Nutrient and salt depletion synergistically boosts glucose metabolism in individual *Escherichia coli* cells

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The interaction between a cell and its environment shapes fundamental intracellular processes such as cellular metabolism. In most cases growth rate is treated as a proximal metric for understanding the cellular metabolic status. However, changes in growth rate might not reflect metabolic variations in individuals responding to environmental fluctuations. Here we use single-cell microfluidics-microscopy combined with transcriptomics, proteomics and mathematical modelling to quantify the accumulation of glucose within *Escherichia coli* cells. In contrast to the current consensus, we reveal that environmental conditions which are comparatively unfavourable for growth, where both nutrients and salinity are depleted, increase glucose accumulation rates in individual bacteria and population subsets. We find that these changes in metabolic function are underpinned by variations at the translational and posttranslational level but not at the transcriptional level and are not dictated by changes in cell size. The metabolic response-characteristics identified greatly advance our fundamental understanding of the interactions between bacteria and their environment and have important ramifications when investigating cellular processes where salinity plays an important role.

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All core metabolic networks require the uptake and utilization of carbon sources. Such function is variant, adaptable and in the process of being precisely shaped by natural selection as many heterotrophic organisms need to adapt their metabolic capabilities to dwell in scarce nutrient environments1–3. In the case of bacteria, sugars often represent the primary driving force for growth; they are used to power replication, to make storage compounds, and/or for the production of secondary metabolites that further dictate metabolic function4–6. Glucose is commonly employed to investigate the regulation of sugar uptake and metabolism in bacteria7. In fact, many bacterial species primarily use glucose when exposed to nutrient mixtures8,9 and have evolved several independent ways of acquiring glucose from the environment, as characterised by hundreds of variant glucose transport systems8.

In gram-negative bacteria, such as *Escherichia coli*, glucose passively diffuses through outer membrane porins whose expression is regulated both at the transcriptional and translational level10–12. Glucose then crosses the *E. coli* inner membrane via five different permeases including the glucose and mannose phosphotransferase systems (PTS)13. Once in the cytoplasm, glucose is phosphorylated to glucose-6-phosphate that is broken down to pyruvate, this in turn is metabolised to acetyl-CoA which then enters the citric acid cycle14 generating ATP. At micromolar extracellular concentrations, glucose and its fluorescent analogue 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) transiently accumulate intracellularly as a result of concomitant uptake and degradation processes15. Seminal experiments carried out in chemostats suggest that glucose uptake increases with cell surface area16–18 and it is heterogeneous within clonal *E. coli* populations.

These insights were obtained by employing bacteria growing in optimal conditions or at low nutrient levels15. In contrast, in natural environments changes in other parameters are common19–21. For example, salinity can dramatically change across different bodily environments with a 6-fold decrease in salinity content from the ileum to the colon (i.e. from 6 to 1 g/L NaCl in simulated ileal and colonic environment media, respectively) that affect the proteome of pathogens such as enterohemorrhagic *Escherichia coli*21. Salinity can also vary across different humans with a 40% increase in the lungs of cystic fibrosis patients22 or across different aquatic ecosystems (i.e. <0.5 g/L salt in freshwater and >40 g/L salt in hyperhaline water)23. These salinity variations often occur concomitantly other environmental variations, such as changes in temperature, pH or nutrient levels. In fact, both nutritional and salinity levels decrease from the ileum to the colon24. These variations can result in additive, antagonistic or synergistic effects on microbes25,26. Understanding and predicting the effects of multiple environmental variations on the phenotypic diversity in microbial traits, such as metabolic rates, is critical for unravelling how microbes interact with their environment.

Here we study the diversity in glucose metabolism within clonal *E. coli* populations and show that simultaneous extracellular nutrient and salt depletion synergistically enhance glucose metabolism. These single-cell traits are not displayed when bacteria are exposed to nutritional or salinity depletion alone, demonstrating that the effect of these environmental changes on glucose metabolism is not additive. These changes in metabolic function are underpinned by variations at the translational and posttranslational level but not at the transcriptional level and are not dictated by changes in cell size. These findings offer new understanding of the interaction between a cell and its environment and will inform modelling metabolic flux across bacterial populations6, adjusting process parameters in biotechnology, food preservation or metabolic engineering27 and optimizing treatment with antibiotics that utilize sugar uptake pathways to reach their intracellular target28–30.

**Results**

**Experimental assessment of glucose accumulation and degradation in individual bacteria.** In order to quantify glucose uptake and accumulation in individual bacteria, we introduced a clonal *E. coli* population into a microfluidic mother machine device31. This device is equipped with a large microfluidic chamber for bacteria loading and media delivery via pressure-driven microfluidics, connected with thousands of bacteria hosting channels with cross section comparable to individual bacteria (inset in Fig. 1).

We then added 30 μM 2-NBDG (i.e. a fluorescent glucose analogue) dissolved in glucose-free M9 medium32,33 in the microfluidic chamber while measuring its diffusion in the bacteria hosting channels over time (dashed line in Fig. 1). We measured a progressive increase in the fluorescence of individual bacteria that quickly became brighter than the channel fluorescence (solid lines in Fig. 1 and insets) demonstrating higher intracellular compared to extracellular 2-NBDG concentration. This was due to the uptake of 2-NBDG and subsequent accumulation in single bacteria up to a steady-state32,33,34. This hyperbolic accumulation kinetics indicated the presence of a sink term, namely intracellular phosphorylation of 2-NBDG and its degradation by *E. coli*, processes that are comparable between glucose and 2-NBDG32,34.

To gain further insight of the dynamics of this degradation process, the extracellular 2-NBDG was washed away from the microfluidic chamber at $t = 1200$ s and replaced with fresh LB medium. Consequently, we measured an exponential decrease in the fluorescence of both the bacteria hosting channels and of each bacterium (dashed and solid lines, respectively, in Fig. 1 and insets). This rapid decrease could not be accounted for by dilution due to cell growth35,36.

In order to quantify phenotypic heterogeneity in glucose accumulation, we evaluated the coefficient of variation (CV, the ratio between the standard deviation and the mean) of single-bacterium 2-NBDG fluorescence values across the clonal population.
at each time point. We verified that this heterogeneity could not be attributed to anisotropy in 2-NBDG concentration within the bacteria hosting channels (Supp. Figure 1 and Supp. Table 1)\textsuperscript{33}.

Glucose accumulation is maximal under simultaneous nutritional and salinity depletion. We then used the experimental approach above to determine the impact of nutritional and salinity depletion on glucose accumulation. We pre-cultured \textit{E. coli} in three environments with different salt contents broadly recapitulating the salinity encountered by bacteria in mesohaline (10 g/L NaCl), oligohaline (5 g/L NaCl) or fresh (0.5 g/L NaCl) water\textsuperscript{34}. These salinity variations also approximate the NaCl concentration faced by bacteria in the ileal or the colonic environment (6.1 and 0.9 g/L NaCl, respectively)\textsuperscript{31}. Moreover, at the lowest salinity, ion availability can become a rate-limiting factor\textsuperscript{35,36}. In each environment, \textit{E. coli} were firstly pre-cultured overnight in LB (at the appropriate salinity) and then for either 3 or 17 h in fresh LB (or M9 medium at the same salinity, see below) for optimal growth or nutrient depletion\textsuperscript{37}, respectively (see Methods).

Exposing \textit{E. coli} to nutrient depletion alone (i.e. 17 h growth in 10 g/L NaCl LB), favoured a steeper increase in intracellular 2-NBDG, compared to optimal growth conditions (i.e. 3 h growth in 10 g/L NaCl LB) with a mean fluorescence of 417 and 250 a.u. after 300 s incubation in 2-NBDG (Fig. 2b, a, respectively, ****Supplementary Tables 2 and 3). However, nutrient depletion alone did not significantly affect 2-NBDG accumulation at steady state with a mean fluorescence of 740 and 733 a.u., respectively, at \textit{t} = 900 s (n.s., Supp. Tables 2 and 3).

Exposing \textit{E. coli} to salinity depletion alone (i.e. 3 h growth in 0.5 g/L NaCl LB), caused a less steep intracellular 2-NBDG increase, compared to optimal growth conditions, with a mean fluorescence of 148 and 250 a.u. after 300 s incubation in 2-NBDG (Fig. 2c, a, respectively, ****Supplementary Tables 2 and 3). Moreover, salt depletion also significantly reduced 2-NBDG accumulation at steady state with a mean fluorescence of 373 and 733 a.u., respectively, at \textit{t} = 900 s (****).

In striking contrast with the findings above, simultaneous exposure to nutritional and salinity depletion (i.e. 17 h growth in 0.5 g/L NaCl LB) caused a steeper intracellular 2-NBDG increase, compared to optimal growth conditions, with a mean fluorescence of 720 and 250 a.u. after 300 s incubation in 2-NBDG (Fig. 2d, a, respectively, ****Supplementary Tables 2 and 3). Moreover, combined nutrient and salt depletion also significantly enhanced 2-NBDG accumulation at steady state with a mean fluorescence of 1714 and 733 a.u. at \textit{t} = 900 s, respectively (****Supplementary Tables 2 and 3). These findings were further confirmed via separate flow cytometry measurements (red and black bars in Supplementary Fig. 2, ****).

Moreover, we found that in nutrient-rich conditions 2-NBDG accumulation increased with salinity (Fig. 2e); on the other hand, under nutrient depletion 2-NBDG accumulation decreased with salinity (Fig. 2f). In fact, a 50% reduction in salinity (from 10 g/L down to 5 g/L NaCl) led to a 145% increase in 2-NBDG accumulation with a mean fluorescence of 740 and 1072 a.u. at \textit{t} = 900 s, respectively (**); a further 90% reduction in salinity (from 5 g/L down to 0.5 g/L NaCl, resembling the salinity change encountered during transition from the colon to the ileum\textsuperscript{21}) led to a further 160% increase in 2-NBDG accumulation with a mean fluorescence of 1072 and 1714 a.u. at \textit{t} = 900 s, respectively (**). Taken together these data demonstrate that reducing the salinity content of the environment favours 2-NBDG accumulation in nutrient-poor but not in nutrient-rich environments.

Next, we verified that nutrient and salt depletion also synergistically boosts glucose accumulation (as a result of uptake and degradation), and not only the glucose analogue 2-NBDG. We performed plate reader based colorimetric assays on \textit{E. coli} populations exposed to either nutrient, or simultaneous nutrient and salt depletion. We found that after 30 s incubation, the extracellular glucose concentration became significantly lower in \textit{E. coli} that had been exposed to simultaneous nutrient and salt depletion compared to \textit{E. coli} that had experienced nutrient depletion alone with a decay time constant \textit{Tau} of (9.5±0.4) s and (16.7±1.2) s, respectively (** red squares and green diamonds, respectively, in Fig. 2g). These data therefore confirm that \textit{E. coli} accumulate glucose significantly faster and to higher levels after exposure to simultaneous nutrient and salt depletion compared to exposure to nutrient depletion alone.

We further confirmed that the findings above were not affected by molecular leakage through the cell membrane, cell integrity being essential for 2-NBDG uptake\textsuperscript{38}, by performing separate experiments using thioflavin T (ThT). This compound stains intracellular macromolecules\textsuperscript{38,39} and is of comparable size to 2-NBDG. We found that, in contrast to 2-NBDG, ThT accumulated to a significantly lesser extent in \textit{E. coli} exposed to combined nutritional and salinity depletion compared to optimal growth conditions (Supplementary Fig. 3a, b, red and black bars in Supplementary Fig. 3c, respectively, and Supp. Table 4). Furthermore, \textit{E. coli} exhibited similar growth curves in both LB formulations (i.e. 0.5 g/L or 10 g/L NaCl). This data confirmed that increased 2-NBDG accumulation under combined nutritional and salinity depletion was not due to molecular leakage through compromised bacterial membranes and that growth rate alone does not necessarily reflect important changes in bacterial metabolism\textsuperscript{40}. Finally, we sought to rule out the possibility that increased glucose accumulation under combined nutritional and salinity depletion was a result of (i) a nutritional shift from LB to M9 (used for growth and 2-NBDG measurements, respectively), (ii) low abundance of divalent cations in LB medium or (iii) changes in extracellular pH\textsuperscript{35,41}. In order to do so, we performed flow cytometry experiments on \textit{E. coli} grown for 17 h in M9 with limited (i.e. 0.1 g/L) glucose or ammonium (i.e. carbon or nitrogen limitation, respectively\textsuperscript{42}, see Methods) and either 0.5 or 10 g/L NaCl. Consistently with the data in Fig. 2, we found that \textit{E. coli} exposed to combined nutritional and salinity depletion in M9 accumulated 2-NBDG to a significantly larger extent than \textit{E. coli} exposed to nutritional depletion alone in M9 both with glucose (mean fluorescence of 3007 and 1045 a.u. at \textit{t} = 900 s, red and green violins, respectively, in Supplementary Fig. 3a, ****) or ammonium as limiting factor (mean fluorescence of 3249 and 1776 a.u. at \textit{t} = 900 s, red and green violins, respectively, in Supplementary Fig. 3b, ****). Furthermore, the measured extracellular pH values were the same (8.0 ± 0.1) for the nutrient depleted and nutrient and salt depleted environments. Finally, \textit{E. coli} displayed similarly large cell-to-cell differences in the accumulation of 2-NBDG after pre-culturing in LB or M9 (coefficient of variations of 38 and 60% after pre-culturing in 0.5 g/L or 10 g/L LB; coefficient of variations of 66 and 69% after pre-culturing in 0.5 g/L or 10 g/L glucose-limited M9; coefficient of variations of 43 and 64% after pre-culturing in 0.5 g/L or 10 g/L ammonium-limited M9). Therefore, the observed heterogeneity in 2-NBDG accumulation was not driven by the nutritional shift from LB to M9. Taken together this data demonstrate that when these two environmental changes come together, they manifest a synergistic effect on intracellular processes, such as glucose metabolism, that is greater than the effect of each environmental change alone.

Glucose accumulation is heterogeneous under different environmental variations in a cell size-independent fashion. Besides the above described different 2-NBDG accumulation traits across variant environments, we also found substantial phenotypic
heterogeneity within *E. coli* populations in the same environment. This heterogeneity increased in the presence of nutrient or salt depletion compared to optimal growth conditions (CV values of 2-NBDG intracellular fluorescence in Supplementary Table 2), suggesting specialization in metabolic functions to endure environmental variations.

In order to gain a mechanistic understanding of these glucose accumulation traits in variant environments, we firstly investigated the role played by cell area in 2-NBDG accumulation. We studied the correlation of the total intracellular fluorescence of each bacterium at steady-state (*t* = 900 s) with the area of each bacterium. We found strong positive correlation between single-cell area and total intracellular 2-NBDG fluorescence both via flow cytometry and via microfluidics-microscopy (Fig. 3a, b, respectively, Pearson correlation coefficient larger than 0.7 in all tested environments).

However, when we calculated the mean 2-NBDG fluorescence for each bacterium, after normalizing by cell size, we found

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**Fig. 2 Glucose accumulation is greatest under combined nutritional and salinity depletion.** Temporal dependence of the mean intracellular fluorescence of the glucose analogue 2-NBDG in individual *E. coli* under (a) optimal growth conditions, (b) nutrient depletion, (c) salt depletion or (d) combined nutrient and salt depletion. Lines are temporal dependences of the intracellular fluorescence of individual bacteria collated from biological triplicate. Symbols and error bars are the corresponding means and standard error of the means of such single-cell measurements. Noteworthy, we measured the 2-NBDG fluorescence as the mean fluorescent values of each pixel constituting each bacterium, thus normalizing by cell size. Coefficient of variations of these single-cell values and statistical tests of quantitative comparisons of values between different environments are reported in Supplementary Tables 2 and 3, respectively. Corresponding 2-NBDG intracellular fluorescence values during the removal of extracellular 2-NBDG are reported in Supplementary Fig. 5. Insets: corresponding fluorescence images at *t* = 900 s when the intracellular 2-NBDG accumulation has reached saturation levels in individual bacteria. Measurements were carried out on *N* = 76, 38, 90 and 46 individual bacteria, in (a)-(d), respectively. Salinity-dependent distribution of single-cell fluorescence after 900 s incubation in 2-NBDG in (e) nutrient-rich or (f) nutrient-depleted environments. Dashed and dotted lines indicate the median and quartiles of each distribution, respectively. (g) Temporal dependence of the extracellular glucose concentration for *E. coli* under nutrient (green diamonds) or nutrient and salt depletion (red squares). *N* = 3 biological replicates for each environment. The dashed dotted and dashed lines are one phase exponential decay fittings to the data yielding a significantly smaller time constant *Tau* for *E. coli* under combined nutrient and salt depletion compared to nutrient depletion alone (*Tau* = (9 ± 1) s vs (17 ± 1) s, respectively, *). *p value < 0.05, **p value < 0.01, ***p value < 0.001, ****p value < 0.0001.
that, under combined nutritional and salinity depletion, *E. coli* exhibited significantly higher intracellular 2-NBDG fluorescence despite displaying significantly lower cell area compared to optimal growth conditions (squares and circles, respectively, in Fig. 3c). Moreover, we found a remarkable lack of correlation between the mean 2-NBDG fluorescence and cell size under optimal growth, salt depleted or nutrient and salt depleted conditions (Pearson correlation coefficients of 0.18 (ns) and −0.01 (ns), respectively); we found instead a significantly positive correlation between 2-NBDG fluorescence and cell size under nutrient depletion (Pearson correlation coefficients of 0.57, ***). Finally, we still found substantial levels of heterogeneity in the mean 2-NBDG fluorescence values across the different environments (Fig. 3c). Taken together these data demonstrate that changes in cell size alone cannot account for the measured glucose accumulation traits and heterogeneities in variant environments in contrast to the current consensus8,15.

**Combined nutritional and salinity depletion boosts both glucose uptake and degradation.** We then sought to decouple the contribution of uptake and degradation mechanisms on the measured 2-NBDG accumulation. To do this, we used a mathematical model describing 2-NBDG accumulation in single bacteria using ordinary differential equation 1 (see Methods). We inferred the uptake rate from the accumulation data in the presence of extracellular 2-NBDG (0 < t < 900 s, Fig. 2) and obtained an independent estimate of the degradation rate from both the accumulation data in the presence of extracellular 2-NBDG (0 < t < 900 s, Fig. 2) and the degradation data in the absence of extracellular 2-NBDG (t > 1200 s, Supp. Figure 5).

Nutritional depletion caused a significant decrease in 2-NBDG uptake rate but also a significant increase in degradation rate compared to optimal growth conditions (Fig. 4a, d, e and Supplementary Table 5). Moreover, both 2-NBDG uptake and degradation were more heterogeneous under nutritional depletion (Supplementary Table 6), further suggesting specialisation in metabolic functions in stressed bacteria34. Finally, we did not find a significant correlation between uptake and degradation rates at the single-bacterium level under nutritional depletion (Pearson correlation coefficient of 0.27 compared to 0.42 for optimal growth conditions). This suggests that under nutritional depletion, some bacteria specialize in fast uptake at the expense of reduced degradation rates.

Similarly, salinity depletion caused a significant decrease in 2-NBDG uptake rate but also a significant increase in degradation rate (Fig. 4b, d, e and Supplementary Table 5). Furthermore, the CV of both uptake and degradation rate was higher under salinity depletion (Supplementary Table 6). However, differently from nutritional depletion, salinity depletion favoured a strong positive correlation between uptake and degradation rates at the single bacterium level (Pearson correlation coefficient of 0.80). This suggests that salinity depletion favours the emergence of a subset of the population specialising in both taking up and degrading 2-NBDG.

In contrast with the findings above, the combined presence of nutritional and salinity depletion significantly increased both the uptake and degradation rate as well as intra-population phenotypic heterogeneities in both parameters (Fig. 4c, d, e, Supplementary Tables 5 and 6). This environment yielded a significant correlation between uptake and degradation rate (Pearson correlation coefficient of 0.36). However, such correlation was weaker with respect to that measured in the presence of salt depletion alone. This suggests that the additional nutritional depletion favoured specialization in fast 2-NBDG uptake at the cost of reduced degradation rates compared to champion degraders exposed to salinity depletion alone.

Next, we set out to confirm that the degradation of 2-NBDG by *E. coli* is linked to activity of the bacterial glycolytic pathway, responsible for the degradation of glucose. We investigated the degradation of the fluorescence of 2-NBDG in the presence of a competitive and non-metabolizable inhibitor (i.e. 3-O-methyl glucose) or a competitive and metabolizable inhibitor (i.e. glucose). The presence of a metabolizable inhibitor would stimulate bacterial glycolytic activity and thereby enhance the 2-NBDG degradation, while the presence of a non-metabolizable inhibitor would have no effect5. Accordingly, we found that the degradation of the fluorescence of 2-NBDG in the presence of a competitive and metabolizable inhibitor was significantly faster than the degradation of the fluorescence of 2-NBDG in the absence of this inhibitor (brown downward triangles and red squares in Fig. 4f, respectively, time constant Tau of (10.9 ± 0.5) min and (16.3 ± 1.1) min, respectively) since such inhibitor promotes glycolytic activity. In contrast, we found that the degradation of the fluorescence of 2-NBDG in the presence of a competitive and non-metabolizable inhibitor was comparable to the degradation of the fluorescence of 2-NBDG in the absence of this inhibitor (cyan upward triangles and red squares in Fig. 4f, respectively, time constant Tau of (16.3 ± 1.2) min and
and salt depletion (Fig. 5a, Pearson coefficient 0.07, **p value < 0.01). We also found a stronger correlation between differential gene and protein regulation under nutrient and salt depletion compared to salt depletion alone (Clusters 5 and 9 in Fig. 5b, c, **). Cellular homoeostasis biological processes including chemical, ion and cation homoeostasis were significantly upregulated under both nutrient and nutrient and salt depletion compared to salt depletion alone or optimal growth conditions both at the transcriptomic and proteomic level (Cluster 1 in Fig. 5b, c, **). Anaerobic respiration and polyol metabolic processes were significantly upregulated under both nutrient and nutrient and salt depletion at the proteomic level compared to salt depletion alone or optimal growth conditions both at the transcriptomic and proteomic level (Cluster 13 in Fig. 5c, *). To determine biological processes underlying acclimation of sugar metabolism to changes in nutritional availability and environmental salinity, we clustered these combined transcriptomic and proteomic data, identifying 13 distinct patterns of gene and protein regulation (Fig. 5b, c, respectively, and Supplementary data 1).

Molecular mechanisms underpinning glucose accumulation traits under nutrient or salt depletion. We then performed comparative transcriptomic and proteomic analysis between bulk E. coli cultures in the four different environments investigated and measured the log₂ fold change in transcript or protein levels under nutrient or salt depletion compared to those measured in optimal growth conditions. We then restricted our combined transcriptomic and proteomic analysis to genes and proteins whose differential expression had a p value adjusted for false discovery rate smaller than 0.05. We found a significant correlation between gene and protein expression under salt depletion but no significant correlation under either nutrient or nutrient and salt depletion (Fig. 5a, Pearson coefficient R = 0.13, 0.06 and 0.07, **ns, ns, respectively). We also found a stronger correlation between differential gene and protein regulation under nutrient and nutrient and salt depletion compared to salt and nutrient and salt depletion (Pearson coefficient R = 0.92 and 0.34, at the transcriptomic level and 0.89 and 0.45, at the proteomic level, all ****).

To determine biological processes underlying acclimation of sugar metabolism to changes in nutritional availability and environmental salinity, we clustered these combined transcriptomic and proteomic data, identifying 13 distinct patterns of gene and protein regulation (Fig. 5b, c, respectively, and Supplementary data 1).

Cellular homoeostasis biological processes including chemical, ion and cation homoeostasis were significantly upregulated under both nutrient and nutrient and salt depletion compared to salt depletion alone or optimal growth conditions both at the transcriptomic and proteomic level (Cluster 1 in Fig. 5b, c, **). Anaerobic respiration and polyol metabolic processes were significantly upregulated under both nutrient and nutrient and salt depletion at the proteomic level compared to salt depletion alone or optimal growth conditions (Cluster 13 in Fig. 5c, *). In accordance with a previous report.

Crucially for this study, phosphoenolpyruvate-dependent sugar phosphotransferase systems (PTS) were significantly upregulated at the proteomic level under both nutrient and nutrient and salt depletion compared to salt depletion alone (Cluster 12 in Fig. 5c, **). These proteins included the PTS system galactitol- and mannose-specific EIID components GatA

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**Fig. 4 Combined nutritional and salinity depletion boosts both glucose uptake and degradation.** a-c Single-cell correlation between the natural logarithm of 2-NBDG uptake rate and the natural logarithm of 2-NBDG degradation rate as predicted by Equation 1 for optimal growth conditions (circles), nutrient depletion (diamonds), salt depletion (triangles) or combined nutrient and salt depletion (squares), with Pearson correlation coefficients of 0.42 (****), 0.27 (ns), 0.80 (****) and 0.36 (*), respectively. N > 30 for each experimental condition collated from biological triplicates, the large symbols are the medians of each set of single-cell values. Coefficient of variations of these single-cell values and statistical tests of quantitative comparisons of values between different environments are reported in Supplementary Tables 5 and 6, respectively. Corresponding distributions of (d) natural logarithm of 2-NBDG uptake rate values and (e) natural logarithm of 2-NBDG degradation rate values. The bottom and top of the box are the first and third quartiles, the band inside the box is the median, the bottom and top whiskers represent the 10th and 90th percentiles, respectively. Temporal dependence of the fluorescence of 2-NBDG when E. coli populations were incubated in 30 µM 2-NBDG alone (red squares), in the presence of 30 mM 3-O-methylglucose (cyan upward triangles), or in the presence of 30 mM glucose (brown downward triangles). N = 3 biological replicates for each condition. The lines are one phase exponential decay fittings to the data yielding time constant Tau of (16.3 ± 1.1) min, (16.3 ± 1.2) min and (10.9 ± 0.5) min, respectively. *p value < 0.05; **p value < 0.01; ***p value < 0.001; ****p value < 0.0001.
and ManZ, respectively, the latter transporting both mannose and glucose across the E. coli inner membrane. Furthermore, glucose metabolic processes were upregulated at the proteomic level under nutrient or nutrient and salt depletion compared to salt depletion alone (Cluster 12 in Fig. 5c, **). These proteins included the formate acetyltransferase 1 PiIB that is involved in pyruvate fermentation (following glucose conversion to pyruvate) and formate synthesis; the L-lactate dehydrogenase LldD which converts pyruvate to lactate; the glucose-1-phosphatase Agp which converts glucose to glucose-1-phosphate; the putative glucose-6-phosphate epimerase YeaD; as well as catabolic processes of other sugars such as galactitol and deoxyribose.

To further validate these data, we performed clustering analysis on transcriptomic data alone and found that carbohydrate transport and metabolic processes were significantly downregulated under nutrient or nutrient and salt depletion compared to salt depletion alone or optimal growth conditions (Clusters 8 and 12 in Supplementary data 2, ###).
Fig. 5 Molecular mechanisms underpinning glucose accumulation traits under nutrient or salt depletion. a Correlation between the log₂ fold change in gene and protein expression under nutrient, salt or nutrient and salt depletion. Each dot is the log₂ fold change in the number of copies for a single gene or protein, blue lines are linear regressions to the data returning a Pearson correlation coefficient R = 0.06, 0.13 and 0.07, (ns, ** and ns, respectively). Transcript and protein reads were measured via RNA-sequencing and proteomics on samples in biological triplicate and are reported in Supplementary data 2 and 3, respectively. Cluster analysis of the combined transcriptomic and proteomic data above returned 13 clusters with distinct patterns of (b) gene and (c) protein regulation under nutrient, salt or nutrient and salt depletion. Each dot represents the log₂ fold change in the number of copies for a single gene or protein, dashed and dotted lines indicate the median and quartiles of each distribution, respectively, the grey solid lines indicate a log₂ fold change of zero. The lists of genes belonging to each cluster are reported in Supplementary data 1. d Biological processes significantly over-represented in each of the clusters above. For clarity, only clusters displaying significantly over-represented biological processes are reported in figure, data for the remaining clusters are reported in Supplementary data 1.

Our clustering analysis performed on proteomic data alone we found that carbohydrate metabolic processes were significantly upregulated under nutrient or nutrient and salt depletion compared to salt depletion alone or optimal growth conditions (Cluster 8 in Supplementary data 3, ****). Besides the proteins found in Cluster 12 of our combined transcriptomic-proteomic clustering analysis, Cluster 8 of the proteomic analysis included the PTS system glucose-specific EIIA component Crr; the phosphocarrier protein PtsH; the glucose-1-phosphate adenyltransferase GlgC; the acetonitrile hydratase A AcaN; the enolase Eno; the mannose-6-phosphate isomerase ManA; the phosphoglycerate kinase Pggk; the pyruvate kinase PykF; the galactitol 1-phosphate 5-dehydrogenase GatD; the phosphoglucomutase Pgm that is a highly conserved enzyme which functions at a key point in glucose metabolism (the interconversion of glucose-1-phosphate and glucose-6-phosphate46), it is involved in several processes including bacterial pathogenesis47 and was previously found to be upregulated under nutrient depletion in minimal medium45. Here we found that Pgm was further upregulated under combined nutrient and salt depletion compared to nutrient or salt depletion alone (log₂ fold change of 0.7, 0.4 and 0.3, respectively, Supplementary data 3) in accordance with our data on glucose degradation rates (Fig. 4e). It is also worth noting that a previous study found that PykF and ManA were upregulated in E. coli cultured in simulated colonic environment medium (i.e. with low salinity and nutritional content)21. Taken together these data demonstrate that under nutrient or combined nutrient and salt depletion glucose uptake and degradation are regulated at translation rather than transcription level.

Moreover, when we carried out phosphoproteomics on E. coli cultured in the four different environments above, we found that the phosphoglucomutase Pgm was one of the 13 proteins for which a phosphorylation event was detected (rows 8–9 in Supplementary data 4). Other phosphorylated proteins included PpsA and GpmM involved in gluconeogenesis and PpC and Icd (i.e. the first example of a protein phosphorylated on a serine or a threonine residue in bacteria45) involved in the tricarboxylic acid cycle. We measured phosphorylation at two different sites for the phosphopeptide GGPLADGVITTPSHNPPEDGGI that aligns with high confidence to the Pgm amino acid sequence. The first phosphorylation event was recorded on serine 13 of the phosphopeptide above with a site probability of 88% as defined by the phosphoRS node of Proteome Discoverer software (i.e. serine 146 in the full protein sequence in accordance with previous studies35,48). The amino acid residues surrounding this serine are highly conserved in the phosphoglucomutase superfamily to which Pgm belongs49. This stretch of residues is known as the phosphoserine loop and is involved in the transfer of a phosphoryl group from the serine residue above to glucose 6-phosphate to form a biphosphorylated sugar intermediate. A high level of phosphorylation of this serine residue increases Pgm flexibility and its enzymatic activity49. In accordance with our glucose degradation data (Fig. 4e), we measured higher Pgm phosphorylation levels at serine 13 under combined nutrient and salt depletion compared to optimal growth conditions (*row 8 in Supplementary data 4). This indicates that the combined nutrient and salt depletion drives a 12-fold post-translational change as well as a 1.6-fold translational change in Pgm levels, thus explaining the higher glucose degradation rate reported in Fig. 4. Moreover, we measured significantly higher Pgm phosphorylation levels under combined nutrient and salt depletion compared to optimal growth conditions (*row 9 in Supplementary data 4) at a second site within the phosphoserine loop. However, this site could not be defined with any degree of confidence (i.e. site probability <70%). Finally, a significantly higher degree of phosphorylation under nutrient and salt depletion compared to optimal growth conditions was also measured for the phosphoenolpyruvate synthase PpsA and the isocitrate dehydrogenase Icd (* and **,rows 6 and 17 in Supplementary data 4) involved in gluconeogenesis and the tricarboxylic acid cycle, respectively.

Considering the significant variations in Pgm both at the translational and post-translational level, we set out to investigate the role played by Pgm in 2-NBDG accumulation. To do this, we performed single-cell 2-NBDG accumulation measurements on both a Δpgm deletion mutant and the parental strain (PS). We found that under nutrient and salt depletion the Δpgm deletion mutant displayed significantly lower 2-NBDG accumulation compared to the parental strain (purple hexagons and red squares in Supp. Fig. 6, respectively, **). In contrast, under optimal growth conditions the Δpgm deletion mutant displayed 2-NBDG accumulation comparable to the parental strain (magenta diamonds and black circles in Supplementary Fig. 6, respectively, ns). These data further confirm that modifications of Pgm at the translational and post-translational level allow E. coli to enhance glucose accumulation.

Taken together these data demonstrate that simultaneous exposure to nutrient and salt depletion decreases gene expression, translation and biosynthetic processes, while increasing E. coli capability to take up and use glucose (and possibly other sugars) via variations at the translational and post-translational level but not at the transcriptional level, thus corroborating our glucose and 2-NBDG accumulation data presented in Figs. 2–4.

Glucose accumulation is not regulated by molecules secreted in the environment. Finally, we also investigated the possibility that the combined nutritional and salinity depletion caused bacterial secretion of molecules that affect intracellular 2-NBDG accumulation. These molecules could include signalling secondary metabolites, such as putrescine and cadaverine, that affect the functioning of membrane transporters44. In order to test this hypothesis, we exposed E. coli grown in optimal growth conditions to the supernatant collected from E. coli exposed to combined nutritional and salinity depletion. After 1 h exposure to such supernatant, we washed the microfluidic environment, introduced 30 μM 2-NBDG dissolved in glucose-free M9 medium and measured 2-NBDG accumulation in individual bacteria. We
then compared these measurements to those performed without exposing bacteria to such supernatant (squares and circles, respectively, in Supp. Fig. 7). In contrast to the hypothesis above, we found that exposure to the supernatant collected from nutrient and salt depleted E. coli did not enhance 2-NBDG accumulation. Taken together these data suggest that the metabolic changes observed in the simultaneous nutritional and salinity depletion are not due to bacterial secretion of compounds that can alter glucose metabolism.

Discussion

Glucose uptake and utilization in bacteria have been previously linked to cell size, suggesting that both glucose uptake and intracellular conversion are maximal in favourable growth conditions, an idea that has led to the consensus that bacteria dwelling in stressful environments reduce their metabolic conditions, an idea that has led to the consensus that bacteria intracellular conversion are maximal in favourable growth conditions. Taken together these data suggest that the metabolic levels under nutrient depletion 45. Here we complement this correlation. We show instead that the measured metabolic changes are underpinned by variations in glucose transport and metabolism at the translational and post-translational level. Protein phosphorylation, especially on serine, threonine, or tyrosine, is one of the most common post-translational modifications in bacteria 45. Protein phosphorylation controls cell metabolism and enhances cellular fitness under growth limiting conditions; for example, enzymes such as phosphoglycerate mutase, phosphoglucomutase and adenosine 5′-phosphosulfate kinase catalyse the turnover of phosphorylated sugars or metabolite phosphorylation by going through a phosphorylated intermediate state during catalysis 42. A previous study found a global increase of protein phosphorylation levels under nutrient depletion 45. Here we complement this understanding by demonstrating elevated protein phosphorylation levels under nutrient depletion, salt depletion or combined nutrient and salt depletion. Taken together these findings point to a likely role of protein phosphorylation in variant environments. Indeed, we found significantly higher phosphorylation levels of the phosphoglucomutase Pgm (at serine 146), a highly conserved enzyme 39, under nutrient and salt depletion compared to optimal growth conditions offering a mechanistic explanation of the measured glucose degradation rates. Furthermore, Pgm was upregulated at the translational level under nutrient depletion in accordance with a previous study using minimal medium 45, whereas we use LB medium, further confirming that this metabolic response is not dictated by the pre-culturing medium. We also found that Pgm was further upregulated under combined nutrient and salt depletion compared to nutrient depletion alone corroborating our data on glucose degradation rates. Finally, it is conceivable that other previously identified post-translational modifications (e.g. the acetylation of GapA and FbaA 48, both involved in carbohydrate degradation) could further contribute to the observed variations in glucose uptake and degradation rates.

We also showed that exposing E. coli growing in optimal conditions to the supernatant collected from cells under nutrient and salt depletion did not enhance glucose accumulation traits although this data should be corroborated in future via LC-MS metabolomics 52. This finding demonstrates that enhanced glucose accumulation traits are not driven by the impact of physicochemical properties of the nutrient and salt depleted environment on molecular transport, but rather by continuous cellular adaptation to such an environment.

These data corroborate previous work about the impact of salinity on carbon uptake in Vibrio marinus and cyanobacteria in natural environments 54,55, and on the remodelling of E. coli glucose metabolism in the presence of environmental challenges 44,56. Furthermore, NaCl is routinely used in food products as an antimicrobial agent 57, therefore our findings that adding NaCl decreases glucose accumulation in stationary phase bacteria should be taken into account in both food preservation and bioproduction.

Our data also demonstrate that heterogeneity in glucose accumulation traits under combined nutrient and salt depletion cannot be ascribed to cell-to-cell differences in surface area alone neither can be ascribed to recovery from stationary phase 58. In fact, we measured similar levels of heterogeneity in glucose uptake and degradation in exponentially growing and stationary phase E. coli. These data therefore suggest that cellular or environmental parameters other than cell size underpin heterogeneity in glucose metabolism adding to our current understanding of the relationship between cell growth rate and cellular processes 59–63 including those preparing a cell for surviving fluctuations in environmental conditions 64. In this respect, we found that exposure to nutritional or salinity depletion increases heterogeneity in both glucose uptake and degradation. This could be explained by the recently reported heterogeneity in the expression of sugar metabolism genes 65,66. Taken together these data add knowledge to the current understanding on phenotypic noise 67, corroborating the view that cell-to-cell differences are ubiquitous within microbial populations 58,67–69 and that there is substantial heterogeneity in the accumulation of metabolites 53,54,65,66,70–75 or antimicrobials 30,76,77.

The experimental approach introduced here could be applied to other fields of research including medical mycology and crop protection, considering that glucose and its fluorescent analogue 2-NBDG is taken up by pathogenic fungi such as Candida albicans 78. The newly discovered glucose accumulation traits should be considered when investigating cellular processes where salinity plays an important role such as cystic fibrosis associated lung infections 22 and the enteropathogens present in the ileum and colon 21.

Methods

Strains, media and cell culture

All chemicals were purchased from Merck unless otherwise specified. Lysogeny Broth (LB, Melford) media made of 10 g/L Tryptone, 5 g/L Yeast extract and either 0.5, 5 or 10 g/L NaCl, were used for culturing E. coli. Noteworthy, the 5 and 10 g/L NaCl LB formulation are routinely used in microbiology, whereas the 0.5 g/L NaCl LB formulation is generally employed only for selective cultivation with antibiotics that require low salt conditions. The three formulations differ only in salt content, these do not differ in carbon and nitrogen source content that it is known to affect glucose metabolism 39. LB agar plates of respective NaCl concentration with 15 g/L agar (Melford) were used for streak plates. Glucose-free M9-minimal media, used to wash cells and dilute 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) or Thioflavin T (TST), was prepared using 5× M9 minimal salts (Merck), diluted as appropriate, with additional 2 mM MgSO4, 0.1 mM CaCl2, 3 μM thiamine HCl in milliQ water. Glucose or ammonium limited M9 media were prepared by adding to this solution...
0.1 g/L glucose or 0.1 g/L NH4Cl, respectively. NaCl was added as required at a final concentration of 0.5 g/L or 10 g/L, as appropriate. The parental strain E. coli BW25113 was used as the genomic knockout parent for the sirA deletion. For a sirA copy construct, Dharmacon (GE Healthcare) and stored in 50% glycerol stock at −80 °C. Streak plates for each strains were produced by thawing a small aliquot of the corresponding glycerol stock every 2 weeks and using LB agar containing either 0.5, 5 or 10 g/L NaCl. Exponentially growing cultures were obtained by inoculating 100 ml of LB (or M9 medium) of 0.5, 5 or 10 g/L NaCl content with 100 ml of stationary phase liquid culture. E. coli BW25113, then placed in a shaking incubator at 200 rpm at 37 °C for 3 h. Overnight cultures were prepared inoculating a single colony of E. coli BW25113 in 200 ml of LB (or M9 medium) with 0.5, 5 or 10 g/L NaCl. Following the inoculation, the overnight cultures were incubated at 37 °C. Spent LB or M9 media used for resuspension of bacteria in microfluidic assays was prepared by centrifugation of overnight cultures (4000 rpm, 20 °C, 10 min). The supernatant was then double filtered (Medical Millex-GS Filter, 0.22 μm, Millipore Corp) to remove bacterial debris from the solution as previously reported[45].

2-NBDG (Molecular Weight = 342 g/mol, ThermoFischer) was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mg/ml and stored at −20 °C. 100 μl of 2-NBDG was added to 1 mL of LB (or M9 medium) of 0.5, 5 or 10 g/L NaCl content, and incubated for 15 min as for the uptake experiments above. The presence of competitive inhibitors, the bacterial pellet was resuspended in 30 μM D-glucose (metabolizable inhibitor) or 30 μM D-2-deoxyglucose (non-metabolizable inhibitor) was followed by a 15 min incubation at room temperature. After incubation, cells were pelleted at 13,400 rpm for 5 min using a microcentrifuge and resuspended in 1 mL of phosphate buffer solution (PBS) and further diluted as necessary.

Measurements were then performed using a Beckman Coulter CytoFlex S (Beckman Coulter, United States) and cell fluorescence was quantified using the fluorescein isothiocyanate (FITC) channel (488 nm excitation and 525/40 nm band-pass filter), with PMT voltages of FSC 1000, SSC 500, FITC 250 and a threshold value of 10000 for SSC-A to exclude any background noise. These measurements were not normalized by cell size. Data were initially collected using CytExpert software, gated using gating strategy shown in Supp. Fig. Plots were exported and later processed using GraphPad Prism 9. For degradation assays in the presence of competitive inhibitors, the bacterial pellet was resuspended in 30 μM D-2-deoxyglucose and incubated for 15 min as for the uptake experiments above. Following incubation, 1 mL of D-glucose (metabolizable inhibitor) or 30 μM D-2-deoxyglucose (non-metabolizable inhibitor) was added and followed by a 15 min incubation at room temperature. After incubation, cells were pelleted at 13,400 rpm for 5 min using a microcentrifuge. Each pellet was subsequently resuspended in 1 mL 30 μM D-glucose and incubated for different time intervals. Following incubation, centrifugation was carried out as above at 0 °C, to reduce any further glucose uptake by cells. The supernatant of each sample was collected while the pellet was discarded. Using a glucose assay kit (Sigma Aldrich, Montana, United States), 50 μL of each supernatant sample was added to 50 μL master reaction mix (46 μL glucose assay buffer, 2 μL glucose probe and 2 μL glucose enzyme mix) and incubated in the dark at room temperature for 15 min. Oxidisation of any glucose present in the sample occurred during incubation, thus generating a colorimetric product, the absorbance of which was measured at 570 nm using a CLARIOstar PLUS plate reader (BMG Labtech, UK). For each experimental repeat, glucose standards were obtained by performing a serial dilution between 0 and 10 μL of a 1 nmol/μL glucose standard solution added to 50 μL of the master reaction mix above, brought to 100 μL per well with glucose assay buffer as needed. Absorbanes collected for measurement were then used as a standard curve by which sample absorbances were compared and interpolated.

To calculate the concentration of glucose present in each sample, background absorbance (assay blank of standard curve where 0 μL of glucose standard solution was present in the sample) was first subtracted and then the following equation was used:

\[ C = \frac{S - S_b}{S_{std} - S_b} \]

where \( S \) is the amount of glucose in the unknown sample (in nmol) from standard curve, \( S_b \) is the sample volume (μL) added into the well and \( C \) describes the concentration of glucose in the sample.

**Image and data analysis.** Images were processed using ImageJ software as previously described[47] tracking each individual bacterium throughout their incubation in and removal of 2-NBDG. A rectangle was drawn around each bacterium at every time point, obtaining its width, length and mean fluorescence intensity. The mean fluorescence intensity for each bacterium was normalised by cell size, to account for cell cycle related variations in glucose accumulation45. The background fluorescence (i.e. the fluorescence of extracellular 2-NBDG) at each time point was measured by placing a rectangle, of similar dimensions to those drawn around the bacteria, was drawn and positioned, at the same distance from the main channel, in the nearest channel that did not contain bacteria and the mean fluorescence value within this rectangle was extracted via ImageJ. This background fluorescence was subtracted from the corresponding bacterium’s fluorescence value at every time point as previously reported43. These fluorescence data were then analysed and plotted using GraphPad Prism 9.
Transcriptic analysis. RNA isolation, library preparation, sequencing, and transcriptomic data processing was performed as previously reported44,83. Briefly, E. coli cultures in high or low salt experimental (optimal growth conditions or salt depletion, respectively) and stationary phase (nutrient depletion or nutrient and salt depletion, respectively) were prepared as described above and 500 µL aliquots were taken after 3 or 17 h, respectively, in biological triplicate for each of the four environmental conditions. RNAPrekt Bacteria Reagent (Qiagen) was added to each aliquot. Extractions were performed using RNeasy Mini Kit (Qiagen) for exponential phase aliquots and RNeasy Micro Kit (Qiagen) for stationary phase aliquots following manufacturer instructions. Reverse transcriptase and cDNA synthesis was performed using FluctommTM M-MLV reverse transcriptase (Promega, Madison, WI) according to manufacturer instructions. cDNA was purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). cDNA (100 ng) was then amplified using 1 µM of each primer (Table 1), 200 µM dNTPs, and 0.5 U of Taq DNA polymerase (Promega, Madison, WI) in 25 µL reaction volume in a thermal cycler using a touchdown PCR protocol as follows: an initial denaturation step at 95 °C for 5 min, followed by 10 cycles of 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min, and then 25 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, with a final extension step at 72 °C for 10 min. PCR products were gel-purified with a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced on an ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA) using the Big Dye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequencing results were analyzed using SEQMAN (Lambdagen, Largs, Australia) and consensus sequences were aligned using Clustal Omega (EMBL-EBI, Cambridge, UK) and visualized with MUSCLE (Edgar, 2004). Allele frequencies were estimated by the method of Weir and Cockerham (1984) and significance was assessed by a chi-square goodness-of-fit test with 1 df. Genotypes were assigned using PHYLIP (Felsenstein, 1993) and GENEPOP (Raymond and Rousset, 1995) with 10,000 dememorization steps and 1,000 replicates with no randomization. The following parameters were used: observed heterogeneity (Ht), expected heterogeneity (He), fixation index (FIS), and linkage disequilibrium (D) measured with Genalex (Peakall and Smouse, 2006). The population structure was determined using the software Structure (Pritchard et al., 2000). The analysis was performed 20 times for different numbers of clusters (K = 1 to 10) using the Evanno method (Evanno et al., 2005).
solution for inference purposes:

\[ [\text{gluc}]_i = \begin{cases} 
  \frac{k_{\text{gluc}\text{in}}}{V_i} (1 - e^{-d_{\text{gluc}}^i t_i}), & t_1 \geq t_i \\
  \frac{k_{\text{gluc}\text{in}}}{V_i} (e^{-d_{\text{gluc}}^i t_1} - e^{-d_{\text{gluc}}^i t_i}), & t_1 < t_i
\end{cases} \]

However, such an approach misses out information (i.e. non-instantaneous 2-NBDG delivery) that is crucial for providing an accurate description of the kinetics in 2-NBDG accumulation in single cells. Rate constant \( u_{\text{gluc}} (\mu m^{-1} s^{-1}) \) and \( d_{\text{gluc}} (s^{-1}) \) dictate the rate of 2-NBDG uptake and degradation, respectively. Parameter \( V_i (\mu m^3) \) denotes the cellular volume, and its presence in the denominator of the uptake rate term ensures that intracellular 2-NBDG concentration is reduced in cells with larger volumes due to dilution (other parameters being the same). We estimate \( V_i \) using the measured width and length of each cell and assuming a rod-like shape. We use the model and Bayesian inference techniques to extract information regarding rate parameters \( u_{\text{gluc}}, d_{\text{gluc}} \) from our single-cell measurements of intracellular 2-NBDG accumulation and degradation. To capture cell-to-cell heterogeneity we employ a hierarchical Bayes approach where parameters \( u_{\text{gluc}} \) and \( d_{\text{gluc}} \) vary between single cells according to an underlying population distribution. To model this unknown population distribution we use an infinite Gaussian mixture model (iGMM)90, which being a non-parametric Bayesian model offers greater modelling flexibility than standard parametric distributional models. We note that the hierarchical Bayes approach also allows us to naturally incorporate in the model uncertainty (or lack of information) regarding the prior distribution of \( u_{\text{gluc}} \) and \( d_{\text{gluc}} \), as we do not have to specify explicit priors for these parameters but instead specify priors for the hyper-parameters of the iGMM model. Choices of priors for the iGMM hyper-parameters are given in Table 1. To sample from the posterior distribution of the two parameters of the iGMM model, \( \theta_{\text{iGMM}} \), consists of \( \theta_{\text{iGMM}} \) are sampled anew given the current values of \( \theta_{\text{iGMM}} \) and \( \theta_{\text{GAALM}} \). In summary, the algorithm involves sampling iteratively from the following target distributions:

\[ a^{(L)}_{\text{gluc}}, d^{(L)}_{\text{gluc}}, \theta^{(L)}_{\text{GAALM}} \sim P(V_i, [\text{gluc}]_i, CV_{\text{error}}) \text{ for each cell i; } \]

\[ \theta^{(L)}_{\text{iGMM}} \sim P(V_i, [\text{gluc}]_i, CV_{\text{error}}) \]

To sample parameters \( u_{\text{gluc}} \) and \( d_{\text{gluc}} \) in step a) we assume that the 2-NBDG accumulation and degradation measurement, \( Y_{i,t} \), taken cell \( i \) at time-point \( t \) obeys a gaussian distribution, \( Y_{i,t} \sim N([\text{gluc}]_i, \sigma_{\text{error}}) \), with mean \([\text{gluc}]_i\) and standard deviation \( \sigma_{\text{error}} = CV_{\text{error}} \times [\text{gluc}]_i \). Here \([\text{gluc}]_i\) is obtained by solving the model numerically using the corresponding rate parameters, cellular volume, and external 2-NBDG concentration assumption. This assumption allows us to use a single step of the Metropolis-Hastings algorithm to draw samples from the target distribution since this is proportional to a product of densities (gaussians and mixture of gaussians) all of which can be straightforwardly evaluated, i.e.,

\[ P(a^{(L)}_{\text{gluc}}, d^{(L)}_{\text{gluc}}, \theta^{(L)}_{\text{GAALM}} | V_i, [\text{gluc}]_i, CV_{\text{error}}) \]

\[ \propto P(Y_i | a^{(L)}_{\text{gluc}}, d^{(L)}_{\text{gluc}}, V_i, [\text{gluc}]_i, CV_{\text{error}}) \]

\[ = \prod_i P(Y_i | a^{(L)}_{\text{gluc}}, d^{(L)}_{\text{gluc}}, V_i, [\text{gluc}]_i, CV_{\text{error}}) \]

In step b) of the algorithm, we sample from the target distribution using the algorithm proposed by Rasmussen.90

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Author contributions

S.P. designed the research and developed the project. G.G. and U.L. performed the experiments. M.V. and K.T.A. developed and implemented the mathematical model. B.M.I. performed the clustering and gene ontology analysis. D.S., P.O. and K.M performed the transcriptomics analysis. S.R. and D.S.M. assisted G.G. during protein extraction, flow cytometry and colorimetric assays. K.H. performed the proteomics and phosphoproteomics analysis. G.G., M.V., U.L., B.M.I., D.S., N.N., D.S.M., S.R., K.H., P.G.P., T.A.R. and S.P. analysed the data. G.G. and S.P. wrote the paper. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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