

Physiological and regulatory convergence between osmotic and nutrient stress responses in microbes

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Abstract

Bacterial cells regularly confront simultaneous changes in environmental nutrient supply and osmolarity. Despite the importance of osmolarity and osmoregulation in bacterial physiology, the relationship between the cellular response to osmotic perturbations and other stresses has remained largely unexplored. Bacteria cultured in hyperosmotic conditions and bacteria experiencing nutrient stress exhibit similar physiological changes including metabolic shutdown, increased protein instability, dehydration, and condensation of chromosomal DNA. In this review, we highlight overlapping molecular players between osmotic and nutrient stresses. These connections between two seemingly disparate stress response pathways reinforce the importance of central carbon metabolism as a control point for diverse aspects of homeostatic regulation. We identify important open questions for future research, emphasizing the pressing need to develop and exploit new methods for probing how osmolarity affects phylogenetically diverse species.

Introduction

Osmolarity, the number of dissolved solute particles per liter of solution, is an important yet often ignored aspect of cellular physiology (Glossary) [1]. Single-celled bacteria typically cannot control their environment, and hence osmolarity broadly influences phenotypes from cell size [2] to antibiotic sensitivity [3]. Osmolarity dictates the water content of cells, impacting the activity of essential enzymes and modulating metabolic function [4,5]. The osmolarity differential between the inside and outside of the cell (turgor pressure, Glossary), which can be >10 atmospheres in some species [6,7], determines the mechanical force balance between the cell envelope and the environment [8], and maintaining positive turgor can be important for growth.

Under typical growth conditions, proteins, sugars, lipids, nucleic acids, and other small molecules within the cytoplasm collectively constitute a dense, highly crowded environment with concentrations as high as 400 mg/mL [9-11]. A substantial increase or decrease in density impacts molecular diffusion (Glossary) and interactions through the level of crowding [12,13]. Osmolarity is intrinsically related (although not identical) to intracellular density. Since osmolarity is the number of solute particles per liter, reducing intracellular osmolarity can be as simple as polymerizing hundreds of amino acids into a single protein or thousands of nucleotides into a molecule of DNA or RNA, as long as the subunits constitute a sizeable fraction of cellular mass. Thus, nutrient

availability and metabolic rate have the potential to alter cytoplasmic osmolarity, and osmotic regulation may be required to maintain cellular homeostasis. Thus, the physical properties of the cytoplasm are intertwined with growth and directly impact intracellular organization and biochemistry.

The osmolarity of the bacterial cytoplasm is stringently regulated by molecular mechanisms that mediate accumulation or secretion of osmolytes. The importance of osmotic regulation is further underscored by its metabolic cost, involving the synthesis and breakdown of carbon-rich molecules. The osmoprotectants trehalose (a disaccharide of two glucose molecules), glycine betaine (amino acid derivative), and proline betaine (amino acid derivative) are manufactured or imported under hyperosmotic stress [1]. Thus, induction of osmoregulatory pathways requires repurposing nutrients from metabolic pathways that would otherwise provide cellular energy and synthesis capacity.

Just as many osmolytes are repurposed nutrients, nutrients can function as osmolytes, illustrating an intrinsic connection between osmolarity and nutrient availability such that a low-osmolarity environment may generically contain fewer molecules with nutritional potential, and vice versa. This coupling is particularly poignant for bacteria that face sudden shifts into drastically more dilute environments. Human gut

commensals and pathogens face both osmotic shifts and feast-famine cycles as they transition between their host and the environment [14] and hence it would be natural to couple osmotic regulation to other aspects of physiology. Indeed, *Leptospira interrogans* utilizes the inevitable change in osmolarity when entering host tissues as a signal to upregulate virulence factors [15].

In this review, we discuss the impact of osmolarity on bacterial physiology and growth, emphasizing physiological and regulatory overlaps between the response to osmotic stress and to nutrient stress. Hyperosmotic or hypoosmotic stress result from a transition to an environment with higher or lower osmolarity, respectively. Although a low-osmolarity environment due to general dilution signifies a dearth of nutritional potential, under hypoosmotic conditions bacteria nevertheless use glucose to synthesize osmoregulated periplasmic glucans (OPGs, Glossary) [16]. This tension between two coincident stresses suggests that devoting precious resources to producing an osmoprotectant outweighs the benefits of using it for biosynthesis. Despite the intriguing nature of the OPGs and hypoosmotic stress as a whole, links with molecular crowding have made hyperosmotic stress the better studied of the two phenomena, and hence it will be the focus of this review.

Nutrient stress and hyperosmotic stress elicit similar changes in cytoplasmic density

Plasmolysis—cytoplasmic dehydration and shrinkage coupled with enlargement of regions of the periplasm (Glossary)—is a visibly striking and well-known hallmark of the initial stages of hyperosmotic stress (Figure 1). Sudden or gradual nutrient depletion in Gram-negative bacteria similarly leads to plasmolysis, although the regions of periplasmic enlargement can differ from hyperosmotic shock (Figure 1A). During stationary phase (Glossary), a state of metabolic quiescence and cell shortening upon gradual nutrient depletion, the cytoplasm decreases in volume and becomes denser [11] and the periplasm increases in volume [17]. Likewise, during sudden depletion of carbon, nitrogen, or phosphorus, the inner membrane of *E. coli* [11], *K. pneumoniae* [11], and *V. cholerae* [18] retracts, resulting in a smaller, denser cytoplasm and enlarged periplasm. Budding yeast cells undergo a similar process under glucose starvation in which cell volume shrinks without any loss of cell mass, in this case due to vacuolar expansion [19] rather than the periplasmic expansion that occurs in bacteria [11]. The increased periplasmic volume at the expense of cytoplasmic volume during hyperosmotic shock and nutrient stress highlights the interplay between these compartments and raises the interesting possibility that one function of the bacterial periplasm may be to act as a vacuole-like organelle.

Gram-positive bacteria lack a periplasm but still exhibit similar cytoplasmic changes as Gram-negative bacteria during starvation (Figure 1B). The model Firmicute *Bacillus*

subtilis and other related species induce a programmed stress response pathway that culminates in the formation of a dormant, heat-resistant endospore [20]. Spore formation (Glossary) is characterized by nucleoid condensation [21] and dehydration, which contributes to heat tolerance [22]. The frequency of protein-DNA interactions also increases in spores, which provides protection against DNA damage [23]. Thus, a condensed, dehydrated state may generally increase resilience across the bacterial kingdom.

Notably, treatment of *E. coli* cells with DNP, a drug that uncouples the electron transport chain from ATP synthesis and leads first to an increase in metabolic activity and then to metabolic arrest, results in an expanded nucleoid rather than an expanded periplasm [24], highlighting the specificity of physiological responses to starvation. It remains unclear whether cytoplasmic volume fraction is intrinsically linked to growth rate and/or nutrient quality [17]; in *E. coli*, nutrient concentration during exponential growth non-monotonically impacts cytoplasmic volume fraction in stationary phase [11]. Altogether, comparison of cytoplasmic condensation between hyperosmotic shock and various modes of metabolic slowdown suggests that reduction in water content may be broadly beneficial but dependent on many aspects of growth history.

Physical mechanisms of enzymatic activity regulation during hyperosmotic and nutrient stress

The reduction in cytoplasmic volume elicited by nutrient stress and osmotic stress affects molecular crowding (Figure 1), a critical component of cellular physiology. Crowding inhibits many enzymatic reactions by physically slowing their kinetics, as diffusion coefficients decrease and reactant molecules encounter each other less frequently [4], or by inactivating enzymes through steric hindrance resulting from polymerization [25]. The increase in crowding during stationary phase in *E. coli* suggests that cells may face similar physiological challenges as under hyperosmotic stress [11,13], when density also increases, although the contribution of increased cytoplasmic crowding to stationary phase-dependent reductions in metabolic activity is unknown. In the budding yeast *Saccharomyces cerevisiae* (Glossary), the rates of cytoplasmic and nuclear diffusion decrease during glucose starvation, which has been suggested to be due to crowding [19], and mechanical stress-induced crowding inhibits translation and curtails cell growth [26]. Importantly, higher intracellular density is not necessarily associated with slow growth [27], and metabolic slowdown can inhibit molecular diffusion in the absence of obvious changes in cell volume or density [24]. Thus, the relationships among crowding, density, and growth may be more multifaceted than previously appreciated.

In addition to the physical effects of crowding, nutrient and hyperosmotic stress lead to changes in protein synthesis. During nutrient stress, bacteria downregulate protein synthesis through the stringent response and synthesis of the alarmone (p)ppGpp (Glossary) [28,29]. Hyperosmotic stress specifically inhibits translation elongation, leading to compensatory transcriptional upregulation of ribosomes in an attempt to maintain translational capacity [30]. Similarly, in budding yeast, translation and metabolic activity are initially inhibited during salt stress, which enables adaptation that avoids long-term defects in cell growth [26,31]. In addition to the shift in water content between the cytoplasm and periplasm that alters cytoplasmic volume fraction, nutrient and hyperosmotic stress also cause major redistribution of the proteome. Bacterial cells experiencing nutrient stress and cells experiencing hyperosmotic stress both have more biomass in the periplasm and outer membrane and a concomitant decrease in biomass in the cytoplasm and inner membrane compared to steady-state growth (Glossary) (Figure 2) [17]. Interestingly, similar changes are observed during growth at pH 6 as well (Figure 2), suggesting the potential for further overlap with the acid stress response pathway.

While an increase in cytoplasmic density is expected after a hyperosmotic shock due to the physical nature of the perturbation, why the cytoplasm shrinks during nutrient stress is unclear. It may be an active and beneficial response or an inevitable

consequence of the physiological changes inherent in starvation. Regardless, the metabolic shutdown inherent in a dense, dehydrated state conserves energy [13,21] and boosts resistance to antibiotics [3], heat, and desiccation [10]. Nucleoid compaction driven by water efflux [21,29,32] also protects DNA from damage under stress [32-34]. Moreover, the reduction in size may itself be beneficial due to the increase in surface area-to-volume ratio, which decreases the distance molecules have to traverse from uptake to synthesis, utilization, and expulsion as waste [21]. Elucidating the genetic and/or physical mechanisms underlying these benefits will likely provide mechanistic insight into the links between nutrient and osmotic stress responses.

The role of ion trafficking in stress adaptation

While the buildup of osmotic stress typically occurs on a short time scale (e.g., the transition of enteric bacteria from a host into a water supply), nutrient stress can increase quickly (e.g. a sudden shift to a rich or poor carbon source) or slowly (nutrient exhaustion and entry into stationary phase) (Figure 3). Adaptation to hyperosmotic stress is a two-step process initially involving quick and promiscuous import of any available ions and osmolytes to balance osmolarity and prevent loss of viability. This emergency response results in an increased concentration of ions including K^+ , which disrupts DNA-protein interactions and protein folding [1]. Ion trafficking is thus not a permanent solution, and as cells adapt to the new conditions, ions are effluxed as

190 synthesis of compatible solutes such as glycine-betaine and trehalose restores water
191 content [1]. Adaptation typically requires synthesis of proteins to generate and/or
192 import osmolytes, costing energy and time.

193
194 Nutrient stress also involves changes in ion flux (Figure 3B). Ions, particularly Na⁺ and
195 H⁺, are essential for the transport of nutrients into and waste out of the cell as part of
196 symporter and antiporter systems. In stationary phase, bacterial cells favor retention of
197 K⁺, NH₄⁺, and H⁺ [35], and accumulate the compatible solute trehalose [34]. In *E. coli*, the
198 membrane depolarizes as cells transition from early to late exponential phase [36], but
199 membrane potential is maintained during sudden starvation [11], suggesting
200 differential regulation of ion flux. Cells suddenly starved of nutrients [11] maintain ATP
201 levels for ~30 minutes, indicating that they possess the energy to adapt to dormancy
202 and to synthesize proteins for the synthesis or transport of osmolytes. The importance
203 of ion flux during both nutrient and hyperosmotic stress provides further evidence of
204 the interconnectedness between these two stresses and the tension that arises as a result.

205 206 **Overlap in transcriptional regulation between osmotic and nutrient stress responses**

207 Several lines of evidence indicate common transcriptional regulation of the osmotic and
208 nutrient stress responses in bacteria. In *E. coli*, the stationary-phase transcription factor
209 RpoS (σ^S) induces hundreds of genes upon nutrient stress, including several

osmoprotectant pathways [37]. Entry into stationary phase confers cross-protection against osmotic stress [38,39], which may be due to induction of one or more of these pathways but also could reflect the common physiological responses to these two stresses (Figure 1). Many genes under the control of RpoS are induced by hyperosmotic stress (Figure 3A), demonstrating an intrinsic link between osmoregulation and starvation. These genes include *otsBA* [40] and *treA* [41], which encode proteins that synthesize and breakdown trehalose, respectively; *proP* [40], which encodes a transporter for glycine betaine and other osmolytes; *osmY* [41], which encodes a periplasmic chaperone; and *osmE* [42-44] and *osmB* [41], which encode predicted lipoproteins. In addition to increasing cellular osmolarity, trehalose and glycine betaine also stabilize proteins [34,45], of notable benefit due to the increase in protein destabilization during both hyperosmotic stress and stationary phase. Interestingly, osmoregulated genes are highly induced by a small amount of RpoS, by contrast to the expression of canonical stationary phase genes that tend to require higher levels of the transcription factor [46].

The Rcs envelope stress response (Glossary) is also directly coupled to both nutrient and osmotic stress response pathways. The Rcs pathway is activated in response to changes in cell width and periplasmic dimensions [47], which are strongly affected by osmolarity and by nutrient stress (Figure 3) [48,49]. Rcs pathway activation also leads to

expression of *osmB* and *osmC* [50], which are regulated by both osmotic stress and stationary phase [51] and encode enzymes that reduce organic hydroperoxides. Taken together, the co-regulation of stationary phase and osmotic genes may reflect a frequent need to adapt to both osmotic and nutrient stresses concurrently.

The Gram-negative cell envelope consists of an inner membrane, a thin layer of peptidoglycan cell wall, and an asymmetric outer membrane. The outer membrane plays a key role in mediating resistance to desiccation and antibiotics [14], and its capacity to bear substantial mechanical stress due to both its protein and lipopolysaccharide (Glossary) content was recently discovered [52]. Interestingly, the outer membrane of *E. coli* does not appear to bear mechanical stress during steady-state growth, but becomes critical during osmotic shock [53]. Production of the outer membrane lipoproteins OsmB and OsmE during osmotic stress may play a role in maintaining cell envelope integrity, although the mechanism is unknown. More generally, how transcriptional responses counteract the physical effects of osmotic and nutrient stress remains to be determined.

Prospects for future interrogation of the coupling between osmotic and nutrient stress responses

Progress in understanding the osmotic and nutrient properties of the cytoplasmic environment and their regulation has thus far largely relied on inferences. It is typically assumed that, like pH, there is an optimal cytoplasmic osmolarity. On the other hand, the turgor pressure of Gram-positive bacteria such as *B. subtilis* has been inferred to be an order of magnitude larger than that of *E. coli* [54,55], indicating a huge range of potential cytoplasmic osmolarity. Without the ability to measure cytoplasmic osmolarity, questions about its magnitude and that of turgor pressure will be difficult to address. Moreover, the functions of periplasmic contents remain largely mysterious, particularly during stress and across growth phases. Notably, for most organisms, no estimates of turgor or intracellular density have been made. Recent methodological and computational advances in quantitative phase imaging (QPI) have simplified the measurement of intracellular density [56,57], which should help to clarify the relationship between osmolarity and intracellular contents. Application of QPI and direct measurements of turgor through nanoscale probing [58] or other methods would expand our understanding of the relationship between osmolarity and physiology across growth conditions. Advances in metabolomics may also provide a window into osmolyte concentrations during perturbations (osmotic and otherwise); phenotypes of interest should generally be compared across media, which may have different osmolarities and/or contain different amounts of compatible solutes (trehalose, betaines, proline).

Beyond tools, we lack general principles regarding how osmolarity affects physiology, motivating a broad comparison across organisms to uncover both general and specific behaviors. The field has mainly focused on one organism (*E. coli*), resulting in narrow understanding of a challenge faced universally across the bacterial family tree. Moreover, the lack of insight into how osmolarity homeostasis during steady-state growth is re-established long after adaptation to an initial shock means that well-studied genes may have unrecognized osmoregulatory roles in the absence of osmotic stress. It will also be fascinating to understand the intersection of osmotic and nutrient stress in microbes that inhabit environments highly distinct from the mammalian gut, from marine cyanobacteria to halophiles, to determine whether an organism's behaviors are dictated by the osmotic variation in its natural environment. Application of oscillatory osmotic shocks has revealed qualitatively distinct phenomena linking osmolarity to growth behaviors in every organism studied thus far [2,27,59,60], indicating that there is likely much to be learned simply by looking. Addressing the crucial knowledge gaps discussed in this review would greatly enhance our understanding how nutrient and osmotic stresses are mechanistically coupled, and of cellular physiology more broadly.

Glossary:

- Budding yeast (*Saccharomyces cerevisiae*): a unicellular fungus used as a model eukaryote.
- Diffusion: the random movement of particles that serves to smooth variations in concentration.
- Guanosine penta-phosphate and guanosine tetraphosphate ((p)ppGpp): an alarmone that induces the stringent response under carbon or amino acid starvation.
- Lipopolysaccharide (LPS): an anionic component of the outer leaflet of the outer membrane of Gram-negative bacteria, important for envelope stiffness and resistance to expansion by turgor.
- Osmoregulated periplasmic glucans (OPGs), formerly known as membrane derived oligosaccharides (MDOs): polymers of D-glucose produced by Proteobacteria in hypoosmotic conditions with predicted roles in envelope and osmotic homeostasis.
- Periplasm: typically used to refer to the space between the inner and outer membrane of Gram-negative bacteria, which is less protected from the extracellular environment than the cytoplasm and contains a distinct enzymatic repertoire. Note that Gram-positive bacteria can also have a periplasm-like space between the cytoplasmic membrane and cell wall.

- 307 • Physiology: processes important for cell viability such as metabolic and stress
308 response pathways.
- 309 • Rcs pathway: induces the expression of genes involved in processes such as
310 capsule synthesis in response to envelope damage.
- 311 • Sporulation: a developmental strategy for adapting to unfavorable conditions
312 through spore formation.
- 313 • Stationary phase: a state of metabolic quiescence entered as cell density increases
314 and nutrient levels decrease, characterized by cell shortening and regulation by
315 the sigma factor RpoS.
- 316 • Steady-state growth: a state with constant rate of cell expansion without shifts in
317 the environment.
- 318 • Turgor: the pressure exerted on the cell envelope as a result of higher
319 intracellular osmolarity compared with the environment.

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325

Figures

Figure 1: Nutrient and hyperosmotic stress have similar physiological effects on microbes.

- A) In the model Gram-negative bacterium *Escherichia coli*, similar features distinguish cells under steady-state growth conditions from those under nutrient stress or hyperosmotic stress: protein instability, intracellular density, DNA-protein interactions, water content, and metabolic rate.
- B) *Escherichia coli* (a model Gram-negative bacterium), *Bacillus subtilis* (a model Gram-positive bacterium), and *Saccharomyces cerevisiae* (a model unicellular eukaryote) all experience dehydration, cytoplasmic volume reduction, increased protein instability, and metabolic slowdown upon nutrient depletion. The crowded cytoplasm decreases molecular diffusion, and in bacteria this crowding increases the levels of DNA-protein interactions and nucleoid condensation.

Figure 2: Proteome redistribution among cellular compartments and membranes upon stress.

A) During exponential growth in LB, the cytoplasm of *E. coli* cells contains 73% of the total protein mass, while the periplasm contains only 6%. During stationary phase (SP) or upon osmotic (Osm) stress in minimal medium supplemented with 50 mM NaCl, the cytoplasm accounts for only 57% and 64% of the proteome, respectively, and the expanded periplasm (Figure 1) accounts for 15% and 11% of the proteome, respectively.

B) The ratio of protein fraction between the periplasm and cytoplasm is greater during stationary phase, hyperosmotic stress, and pH stress than during exponential growth in LB.

Data plotted are from [17].

Figure 3: The timelines of hyperosmotic and nutrient stress.

- A) Hyperosmotic shock involves rapid onset of stress. Water efflux causes immediate plasmolysis, and cells initially respond with a rapid influx of ions, and later with synthesis or import of compatible solutes. The genes responsible for re-establishing osmotic homeostasis at steady state are not well understood.
- B) Nutrient depletion can be sudden or gradual. In stationary phase, cells accumulate positively charged ions and trehalose in the cytoplasm and induce transcriptional changes. By contrast, cells that are starved by sudden removal of nutrients accumulate positively charged ions outside of the cytoplasm and the response does not rely on transcriptional regulation.

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