



Analysis of proteome adaptation reveals a key role of the bacterial envelope in starvation survival

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Abstract

Bacteria reorganize their physiology upon entry to stationary phase. What part of this reorganization improves starvation survival is a difficult question because the change in physiology includes a global reorganization of the proteome, envelope, and metabolism of the cell. In this work, we used several trade-offs between fast growth and long survival to statistically score over 2,000 *Escherichia coli* proteins for their global correlation with death rate. The combined ranking allowed us to narrow down the set of proteins that positively correlate with survival and validate the causal role of a subset of proteins. Remarkably, we found that important survival genes are related to the cell envelope, i.e., periplasm and outer membrane, because the maintenance of envelope integrity of *E. coli* plays a crucial role during starvation. Our results uncover a new protective feature of the outer membrane that adds to the growing evidence that the outer membrane is not only a barrier that prevents abiotic substances from reaching the cytoplasm but also essential for bacterial proliferation and survival.

Keywords outer membrane; proteomics; starvation; stationary phase; trade-off

Subject Categories Microbiology, Virology & Host Pathogen Interaction; Proteomics

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Introduction

Nutrient limitation is a defining part of the lifecycle of microorganisms. In the absence of external nutrients, the only energy source for heterotrophic organisms is either internal storage (Strange, 1968; Hengge-Aronis & Fischer, 1992; Fung & Kwong, 2013) or nutrients retrieved by recycling dead biomass (Steinhaus & Birkeland, 1939; Zambrano & Kolter, 1996; Finkel, 2006; Schink *et al.*, 2019). These finite energy sources can be temporarily used to maintain the cell

but will eventually deplete, exposing cells to a slow deterioration process driven by entropic forces. It was previously shown that the survival kinetics during starvation, i.e., how many cells will be still alive after a certain time, is determined by the consumption rate of these nutrients, called the maintenance rate, and the availability of nutrients (Schink *et al.*, 2019). Because maintenance rate is a major determinant of bacterial lifespan, we expect bacteria to minimize their maintenance cost to maximize fitness in starvation.

But what determines how much maintenance bacteria need to perform, and how they can reduce it is still largely unclear. The question of maintenance is particularly puzzling because sporulating organisms can minimize the number of active processes without losing their viability. The maintenance rate of nonsporulating bacteria is similarly not an engraved biophysical constant. Instead, *Escherichia coli*, for example, can decrease its maintenance rate and death rate over 5-fold in response to environmental cues sensed during the prior growth phase (Biselli *et al.*, 2020).

But identifying the part of the adaptation that determines lifespan is tricky as the bacteria globally reorganize their proteome (Houser *et al.*, 2015a; Hui *et al.*, 2015a; Schmidt *et al.*, 2016a), macromolecular composition (Schaechter *et al.*, 1958; Bremer & Dennis, 2008) and even morphology (Schaechter *et al.*, 1958) when nutrients get depleted and growth slows down. This means that most properties of bacteria correlate or anticorrelate with lifespan. In addition, while several regulators and genes have been identified that react to nutrient depletion and are essential for survival, such as the general stress response regulator *rpoS* (Hengge-Aronis & Fischer, 1992), cAMP-Crp (Makman & Sutherland, 1965; Notley-McRobb *et al.*, 1997), stringent response (Magnusson *et al.*, 2003; Irving *et al.*, 2021) or DNA protection in starvation *dps* (Almirón *et al.*, 1992), there is no evidence that these systems are the limiting factors for survival.

In this paper, we analyzed diverse growth conditions and find that they heavily influence lifespan after nutrients run out. Due to the diversity in proteomic responses, we can use this information to narrow down the set of proteins that correlate with better survival and validate which parts of the adaptation determine the lifespan of *E. coli* in starvation.

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Results

Proteome analysis identifies proteins and processes that correlate with long lifespan

We studied *E. coli* K-12, first grown in a single carbon source N⁺C⁺ minimal medium, followed by a wash and resuspension in medium with the carbon source missing. The washing step introduces bacteria suddenly to starvation and prevents their complete adaptation. As a result, during the ensuing starvation, viability decreases exponentially (Phaiboun et al, 2015; Schink et al, 2019), with the death rate depending on the previous growth condition. *Escherichia coli* grown on carbon substrates supporting faster growth die faster than those on poor carbon substrates (Biselli et al, 2020), see Fig 1A (“blue”). Alternatively, letting *E. coli* adapt to stationary phase on glucose minimal medium results in a death rate similar to sudden starvation from very low growth rates (Fig 1A, open symbols placed

at growth rate zero). We suspected that changes in the proteome composition, which is known to vary highly across growth conditions (Hui et al, 2015a; Schmidt et al, 2016a), are at least partially responsible for the observed changes in death rate. However, in a single growth perturbation, such as varying the carbon substrates, over half of all proteins were up- or downregulated (Hui et al, 2015a; Schmidt et al, 2016a), making it impossible to pinpoint individual proteins or processes as being responsible.

To narrow down the search for the proteins that determine lifespan, we decided to use additional growth conditions that show different changes in proteome. We identified a total of six independent perturbations, which we collectively call “CARLOS conditions” that affect proteome composition during growth and death rate after washing and resuspension in the carbon-free medium. Among these, those that reflect a change in nutrient availability (C: catabolic limitation, A: anabolic limitation, L: rich media (LB), and S: stationary phase) collapse onto a single exponential increase in

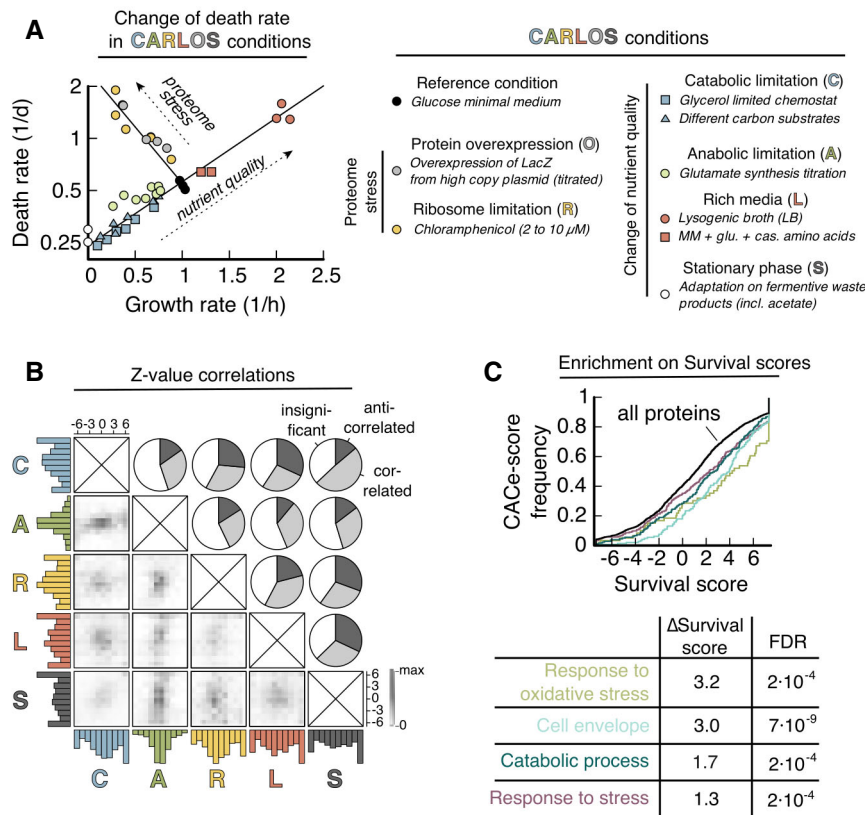


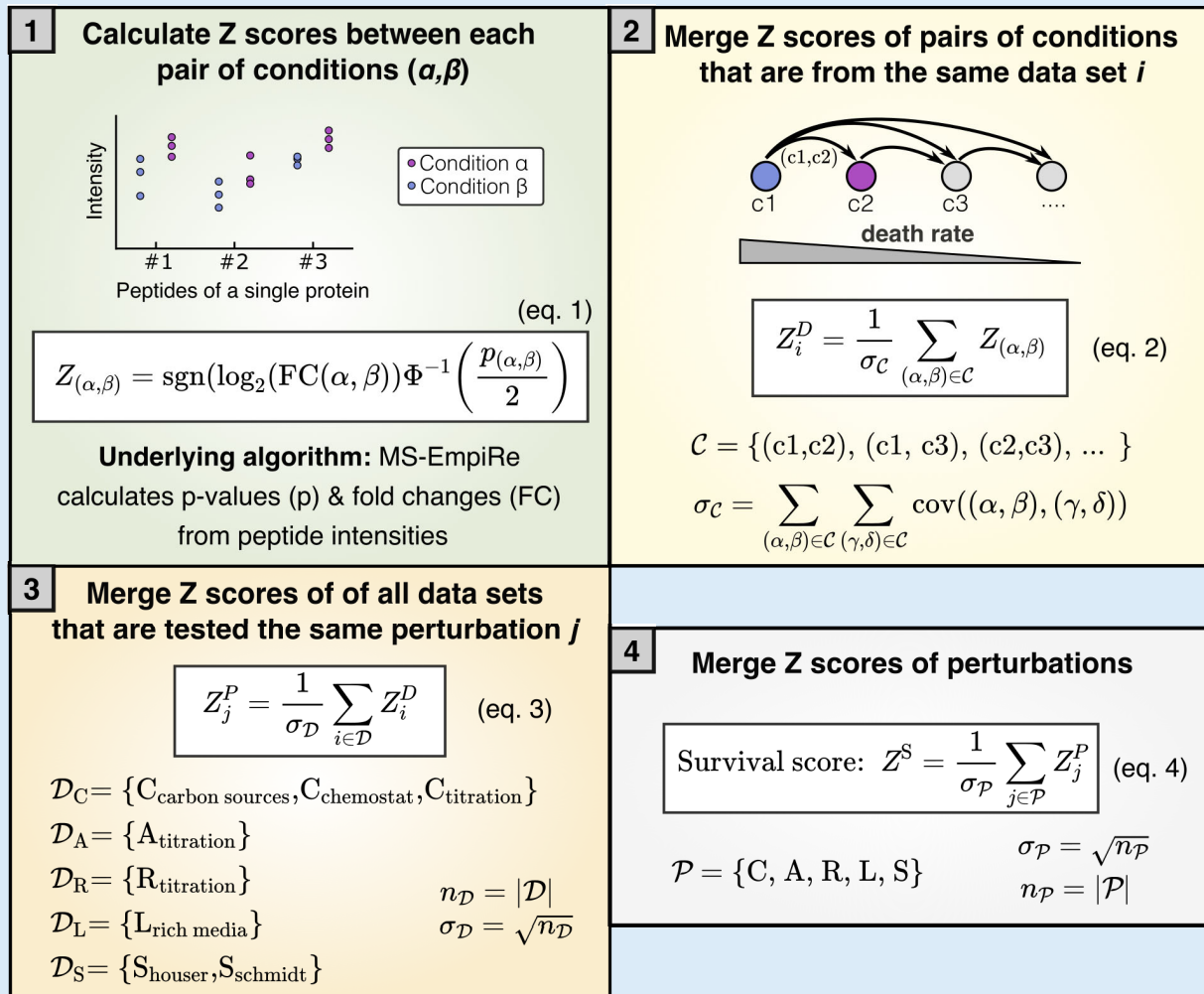
Figure 1. Proteome composition and death rates in CARLOS conditions.

A Death rate versus prestarvation growth rate on a semi-log plot. CARLOS conditions are defined in the legend. CARLOS conditions that perturb nutrient quality (C, A, L, and S) lead to an exponential increase in the death rate. Conditions that stress the proteome (R and O) show an orthogonal exponential increase with slower growth. Each data point is a single experiment. Chemostat (C) and parts of (L) are from Ref. (Biselli et al, 2020). See Dataset EV1 for data.

B Pair-wise comparison of protein Z-values between conditions. Histograms of Z-values of individual conditions (colored) and gray values of the 2D histogram between pairs of conditions are normalized to their respective maximum value. The fraction of proteins that correlate, anticorrelate, or are insignificant is calculated by thresholding in the 2D histogram, see Fig EV1A for an illustration.

C Z-values are merged into a single “Survival score” for each protein that measures the correlation of abundance with survival across all experiments. A detailed description of the construction of the score is given in Box 1. Several GO processes are significantly enriched (FDR levels of the Benjamini-Hochberg step-up procedure) towards higher scores. Δ Survival score is calculated as the difference in median Survival score between a process and the background of all proteins.

Source data are available online for this figure.

Box 1. Calculation of Survival scores from multicondition proteomics data sets.

The Survival scores Z^S quantify how well a protein correlates with lifespan over all CARLS perturbations and is calculated in the steps listed below. Jupyter Notebooks implementing the procedure are provided in the SI (see also Data Availability section).

- Using the MS-Empire statistical software package, we calculated P-values and fold changes (FC) of proteins between a pair of conditions (α, β) based on the underlying peptide intensities across replicates (blue, purple). We use this information to calculate the Z score of a pair of conditions, $Z_{(\alpha, \beta)}$, in equation (1) using the direction of the fold change, where “sgn” is the sign function, and the significance of the regulation, using the inverse cumulative standard normal distribution function Φ^{-1} .
- Next, we merge all $Z_{(\alpha, \beta)}$ that are from the same data set C , into a new Z-value of a data set Z_i^D in equation (2), where the subscript i stands for the specific data set. An example of a data set is $Z_{(\alpha, \beta)}$ scores of different carbon sources derived from the same data repository. The pairs of conditions (α, β) are sorted from high to low death rate, such that a positive Z-value corresponds to a correlation with a lower death rate. Because the merged score Z_i^D should follow a standard normal distribution with a standard deviation of 1, merging $Z_{(\alpha, \beta)}$ scores requires normalization by the standard deviation across the whole data set, σ_C . The standard deviation can be derived from the covariance matrix, where $\text{cov}((\alpha, \beta), (\gamma, \delta))$ is 1 if ($\alpha = \gamma$ AND $\beta = \delta$), 0.5 if ($\alpha = \gamma$ XOR $\beta = \delta$) and 0 otherwise (Ammar et al, 2019).
- Next, we merge all Z_i^D that belong to the same perturbation into a single score for each perturbation Z_j^P , where the subscript j stands for any of the CARLS conditions. Because data sets are independent of each other, the standard deviation σ_D depends only on the number of data sets n_D . For A, R and L there is only a single data set, such that $Z_j^P = Z_i^D$. Figure EV1 visualizes this merging step for C and S conditions.
- Finally, all Z_j^P are merged into a single Z^S , which we call “Survival score.” As in panel 3, the standard deviation σ_P depends only on the number of perturbations n_P that are measured.

death rate with growth rate, while conditions that perturb the proteome by expression of excess proteins (R: ribosome limitation, O: LacZ overexpression) fall onto an orthogonal exponential increase (Fig 1A).

CARLOS conditions result in substantial changes in proteome composition. We analyzed these changes in the proteome using published proteomics data from three different repositories (Data ref: Houser et al, 2015b; Data ref: Hui et al, 2015b; Data ref: Schmidt

et al., 2016b), which individually cover a subset of the CARLOS conditions (C, L, and S in (Data ref: Schmidt *et al.*, 2016b), C, A, and R in (Data ref: Hui *et al.*, 2015b) and S in (Data ref: Houser *et al.*, 2015b)). To be able to compare proteins across conditions and across data sets, we created a composite statistical score, termed “Survival score,” explained in detail in Box 1 and the Materials and Methods section.

In short, we compare protein abundance levels between data sets and assign a positive score (in the form of a Z-value), if the protein is correlated with better survival and a negative score if the protein is anticorrelated (Box 1, step 1). We collect these scores over the variety of conditions and data sets and iteratively merge the scores while correcting for statistical dependencies and ignoring missing values (steps 2–4).

Z-values calculated from individual data sets of similar perturbations or different sources (Box 1, step 2) are highly correlated (Fig EV1A and B) and are used to create merged Z-values for each protein (Fig EV1C and Box 1, step 3). Histograms of these merged Z-values on the condition level (step 3) are shown as colored 1D histograms in Fig 1B, normalized to the highest value. We had to exclude “O, LacZ overexpression” from this analysis due to a lack of sufficient statistical data, shortening CARLOS to CARLS, but we will integrate qualitative information from this condition below.

Next, we compared Z-values of the conditions in a pair-wise manner by plotting 2D histograms of Z-values detected in both respective conditions (Fig 1B, bottom left of matrix). By classifying proteins that score above $|Z| > 1.28$, which corresponds to $P < 0.1$, as significantly correlated or anticorrelated, depending on the direction, we confirmed that proteome changes across different conditions are partially orthogonal to each other (Fig 1B, top right of matrix). This means that each condition has additional information that can help us narrow down the target set of proteins. Finally, we merged the Z-values of the individual CARLS conditions into a single “Survival score” that measures the global correlation strength of proteins with lifespan (Box 1 step 4). The resulting list of CARLS Z-values and “Survival scores” for over 2000 proteins is available in Dataset EV2.

Fractions of the proteome determining death rate can be either beneficial or harmful. To understand which of these fractions is dominant, we used the O’ condition, where overexpression of a useless protein leads to a decrease in the abundance of virtually all proteins simultaneously (Fig EV2). This condition led to a 5-fold increase in death rate (Fig 1A, gray symbols), as we would expect for largely beneficial proteins determining death rate. Thus, we restricted our further analysis to positive Survival scores, where a higher abundance correlates with a longer lifespan across conditions.

Finally, we used Survival scores of all proteins measured in at least three of the five CARLS conditions to identify gene ontology (GO) processes and cellular compartments that contain proteins with significantly larger Survival scores than average. Using a Kolmogorov–Smirnov test, we evaluated the enrichment of proteins in GO processes and compartments, which includes “oxidative stress response,” “response to stress,” “catabolic process,” and “cell envelope” as some of the highest scoring GO processes (Fig 1C, see Dataset EV3 for full list). Note that GO processes are not mutually exclusive and can contain the same proteins, which is particularly true for “oxidative stress response,” which substantially overlaps with “response to stress.”

Death rate depends on the integrity of the cell envelope

We next wanted to test whether any of these processes/compartments causally affect bacterial lifespan. The response to oxidative stress had the highest Survival score in our analysis, with proteins such as catalases KatE (Survival score: 7.3) and KatG (7.3), superoxide dismutase *sodA* (6.5), and hydroperoxidase *AhpC* (6.2), all of which are essential to *E. coli*’s adaptation to stress (Farr *et al.*, 1988; Farr & Kogoma, 1991; Dukan & Nyström, 1999; Chiang & Schellhorn, 2012), correlating well with survival across CARLOS conditions. Therefore, we first tested whether oxidative stress limits the lifespan of starving bacteria by starving bacteria in anaerobic conditions, where oxidative damage should be absent. It was previously reported that starved *E. coli* suffer from protein carbonylation and that anaerobic conditions can prevent carbonylation and stop death (Dukan & Nyström, 1999). However, to our surprise, our results showed the opposite. We found that death in anaerobic conditions was even faster than in aerobic conditions (Fig EV3A). This did not depend on whether growth prior to starvation was in aerobic or anaerobic conditions. Similarly, adding antioxidants glutathione, ascorbic acid, or mercaptoethanol, which could prevent carbonylation during starvation, did not decrease the death rate of starvation *E. coli* (Fig EV3B).

Also, inducing the “response to stress” did not decrease the death rate, despite the high score. Neither inducing its expression using heat shocks, low pH nor high osmolarity during growth, all of which significantly increase the abundance of “response to stress” proteins (Fig EV4A–C), resulting in changes in the death rate of *E. coli* in reference starvation conditions (Fig EV4D). One possible explanation is that our wild-type strain NCM3722 is insensitive because one of the master regulators of the stress response, *rpoS*, contains a premature amber stop codon, which reduces the expression of *rpoS*-dependent genes (Mori *et al.*, 2021). But restoring *rpoS* did not decrease the death rate (Fig EV4E). In contrast, knock-outs of *rpoS* still showed significantly increased death rates compared with NCM3722 (Fig EV4E), which indicates that the *rpoS* gene is sufficiently functional in our strain. Only a knock-out of *rssB*, a gene responsible for the degradation of RpoS (Muffler *et al.*, 1996) leads to a significant decrease in the death rate (Fig EV4E), similar to previous reports (Fontaine *et al.*, 2008). These conflicting observations warrant further investigation into the physiological role of *rpoS* and *rssB* in starvation survival. However, it is known that *rpoS* knock-outs do not remove the growth-death dependence in carbon limitation (Biselli *et al.*, 2020) that forms the basis of our analysis.

Another high-scoring class of proteins identified by our analysis was proteins of the cell envelope, which includes the periplasm, cell wall, and outer membrane (Fig EV5 for graphical summary). Proteins associated with the cell envelope make up about 13% of the proteome in rich nutrient conditions, and up to 23% for bacteria adapted to the stationary phase (Fig 2A and Dataset EV4). In addition to the GO analysis, we also noticed the cell envelope in a knock-out screen of high-scoring, nonessential genes. The fastest dying knock-out in the screen, Δlpp , encoded a protein located in the cell envelope (Dataset EV5). In contrast, knock-outs of most other genes, especially those belonging to stress response and oxidative damage, had little or no impact on death rates in starvation (Dataset EV5), further corroborating that many of them are nonlimiting in our conditions.

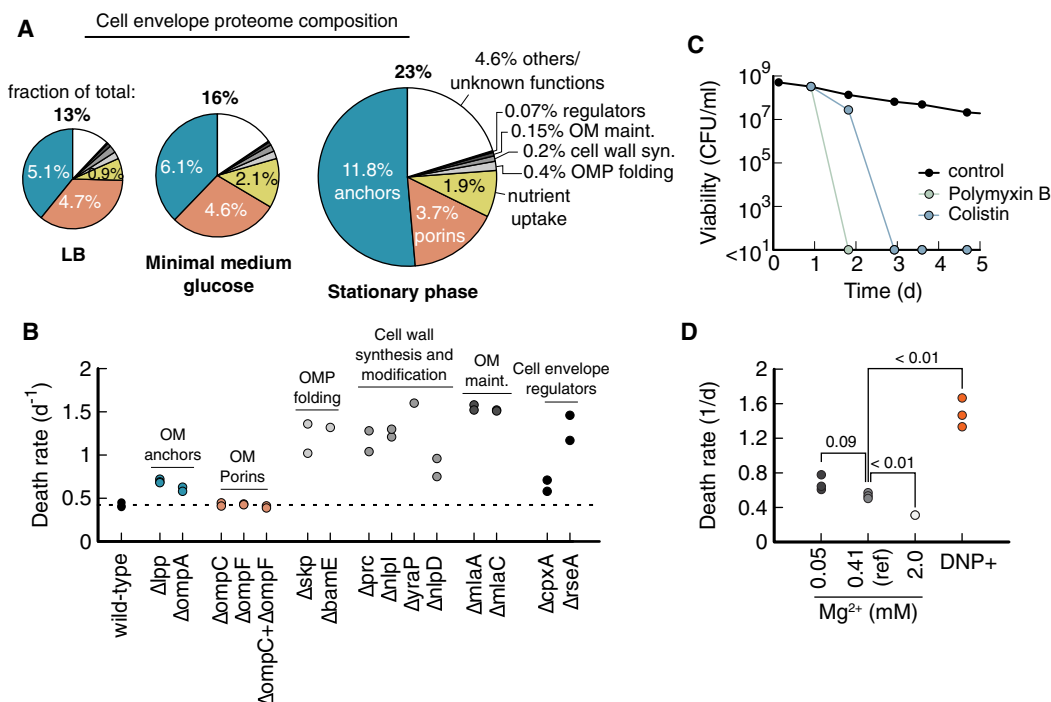


Figure 2. Cell envelope integrity plays a major role in starvation survival.

- A Proteome composition of the cell envelope (see Fig EV5 for details on the cell envelope structure) for cultures during exponential growth on LB, glucose minimal medium, and stationary phase. Size of pie indicates the fraction of total proteome that is associated with the envelope. Outer membrane anchors (Lpp and OmpA) constitute half of the cell envelope proteome. Major porins (OmpC and OmpF) and nutrient uptake (large number of binding proteins) constitute other major fractions. Quantification of abundancies is in Dataset EV4 using data from Ref. (Data ref: Schmidt *et al*, 2016b).
- B Knock-out of several genes with different functionalities in the cell envelope increases death rates. Growth in glycerol minimal medium, starvation induced by glycerol depletion.
- C 8 μ g/ml Polymyxin B and 4 μ g/ml Colistin, added daily to cultures previously grown on glucose minimal medium (reference condition) with the first dose at 24 h. Antibiotic concentration was 10 $\times$ minimum inhibition concentration during growth (MIC). Each data point is the average of two biological repeats. Error bars are not shown because the variation is smaller than the symbol size. Viabilities indicated at $< 10^1$ mean no colonies were detected from 100 μ l culture.
- D Death rates for different Mg^{2+} concentrations. Reference condition (black circle in Fig 1A) has 0.41 mM Mg^{2+} . DNP+ cultures were supplemented with 1 mM 2,4-dinitrophenol during starvation in the reference medium. Each data point is from a single experiment. All cultures were grown in a glucose minimal medium. *P*-values (Student's *t*-test, two-tailed) are indicated in comparison to the reference condition.

Source data are available online for this figure.

The physiological role of Lpp (Survival score: 7.2), together with OmpA (5.8), is to physically link the outer membrane and cell wall. These proteins are essential for the cell envelope's mechanical integrity and make up around half the cell envelope proteome and up to 12% of the total proteome (Fig 2A). Motivated by this finding, we tested more cell envelope proteins, independent of their Survival scores, and found that starvation survival is highly sensitive to knock-outs across different functionalities of the cell envelope (Fig 2B), from outer membrane protein folding (BamE and Skp), cell wall synthesis and modification (Prc, NlpI, DolP, and NlpD), phospholipid recycling (MlaA and MlaC) to cell envelope regulators (CpxA and RseA).

Conversely, only knock-outs of porins, which facilitate diffusion of biomolecules across the outer membrane, have high Survival scores, and are highly abundant (Fig 2A), did not change the death rate (Fig 2B).

This sheer abundance of cell envelope proteome makes it a compelling candidate for determining the death rate in carbon starvation, as it would mean adaptation to optimal starvation survival

would require a major reorganization of the proteome, rather than simply synthesizing a small set of proteins that could be achieved with a limited amount of stored nutrients. This could explain why the prior growth condition has such a big influence on starvation survival.

Many of the knock-outs that we identified are known to impair the mechanical integrity of the cell envelope (Suzuki *et al*, 1978; Yem & Wu, 1978; Schäfer *et al*, 1999; McBroom *et al*, 2006; Malinverni & Silhavy, 2009; Tsang *et al*, 2017; Rojas *et al*, 2018; Bryant *et al*, 2020). Therefore, we hypothesized that the mechanical integrity of the cell envelope plays a role in survival. To test this hypothesis, we applied antibiotics that are known to compromise the cell envelope, polymyxin B and colistin. Both antibiotics were highly effective when added 24 h into starvation, killing the entire population within 24 and 48 h, respectively (Fig 2C). This is unusual, as most other antibiotics are ineffective on starving bacteria (Nguyen *et al*, 2011; preprint: Schink *et al*, 2022). Effective killing by outer membrane targeting antibiotics was also reported for persister cells (Grassi *et al*, 2017). Polymyxin B and colistin insert into the outer

membrane between lipid A molecules and displace Mg^{2+} ions that shield repulsive negative charges between lipid A (Moore *et al.*, 1986). Similarly, supplementing the starved culture with DNP, which acts as an ionophore and permeabilizes bacteria (McLaughlin, 1972) increased the death rate 2.8-fold (Fig 2D).

These results show that cell envelope integrity is essential for survival. In addition, we wanted to know whether cell envelope integrity is also limiting for survival and tested the reverse effect by increasing Mg^{2+} concentrations. Indeed, we found a 40% drop in death rate at high Mg^{2+} concentrations compared with the reference condition (Fig 2D), suggesting that cell envelope integrity is both essential and limiting.

Ion homeostasis and cell envelope integrity account for the variation of starvation survival

But how does cell envelope integrity influence survival? We recently showed that the cost of ion homeostasis is a major determinant of the lifespan of bacteria in carbon starvation (preprint: Schink *et al.*, 2022). Based on this work, we hypothesize that changes in death rates in the CARLOS conditions were due to changes in the cost of ion homeostasis, mediated by the envelope proteome affecting membrane permeability. If membranes are less permeable, less energy is required to actively pump ions across the cytoplasmic membrane to remain in ion homeostasis. This reduction in maintenance should then result in a lower death rate (Schink *et al.*, 2019).

To test this hypothesis we used an “osmo-balanced” medium with low salt concentrations, designed to minimize the content of inorganic ions that can diffuse across membranes (preprint: Schink *et al.*, 2022). This medium reduces the death around 4-fold in standard glucose minimal medium conditions due to a reduction in the maintenance rate required for ion homeostasis (preprint: Schink *et al.*, 2022). If changes in the membrane permeability were responsible for the modulation of the death rate in Fig 1, we predict that the osmo-balanced medium should remove the growth-death dependence. In a perfect scenario, in which loss of ion homeostasis is the only possibility to die we would predict the death rate to decrease to zero for all conditions, but we already know from previous work (preprint: Schink *et al.*, 2022) that the death rate does not decrease to zero with this medium, presumably because at some point lifespan will be limited by other causes.

On the other hand, if the growth-death dependence is independent of the permeability of ions, for example, if the modulated proteins and processes are preventing some type of damage or are influencing nutrient recycling, then we would expect the growth-death dependence to remain in “osmo-balanced” medium but be shifted to a lower death rate.

Because the osmo-balanced medium allows us to distinguish whether the growth-death relation is due to a modulation of the ability to deal with ions in the medium, we grew *E. coli* in the CARLOS conditions followed by washing and resuspension in “osmo-balanced” medium. We found that the characteristic growth rate dependence of the CARLOS condition was indeed abolished and no significant correlation between growth rate and death rate remained (Fig 3). This confirms that differences in the cell envelope integrity and membrane permeability underlie the variations in death rate seen in Fig 1A.

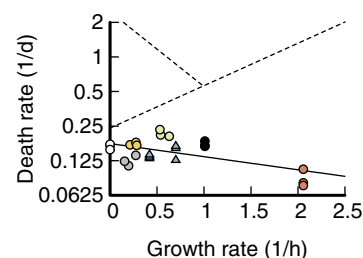


Figure 3. Death rates in osmo-balanced medium.

Death rate plotted versus growth rate on a semi-log scale for experiments in “osmo-balanced” medium. Colors indicate carbon limitation (C, “blue,” acetate and glycerol), anabolic limitation (A, “green,” 3 μ M IPTG), ribosome limitation (R, “yellow,” 4 μ M Chloramphenicol), rich medium (L, “red,” LB), LacZ overexpression (O, “gray,” 10 ng/ml cTc), stationary phase (S, “white”) and the glucose reference condition (“black”), as defined in Fig 1A. The data shows weak anticorrelation with growth rate; solid black line is an exponential fit to the data ($R^2 = 0.34$). Dashed lines are the exponential fits to the CARLOS data in Fig 1A.

Source data are available online for this figure.

Discussion

We performed an analysis of proteomics data to identify proteins, processes, and cellular compartments that contribute to improved survival of carbon starvation. We identified the cell envelope proteome and the membrane permeability as a key determinant of survival.

Limitations of the study

Before discussing the implications of our work, we should spell out the limitations of our search for proteins that determine the death rate. First, because we assume that the same proteins determine the modulation of the death rate across all CARLOS conditions, we are prone to miss proteins that contribute to the death rate in a single (or small subset) of conditions. Secondly, because we focused our analysis on the level of processes and cellular compartments, we will miss proteins and mechanisms that are not connected to significant processes and compartments. Therefore, these proteins could be undetected, even though they should in principle pop up with a high Survival score (Dataset EV2). It is thus possible that we missed additional mechanisms not connected to the cell envelope. Thirdly, the quality of the data sets varies across sources. The Schmidt *et al* data set, used for C, S, and L, has a deep coverage that includes many low-abundance proteins, and as a result, there is the large number of proteins missing values in A and R (Dataset EV2), which are derived from data sets with less coverage. This leads to some limitations in the scoring and the construction of the overall Survival score. In order to retain the information available in the data set, we constructed our scores in such a way, that we omit missing values in the Survival score, which leads to larger contributions of the available scores to the overall score. This can have two opposing types of effects: (i) Globally correlating proteins that are only measured in a subset of perturbations (which are generally of lower abundance) will have a lower overall score, which introduces a bias towards proteins of high abundance. (ii) If a protein has a strong

anticorrelation in one of the missed conditions, the estimated Survival score will be higher than it should be. To mitigate the effects of this in the downstream analysis, we perform our final enrichment analysis on the subset of proteins that were measured in at least three of the five CARLS conditions. In addition, proteins can be present in the data but measured with so much underlying noise, that the direction of regulation cannot be determined with high confidence. This results in low Z-values and the effects are like the effects of missing values mentioned above. Finally, the number of individual data sets for each perturbation varies. While we compensate the impact of the number of data sets on the final Survival score by weighing the scores appropriately, more data sets per perturbation will reduce noise and improve the scoring. This creates a bias towards perturbations with many data sets, such as C, at the expense of those with few, such as A and R. It should be noted that the challenges of missing values, bias towards higher abundant proteins, and measurement noise are common in the analysis of proteomics data and we have strived to find a compromise between stringency and comprehensiveness in the construction of our score.

Nevertheless, we have presented a variety of results that allow us to be confident in our main conclusions. In the construction of our score, we have shown a good correlation between identical types of perturbations on independent data sets (Fig EV1). We see enrichment in a canonical set of processes (Fig 1C) and an increase in death rate for knock-out of key genes in the cell envelope (Fig 2B). We additionally validated that both weakening and strengthening the cell envelope affect death rate (Fig 2C) and established that the growth-death relation can be abolished in the absence of ions in the medium (Fig 3), which is a strong indication that the mechanism identified by our analysis, i.e., the integrity of the cell envelope, is the major contribution to the growth-death dependence of Fig 1.

Cost of building a strong cell envelope

Building a strong cell envelope is a costly endeavor. A third of the dry weight of a typical cell consists of cell envelope biomass, half of which consists of protein, and the other half phospholipids, LPS, and cell wall (Neidhardt, 1996). Given the substantial fraction of biomass devoted to the cell envelope, it is reasonable that bacteria reduce this biomass investment into the cell envelope in favorable growth conditions, and instead focus on maximizing their growth rate. However, because of the magnitude of the biomass investment into the cell envelope, bacteria cannot easily adapt to starvation using carbon stores after sudden nutrient depletion, which would explain why pregrowth conditions have such a big effect on starvation survival, and why optimal survival requires prolonged adaptation.

Role of the outer membrane in cell envelope integrity and starvation survival

Surprisingly, many of the proteins that we found to affect survival rates in carbon starvation are related to the outer membrane and overall, our data demonstrate an important role of the outer membrane in starvation survival. While the outer membrane is known to be a permeability layer, e.g., to antibiotics, it is less clear how it could achieve a similar effect for inorganic ions, which would explain improved survival in starvation. Ion export pumps, such as

NhaA, are thought to only span the cytoplasmic membrane, meaning that ion gradients across the outer membrane should equilibrate. In this case, the outer membrane should offer little protection from inorganic ions. Indeed, in “plasmolysis” from osmotic shocks, the inner membrane contracts as a result of osmotic pressure, while the outer membrane largely maintains its shape (Rojas *et al*, 2018), indicating that osmolytes readily diffuse into the periplasm. There is, however, the considerable biochemical and mechanical interplay between inner and outer membranes that could contribute to permeability changes. Both membranes share phospholipids, and the outer membrane is involved in recycling lipids that have accumulated on the outer leaflet of the outer membrane (Malinverni & Silhavy, 2009). Recently, the outer membrane has been found to mechanically stabilize the inner membrane, e.g., via the tol-pal system, which is essential for bacteria to reach plasmolysis in starvation (Rojas *et al*, 2018; Shi *et al*, 2021). Integrity of the cell envelope could also protect periplasmic proteins from damage. We therefore argue that a mechanically strong cell envelope allows Gram-negative bacteria to stabilize and protect their cytoplasmic membrane, allowing bacteria to reduce their maintenance cost and increase their lifespan.

Materials and Methods

Strains

All strains used in this study are derived from wild-type *E. coli* K-12 strain NCM3722 (Soupene *et al*, 2003). Strains NQ381 (attB:Plac-xylR, Km-Pu-lacY) was used for “catabolic limitation” and was described in Ref. (You *et al*, 2013). In NQ381 strain, the expression of the lactose transporter lacY is induced using 3-Methoxybenzamide (3MBA). NQ393 (Δ gdhA+plac-gltBD, attB(phage):lacIQ-tetR::Sp, Δ lacY) was used for “anabolic limitation” and is described in Ref. (Hui *et al*, 2015a). Using isopropylthio- β -galactoside (IPTG), the expression of gltBD is induced, which encodes glutamate synthase. NQ1389 (Ptet-tetR on pZA31; Ptetstab-lacZ on pZE1) was used to overexpress LacZ using chlortetracycline (cTc) as inducer and is reported in Ref. (Basan *et al*, 2015a). All knock-outs shown in Fig 2 were transferred from the Keio collection (Baba *et al*, 2006) to NCM3722 via P1 transduction to yield strain. Knock-outs from Dataset EV5 are taken from the Keio collection (Baba *et al*, 2006) and compared with the ancestor wild-type BW25113.

Culture medium

The culture medium “N[−]C[−] minimal medium” (Csonka *et al*, 1994), contains 1 g K₂SO₄, 17.7 g K₂HPO₄, 4.7 g KH₂PO₄, 0.1 g MgSO₄·7H₂O, and 2.5 g NaCl per liter. The medium was supplemented with 20 mM NH₄Cl, as a nitrogen source, and varying carbon sources. The “reference glucose condition” contained 0.2% glucose. All chemicals were purchased from Sigma Aldrich (St. Louis, Mo, USA). The “osmo-balanced” medium is described in Schink *et al* (2019), and contains 0.2 M MOPS (3-(N-morpholino) propanesulfonic acid), titrated to pH 7 with KOH, and 1 mM MgCl₂, 0.1 mM CaCl₂, 0.16 mM K₂SO₄, 0.5 mM K₂HPO₄, 22 mM NH₄Cl and lacks a carbon source. Cultures starved in the “osmo-balanced” medium were previously grown in N-C- medium supplemented with NH₄Cl and glucose.

Culture conditions

Prior to each experiment, bacteria were streaked out from -80°C glycerol stock on an LB agar plate supplemented with antibiotics if necessary. Bacteria were cultured in three steps. First, a seed culture was grown in lysogenic broth (LB) from a single colony. Second, the seed culture was diluted in N^{-}C^{-} minimal medium supplemented with 20 mM NH_4Cl and a carbon source and grown overnight for at least five doublings to the exponential phase. The next morning, the overnight culture was diluted into fresh, prewarmed N^{-}C^{-} minimal medium supplemented with 20 mM NH_4Cl and a carbon source and grown for another 5–10 doublings. At an optical density of 0.5 or below, the culture was washed by centrifugation (3 min at 3,000 g) and resuspension into fresh, carbon-free, prewarmed N^{-}C^{-} minimal medium supplemented with 20 mM NH_4Cl . This washing step removes excreted byproducts such as Acetate. For growth conditions known to fully respire carbon, e.g., wild-type NCM3722 grown on glycerol (Basan *et al*, 2015b), this washing step was omitted.

NQ381 was grown overnight and during the experimental culture with the inducer concentrations indicated in Dataset EV1. NQ1389 was grown without inducer overnight, with indicated inducers added after dilution of the overnight culture, to prevent escape mutations and plasmid loss. NQ399 was grown with 100 μM IPTG overnight and the indicated IPTG concentrations after dilution of the overnight culture.

For culturing we used 20 mm $\times$ 150 mm glass test tubes (Fisher Scientific, Hampton, NH, USA) with disposable, polypropylene Kim-Kap closures (Kimble Chase, Vineland, NJ, USA) filled with 5–7 ml of medium. Cultures were sealed with Parafilm “M” (Bemis Company, Neenah, WI, USA) to prevent evaporation.

Death rate measurements

Death rates were extracted using a linear fit of log-transformed viability measurements during the first 10 days of starvation, or until the viability reached below 10^7 CFU/ml, whichever came first. Note that after the initial, exponential phase of starvation that we study, mutants take over, viability stabilizes, and cultures enter the “long-term stationary phase.” For viability measurements, cultures were diluted in untreated, sterile 96 well plates (Celltreat, Pepperell, MA, USA) in three to four steps using a multichannel pipette (Sartorius, Göttingen, Germany) to a target cell density of about 4,000 CFU/ml. 100 μl of the diluted culture was spread on LB agar plates supplemented with 25 $\mu\text{g}/\text{ml}$ of 2,3,5-triphenyltetrazolium chloride to stain colonies bright red using Rattler Plating Beads (Zymo Research, Irvine, CA, USA), and incubated for 12–24 h. Images of agar plates were taken with a Canon EOS Rebel T3i (Tokyo, Japan) mounted over an LED light box “Lightpad A920” (Artograph, Delano, MN, USA), and analyzed using a custom script in Cell Profiler (Carpenter *et al*, 2006). Colony forming units per volume (CFU/ml) were calculated by multiplying the number of colonies per agar plate by the dilution factor.

Stress conditioning

For prestressing, wild-type *E. coli* NCM3722 was grown in glucose minimal medium, either in a water bath at 40°C (“heat stress”), in a medium supplemented with 50 mM NaCl (“osmotic stress”) or in

N^{-}C^{-} medium adjusted to pH 6 using KOH (“pH stress”). At an optical density OD_{600} of about 0.5, cultures were washed and transferred to prewarmed, carbon-free N^{-}C^{-} supplemented with 20 mM NH_4Cl , and the decay of viability was recorded for about 10 days.

Anaerobic culturing

For anaerobic growth and starvation, cultures were grown in 0.05% glucose minimal medium in a vinyl anaerobic chamber (COY Lab Products, Grass Lake, MI, USA), in Erlenmeyer flasks (Chemglass, Vineland, NJ, USA) on a magnetic stirrer (IKA RO10, Staufen, Germany), and not washed after the end of growth. For aerobic growth and anaerobic starvation, cultures were grown in 0.05% glucose minimal medium in an air incubator. At an optical density of about 0.5, cultures were centrifuged, supernatants were discarded, and pellets were introduced to the anaerobic chamber. In the anaerobic chamber, pellets were resuspended in a prewarmed, carbon-free minimal medium. All media were degassed prior to being introduced to the anaerobic chamber and left with an open lid to be equilibrated for 1 week.

Proteomics data processing

The MS proteomics data were downloaded from the corresponding PRIDE partner repositories (Schmidt *et al*, 2016b: PXD000498, Hui *et al*, 2015b: PXD001467, Houser *et al*, 2015b: PXD002140). For the Schmidt and Houser data sets, the .raw files were downloaded; for the Hui data set, peptide-intensity mappings were downloaded. The Schmidt and Hui data sets were searched MaxQuant (Cox & Mann, 2008) v. 1.5.7.4 using standard settings and “Label-Free Quantification” (LFQ) and “Match between runs.” The Schmidt and the Houser data sets were searched against the reviewed Uniprot *E. coli* database (03/2019).

Differential expression analysis of proteomics data

All data sets were processed with the MS-Empire (Ammar *et al*, 2019) algorithm for differential quantification, which assigned fold changes and significance scores to each protein in a conditioned pair. For the Schmidt and Houser data sets, the MaxQuant “peptides.txt” and “proteinGroups.txt” files were used as input. The Hui data set was preprocessed as follows: As in the experimental setup of Hui *et al*, the data were quantified relative to a ^{15}N -labeled spike-in and a direct assessment of LFQ values was not optimal. Similar to the approach of Ref. (Geiger *et al*, 2010), we first assessed the fold changes of each peptide relative to its heavy labeled spike-in. To preserve the intensity information, we re-scaled each spike-in fold change by the median intensity of the spike-in peptide over all conditions. This resulted in pseudo-intensities, which we further processed in a “LFQ-like” manner using MS-Empire.

Z-value-based ranking of the proteome perturbations

As displayed in Fig 1 and discussed in the main text, each growth condition has a corresponding death rate. Additionally, when we compare two conditions with one condition having a lower death rate than the other, we expect the lower death rate to be caused by increased expression of proteins. In our ranking, we hence had to

identify proteins that show increased expression for decreased death rate over all conditions.

Our initial data set consisted of “Transporter Titration” (C), “Ribosome Limitation” (R) and “Anabolic Limitation” (A) in the Hui set, “Carbon Substrates” (C), “Chemostat” (C), “Rich Media” (L), “Stationary Phase” (S), “Osmotic Shock,” “Heat Stress,” and “PH Stress” in the Schmidt set and “Stationary Phase” (S) in the Houser set. Each data set had a glucose reference condition.

The “Stationary Phase,” “Osmotic Shock,” “Heat Stress,” and “PH Stress” perturbations were compared with the corresponding glucose reference using differential expression analysis with MS-Empire. This assigned to each protein a log fold change denoting the change in expression and a significance score denoting the confidence in an expression change. The remaining perturbations were compared differently, because they consisted of multiple experiments with varying perturbation strength, e.g., different levels of transporter titration in the “Transporter Titration” data set. The individual conditions within a data set were ranked by their corresponding death rate and all combinations were compared in an increasing manner (e.g., c1 vs. c2, c2 vs. c3, c1 vs. c3). This way, positive fold changes always correlate with better survival.

Concerning the Survival score calculation, our data were structured as follows: We have the five different CARLS perturbations, which we denote here as the set of perturbations P . Each of the perturbations in P has one or more data sets D , which are measurements of this perturbation type. For example, there are three data sets that belong to the perturbation of type C: different carbon sources (Data ref: Schmidt *et al.*, 2016b), the chemostat data set (Data ref: Schmidt *et al.*, 2016b), and the data set, where carbon uptake was regulated by transporter titration (Data ref: Hui *et al.*, 2015b). In other words, $D_C = \{C_{\text{carbon sources}}, C_{\text{chemostat}}, C_{\text{titration}}\}$, where C is the respective set of condition pairs. A condition pair (α, β) is the comparison of a condition α (e.g., glucose) against another condition β (e.g., acetate).

We started the calculation of the score at the level of a single condition pair and the score is calculated separately for each individual protein X. First, Box 1, step 1, we applied a Z-value transformation to each condition pair (α, β) of protein X as follows:

$$Z_{(\alpha, \beta)} = \text{sgn}(\log_2 \text{FC}(\alpha, \beta)) \cdot \phi^{-1} \left(\frac{P_{(\alpha, \beta)}}{2} \right); \quad (1)$$

where $\log_2 \text{FC}$ is the log2 transformed fold change, p is the P -value determined by MS-Empire, sgn is the sign function, and ϕ^{-1} is the inverse cumulative standard normal distribution function. The Z -value $Z_{(\alpha, \beta)}$ is the distance from zero in a standard normal distribution and can immediately be transformed back into a significance score. The higher the absolute value of the Z -value is, the more significant a measurement is. The Z -value carries both the direction of regulation (via its sign) and its significance (via its value).

Next, Box 1, step 2, we merged the set of all condition pairs C which belonged to the same data set D via the formula:

$$Z_i^D = \frac{1}{\sigma_C} \sum_{(\alpha, \beta) \in C} Z_{(\alpha, \beta)} \quad (2)$$

where Z_i^D corresponds to the normalized Z -value of the i -th data set. Normalization was performed by dividing by the standard

deviation of the summed random variables denoted as σ_C , which is the square root of the variance.

Generally, the variance of the sum of multiple random variables is the sum over the corresponding covariance matrix. As we combine variables that follow a standard normal distribution, we can derive the individual covariances in our setup as follows: When random variables from two independent standard normal distributions are compared, (e.g., Z1 comes from the comparison C1 vs. C2 and Z2 comes from the comparison C3 vs. C4) the covariance is equal to 0. When random variables that share one sample are compared (e.g., Z1 comes from the comparison C1 vs. C2 and Z2 comes from the comparison C1 vs. C3) it can be shown that the covariance is equal to 0.5 (Ammar *et al.*, 2019). This then allows us to determine σ_C and therefore Z_i^D .

Subsequently, Box 1, step 3, we merged the Data set $s D$ that were available for each type of perturbation P (i.e., each of the CARLS perturbations). Depending on the perturbation, the number of available data sets varied—for example, for the C perturbation, we had three data sets ($D_C = \{C_{\text{carbon sources}}, C_{\text{chemostat}}, C_{\text{titration}}\}$), while for the A perturbation, we had only one data set ($D_A = \{A_{\text{titration}}\}$). To obtain a normalized Z -value for each perturbation, we used the equation:

$$Z_j^P = \frac{1}{\sigma_D} \sum_{i \in D} Z_i^D \quad (3)$$

where Z_j^P corresponds to the normalized Z -value of j -th perturbation P . The value is normalized by the standard deviation over the data sets D , denoted as σ_D . The calculation is again carried out via the summation over the covariance matrix. However, because the random variables Z_i^D are independent from each other, all covariances are zero except for the covariances of the random variables with themselves (i.e., their variances). Because each Z_i^D is (0,1) normalized according to equation 2, the resulting variance corresponds simply to the number of elements in D and σ_D is therefore the square root of the number of elements n in D . In the case that D contains only one element, for example in the D_A case, $Z_j^P = Z_i^D$.

For the final calculation of the Survival score Box 1, step 4, the set of perturbations P is summarized to the Survival score Z^S via the equation:

$$Z^S = \frac{1}{\sigma_P} \sum_{j \in P} Z_j^P \quad (4)$$

The standard deviation σ_P is the square root of the number of elements in P , following the same reasoning as above. The overall score is robust against missing values, as perturbations that are not available (e.g., if for a certain protein X, we have only $P = \{C, R, L, S\}$ available) also do not contribute to σ_P . The available Z -values then contribute more strongly to the overall score.

Absolute quantification of proteins

For absolute quantification, we used protein synthesis rates derived by (Li *et al.*, 2014) from ribosomal sequencing data of an MG1655 glucose reference condition. Synthesis rates were used as proxies for copy numbers and multiplied by the respective molecular

weights to obtain mass estimates. Further conditions were compared relative to the reference with MS-Empire and the mass estimates were scaled by the respective fold changes. To determine the mass fraction of a gene set, the genes of the set were summed and divided by the summed mass of all genes.

GO enrichment analyses

The Gene Ontology (GO) was downloaded from <http://geneontology.org> (03/2019) together with the *E. coli* “ecocyc.gaf” annotation. The relations “is_a” and “part_of” were used for the construction of the gene sets. Score-based analysis was carried out using the Kolmogorov–Smirnov test with signed scores. Multiple testing correction was carried out via the Benjamini–Hochberg procedure.

Data availability

The uploaded Source Data contain the data underlying each figure. The proteomics raw data can be found under the repositories specified in the references. Code and data for bioinformatics analyses are available on Github (https://github.com/ammarsj/ecoli_survival_scoring).

Expanded View for this article is available [online](#).

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Author contributions

Severin Schink: Conceptualization; formal analysis; funding acquisition; investigation; visualization; writing – original draft; writing – review and editing. **Constantin Ammar:** Conceptualization; software; formal analysis; investigation; visualization; writing – original draft; writing – review and editing. **Yu-Fang Chang:** Investigation. **Ralf Zimmer:** Resources; supervision; writing – review and editing. **Markus Basan:** Conceptualization; resources; supervision; funding acquisition; investigation; writing – original draft; project administration; writing – review and editing.

Disclosure and competing interests statement

The authors declare that they have no conflict of interest.

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