, is heavily repressed by MetJ in the presence of excess methionine; Fig. 7a) in wild-type, ΔmetJ, wild-type metJ-FLAG and ΔyeaG metJ-FLAG strains grown in LB broth supplemented with 100 µg ml⁻¹ methionine. As shown in Fig. 7(b), the FLAG epitope did not detectably affect the activity of MetJ. We then used an antibody against the FLAG epitope to measure the relative intracellular levels of MetJ-FLAG protein in whole-cell extracts prepared from wild-type metJ-FLAG and ΔyeaG metJ-FLAG cells as a function of time (up to 24 h) under N starvation. Results shown in Fig. 7(c) reveal that the intracellular levels of MetJ-FLAG protein in wild-type metJ-FLAG and ΔyeaG metJ-FLAG cells do not sufficiently differ to warrant the increased levels of metF transcription (and by inference, transcription of other MetJ-controlled genes) in the ΔyeaG bacteria at N-24. In fact, we note that the intracellular level of MetJ-FLAG protein is ~1.6-fold higher in ΔyeaG bacteria than wild-type bacteria at N-24.

We next investigated whether the affinity of MetJ to bind to the met boxes is adversely affected in ΔyeaG bacteria at N-24. To do so, we used chromatin immunoprecipitation to obtain MetJ-FLAG bound DNA fragments using anti-FLAG antibodies and subsequent rate-limiting semi-quantitative PCR for specific enrichment of a MetJ binding sequence. To overcome the limited sensitivity of this method, we chose to enrich the metF regulatory region because metF (i) is the only MetJ repressed gene that contains five met boxes (Fig. 7a), (ii) is highly de-repressed in our RNA-seq analysis in ΔyeaG bacteria (Fig. 4c and Table S4), and (iii) is widely used for in vitro analyses of MetJ-DNA interactions [24, 25]. As shown in Fig. 7(d), the binding of MetJ to the metF regulatory region in ΔyeaG bacteria was only slightly decreased (by ~1.3- (at N/C₀) and ~1.5- (at N-24) fold) (compare lane 5 with 14 and 8 with 17, respectively), suggesting that the affinity of MetJ to bind the met boxes is only subtly compromised in ΔyeaG bacteria compared to wild-type bacteria. Since the met boxes at most MetJ controlled promoters (as predicted or experimentally validated on EcoCyc – www.ecocyc.org) are located with respect to the core promoter sequences (i.e. −35 and −10 regions) such that MetJ

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**Fig. 5.** A role for YeaG in regulating methionine biosynthesis genes in *E. coli* experiencing sustained N starvation. Graphs showing the log₂ fold change in expression levels of genes of the MetJ regulon (as defined by [15]) in wild-type (left) and ΔyeaG (right) bacteria at N− relative to N+ (a), N-24 relative to N− (b), and N-24 relative to N+ (c). The schematic representation of the bacterial growth curve at the top indicates the time points: N+, N− and N-24 with respect to the N consumption ([NH₄]) and growth (OD₆00 nm).
binding would occlude RNAP binding, we immunoprecipitated RNAP and repeated the experiment described in Fig. 7 (d) to determine how efficiently RNAP bound to the \textit{metF} promoter in wild-type and mutant bacteria. As shown in Fig. 7(e), a ~4.8-fold increase in RNAP binding to the \textit{metF} promoter was detected in \textit{DyeaG} bacteria compared to wild-type bacteria at N-24 (compare lanes 2 and 4). It thus seems that, despite MetJ being present at the \textit{metF} promoter, and by extension, other MetJ-repressed promoters, RNAP can access and initiate transcription from these promoters in \textit{DyeaG} bacteria at N-24. Overall, we conclude that the aberrant upregulation of methionine biosynthesis genes in \textit{DyeaG} bacteria at N-24 occurs because MetJ is unable to effectively prevent RNAP from accessing the promoter, thus leading to transcriptional derepression of MetJ-regulated genes.

**Transcriptional dysregulation of methionine biosynthesis genes does not appear to be a contributing factor for compromised viability of \textit{DyeaG} bacteria experiencing sustained N starvation**

Since MetJ-repressed genes clearly become upregulated in \textit{DyeaG} bacteria experiencing sustained N starvation, resulting in the aberrant biosynthesis of methionine and associated intermediates, we considered whether this energetically costly process is a contributing factor towards the compromised viability of \textit{DyeaG} bacteria experiencing sustained N starvation. To investigate this, we compared the viability of \textit{DyeaG}, \textit{DmetJ} and \textit{DyeaGΔmetJ} bacteria at N− and following 5 days under N starvation with that of wild-type bacteria by counting viable c.f.u. No difference in the viability was detected for \textit{DyeaG}, \textit{DmetJ} and \textit{DyeaGΔmetJ} bacteria compared to wild-type bacteria at N− (data not shown).
Consistent with the data shown in Fig. 1(b), the viability of ΔyeaG and ΔyeaGΔmetJ bacteria decreased by ~58 and ~46%, respectively, compared to wild-type bacteria following 5 days under N starvation (Fig. 8). However, surprisingly, the viability of ΔmetJ was slightly higher than that of wild-type bacteria (~127%) following 5 days under N starvation. Overall, we conclude that the transcriptional dysregulation of methionine biosynthesis genes, and the consequential aberrant biosynthesis of methionine, is not a contributing factor for the compromised viability of ΔyeaG bacteria experiencing sustained N starvation.

**Conclusions**

Many bacterial adaptive responses begin with, and are accompanied by, large-scale changes to the transcriptome. The coordination and the scale of the changes that occur in the transcriptome are often underpinned by multiple and complex control layers, which ensure the appropriateness of the adaptive response to benefit the cell. The initial adaptive transcriptional response to N starvation in *Escherichia coli* and related bacteria is orchestrated by the global transcription factor, NtrC. This initial response, which is energetically costly, allows the cells to adjust their physiology to
actively import and assimilate N from alternative resources. How the transcriptome changes and the regulatory basis underpinning such changes during sustained N starvation however, are not well understood. Although expression of the NtrC regulon does not considerably differ between wild-type and ΔyeaG bacteria, the results of this study indicate that the transcriptome of E. coli experiencing sustained N starvation is likely to be dynamic and subject to temporal regulation. Surprisingly, despite σ38 levels being clearly lower in ΔyeaG bacteria compared to wild-type bacteria at N-24, our transcriptomics data show that the σ38 regulon is only subtly differentially expressed in ΔyeaG bacteria.

The initial adaptive response to N starvation is largely a scavenging response and does not involve chemotactic-like behaviour [26]. Intriguingly, some cells in the ΔyeaG population synthesize functional flagella and display motility upon entry into N starvation. This observation implies a potential role for YeaG in regulating flagellum biosynthesis genes and motility during the initial adaptive response to N starvation, which warrants further investigation. We further note that a study by Dong et al. [27] revealed that a ΔrpoS E. coli strain displays enhanced motility due to the increased expression of FliA (the RNAP σ factor associated with the transcription of motility-associated genes, including those upregulated in ΔyeaG bacteria at N−; Fig. 4d and Table S3). Thus, it is possible that the increased motility of the ΔyeaG bacteria at N− is somehow connected to the reduction in σ38 levels in ΔyeaG bacteria at N-24 (Fig. 3a). However, it is unlikely to be the case since motility genes are not differentially expressed at N-24.
We have uncovered that YeaG is part of the regulatory sub-circuit controlling the expression of methionine biosynthesis genes in sustained N starved E. coli and furthermore, have demonstrated that the absence of YeaG results in transcriptional derepression of these genes and subsequent activation of the methionine biosynthesis pathway. It appears that YeaG is required for the ability of MetJ to fully repress the transcription of genes within its regulon in E. coli experiencing sustained N starvation. Strikingly, however, the aberrant activation of the methionine biosynthesis pathway is not a contributing factor for the decreased viability of ΔyeaG bacteria experiencing sustained N starvation.

We note that the intracellular levels of SAM are ≈threefold higher in ΔyeaG bacteria compared to wild-type bacteria at N-24. SAM is generated from methionine and ATP to be used as a major cofactor (reviewed in [28]) within the cell, and an important nucleoside serving as a methyl donor in a broad array of metabolic and biosynthetic reactions, including methylation of DNA, RNA, proteins and lipids. Methylation can have direct biochemical relevance in metabolic reactions and is involved in transcription regulation at the epigenetic level. Thus, it is tantalizing to speculate whether epigenetic control of the transcriptome, through methylation or phosphorylation, happens during sustained N starvation and the role of YeaG in this process. In other words, based on our data that YeaG affects the transcriptome of E. coli experiencing N starvation, we hypothesize that dysregulated epigenetic control of the transcriptome in the absence of YeaG could adversely affect the viability of E. coli experiencing sustained N starvation.

In summary, we consider that the yeaGH operon represents a new branch in the transcriptional response to N starvation in E. coli and related bacteria (Fig. 9). Although the transcriptional responses to transient growth arrests or during growth transitions due to nutrient starvation (e.g. diauxic growth) has been previously explored, this study uniquely demonstrates that the transcriptome of growth attenuated bacteria experiencing sustained nutrient starvation is plastic, and in the case of the N stress response, this plasticity is affected by the kinase, YeaG.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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