# *E. coli* leverages growth arrest to remodel its proteome upon entry into starvation

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## Abstract

It is widely believed that, owing to the limitation of nutrients in natural environments, bacteria spend most of their life in a non-growing state. However, despite its major clinical and ecological implications, very little is known about what determines the phenotype of starved bacteria, in particular what controls the concentration of different gene products inside the cells. Using microfluidics and quantitative fluorescence microscopy, we monitored growth and gene expression in many independent *E. coli* lineages as we switched them from exponential growth to starvation. We observed that all cells stopped growing immediately and that no cell death occurred for more than two days. At the same time, gene expression undergoes a dramatic remodeling upon entry into starvation in a promoter-dependent manner. Some promoters, including ribosomal protein promoters, arrest gene expression immediately, others show a slow exponential decay of expression on a 10 h time scale, while a third category exhibits a transient burst of activity before decaying exponentially. Remarkably, the time dynamics of these changes are highly homogeneous across single cells. In addition, we demonstrated that the gene expression response does not qualitatively depend on the dynamics of starvation entry. Combining the observed time-dependent protein production and decay rates, we showed with mathematical modeling that a delay between growth arrest and shutdown of gene expression allows a massive increase in the concentration of certain proteins without requiring an upregulation of their expression. Moreover, protein concentrations deep in starvation appeared to be mainly determined by the expression dynamics during the first 10 h of starvation. Finally, we established that this expression program at the onset of starvation is critical for cell viability. In particular, by inhibiting gene expression during different periods of starvation, we were able to show that the tolerance to stress after 2 days is determined by gene expression occurring during the first 10 h, which thus constitutes a preventive response. These results provide a starting point for quantitative studies of cell maintenance and the emergence of specific phenotypes when nutrients become scarce.

## Introduction

Bacteria are present in most ecosystems, and some bacterial species are capable of growing very fast. This rapid growth is an important ingredient of their ecological importance, as it enables them to consume available resources before other competing species. In the laboratory, conditions that support fast bacterial growth are common because they can be used to prepare cultures in which

cells are in a steady state of growth, and hence have a defined composition. In quantitative biology, this is a common assumption of mathematical models which must be fulfilled to analyse experimental observations in their light. More generally, having cells in a defined state constitutes a key ingredient of reproducibility between experiments. As a consequence, bacteriology knowledge mainly covers phenomena related to growth. Noteworthily this focus on fast growth has also led to ignoring that other traits related to slow growth or survival during growth arrest may be equally important over evolutionary timescales.

Bacteria in their natural environments are often starving for nutrients and thus growing very slowly, if at all. Although the physiological state of bacteria in the wild is very difficult to characterize, we note that if the bacterial population at the global scale remains roughly constant in size, growth and death must be balanced. Among the many ways that bacteria die (*e.g.* predation by phages), it is well-established that starvation unavoidably leads to rapid death of the majority of the population, although the mortality dynamics vary much between species and between conditions [1,2]. Hence, one would *a priori* expect the selection pressure for survival, and for surviving starvation in particular, to be as important as the selection for fast growth.

When a missing nutrient becomes available, bacteria can grow and divide until it is exhausted. For instance, in the case of standard laboratory cultures, bacteria are inoculated at low concentration, grow exponentially as long as all nutrients are available, and subsequently enter a 'stationary phase' where the population size initially remains constant and then decreases due to cell death. Stationary phase is therefore a practical setting for studying bacterial starvation. Previous studies of stationary phase have revealed that the physiological state of the cells and the survival rate of the population depend strongly on the precise physico-chemical conditions (*e.g.* limiting nutrient, osmolarity, pH, *etc.*) [3,4]. The medical significance of growth-arrested bacteria has also been clearly established since various pathogenic bacterial species induce their virulence genes at the transition to stationary phase [5–8]. Moreover, bacteria in stationary phase have higher levels of tolerance to antibiotics [9–11], and the fraction of persister cells is much higher in stationary phase cultures than in exponentially growing ones [12,13].

Despite the medical significance of bacteria in stationary phase, their physiology remains relatively unknown. Several observations indicate that cells become much less active: their energy level sinks [14–16], the DNA is coated by the protein Dps which is not expressed in growing cells [17], and the composition of the cytoplasmic membrane and the cell wall changes to make them sturdier [3,18]. In contrast to these signs of decreased activity, a study of the expression of a synthetic inducible promoter revealed that there is continued gene expression during 50 h [19], at $\approx 10\%$ of the level of growing cells. This indicates sustained metabolic activity and raises the question of how bacteria shape their phenotype (e.g. virulence, or tolerance to antibiotics) during stationary phase. It should be noted that the term 'stationary phase' is loosely defined. The state in which bacteria are found depends on multiple factors, such as which nutrient is depleted, the duration spent in stationary phase, but also on how quickly bacteria transition from growth conditions to growth arrest. Indeed, while many bacterial species are likely to enter stationary phase after gradual nutrient depletion in the wild, the typical speed of such transitions

remains unknown. Interestingly, the rate of the depletion can be modulated by changing the bacterial population density at the entry into stationary phase, and the subsequent death kinetics are delayed when depletion takes longer [20]. However, the overall importance of the gradual depletion of nutrients upon entry into stationary phase, compared to an abrupt transition to nutrient-free media, remains to be elucidated.

Noticeably, stationary phase is a complex state involving not only starvation, but also cell-to-cell communication, chemical changes of the media by the bacteria, etc. We focus on starvation since it is a generic feature of stationary phase, and in particular on the case of carbon starvation where energy carrier molecules fueling cellular activity (such as ATP) cannot be replenished from external nutrients. In this context, many aspects of *E. coli*'s response to starvation remain to be elucidated, in particular how protein production continues after nutrients are depleted, and whether gene expression reaches a steady state before hitting zero. This also raises the question of how this continued gene expression during starvation impacts phenotypes, more specifically protein concentrations. Finally, since cell-to-cell variability is reportedly higher during stationary phase than during growth [4], understanding how bacterial phenotypes are established during starvation requires characterizing the variability of these processes across individual cells and comparing the variability in gene expression between growth and starvation.

In this article, we report on experiments in which bacteria were monitored with single-cell resolution throughout starvation in a microfluidic device. The continuous flow during the experiments allowed cells to be switched to starvation in a well-controlled manner and to maintain the environment constant afterward. Although our experimental design allows us to measure gene expression during prolonged starvation, we observed the largest changes in gene expression immediately upon entering starvation, which has not been reported previously. While some genes cease to be expressed immediately, some gradually fade within the first 10 h of starvation, and others show a transient increase in expression. We further characterise how gene expression immediately following growth arrest has a major impact on the state of the cell during starvation; in particular, the concentration of genes whose expression continues for a short period after growth arrest increases markedly compared to their concentration during growth. Comparing to another setup where a batch culture is flown through the microfluidic chip as it progresses from exponential phase to stationary phase, we show that the gene expression response is qualitatively similar between abrupt and gradual growth arrest. Finally, we demonstrate that this transient expression upon entry shapes phenotypes during starvation for several days and, in particular, confers resistance to stress late in starvation, preparing the cells for future challenges.

# Results

In order to study gene expression under starvation with single-cell resolution, we used an integrated setup which combines microfluidics, time-lapse microscopy and image analysis [21]. After inoculation in the Dual Input Mother Machine (DIMM) microfluidic chip, *E. coli* were grown in M9 minimal media supplemented with 0.2% glucose during 10 h, then exposed to M9 minimal media

without carbon source (referred to as "M9 Zero") for 60 h. If relevant, viability at the end of the starvation period was assessed by regrowing cells in fresh media during 10 h. We used a multiplexed version of the DIMM chip in order to measure up to 8 strains in parallel, each carrying a different transcriptional fluorescent reporter (Fig. 1A).

The cell length and the total fluorescence were measured at each time point and for every individual cell in the experiment using our image analysis software DeepMoMA (see SI. 3). From this we infer the instantaneous exponential growth rate $\lambda(t)$ and the volumic production rate $q(t)$ for our reporter, defined as the rate of production of fluorescent protein per unit volume, assuming the total amount of fluorescence $F$ evolves as $\frac{dF}{dt} = q(t)V(t) - (\beta + \gamma(t))F(t)$ where $\beta$ is the fluorescence photobleaching rate and $\gamma(t)$ is a time-dependent degradation rate. The volumic production rate is expected to reflect the product of the promoter-specific transcriptional activity and of the global ribosome activity. We use this rate as a measure of gene expression activity, and use the two terms interchangeably hereafter. In practice, estimating *instantaneous* growth rates and volumic production rates from time series of cell size and fluorescence is challenging because these are affected by significant measurement noise. For example, simply taking discrete derivatives between neighboring measurement time points would result in estimates that are far too noisy to be useful. To address this challenge, we recently developed an advanced Bayesian inference procedure that models the fluctuating growth and production rates using maximum entropy process priors that are characterized only by their mean, variance, and correlation time. The inference additionally requires knowledge of the photobleaching and degradation rates of the fluorescent proteins, which we estimated using independent calibration experiments (see SI. 7 and 6; Fig. S3). With the fitted prior, we then inferred posterior distributions for the instantaneous growth and production rates of every cell at every time point. Although this approach was primarily designed for measurements at steady state, we have established that it also reliably estimates growth and production rates during transients, *e.g.* upon entry into starvation (Fig. S1, and SI section 5).

The most readily observable signatures of starvation are the arrest of cell growth and the decrease of cell size. Interestingly, none of those two was described by directly observing cells with a high time resolution as they get abruptly switched from exponential growth to a carbon-free media. It has been reported recently that *E. coli* cells are all growth-arrested during stationary phase [22]. However, when bacteria in microfluidic chips are starved by exposing them to a continuous stream of nutrient-free media, it can be challenging to arrest growth since chemicals present at low concentration in media components may be used for growth given the high supply rate. In our setup, upon the switch from M9 + 0.2% glucose to M9 zero, we observe that all bacteria stop growing immediately (within the 3 minute microscopy acquisition time, see Fig. S4A,B). In addition, although growth has completely ceased, within the first 10 h a large fraction of cells (38%) undergoes division, with most of these reductive divisions occurring early and their frequency decreasing with time (Fig. S4C). This leads to a decrease of the average cell size with time (Fig. 1B). It is well-known that the average cell size decreases upon entry into stationary phase [22, 23], and this has been attributed to a different mechanism of cell size

control during the last cell cycle(s). In contrast, our data show that reductive division can happen even in absence of cell growth. Moreover, whether a cell will divide or not after entering starvation is very well predicted by its size at the time of entry, with larger cells more likely to divide (Fig. S4D). Interestingly, although the added volume should be a better predictor of cell cycle progression assuming an adder control mechanism for the division, we find that both cell length and added length since birth measured at the moment of the switch are highly predictive of whether a reductive division occurs. Taken together, the sharp growth arrest and the frequent occurrence of reductive divisions are insights into the mechanism behind cell size changes upon entry into starvation. This indicates that our setup used with M9 zero constitutes a powerful platform to study starvation at high time resolution, under controlled conditions.

We measured gene expression dynamics during starvation using transcriptional fluorescent reporters for a set of native and synthetic promoters selected as follows. For native promoters, we took advantage of previous flow cytometry measurements [24] to pick 18 promoters spanning a broad range of expression and of fold-changes between growth and stationary phase (Fig. S5). In order to quantify expression of constitutive genes, we also used 4 synthetic promoters that were obtained by screening random sequence fragment for their expression level during exponential growth (either "medium" or "high") [25], and for which we verified the absence of regulatory inputs other than sigma factor binding sites (RpoD/RpoS). During the course of our study, we established that while high expressors are primarily driven by RpoD and thus good reporters of constitutive gene expression, some medium expressors instead primarily report on RpoS activity (cf SI. 10).

Fig. 1C shows the average and standard-deviation of volumic protein production rate across time for 4 native promoters representative of the diversity of the dynamics that we observe across the 22 promoters (Fig. S7A). Note that values are scaled to the average volumic production rate during exponential growth and that our set of promoters covers a range of absolute volumic production rates that spans more than 2 orders of magnitude during growth, and almost 4 orders of magnitude during starvation (Fig. S7B). Overall, protein production rates change dramatically at the onset of starvation. As expected, we observed that mean volumic protein production rate deep in starvation is always lower than it was during exponential growth. As can be seen from Fig. 1C, production patterns are diverse and rather complex upon entry in starvation: Certain promoters, such as the ribosomal promoter rplN, undergo a sharp down-regulation of production that almost stops their production within a short time scale. Other promoters, such as hupA, see a sharp initial drop in production, followed by a slower decay, and even a very long sustained production at a lower level. Finally, promoters such as hslV and bolA show a transient burst of variable amplitude in production, that increases production over what it was during exponential growth, followed by a slower decay in production that proceeds at different rates for different promoters.

Although there might have been large variations in the timing and magnitude of the response in individual cells, we observe on the contrary that such bursts are very uniform across all cells (Fig. 1D), which suggests that they are part of a deterministic response of bacteria to starvation. Given how reproducible the time-dependent responses are across single cells, we further analyse the

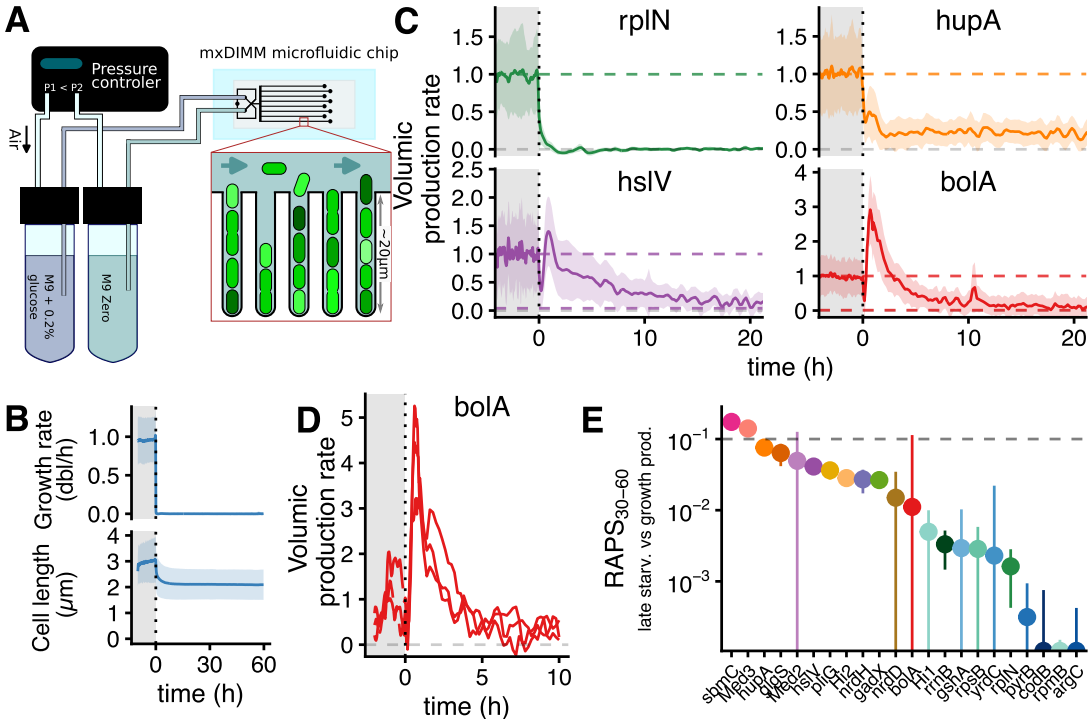


**Figure 1. Characterisation of gene expression during starvation using single-cell measurements in microfluidics**. (A) Length and fluorescence of individual *E. coli* bacteria was monitored as cells were switched from exponential growth (M9 minimal media + 0.2% glucose) to starvation conditions ("M9 Zero" without carbon source) in a modified DIMM microfluidic chip [21], allowing to measure up to 8 strains independently. (B) After switching to "M9 zero", elongation stops immediately (lower panel, Fig. S4), and average cell size decreases due to reductive divisions (bottom panel). Lines and ribbons show mean and mean ± s.d. across all single cells, respectively; time 0 was defined as the time of the media switch. (C) Gene expression is measured as the protein production rate per unit volume $Q$ of a transcriptional reporter, inferred for individual cells from time series of length and fluorescence levels. Four example promoters (out of 22) are shown in order to depict the diversity of the responses upon entry into starvation (only the first 20 of 60 h are shown); lines and ribbons show mean and mean ± s.d. across all single cells, respectively, normalized by the average value during exponential growth. Coloured dashed lines are guides for the eye that show 1 (to compare the relative changes with exponential growth) and the average production rate in the last 30 h of starvation relative to the exponential growth average (Relative Average Production in Starvation between 30 and 60h, $\mathrm{RAPS}_{30\text{-}60}$). (D) Sample single-cell traces of volumic production $Q$ for the bolA promoter show a consistent response across the population upon entry into starvation. (E) Levels of continued gene expression during starvation ($\mathrm{RAPS}_{30\text{-}60}$) for 18 native and 4 synthetic promoters vary over more than 3 orders of magnitude. The horizontal dashed line indicates the value reported previously for a synthetic promoter in [19].

responses of different promoters based on their time-dependent means and standard-deviations across the population. The coherence of this response across single cells is truly remarkable, and rarely observed, due to the stochastic behavior often observed across single cells. In comparison, changes occurring due to gene regulation often result in different timing of the response between cells [26,27]. In such situations, taking population average would dampen the individual response patterns, and often make them invisible. The distribution of responses observed across individual cells for volumic production will naturally propagate to the distribution of concentration of the corresponding proteins during starvation. We analyse this phenotypic variability in more details later on.

For many promoters, the production is sustained (i.e. significantly higher than zero) until the end of our experiments (Fig. S8A). This is in line with the *Constant Activity in Stationary Phase* (CASP) reported earlier at the population level over 50 h [19] although, rather than a constant production, the activity of all promoters keeps decreasing slowly over this timescale (see SI 9). In this context, to compare the activity of different promoters during starvation, we decided to measure the average volumic production rate over a defined time window, relative to the average rate during growth (referred to as $\mathrm{RAPS}_{30\text{-}60}$, for Relative Average Production in Starvation between 30 and 60 h). It is conceivable that the gene expression response during starvation is driven primarily by the overall reduction of energy or of building blocks availability, in which case all promoters would have similar levels of $\mathrm{RAPS}_{30\text{-}60}$ (estimated at approx. 10% in [19]). To the contrary, our data reveal that $\mathrm{RAPS}_{30\text{-}60}$ is very variable across promoters and varies by more than 3 orders of magnitude (from 20% down to 0.01%) in our set of 22 promoters (1 E). Thus gene expression during starvation is not only governed by the decrease of the overall gene expression capacity of the cell, but also by profound changes of the state of the gene regulatory network.

In order to assess the integrated effects of the protein production rate changes that occur during starvation, we also investigated how protein concentrations change across time for these promoters. While protein production rates are the best measure of the *current* gene expression state of the cell, protein concentrations are also very important from a physiological perspective, as they dictate the rates of biochemical reactions that occur within the cell.

Based on our careful measurements of instantaneous production rate and growth rate, we obtain the time series of fluorescent protein concentration by numerical forward integration (Fig. 2A). In order to be able to account for protein degradation during starvation, we performed additional calibration experiments (Fig. S2, SI.6, SI.8). These experiments indicate that the degradation of fluorescent proteins decelerates markedly during the first 2 days of starvation, with the degradation *rates* decreasing exponentially with time. Our estimates of the initial values of degradation rate (0.02 to 0.03 $\mathrm{h}^{-1}$, see Table S5) are compatible with the overall protein degradation rate at vanishingly slow growth [28], and the marked slow-down of degradation during starvation is consistent with the fact that the vast majority of protein degradation in *E. coli* is performed by ATP-dependent proteases [29,30] and that ATP levels decrease dramatically during starvation [15,16,31]. Furthermore, changes in the levels of metabolites related to protein degradation upon entry into carbon starvation

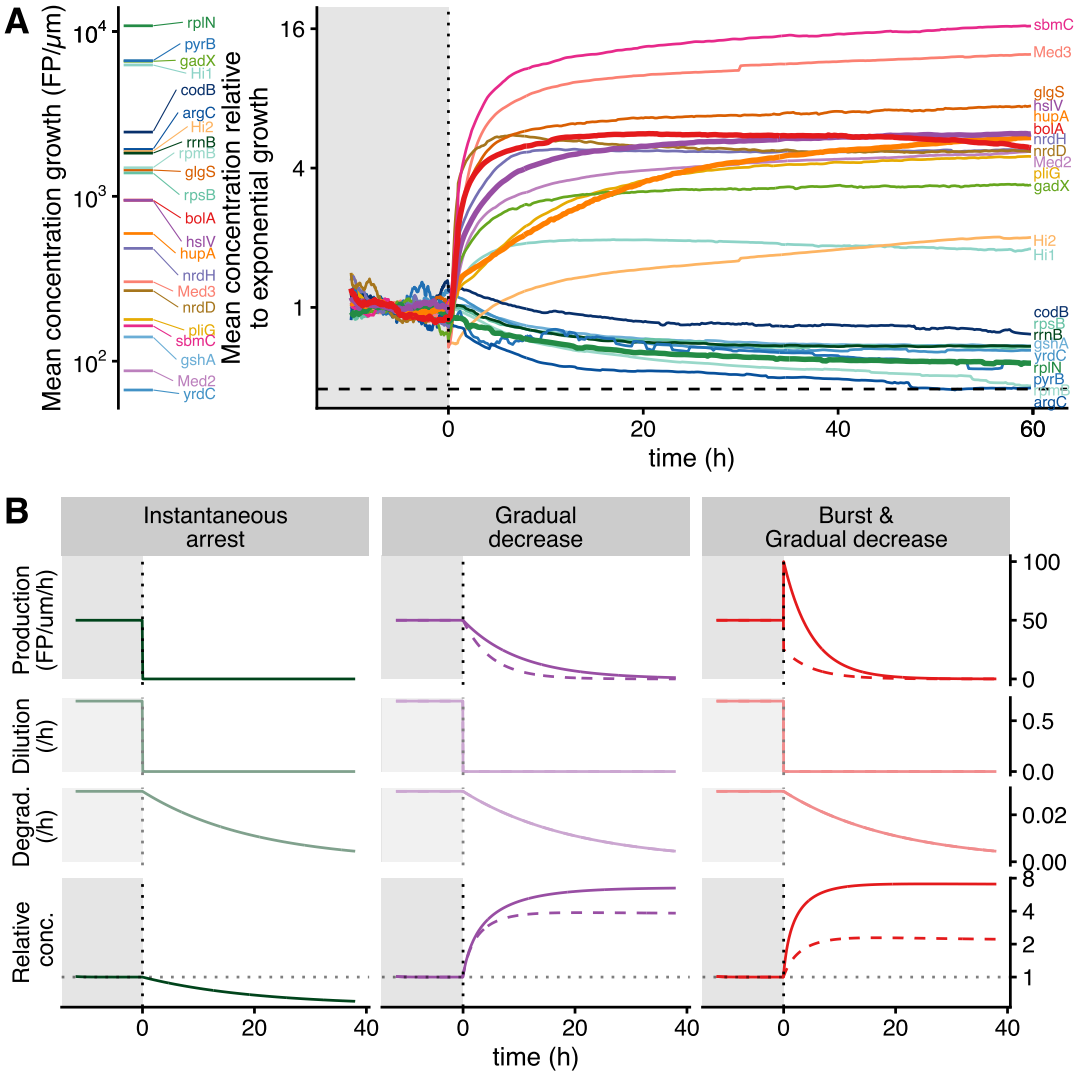


**Figure 2. Dynamics of protein concentration during the switch from growth to starvation.** (A) The protein concentrations corresponding to promoters used in this study span a broad range (ca. 100 ×) during exponential growth (left), and change in a promoter-dependent manner upon entry into starvation (right). Protein concentrations are measured in fluorescent protein per $\mu$m (FP/$\mu$m) and corrected for photobleaching (see SI.8). Time traces show averages across all cells, scaled to the average concentration during exponential growth; promoters shown in Fig. 1C are indicated with thick lines. The horizontal dashed line shows the minimal concentration that would be reached, theoretically, in the case of instantaneous arrest of both production and growth upon entry into starvation (see SI.12). (B) Predictions of a simple model of gene expression dynamics under three different scenarios (columns). Protein concentration dynamics (bottom row) is determined by the dynamics of protein production (top row), dilution (second row) and protein degradation (third row). For all different scenarios of production dynamics, dilution drops to zero as growth stops completely after the switch (Fig. 1.B) and degradation rates decay exponentially during starvation as measured experimentally (Fig. S2). All parameters of the model are set after experimental observations (see SI.12). Note that the predicted fold changes are comparable to those measured experimentally in A.

also indicate that degradation slows down during starvation [14]. Since we rely on transcriptional reporters to measure changes in gene expression from growth to starvation conditions, it is also important to take into account how degradation changes for the proteins of interest. For this, we set out to measure the temporal dynamics of native protein levels in batch cultures using quantitative proteomics (see SI 11). However, due to the limited accuracy in measuring individual protein levels, these experiments were inconclusive (Fig. S9). Similarly, publicly available proteomics datasets comparing growth and starvation also show too much variability between replicates and datasets to reliably estimate the change in concentration (Fig. S9). Based on available knowledge, we thus assume that the overall decrease of degradation due to reduced energy levels is the dominating effect for most genes, and that concentrations of fluorescent proteins used as transcriptional reporters are representative of the corresponding proteins.

It is noteworthy that, among our set of promoters, there are almost none whose protein concentration remains stable across the switch, i.e. concentration either mildly decreases due to degradation after a sharp production arrest, or increases more than 2-fold (Fig. 2A). Notably, synthetic promoters reporting constitutive gene expression (high expressors) are the closest to staying constant in concentration. For downregulated promoters the fold-change is always moderate, even if production is completely arrested upon the switch (theoretical dashed line in Fig. 2A), because dilution stops and protein degradation is relatively slow. In contrast, for promoters whose concentration increase, the relative change can be much larger, up to 12-fold across our set of 22 promoters. Strikingly, for those promoters, most of the changes seem to happen within the first 10h of starvation, suggesting that the observed differences production deep into starvation, *i.e.* $\mathrm{RAPS}_{30\text{-}60}$, are not playing a fundamental role in the final concentrations reached at the end of the experiment.

Quite counter intuitively, promoters whose activity does *not* increase upon entry into starvation can nonetheless increase markedly in concentration (i.e. more than 2-fold). Among the 4 examples of Fig 1C, this is the case for hslV, and for hupA although with a slower increase (Fig. 2A right). Mechanistically, this increase comes from the delay between growth arrest (which is almost instantaneous) and arrest of protein production (which is gradual and promoter-dependent). As a consequence, although protein production is reduced to very low levels during starvation, the concentration of various proteins nonetheless increases during starvation.

We explained in the introduction why the speed of the transition between growth and starvation is expected to shape the response to starvation. Since very fast depletion of nutrients might be rare in the natural environments of *E. coli*, the cellular physiology might not have been selected to function robustly in response to rapid changes. It is thus conceivable that our findings described so far do not generalize to starvation being induced more gradually, in particular the facts that the relative gene expression in starvation spans several orders of magnitude and that many genes are increasing in concentration upon entry into starvation even without upregulation of their expression.

To address how the dynamics of entry into starvation impacts the gene expression, we did another series of microfluidic experiments where a culture growing in M9 + 0.02% glucose is flown through a mother machine microfluidic

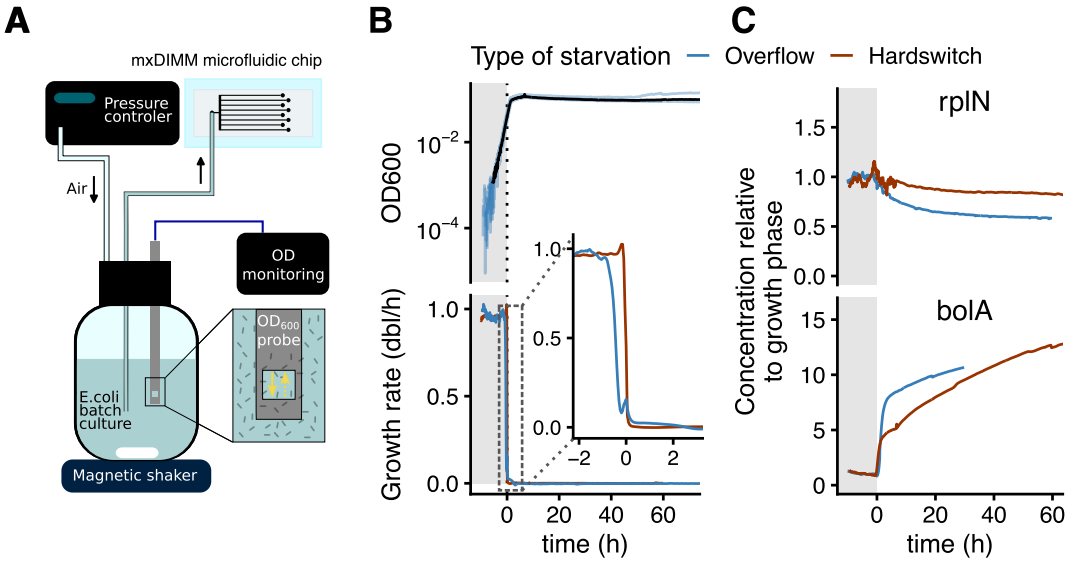


**Figure 3. Temporal dynamics of gene expression is similar when the entry into starvation is gradual** (A) A batch culture of *E. coli* growing in M9+0.02% glucose was connected to a microfluidic chip to induce starvation of cells trapped in the growth channels of the mxDIMM. (B) The $OD_{600}$ in the flask was followed along with the average growth rate of cells in the chip to verify that the growth arrest was synchronized between flask and chip. Time 0 was defined as moment where average growth rate becomes lower than 0.05 dbl/h. Top: individual blue lines correspond to replicates, while the black line is the mean of those replicates. Bottom: average instantaneous growth rate across all cells, along time, measured in the two experimental setups, indicated with colors. The inset is a zoom on a 5 h time window spanning the entry into starvation. The growth arrest is more progressive in the overflow setup (blue line, duration ≈ 50min, see Fig. S11A,B) than in the hard-switch setup (red line, duration ≈ 6min see Fig. S4B). (C) The temporal dynamics and magnitude of the relative concentration, computed as in 2 is similar between the two mode of starvation entry for promoter studied in both (see also Fig. S11B and S10).

chip, so as to expose bacteria to the same physico-chemical conditions in the chip as in the culture flask where nutrients are depleted due to the uptake by bacteria (Fig. 3A).

In order to compare the growth and gene expression dynamics between the two types of transitions, we define the entry into starvation using a criterion on single-cell behaviour, the time when the instantaneous growth rate (averaged over all individual cells) dips below 0.055 dbl/h. At this point, most of the decrease of growth rate has already happened, and it eventually goes to zero on a longer time scale afterwards (see Fig. 3B and Fig. S11A,B). Importantly, while the growth rate dropped from its maximum value to zero in ca. 5 min in experiments with instantaneous switching, this transition takes ca. 45 min in these culture overflow experiments, which almost certainly indicates that nutrients are exhausted more gradually (Fig S11A,B). It is interesting to contrast this timescale with the sharp angle of the growth curve observed at the population level in this media (Fig 3B), and to note that the transition is uniform across all cells *i.e.* there is no subpopulation of cells which can grow at lower nutrient concentration and thus keep growing longer during the transition. It should nevertheless be noted that the growth variability across cells increases during the transition to growth arrest (Fig S11B), which does not seem to happen when cells are switched abruptly (Fig S4B).

Despite this much more gradual entry into starvation, the dynamics of protein production rates (Fig S11C) as well as the resulting dynamics of protein concentration (Fig 3B and Fig S10) are remarkably similar to those measured in experiments with instantaneous switching. This suggests that our observations on gene expression in the experiments with instantaneous switching likely generalize to a broad range of environmental transitions from growth to starvation (among others to the entry into stationary phase in glucose-limited media).

Although the protein production time courses are very diverse between promoters, it seems that most of these dynamics can be captured by two main variables: the amplitude of the initial burst of production and the delay between growth arrest and production arrest. To assess whether the diversity of concentration dynamics seen in our experiments is actually driven by these two parameters, we developed a simple mathematical model of gene expression during starvation. In this model, dilution and degradation are assumed to be the same for all promoters; the former drops instantaneously to zero upon entry into starvation, while the latter decays exponentially as measured in our experiments (Fig. S2). Protein production is promoter-dependent and several cases are considered (Fig. 2B): in a first scenario, production stops instantaneously upon entry into starvation. A second case consists of a simple exponential decrease of the production rate after entering starvation, corresponding to a delayed production arrest. Finally, we also consider the case of a transient burst of production followed by an exponential decrease of the production rate.

We observe that this simple model is able to quantitatively recapitulate the main features of the protein concentration dynamics upon entry into starvation (during the first day): First, for promoters with rapid production arrest, the concentration decreases by 30 to 50%. We also derived analytically the lowest possible relative concentration for GFP, $c_{\min} = 0.444$ (shown with a dashed line in Fig. 2A, see SI. 12 for calculation), attained in the case where growth and production stop immediately upon entry into starvation. Second, introducing

a delay in the arrest of production is enough to produce a large increase of concentration (e.g. there is a 4-fold increase when the production decays with a characteristic time of 2h). Third, the early burst of production can further tune the concentration fold-change observed between growth and starvation (*e.g.* 7-fold for bolA).

Because bolA is a known reporter of RpoS activity, we hypothesize that the increase in concentration we observe for this promoter, and by extension for others that display the same dynamics, are the results of rpoS activity. To test this hypothesis, we studied the concentration levels and fold change for some of our promoters in a ΔrpoS background. We find that promoters going down in concentration in the wild type display a slightly elevated concentration, while promoters going up in concentration during starvation in the wild type display a great decrease of concentration in the ΔrpoS background (Fig. S6A). Surprisingly, knocking out rpoS did not always results in a decrease of the concentration fold change upon entry into starvation (Fig. S6B). Together, those results demonstrate the importance of RpoS on gene regulation during starvation, but suggest that other important regulation mechanisms are at play in this condition.

We discussed above that the dynamics of volumic protein production tends to be highly reproducible across single cells, and this reproducibility is also observed for the protein concentration dynamics (Fig. 4A). To characterize the homogeneity of the response to starvation, we quantify how much of the single-cell response is explained by the population average. For this, we measure the ratio $\rho$ of the variance in the dynamics of the population average and the variance in all observations across all cells $\rho = \mathrm{Var}(\bar{X}_t)/\mathrm{Var}(X_{c,t})$, where $X_{c,t}$ is the observation in individual cell $c$ at time point $t$ and $\bar{X}_t$ is the population average at time $t$. When the concentration fluctuates around a fixed value, $\rho$ is close to zero, and in the limit that changes in the population average are much larger than single-cell variations $\rho$ may approach its maximum value of 1 (Fig. 4B). For promoters whose gene product concentration increases upon entry into starvation, we note that $\rho$ is much larger during the first 10 h of starvation than during exponential growth or later in starvation (Fig. 4C, Fig. S12). For bursting promoters (*e.g.* hslV, bolA), more than 50% of the observed variation across all cells during early starvation is captured by the population average behavior, while $\rho$ remains smaller for promoters only displaying a delayed arrest of production (e.g. 25% for hupA). Altogether, this analysis demonstrates that the gene expression response upon entry into starvation is highly uniform across the population, which in turns supports that the dynamic properties of the response (amplitude of the burst and timescale of the arrest of volumic production for a given promoter) have been tuned by natural selection.

An important consequence of the fact that volumic production rates drop to low levels is that protein concentrations late in starvation will strongly depend on previous gene expression, *i.e.* on the protein production early in starvation, and perhaps even on production during the exponential growth. To quantify the relative contributions from gene expression in different periods to the protein concentrations late in starvation we measured, for each time point in growth arrest, the fractions of proteins that derive from production in the exponential growth, during the first 10 h of starvation, and after the first 10 h of starvation. We use Müller plots to show these relative contributions as a function of time for

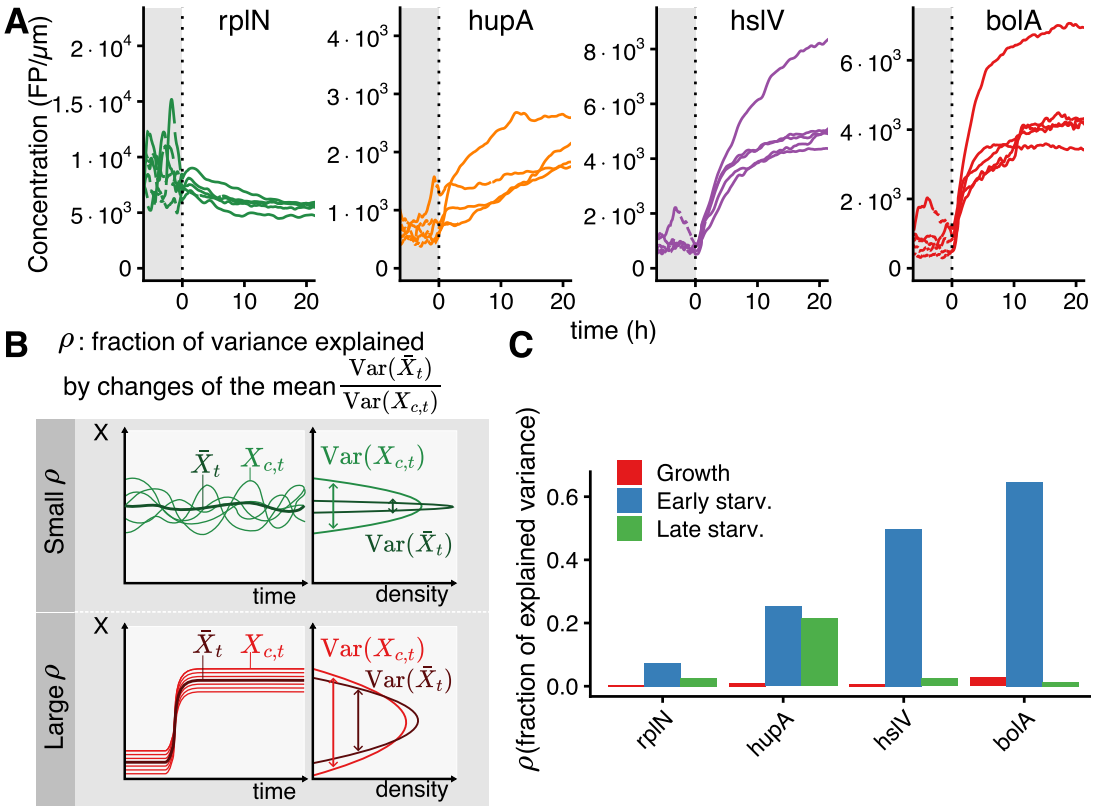


**Figure 4. Concentrations of proteins change rapidly and homogeneously across bacterial population at starvation onset.** (A) The protein concentration dynamics is uniform across single cells and underlies the average population dynamics; lines show traces for randomly chosen single cells for 4 representative promoters. (B) To quantify how uniform changes of concentration are across cells, we calculate $\rho$, the ratio of the variance in the population average protein concentration across time to the variance of all individual measurements. Two limit cases illustrate the meaning of $\rho$. When individual cells fluctuate around a constant value, the population average variance is small compared to total variance ($\rho \approx 0$; top row). In contrast, when all cells follow the same dynamics starting from slightly different values, the total variance is close to population average variance ($\rho \approx 1$; bottom row). (C) For fluorescence concentration, the fraction of explained variance $\rho$ is systematically higher during the first 10h of starvation ("Early starv.", blue), compared to exponential growth ("Growth", red) and late starvation ("Late starv.", green) behavior; values for all promoters are shown in Fig. S12.

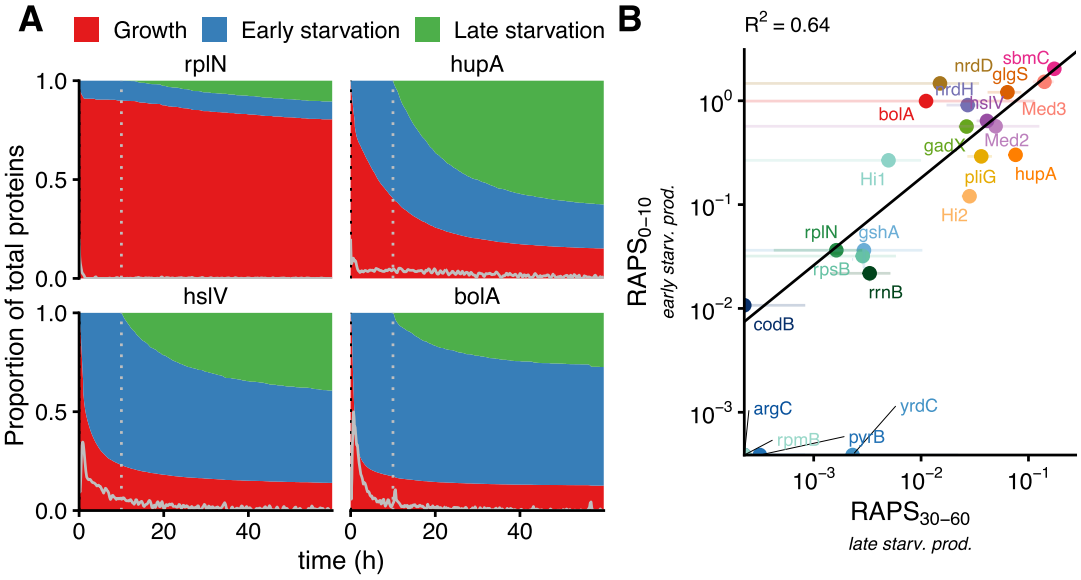


**Figure 5. The gene expression state late into starvation is set early on and maintained for days.** (A) Müller plots showing the proportion of total fluorescent proteins coming from protein production in different time periods, as a function of time in starvation. The proportion is calculated by integrating forward in time the volumic protein production rate and accounting for protein degradation (see 8); "early starvation" (blue, before the vertical dotted lines) corresponds to the first 10 h of starvation where bursts of production are observed for some promoters, while the production tends to stabilized during "late starvation" (10-60 h, green, after the vertical dotted lines). The volumic protein production time courses from Fig. 1C are shown as light grey lines (rescaled such that the production during growth is 0.2). (B) The level of continued gene expression during late starvation, quantified by $RAPS_{30\text{-}60}$, is correlated with the initial production during early starvation as quantified by $RAPS_{0\text{-}10}$(Pearson correlation coefficient $R^2 = 0.64$). Some promoters that fluctuate near 0 at the entry in starvation have negative $RAPS_{0\text{-}10}$. These are set to 0 (bottom) and excluded from the fit.

the same 4 example promoters displayed in previous figures (Fig. 5A). We see that, while for a promoter that is downregulated such as rplN, the expression late in starvation is still dominated by proteins produced during the exponential growth, for promoters with delayed production arrest (*e.g.* hslV) or with a burst (*e.g.* bolA), more than half of the proteins derive from production that happened during the first 10 h of starvation (blue segments in Fig. 5A). For promoters with significant sustained expression (e.g. hupA), the contribution from proteins produced later in starvation eventually increases (green area in Fig. 5A). This observation holds for all promoters displaying similar volumic production temporal dynamics, and we find that here as well individual cells display remarkably homogeneous patterns (see Fig. S13). Interestingly, we find that the ratio of the initial expression ($RAPS_{0\text{-}10}$) and the continued expression during starvation ($RAPS_{30\text{-}60}$) are strongly correlated ($r^2 = 0.64$; Fig. 5B). In particular, promoters that display high relative protein production early in starvation also tend to have sustained expression. This suggests that the mechanism controlling gene expression during the entry into starvation also determines the level of continued expression late in starvation. Moreover, this correlation also implies that the concentrations reached during the first 10 h will be maintained later on by a corresponding level of continued protein production to balance degradation. Thus, the protein production dynamics during the entry and early period of starvation is a major determinant and predictor of protein concentrations deep into starvation, which is reinforced by the correlation between production during early and late starvation. Together, this causes cells to maintain an approximately fixed phenotypic state for days.

We have shown that protein levels during starvation, and hence the state of the cell, is set early on by the initial production: due to the low degradation and low production rate after the first half day of starvation, protein concentrations are rather stable. Moreover the ability to respond to environmental changes during starvation is fundamentally limited by low gene expression levels. This raises the question of the importance of the initial production for survival and stress tolerance during starvation. A straightforward experimental design would be to compare the ability to cope with starvation (assessed as mortality and/or duration of the lag in fresh medium) between an experiment where protein synthesis is inhibited by low dose of chloramphenicol during the first 5 h of starvation and a control experiment (without chloramphenicol). However, mortality after 1 day of starvation is too low to be measured in practice (Fig. S15C). We thus decided to apply a strong oxidative stress (0.65 or 0.75 mM hydrogen peroxide) between 20 and 25 h of starvation. Moreover, the addition of chloramphenicol during the first 5 h of starvation did not prevent the burst of expression observed in RpoS regulated genes, but simply postponed it until the end of the chloramphenicol treatment (Fig. S14).

For this reason, we decided to compare the effect of strongly inhibiting protein synthesis with 16 $\mu$M chloramphenicol during the entire starvation period (stress included) to the effect of the same treatment starting 5 h after the entry into starvation (Fig. 6A). In the latter, only the initial production happens while the former constitutes a control where gene expression is strongly inhibited during the entire starvation period. We measured the fraction of cells surviving the oxidative stress (assessed as regrowing in fresh media afterward within less than 20 h). A negative control without chloramphenicol shows

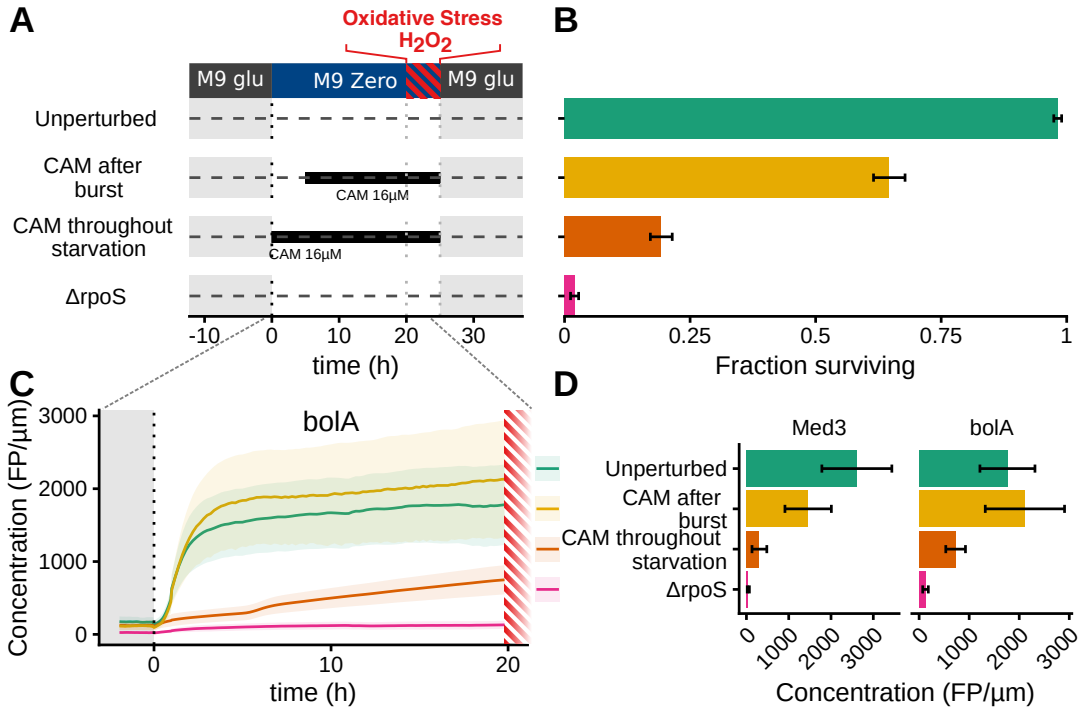


**Figure 6. Gene expression at the onset of starvation is essential for survival to oxidative stress during starvation.** (A) *E. coli* cells inoculated in the DIMM microfluidic chip are switched from M9 glucose to starvation in M9 zero and, after 20 h, submitted to oxidative stress during 5 h (0.65 - 0.75 mM $H_2O_2$), before being switched back to fresh media in order to assess survival. In addition to this default treatment ("Unperturbed"), gene expression can be inhibited using chloramphenicol, either starting after 5h of starvation ("CAM after burst"), or during the entire starvation time ("CAM throughout starvation"). Moreover, we studied a ΔrpoS mutant, in which the response to carbon starvation is strongly reduced. (B) Survival to stress is measured for the different treatments as the fraction of cells regrowing and dividing within the time of observation in fresh media (20 h). (C) Concentration profiles of the bolA fluorescent reporter (as described in Fig. 2) up to the point of the oxidative stress challenge for the various treatments (in colors). (D) Concentration levels of RpoS-regulated reporters (bolA and Med3) at the moment of the oxidative stress challenge, for the different treatments (colours). Data from 2 independent experiments and 2 similar $H_2O_2$ concentrations; replicates shown separately on Fig. S15.

limited mortality (98.2% survival), which suggests that the stress is strong enough to become lethal upon additional perturbations (Fig. 6B). Comparing the treatment with chloramphenicol added after 5 h in starvation and the control where chloramphenicol is present from the start reveals that the initial production significantly decreases the sensitivity to oxidative stress (65% *vs* 19% survival; Fig. 6B). The gene expression observed upon entry into starvation thus prepares the cell for stresses that can happen during starvation.

Using the transcriptional fluorescent reporters described previously, we can assess the effect of the temporary or permanent protein synthesis inhibition on the concentration of RpoS regulated promoters (bolA and med3; Fig. 6C and Fig. S15A,D). As expected, starting the inhibition after 5 h does not significantly affect the levels of these reporters during starvation. In contrast, upon chloramphenicol treatment during the entire starvation, their concentration is reduced more than 2-fold after 20 h. Interestingly, the concentrations at the time of the oxidative stress for the different treatments have the same order as the fraction surviving (Fig. 6D), hinting that expression of RpoS regulated gene underlies a large part of the protective effect. To test this further, we submitted a ΔrpoS strain to this assay of resistance to oxidative stress during starvation (without chloramphenicol treatment). As expected, rpoS-regulated reporters were not expressed anymore, neither during growth nor during starvation (Fig. 6C and D). Remarkably the survival after oxidative stress dropped almost to zero and was much lower than in the wild-type strain with chloramphenicol during the first 5 h of starvation. The role of RpoS in gene expression upon entry into starvation is complicated; for instance bolA, a well-established reporter of RpoS activity, shows a much reduced expression both during growth and starvation in the ΔrpoS mutant, but is still increasing markedly upon entry into starvation (Fig. S6). Our experiments with the ΔrpoS mutant indicate overall that RpoS regulated genes are crucial for survival to oxidative stress during starvation. The fact that deleting rpoS had a stronger impact on the sensitivity to oxidative stress than inhibiting the gene expression globally during the whole starvation might be due in part to the incomplete inhibition by chloramphenicol at this concentration, but also probably from the lower levels of rpoS-regulated genes before starvation (Fig. S6A). Overall, these experiments show that early gene expression upon entry into starvation sets phenotypic traits which are ecologically important, such as resistance to stress, much later into starvation.

# Discussion

In this work, we have studied at the single-cell level the temporal dynamics of *E. coli*'s growth and gene expression and their consequences when bacteria are switched from exponential growth to starvation. This brought several important new insights on how *E. coli* handles starvation conditions.

First, upon entry into stationary phase, bacterial size decreases due to reductive division. While this process is generally thought to occur as cells slow down prior to growth arrest [22,32], we show here that reductive division also occurs when cells are already growth arrested. Providing a mechanism to explain this observation is beyond the scope of this work, but we note that cells are more likely to undergo a reductive division when their cell cycle is already

well advanced, as hypothesized in the past [23].

Regarding gene expression, Gefen *et al.* reported that bacteria can sustain expression over an extended period of time ($\approx$ 2 days) in stationary phase [19]. We further showed that the levels of this activity, as well as its temporal dynamics, are promoter-dependent. In particular, in our sample of $\approx$ 20 promoters, the levels of expression during starvation (relative to during exponential growth) span more than 3 orders of magnitude. The level of about 10% reported by Gefen *et al.* is within this range, at the upper end. However, in contrast to them, we do not see a clear sign that the activity stabilizes during starvation, but rather that it decreases at different rates for different promoters.

Importantly, while Gefen *et al.* results suggested that *E. coli* is capable to reshape its phenotype during starvation, the high sensitivity and time resolution of our measurements revealed that most of the phenotypic changes take place early in starvation. This is a result of the combined effects of the transient increase in expression of different genes, and of the time shift observed between the arrest of growth and of gene expression, which varies from gene to gene. It is worth noting that, although bacteria are generally thought to handle starvation better when nutrients are depleted gradually, we observed no qualitative difference between the gradual transition occurring over 30 min in a standard batch culture and an instantaneous starvation. Finally, we have demonstrated that these early changes in proteome composition determine the phenotypic state of the cells late in starvation, and are crucial for stress resistance during this period.

In this study, we relied on a microfluidic device allowing to monitor bacterial growth and gene expression with single-cell resolution [21]. Although the common belief is that phenotypic variability is higher during stationary phase [4], the gene expression response to starvation which we have discovered is remarkably homogeneous across individual cells. Note that the combination of microfluidics and quantitative fluorescence microscopy was also critical for measuring gene expression with high sensitivity and for studying bacteria in constant starvation conditions. In comparison, after a batch culture reaches stationary phase, bacteria modify the composition of the media over time, which can be an important confounding effect.

Our findings provide new insights into various aspects of *E. coli*'s response to starvation. We discuss hereafter their key implications. During starvation in *E. coli*, intracellular ATP levels drop rapidly, with 70-90% depletion occurring within minutes. The level slowly decreases afterwards and stabilizes at around 10% of the exponential growth levels for an extended period of time [14,31,33,34]. The sustained gene expression activity which we report therefore raises an apparent paradox: how can cells maintain this energy-demanding process in the absence of carbon source? While a large fraction of gene expression is allocated to ribosomal genes during exponential growth [35,36], we observe that ribosomal gene expression stops immediately upon entering starvation. We hypothesize that this sudden stop frees up resources that can be used for the expression of other genes during early starvation.

In addition, intracellular energy storage such as glycogen is depleted within minutes at the onset of starvation [37], which could also fuel the early gene expression activity we observed. The lower - but longer sustained - levels

of ATP and of gene expression activity might be explained by endogenous metabolism [38], even though this hypothesis remains to be investigated.

In parallel to the energy decrease, it has been noted in the past that growth arrest implies that cellular compounds are no longer diluted by growth, which precludes rapid protein turnover. This led to the conclusion that bacteria cannot adapt their phenotype once they are starved [39], and that adaptation to starvation only occurs when cells progressively deplete nutrients and sense the impending starvation as their growth slows down [20]. On the contrary, we have shown here that the absence of dilution actually facilitates the increase of concentrations, even when protein production simply peters out due to the decrease of available energy. Furthermore, we were able to show that the temporal dynamics as well as the amplitude of the adaptation were not different when we allowed the cells to progressively enter starvation as in a batch culture, further highlighting the ability of bacteria to adapt after growth arrest.

The homogeneity of single-cell behavior at the onset of starvation suggests the existence of a regulatory program that allows energy-intensive processes to be sharply stopped and available resources to be allocated to reshape the cell phenotype before energy runs out completely. How bacteria commit to this new state remains to be elucidated, but our study already provides relevant insights. It is striking that, even when gene expression is inhibited with chloramphenicol during the first 5 h of starvation, the concentration still increases to levels similar to those achieved without perturbation (Fig. S14). This implies that the decrease in production rate we observe during the first 10 h of starvation is likely the result of energy depletion by expression itself, rather than a specific downregulation mediated by transcription factors. The similarity of the final concentration levels with and without chloramphenicol inhibition suggests that the time dynamics of production and decay could be tuned to reach a certain concentration of gene products given an initial amount of energy. Altering the metabolic capacity of the cells at the moment they enter growth arrest by varying the pre-starvation conditions will inform us about the factors limiting phenotypic adaptation in this context. In addition, dual reporter experiments, where two fluorescent protein genes are driven by promoters reporting on (the same or different) regulators in the same cell, would further help to disentangle global and regulator-specific effects.

Finally, we have shown that the gene expression occurring during the first 5 h of starvation is crucial for *E. coli* bacteria to survive a stress coming 15 h later. At the same time, bacteria could easily survive 25 h of starvation in our setup, even when their gene expression was inhibited throughout the whole starvation period. Together, this suggests that gene expression occurring at the onset of starvation is important for surviving stress concurrent with starvation, but not crucial for surviving starvation itself, at least on those timescales. This can be interpreted as a preventive response or as anticipatory regulation. In a preventive response, bacteria express early on genes that are necessary to survive unforeseeable future stresses, but that are not necessarily required at the moment of their expression. In contrast, anticipatory regulation consists in expressing, in response to an environmental change, genes that will only be required later in a deterministic sequence of events following the perturbation.

Differentiating a preventive from an anticipatory response is challenging in

practice since it requires very detailed knowledge of the evolutionary history. Moreover, a given regulatory response may combine preventive and regulatory aspects; in particular an anticipatory response will work as preventive in other environmental successions as soon as it is beneficial (and despite not being the ones on which this response has been selected). In the case of bacterial starvation, gene expression upon entry is not necessary for survival during the first day of starvation or for rapidly resuming growth afterward. Nevertheless, it may help the cells survive intracellular stress that develop over the next few days of starvation, making it anticipatory. As for the preventive response, it is expected to be more efficient when its effects have a broad spectrum. Starved bacteria do exhibit increased tolerance to various types of stress, including osmotic and oxidative stress, heat shock, antibiotics, phages [40–42]. Moreover, since bacteria are not able to mount an adaptive response later in starvation, it should be selectively beneficial to respond immediately to environmental stresses happening during starvation. Altogether, it is therefore likely that most of the phenotypic changes occurring at the onset of starvation are preventive.

To better understand *E. coli*'s regulatory strategies for coping with starvation and the selective pressures that have shaped these responses, it will be very instructive to identify the types of stress for which early gene expression is protective and to compare how the response to starvation varies across different *E. coli* strains.

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