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Responses of Microorganisms to Osmotic Stress

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Abstract

The cytoplasm of bacterial cells is a highly crowded cellular compartment that possesses considerable osmotic potential. As a result, and owing to the semipermeable nature of the cytoplasmic membrane and the semielastic properties of the cell wall, osmotically driven water influx will generate turgor, a hydrostatic pressure considered critical for growth and viability. Both increases and decreases in the external osmolarity inevitably trigger water fluxes across the cytoplasmic membrane, thus impinging on the degree of cellular hydration, molecular crowding, magnitude of turgor, and cellular integrity. Here, we assess mechanisms that permit the perception of osmotic stress by bacterial cells and provide an overview of the systems that allow them to genetically and physiologically cope with this ubiquitous environmental cue. We highlight recent developments implicating the secondary messenger c-di-AMP in cellular adjustment to osmotic stress and the role of osmotic forces in the life of bacteria-assembled in biofilms.

Contents

1. INTRODUCTION

The development of the semipermeable cytoplasmic membrane was a key event in the evolution of microbial cells because it provided a confined space for copying genetic material and a reaction vessel with optimized conditions for key cellular functions (125, 142). The cytoplasm is a crowded compartment that possesses considerable osmotic potential because of the high concentrations of its various constituents (134, 144). As a result, water influx is instigated, which in turn causes the buildup of hydrostatic pressure, turgor, in essentially all walled microbial cells (17, 57, 144). However, the semipermeability of the cytoplasmic membrane makes cells vulnerable to variations in the osmolality of the environment, one of the most ubiquitous stress factors encountered by microorganisms in their habitats (33, 73, 93, 108, 124, 144, 145). The exit of water under hyperosmotic conditions triggers cytoplasmic dehydration and a drop in turgor to physiologically nonsustainable values (17, 144). Conversely, excessive water influx under hypoosmotic circumstances threatens cellular integrity as a result of the concomitant increase in turgor (12, 13, 32). Both scenarios impair the ability of the cell to perform vital biochemical tasks and therefore require precise control of water influx and efflux to adjust turgor.

Osmolality: the osmotic pressure at a particular temperature; expressed in molal units (osmoles per kilogram of solvent)

Turgor pressure: hydrostatic pressure difference balancing the osmotic pressure difference between the exterior and interior of walled microbial cells

Due to the small size of bacteria, turgor is experimentally quite difficult to determine, and with the use of different techniques, values for the magnitude of turgor in *Escherichia coli* differing by a factor of ten have been reported; values range between 30 kPa (0.3 atm) (42) and 300 kPa (3 atm) (25). The considerably higher turgor pressure measured for *Bacillus subtilis* (1.9 MPa) (19 atm) (139) is generally thought to be required to stretch the much thicker peptidoglycan sacculus of this gram-positive bacterium when the cell doubles its volume before it divides. Turgor is generally considered essential for growth (47, 109), but there is still considerable debate as to whether turgor presses the cytoplasmic membrane onto the peptidoglycan sacculus or the cytoplasm and periplasm of gram-negative bacteria are actually isosmotic, which would make the outer membrane the turgor-restraining cellular structure (25, 47).

No microorganism can actively pump water into or out of the cytoplasm to compensate for the osmotically instigated water fluxes across the cytoplasmic membrane. Hence, cellular adjustments to both hyper- and hypoosmotic stress must rely on indirect countermeasures that allow the cell

to direct and scale water influxes or effluxes as the environmental osmolality fluctuates (17, 33, 73, 142, 145). Here, we address those biochemical and cellular events that allow the bacterial cell to perceive osmotic stress and to cope with its negative consequences. We focus on those microorganisms that attain in their core osmotic adjustment through the accumulation and expulsion of physiologically compatible organic osmolytes (17, 33, 73, 142). We refer readers to reviews on those bacteria and archaea that achieve osmotic adjustment to high-saline environments by the massive accumulation of ions (K^+/Cl^-) (57) or by using a hybrid strategy involving the accumulation of both ions and organic osmolytes (116). We point readers to other excellent reviews dealing with various aspects of osmotic stress and cells' adaptive responses among bacteria, archaea and fungi (12, 18, 32, 57, 67, 93, 102, 108).

2. PHYSIOLOGICAL AND BIOPHYSICAL BACKGROUNDS

2.1. Biophysics of Osmostress

Numerous reviews and articles are available in which the (bio)physical background of osmostress and its impact on macromolecules and cellular structures are expertly described (10, 104, 142, 144). Consequently, only a brief outline is given here. The osmotic pressure Π of an aqueous solution is given by $\Pi = -(RT/V_w) \cdot \ln\alpha_w$, where R is the gas constant, T the temperature, V_w the partial molar volume of water, and α_w the water activity. The osmolality of a solution is then Π/RT, which is equal to $-V_w \ln\alpha_w$. Osmolality, the osmotic pressure of a given solution, has the dimension of osmoles per kilogram of water (osmolal), whereas the unit of osmolarity, which refers to moles of solute per liter of water, is osmoles per liter of water (osmolar). Since osmolality, in contrast to osmolarity, can experimentally be measured using osmometers, we prefer the former expression here, which has been used in previous articles as well (142, 144).

In the basic equations, the activity and not the concentration of the solvent involved, water, the predominant cellular constituent, is relevant. This makes a significant difference, since the bacterial cytoplasm is far from being an ideal (dilute) solution but is instead a very crowded environment (134, 142, 144, 152). Thus, under native conditions, solute activities (product of solute concentration and activity coefficient of solute) are also far from the value of solute concentration. The osmotic conditions of the cytoplasm affect cellular macromolecules (e.g., proteins) (126), metabolic networks (123), the interaction of RNA polymerase with promoters (20, 54), and other key cellular processes (e.g., by reducing the initiation volume for DNA replication and by slowing translation elongation) (38, 39). Because proteins have charged surfaces, both conformation and function are influenced by interactions with surrounding water and solutes, respectively, processes depending on their activities α (10, 104). Another relevant aspect related to protein-solute interactions is macromolecular crowding. This term refers to the effect of the concentration of macromolecules, e.g., proteins, on other cellular functions, e.g., enzyme activities, based on the space they occupy in a compartment, e.g., the cytoplasm (134, 144). There is increasing evidence that a change in macromolecular crowding in the cytoplasm upon hyperosmotic challenge may have significant consequences for cellular functions (104, 112, 134, 152).

2.2. Physiology of Osmostress and Osmoregulation in Bacteria

A crucial aspect of microbial adjustment to fluctuating environmental osmolalities is the ability of the cell to scale water fluxes and direct them across the semipermeable cytoplasmic membrane to prevent dehydration (at high external osmolality) and rupture (at low external osmolality). Synthesis, import, and release of compatible solutes assume center stage in these processes.

Osmotic pressure: hydrostatic pressure arising in an aqueous solution surrounded by semielastic walls and separated from pure water by a semipermeable membrane

Water activity (α_w): partial vapor pressure of water in a substance divided by partial vapor pressure of pure water at the same temperature; pure water has an α_w of 1, and water migrates from areas of high α_w to areas of low α_w

Osmolarity: the osmotic pressure at a particular temperature; expressed in molar units (osmoles per liter of solution)

Macromolecular crowding: Impact of high concentrations of macromolecules, e.g., proteins, in a solution on properties of functional elements of the cell

Osmoregulation: physiological processes that alleviate changes in cell structure(s) and physiological processes caused by changes in extracellular osmotic pressure

Compatible solute: an organic osmolyte that can attain exceedingly high cytoplasmic concentrations while being fully congruent with cellular biochemistry and physiology

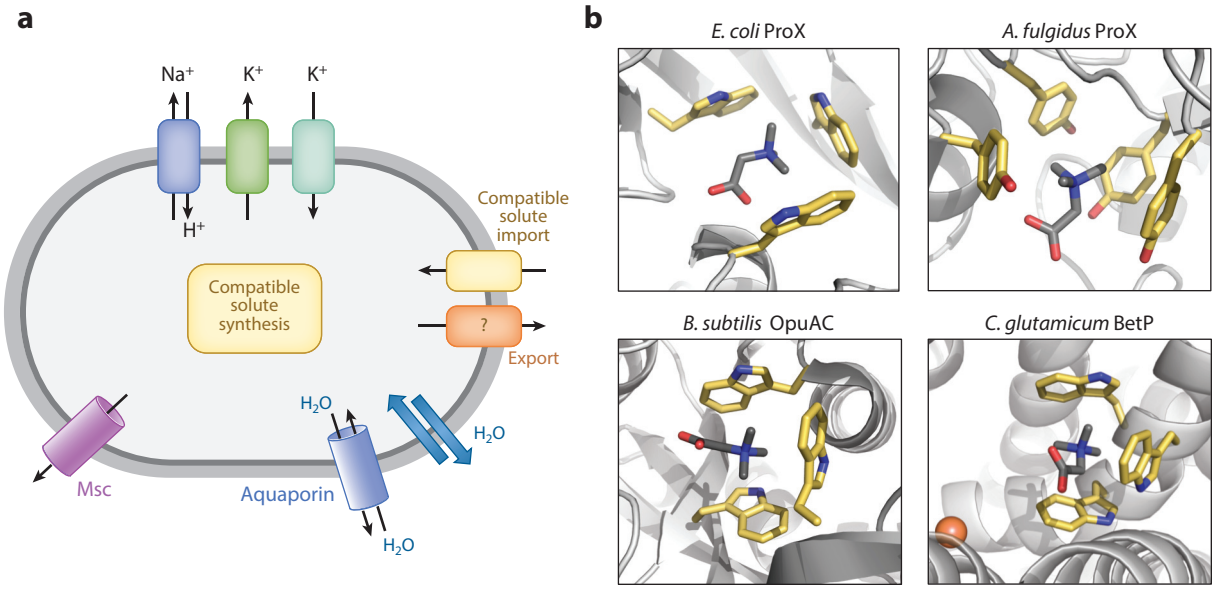

Figure 1

General scheme of osmostress response systems and structural views into glycine-betaine-binding sites present in transport systems for compatible solutes. (*a*) Bacteria using the salt-out osmostress response accumulate compatible solutes via either synthesis or import, and they tightly control their intracellular K^+ and Na^+ pools (33, 73, 142). Under hypoosmotic conditions, the cells rapidly jettison, in a nonspecific fashion, organic and inorganic compounds via mechanosensitive channels (Msc) to reduce the osmotic potential of the cytoplasm to curb water influx (12, 32). An aquaporin is a water-conduction channel (e.g., AqpZ). (*b*) Architectures of glycine-betaine-binding sites in extracellular substrate-binding proteins [*Archaeoglobus fulgidus* ProX (PDB accession code 1SW2), *Escherichia coli* ProX (PDB accession code 1R9L), *Bacillus subtilis* OpuAC (PDB accession code 2B4L)] operating in conjunction with ABC transporters, or which are present in the membrane-embedded BetP transporter from *Corynebacterium glutamicum* (PDB accession code 4AIN). The main chain of the indicated proteins is shown in gray. The side chains of amino acids relevant for binding of the substrate(s) are shown as colored rods, as is the glycine betaine ligand (oxygen is red, nitrogen is blue, and carbon is yellow or dark gray). In the substrate-binding site of BetP, the carboxylate of glycine betaine interacts with a Na^+ ion (*orange sphere*).

2.2.1. Aquaporins and transmembrane water fluxes. The discovery of aquaporins (AqpZ) in *Escherichia coli* (22) by the Nobel laureate Peter Agre and his coworkers raised the expectation that these types of dedicated water channels might be an important component of the cells' management of osmotic stress, as they can mediate accelerated water fluxes along osmotic gradients (**Figure 1*a***). However, a physiological function of AqpZ during osmotic up- and downshifts is by and large unclear, and its absence in many bacteria makes a central role of AqpZ-type aquaporins in osmotic adjustment unlikely (62, 132) [see, however, data on *Synechocystis* sp. PCC 6803 (1) and *E. coli* (40) that could support such a function]. Apparently, the water permeability of the cytoplasmic membrane is generally high enough (9, 147) that the cell can react in a timely way to fluctuations in environmental osmolality by adjusting the osmotic potential of the cytoplasm to direct water fluxes (12, 13, 17, 32, 144). These adjustment processes occur primarily through the amassing of a selected group of physiologically compliant organic osmolytes, the compatible solutes, when the environmental osmolality is raised (17, 33, 73, 144). Conversely, rapid expulsion of these compounds, ions, and metabolites occurs through the transient opening of mechanosensitive channels when the external osmolality is suddenly reduced (12, 13, 32).

2.2.2. Synthesis and import of compatible solutes. Those bacteria that employ the salt-out adjustment response will not increase their cellular ion pool on a sustained basis to counteract the negative effects of high external osmolality on cellular hydration (17, 33, 73, 144, 145). While they temporarily increase their K^+ content as an emergency stress reaction to limit water efflux (14, 44, 138), these bacteria prefer the accumulation of compatible solutes as a sustained stress response. The amassing of compatible solutes, a process that can be attained through synthesis and uptake (17, 73, 145), allows the reduction of the ionic strength of the cytoplasm through export of K^+ (**Figure 1*a***) without compromising the osmotic potential of the cytoplasm; hence, physiologically appropriate levels of cellular hydration and turgor can be maintained.

Ionic strength: a measure of the total ion content of a dilute solution

Halotolerant: able to tolerate high salinity

Preferential exclusion: the thermodynamically unfavorable exclusion of a solute from the immediate hydration shell of a protein

Compatible solutes are operationally defined as highly water-soluble organic osmolytes whose physicochemical properties make them physiologically compliant with cellular biochemistry (10, 26). Representative types of compatible solutes synthesized or imported by members of the Eubacteria are proline, glycine betaine, carnitine, proline betaine, dimethylsulfoniopropionate, ectoine/hydroxyectoine, trehalose, and glucosylglycerol (33, 37, 73, 93, 108, 145). The accumulation of compatible solutes, although they are energetically costly to produce (90), provides a flexible means for the cell to adjust to a broad range of environmental salinities and osmolalities (17, 57, 108, 145).

Both the synthesis and the import of compatible solutes are typically subject to osmoregulation (17, 73, 142, 145). Characteristically, expression levels of genes for compatible solute synthesis are tuned to the degree of osmotic stress imposed on the cell (17, 33, 73, 142, 145). However, despite decades of research, the biophysical and genetic sequence of events that leads to osmoregulated transcriptional regulation is still insufficiently understood. An interesting exception might be BusR, a repressor protein for the genes encoding an ABC-import system (BusAB/OpuA) for glycine betaine in *Lactococcus lactis*, as its DNA-binding activity is dictated by the ionic strength of the cytoplasm (111). A mechanistically interesting example for the activity control of a compatible solute biosynthesis route is that for glucosylglycerol (93). The glucosylglycerol synthase from the halotolerant cyanobacterium *Synechocystis* sp. PCC 6803 is regulated by the reversible binding (low cytoplasmic Na^+) and detachment (high Na^+) to and from the chromosomal DNA, thereby switching between the inactive (bound) and active (free) forms of the enzyme (89). The alternative sigma factor RpoS, the master regulator of the general stress response network of *E. coli*, plays an important role for the transcriptional regulation of many osmostress-responsive genes (61), including those encoding the biosynthetic enzymes for trehalose, the dominant compatible solute produced de novo by *E. coli*.

Acquisition of compatible solutes from environmental sources is particularly useful, as it allows the cells to preserve precious energetic and biosynthetic resources. For example, synthesis of a single ectoine molecule requires the expenditure of about 40 high-energy bonds when cells grow as heterotrophs with glucose as the carbon source, and the expenditure of even about 50 high-energy bonds is needed when they grow as autotrophs with CO_2 as their carbon source (90). In contrast, the hydrolysis of just two ATP molecules is required to import an ectoine molecule via an ABC transport system (80). Both principles of compatible solute accumulation (osmoregulated activity control and osmoregulation of transcription) are particularly important for various types of compatible solute importers. These two levels of stress control provide the osmotically upshocked cell with practically instantaneous increased transport capacity for osmostress-relieving compounds through the activation of preexisting transporters and the subsequent, slower, de novo synthesis of additional copies of these import systems (17, 102, 142, 143, 153).

2.2.3. Compatible solutes: preferential exclusion and high-affinity binding. A hallmark of compatible solutes is their preferential exclusion from the surface, which in the case of proteins,

is primarily mediated by unfavorable interactions with the protein backbone (10, 24, 126, 129, 149). For thermodynamic reasons, this acts against the unfolded state and leads to a preferential hydration of the protein surface and the preservation of the native conformation (10, 24, 26, 126). However, high-affinity and specific interactions between compatible solutes and transporters have to take place when the cell scavenges these compounds from scarce environmental sources. How is this accomplished?

The crystallographic analysis of extracellular high-affinity substrate-binding proteins from ABC transporters for osmostress protectants provided an answer to this question. The first view into the architecture of the ligand-binding site for a glycine-betaine-binding protein was attained by the crystal structure of the periplasmic ligand-binding protein (ProX) from the ProU transporter from *E. coli* (117). Three Trp residues form an aromatic cage in which the negatively charged surface of these side chains partner via cation-π interactions with the positively charged trimethylammonium head group of glycine betaine (**Figure 1*b***). The carboxylate of the ligand protrudes out of the aromatic cage and is stabilized within the binding site through additional contacts (117). Similarly configured glycine-betaine-binding sites, and those for its biosynthetic precursor choline, were found in substrate-binding proteins from the hyperthermophilic archaeon *Archaeoglobus fulgidus* (118) and those from the gram-positive bacterium *Bacillus subtilis* (45, 68, 100) and other microorganisms (91). Strikingly, a substrate-binding site with an architecture similar to that of the OpuAC substrate-binding protein from *B. subtilis* is also present in the transmembrane segment of the Na^+-coupled BCCT-type (153) glycine transporter BetP from *Corynebacterium glutamicum* (95, 96) (**Figure 1*b***). In the case of glycine betaine, the interaction of the positively charged trimethylammonium head group with aromatic hydrocarbons (24) enables high-affinity ligand binding via cation-π interactions. Hence, building upon the physicochemical parameters of compatible solutes (10, 126, 129), nature has found a common solution for how these compounds can be specifically recognized. Minor variations on this theme allow the design of ligand-binding sites for compatible solutes that will yield transporters with either narrow or broad substrate specificity (45, 64, 100, 133).

2.2.4. Expulsion and excretion of compatible solutes. When bacteria are faced with a sudden osmotic downshock, they must react instantaneously (19), as turgor will increase within milliseconds (12, 13) to a degree that cannot be restrained by the cell wall (79). This emergency reaction to a sudden drop in the environmental osmolality relies on the transient opening of different types of mechanosensitive channels (13, 32). Microorganisms typically harbor several types of these channels (MscM, MscS, MscL) (46), and these possess different electrophysiological properties and gating behavior and, in their open forms, different diameters. These attributes of mechanosensitive channels permit a graded response of the cell to the severity of the osmotic downshift (12, 13, 32). MscL- and MscS-type channels typically do not possess substrate specificity, but the unusual MscS-type channel MscCG from *C. glutamicum* also specifically mediates the efflux of glutamate under particular conditions in the absence of an osmotic downshock (8, 32).

Microorganisms often possess transporters for the same type of compatible solutes that they synthesize. When these are genetically disrupted, considerable quantities of newly synthesized compatible solutes can be found in the growth medium (55, 65), indicating that these osmoregulated transporters also function as recycling systems for newly synthesized osmostress protectants that are either leaking or actively extruded from the cell. In *C. glutamicum*, glycine betaine that had been imported via BetP under sustained high-osmolality growth conditions is partially released again by the MscCG channel (the so-called pump-and-leak model) (15). In contrast, hydroxyectoine secretion/efflux was observed in a synthetic *E. coli* cell factory lacking all known MscL-, MscS-, and MscM-type channels (36), and in another study ectoine was released/secreted from

an *E. coli* cell factory independent of the ProU and ProP transporters that can mediate its uptake (35). These findings, and data reported on the release of various compatible solutes in different bacteria (55, 63, 84), raise the question of whether this synthesis/import-release-recapture process under steady-state high-osmolality growth conditions is simply an accident or serves a useful physiological function (e.g., for the fine-tuning of turgor). In addition to mechanosensitive channels (15), extrusion systems for compatible solutes could perhaps involve multidrug-resistance-type exporters with a broad substrate profile [e.g., EmrE (7)] or substrate-restricted efflux systems (53) similar to those used for the export of amino acids (81) and other types of compounds from microbial cells.

3. OSMOSTRESS-RESPONSIVE CELLULAR SYSTEMS

3.1. Basic Requirements for Systems Responding to Osmostress

Osmoresponsive systems compensate for the adverse effects of changing osmolalities on cellular fitness (142). Mechanistic requirements to achieve this aim are as follows:

1. The cell must be capable of sensing osmostress in both quality (nature) and quantity (extent). This is reflected by a linear correlation between extent of stress and extent of related cellular response, e.g., concentration of accumulated compatible solutes (66).
2. The cell must be able to switch stress response systems according to the actual need. Besides varying in type, osmostress may also differ in onset (fast/slow) and duration (transient/persistent) (17, 144).
3. Osmoresponsive systems are optimized with respect to carbon and energy demand. Stress response is costly; cells will thus optimize both its efficiency and economy. Cells will choose whether a response system is constitutive or inducible, i.e., whether response will be at the level of activity or synthesis. Accordingly, as a frequent decision, uptake of compatible solutes is preferred over de novo synthesis (17, 73, 145), thereby saving both carbon and energy (90).

3.2. Sensing, Signal Transduction, and Cellular Responses

For a basic understanding of microbial responses to osmotic stress on a mechanistic level, the complex scenario will be dissected into different aspects, e.g., the nature of relevant external stimuli and the way they are sensed, the conversion of stimuli into cellular signals, and, finally, the generation of useful functional responses in the microbial cell.

3.2.1. Sequence of events. For proper classification and as a basis for mechanistic understanding, the response to osmotic stress can be discriminated into consecutive steps (**Figure 2**).

1. Change in external and internal osmolality. The former is the primary event and occurs in the environment, whereas the latter is an inevitable consequence of the high water permeability of membranes (9, 147). This causes instant water influx in hypoosmotic conditions and water efflux in hyperosmotic conditions and occurs in a time range of milliseconds (142). Transmembrane water flux may depend on membrane composition; however, there are no systematic studies on this topic available for bacteria.
2. Generation of a functionally relevant physical stimulus. A change in external osmolality, i.e., a change in the concentration of solute molecules, is per se not a relevant physical stimulus; at least it is not used by the cell. Consequently, the cell's ability to properly react will depend

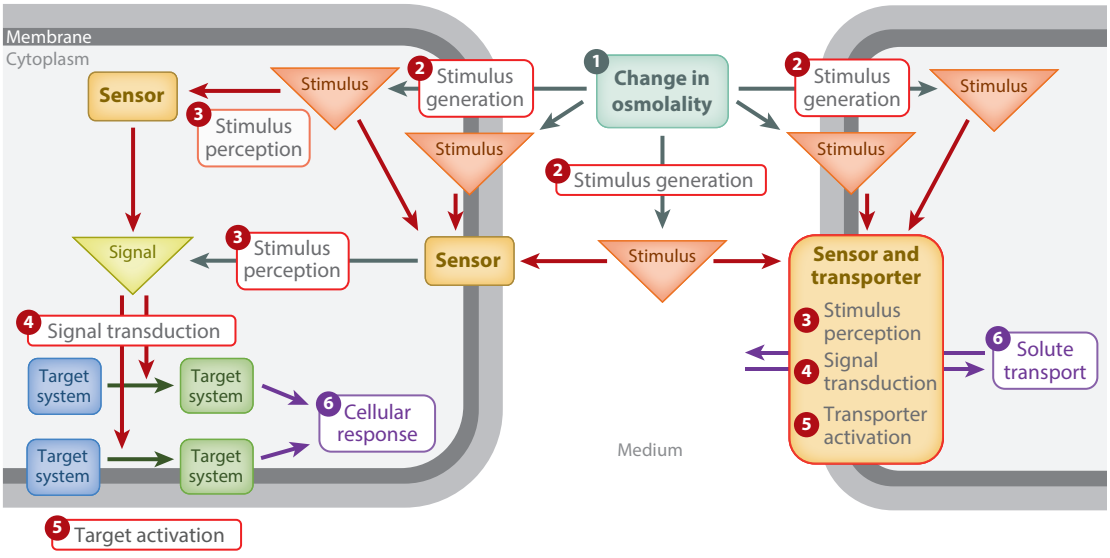

Figure 2

Stimulus perception and signal transduction in bacterial osmostress response. The figure discriminates between a full-scale signal transduction cascade (*left*) and a shortcut version for carriers and channels (*right*). The primary event, a change in external osmolality (❶), generates a physical stimulus in the medium, the cytoplasm, or the membrane (❷). This stimulus is perceived by a sensor (❸) and converted into a useful signal (❹), which in turn is transduced to specific targets, leading to their activation (❺). This ultimately results in an appropriate response of the bacterial cell (❻) to the basic change in osmolality.

on the availability of a physical stimulus that the cell is able to perceive by a sensor and that closely corresponds to the change in external osmolality.

3. Stimulus perception by a sensor system. A physical stimulus becomes relevant for the cell by the presence of an appropriate sensor system located either in the membrane or in the cytoplasm. The consequence of stimulus perception is a structure/function change in the respective sensor protein. Although various types of sensor systems can be recognized, the mechanisms of stimulus perception by them are still unknown in most cases.
4. Generation of an appropriate cellular signal and signal transduction. Only if converted into a mechanistically relevant signal is the cell able to make use of the physical stimulus perceived by the sensor. Typical examples for such kinds of signals are conformational or structural changes of signal-transducing components, e.g., protein phosphorylation and/or a conformational switch of these components. The cellular signal is then transduced to an appropriate target system (signal transduction).
5. Structure/function change in the target system. As a consequence of signal transduction, the target system changes its functional state, e.g., by switching its enzymatic activity or its affinity for binding to further components of the cell, such as specific DNA motifs or target proteins.
6. Cellular responses. Functional alterations of the target system will finally cause appropriate cellular responses to the primary osmotic event, e.g., gene expression, metabolite synthesis, protein synthesis, or activation of an enzyme or a transport protein. The diversity of typical cellular responses has explicitly been discussed in several review articles (17, 64, 142, 144).

3.2.2. Examples of signal-transduction systems. In transporters and channel proteins not all these steps are realized, and the signal pathway is cut short. These membrane proteins integrate three steps, namely, stimulus perception, signal transduction, and target (carrier or channel) activation (**Figure 2**).

The most elaborate examples of extended cascades of events are bacterial two-component systems (TCSs) involved in osmostress response. TCSs are often transmembrane signaling systems receiving an external physical stimulus and transducing this information into an intracellular signal (127, 154). They consist of a membrane-bound sensor histidine kinase and a corresponding response regulator (transducer). Stimulus input into TCSs involved in osmoregulation may also occur from the cytoplasmic side.

A well-studied osmoresponsive TCS is EnvZ/OmpR of gram-negative bacteria (49). EnvZ is a sensor histidine kinase anchored in the plasma membrane. At elevated osmolality, EnvZ becomes autophosphorylated, resulting in an increased phosphorylation of the response regulator OmpR, which in turn activates transcription of the genes encoding the outer membrane porins OmpF and OmpC. The nature of the stimulus is not known. The fact that stimulus input into EnvZ happens at its cytoplasmic domain indicates that it relates to the cytoplasmic concentration of a relevant solute. Another example is the MtrAB system, a highly conserved stress-responsive TCS from *Actinobacteria*, e.g., *Mycobacterium* and *Corynebacterium* (16, 94). The involvement of the membrane-bound sensor kinase MtrB from *C. glutamicum* in osmoregulation was analyzed in proteoliposomes. Stimulus input was located at its cytoplasmic domain, and activation was linked to changes in the hydration state of this domain in response to cytoplasmic solute concentration (86, 87). Recently, PhoQ/PhoP, a TCS playing important roles in the response of enterobacteria to various stimuli of their mammalian hosts, was found to also perceive osmotic upshifts. Sensory input into the sensor kinase PhoQ relied on conformational changes in the transmembrane domains of PhoQ prompted by perturbation in membrane thickness and lateral pressure in the cytoplasmic membrane (148).

KdpDE, a TCS from *E. coli* with special features, is associated with the KdpFABC K^+ transport ATPase, which is critically involved in K^+ uptake and thus indirectly related to osmoregulation. Sensing and signal transduction by the sensor kinase KdpD have been studied intensively for a long time (2), and only recently has a sophisticated mechanism involving both extracellular and intracellular sensor elements for K^+ in KdpD been unraveled, showing a dual-sensor mechanism for effective K^+ homeostasis in *E. coli* (120).

3.2.3. Nature of relevant physical stimuli and sensor principles. Cells need useful physical stimuli as a proxy for the change in external osmolality, which is not directly detectable, because of the lack of appropriate sensor mechanisms. Thus, the question is what kind of stimuli bacterial cells recognize when exposed to osmotic stress. Plausible candidates are the concentration of specific internal indicator solutes, e.g., monovalent cations; cytoplasmic water activity via its impact on protein conformation; macromolecular crowding; alteration of forces in the cell membrane (lateral pressure); and alteration of membrane surface properties (surface charge density). The availability of a suitable sensing principle will define what kind of stimulus is relevant for an individual cell.

Understanding of stimulus perception is rather advanced for mechanosensitive channels (sensing of lateral membrane forces), for a couple of solute transporters (changes in internal solutes and lipid behavior), and for some TCSs (changes in internal conditions). In addition, experimental evidence has been provided for molecular crowding being a primary stimulus for osmoregulation (112, 134, 152).

3.2.4. Significance of lipid environment for osmosensing and osmoregulation. A number of osmoresponsive elements in bacteria are embedded in the cell envelope; the surrounding lipids

are thus an obvious candidate for stimulus input and signal generation. Lipids may contribute at different levels, via bulk phase or via direct (specific) interaction (78). Although the specific lipid requirement is known for several membrane proteins, mainly bulk-phase effects seem to be relevant for osmoresponsive elements. The paradigmatic examples are mechanosensitive channel (Msc) proteins. Notably, membrane lipid interactions have been analyzed in detail for Piezo1, a structurally unrelated eukaryotic mechanosensitive channel (32, 60).

Recently, also for bacterial membranes, segregation of lipid domains has come into focus (128), although there is no proven example for this phenomenon being relevant for osmoregulated membrane proteins. The observation, however, that ProP from *E. coli* is concentrated into membrane areas with a higher content of cardiolipin, resulting in spatial segregation to the cell poles, may be associated with this concept (110).

Lipid binding is relevant for several osmoregulated transporters. Bound lipid molecules have been identified for members of the BCCT family of carriers (75, 153). For BetP, the best-studied member, it was found that regulation critically depends on lipid composition (92) and, in particular, that the physical state of the surrounding lipid phase determines the response of BetP to hyperosmotic stress (83). Moreover, direct phospholipid-protein interaction was identified by spectroscopic techniques (56).

3.3. Mechanosensitive Channels

Bacterial mechanosensitive channels are pore-forming proteins in the plasma membrane that gate (open) in response to mechanical force originating from transmembrane osmotic stress. In case of emergency, i.e., upon hypoosmotic stress, gating occurs on a millisecond time scale (32). There are two major families of mechanosensitive channels, namely, MscL-like [L for large conductance (131)] and MscS-like [S for small conductance (79)] channels. They differ in conductance and in gating threshold, i.e., the mechanical force at which they open. For the sake of a graded response, bacterial cells in general harbor multiple mechanosensitive channels. For example, *E. coli* possesses one MscL and six MscS-like channels (46).

The structures of several mechanosensitive channels have been solved by X-ray crystallography, e.g., MscL from *Mycobacterium tuberculosis* (27) and MscS from *E. coli* (6). MscL is a homopentamer, while MscS forms homoheptameric channels. The conformational events upon gating have been modeled on the basis of available structures, and gating mechanisms have been proposed for both channel types (32, 97, 130, 137).

Besides being fundamental examples of mechanosensation, mechanosensitive channels are also paradigms for understanding stimulus input and protein–lipid bilayer interaction. It is widely accepted that gating of these channels is achieved by the so-called force-from-lipids mechanism (32, 77). This concept assumes that mechanical force (lateral pressure) is directly transferred to the channel via the lipid bilayer (23). Experimental evidence argues for the validity of this concept, e.g., the fact that reconstituted mechanosensitive channels retain their mechanosensitivity (41), as well as the impact of membrane bending (82), phospholipid composition (85), and acyl chain length (97). Although this concept is based on bulk-phase lipid interaction, earlier observations also suggested specific interactions (103), and a recent study indicated a significant role. Based on a higher-resolution structure of *E. coli* MscS, lipid-binding pockets in the transmembrane region of the channel were identified (101). Depending on the conformational state of MscS, which changes during the gating event, alternatively phospholipid and lysolipid molecules were shown to bind to these pockets.

In spite of broad knowledge of the function of mechanosensitive channels, their structure, and conformational events during gating, the details of stimulus perception, the exact mechanisms of gating, and coupling between these events are still largely unknown.

3.4. Osmoresponsive Transport Systems

Like mechanosensitive channels, osmoregulated transport proteins are characterized by common features; some of them are based on their nature as carriers (e.g., substrate specificity, saturation kinetics, solute flux slower than channels), and some are related to their capacity of being osmoregulated (e.g., fast response to osmotic challenges, ability to adapt their activity to the extent of stress, and integration of the properties of osmosensor, transducer, and transporter) (17, 153).

Osmosensor: a protein monitoring changes in water activity or derived alterations in cells, membranes, or the composition of the cytoplasm

Osmoresponsive transport proteins are regulated at the level of activity and potentially also synthesis. In mechanistic terms activity regulation is achieved by modulating intrinsic properties of the carrier, e.g., substrate affinity, transport capacity (V_{max}), or efficiency of coupling to the driving force. As far as what has been studied, regulation at the V_{max} level, i.e., modulating the activation energy barrier of transport (50), seems to be the common mechanism.

Osmoregulated compatible solute carriers are found in various transporter classes, namely, primary transporters (ABC systems), secondary systems, and binding-protein-dependent secondary systems (TRAP transporters) (17, 55, 64, 142–145, 153). In several organisms, the full setup of osmoregulated transporters has been described, e.g., *E. coli*, *B. subtilis*, and *C. glutamicum* (64, 73, 98, 143). In a few cases, detailed analyses concerning the activation mechanism have been carried out, resulting in the identification of common principles, e.g., direction of stimulus input from the cytoplasm. This mainly refers to the two secondary transporters BetP from *C. glutamicum* (Na^+ coupled) and ProP from *E. coli* (H^+ coupled), and to the ABC transporter OpuA from *Lactococcus lactis* (102). The BCCT transporter BetP stands out because several high-resolution structures are available (96, 106), a mechanistic view of the catalytic cycle was unraveled (95), and interactions with specific lipids were identified (75, 92, 119). For BetP, activation depends on both a membrane trigger and a change in the cytoplasmic K^+ concentration (83, 114), whereas OpuA from *L. lactis* is stimulated by changes in the internal ionic strength (102, 135). Recent work on the MFS-type transporter ProP points to cellular dehydration, which alters membrane properties, as being responsible for osmotic activation (34, 102).

For a full regulation cycle of BetP, mechanistic analysis revealed the requirement of two opposing principles for both activation and deactivation (upon adaptation to stress), taking into account that the situation is not the same before onset of stress and after adaptation to it (15). For deactivation, two different mechanisms have been suggested, coordinated cooperation with mechanosensitive channels and ceasing of the specific (membrane) trigger (15, 83).

Similar to the case of mechanosensitive channels, the question arises as to what extent available structural information provides insight into a mechanistic understanding of regulation. This is independent of the structure of solute-binding sites and of structural aspects of carrier catalysis (95, 96, 106). Although structural elements involved in osmoregulation, e.g., particular domains of BCCT proteins, of ProP, and of OpuA, have been identified (34, 72, 153), the static information obtained from X-ray studies is not sufficient to unravel the process of osmotic activation. Application of dynamic techniques, e.g., single-molecule Förster resonance energy transfer (FRET) spectroscopy or high-speed atomic force microscopy (AFM), will provide more information, as shown for the (not regulated) bacterial glutamate transporter GltPH (107, 113).

4. OSMOREGULATION AT COMPLEX CELLULAR LEVELS

4.1. Impact of Osmotic Stress on Cellular Ion Homeostasis

Transmembrane ion fluxes are an integral part of bacterial osmoregulation. Bacteria have developed elaborate mechanisms of ion homeostasis, mainly concerning the cations Na^+, K^+, and H^+, while anion homeostasis is less well studied. Although the steady-state H^+ concentration in the

cell is orders of magnitude lower than that of other monovalent cations, and the buffer capacity of the cytoplasm is rather low (11), fluctuation of cytoplasmic pH upon osmotic stress is effectively balanced by high-capacity proton flux mediated by the electron transport chain, and by effective Na^+/H^+ antiport systems (76) (**Figure 1*a***).

Na^+ is accumulated either by passive influx in response to high external NaCl concentrations or by active Na^+ cotransport with certain types of compatible solute uptake. A similar pattern might occur for H^+-coupled uptake systems, leading to an increased influx of protons. Prominent examples of osmoregulatory transporters are BetP from *C. glutamicum* for the former case and ProP from *E. coli* for the latter. The cell actively keeps the cytoplasmic concentration of the cytotoxic Na^+ ion at a low level, mainly through the activity of Na^+/H^+ antiporters (**Figure 1*a***). Bacteria in general harbor a variety of these transporters with different structural complexity, mechanisms, and regulation (70).

The connection of K^+ fluxes to cell turgor and osmoregulation is even more complex. Many bacteria, in particular gram-positive species, maintain surprisingly high internal K^+ concentrations (up to several 100 mM) under standard growth conditions, in contrast to their Na^+ concentrations (14, 44, 138). The rapid imposition of high osmolality leads to increased K^+ uptake in many bacteria to counteract the outflow of water, but the K^+ pool is typically only transiently increased, as the cells extrude this cation again through dedicated export systems (44, 51, 138) (**Figure 1*a***).

It is relevant to emphasize in the context of cellular ion homeostasis that there are important differences between salt stress and osmotic stress. Salt stress includes osmostress, but the converse is not necessarily true. Many bacteria can cope with both types of stress; however, cases exist where cells discriminate between the two, a well-studied example being *Synechocystis* sp. (89). Adapted to live in salt water, this cyanobacterium efficiently counteracts salt stress. Osmostress upon increase of a neutral solute, however, causes cell death, a phenomenon with interesting consequences for the mechanism of osmoregulation.

4.2. Osmotic Stress and Biology of the Second Messenger c-di-AMP

The second messenger c-di-AMP (29, 31) has gained considerable attention in connection with K^+ homeostasis and osmotic stress responses (28). c-di-AMP is essential in many bacteria, and both its synthetic depletion and overproduction are detrimental to growth and cellular integrity. Notably, such strains often become osmotically sensitive (28, 99, 140). Mutants that lack c-di-AMP entirely can generally only be obtained under special growth conditions (e.g., in media with low K^+) (58), or through the selection of suppressor mutations in strains with physiologically imbalanced c-di-AMP pools. These suppressors often affect K^+ and ion homeostasis and the uptake of compatible solutes (43, 58, 99, 140, 150, 151).

As an example, *B. subtilis* possesses three transport systems for K^+ (KtrAB, KtrCD, KimA) (58), and each of them is negatively affected by increases in the c-di-AMP pool. Transcription of *ktrAB* and *kimA* is controlled by the YdaO c-di-AMP-sensing riboswitch (88), which upon binding two c-di-AMP molecules triggers premature termination of transcription (52, 88, 105). c-di-AMP also negatively affects the transport activity of the KtrAB and KtrCD systems by binding to the RCK_C domain (RCK for regulator of conductance of K^+) present in their cytoplasmic KtrA/KtrC regulatory component (30, 74), and the KimA K^+ importer has recently also been shown to bind, in a yet to be discovered manner, this signaling molecule to inhibit KimA transporter activity (59). Gundlach et al. (59) also detected c-di-AMP binding to various K^+ export systems in *B. subtilis* and speculated that the activity of these exporters might be activated by c-di-AMP. In this way, c-di-AMP would be a key player in setting homeostasis of K^+ (59), an ion that plays in many cases a crucial role in bacterial osmoregulation.

Notably, a *B. subtilis* strain lacking the ability to synthesize c-di-AMP can only grow in media with high K^+ concentrations when suppressor mutations are present that increase the activity of the cation exporter NhaK (58). Mutations that inactivate K^+ importers are often found among the suppressors that allow growth of bacterial strains with imbalanced c-di-AMP pools (99, 150). The gating component of Ktr-related K^+ transporters from *Streptococcus agalactiae* (43) and *Streptococcus pneumoniae* (5, 150) is also a direct target of c-di-AMP, as is the KdpD sensor kinase of the KdpDE TCS (120) that regulates the expression of the Kdp K^+ importer in *Staphylococcus aureus* (30).

c-di-AMP impacts compatible solute import either by influencing gene expression or by affecting the activity of the encoded transporters. It inhibits the activity of the OpuC carnitine transporter in *S. aureus* and *Listeria monocytogenes* by binding to paired cystathionine-β-synthase (CBS) domains present in the ATPases (OpuCA) of these ABC systems (69, 121). Notably, the CBS domain present in the BusR repressor controlling the expression of the genes for the glycine betaine ABC-type transporters BusAA-BusAB (OpuA) in *L. lactis* and *S. agalactiae* is also the target for c-di-AMP (43, 99).

Judging from the known targets of c-di-AMP in various bacteria (29, 31), and the types of suppressor mutants obtained from strains with a physiologically imbalanced c-di-AMP content (43, 58, 99, 150, 151), the control of cellular K^+ pools, and thus indirectly turgor, seems to lie at the core of the crucial function of c-di-AMP (28, 58, 59). However, its role as an allosteric effector molecule for controlling compatible solute transporters (69, 121) is far from being resolved.

4.3. Osmotic Stress and Biofilm Formation

Biofilms are a major lifestyle of microorganisms, as these single- or multiple-species assemblies protect the cells from predation and various environmental insults (48). In such communities, cells are embedded in a matrix consisting of extracellular polymeric substances (EPSs), often composed of exopolysaccharides and fibril proteins and sometimes also of DNA (48, 63). Complex genetic and physiological regulatory circuits determine biofilm formation, communication between cells, cellular differentiation into various cell types, cooperation and competition between cells, and nutrient acquisition. The genetics of biofilm formation has been particularly well studied in *B. subtilis*, which can form complex structured biofilms on surfaces (**Figure 3*a*,*b***) and pellicles at liquid-air interfaces (21, 71, 136). The surface of the biofilm is covered with the redox-sensitive hydrophobin BslA (3), which provides it with water-repellent, Teflon-like properties (4) (**Figure 3*c***). Water fluxes into the biofilm, and along with it nutrients, driven by osmotic gradients established by the considerable osmotic pressure exerted by copious amounts of the EPS matrix (21). A pioneering study by Seminara et al. (122) revealed that spreading of the biofilm on solid surfaces is crucially dependent on the osmotic pressure generated by the EPS. Such a phenomenon is not specific to biofilms developed by *B. subtilis*; it also drives the expansion of biofilms formed by the gram-negative bacterium *Vibrio cholerae* (146). The osmotic pressure of the EPS generates a physical cue that is perceived in *B. subtilis* by the membrane-embedded histidine sensor kinase KinD (115), which in its activated form determines the phosphorylation status of the master regulator of *B. subtilis* cellular differentiation, Spo0A (136). High-level Spo0A-P represses matrix gene expression (115) and favors sporulation (136). Strikingly, the genetic disruption of RemA, a crucial activator of genes required for biofilm formation in *B. subtilis* (141) (**Figure 3*d***), prevents expression not only of the genes for the matrix components but also of the osmoregulated *opuA*, *opuB*, and *opuC* operons (141), which encode major compatible solute import systems of *B. subtilis* (64). Hence, cells in the biofilm experience osmotic stress as the biofilm expands, and they try to ameliorate it through the import of osmostress protectants.

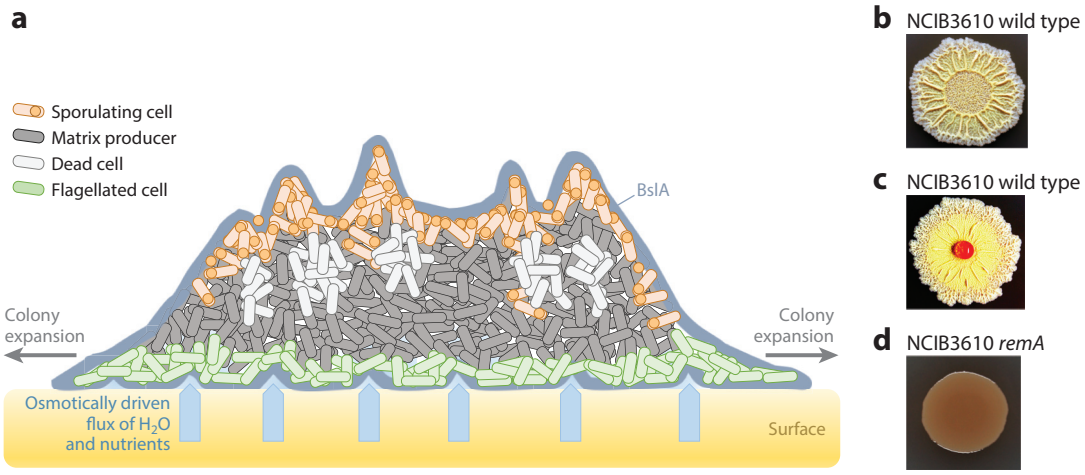

Figure 3

Osmotic forces at work in a *Bacillus subtilis* biofilm. (*a*) Schematic cross section of a biofilm colony formed by the environmental *B. subtilis* isolate strain NCIB3610 on a solid surface (21, 63, 136). Coloring of the cells represents various differentiation states leading to the characteristic surface architecture of *B. subtilis* biofilms grown on MSgg agar plates. BslA is a redox-sensitive hydrophobin (3) covering in different states both the surface and the bottom of the *B. subtilis* biofilm (4). (*b*) Colony morphology of a biofilm developed after four days of growth on MSgg agar plates. (*c*) The hydrophobicity of the biofilm surface can be visualized by placing a droplet of colored water onto the *B. subtilis* biofilm macrocolony (4, 21). (*d*) Colony appearance of a *B. subtilis remA* mutant in which the core biofilm-forming gene clusters are no longer expressed (141). Photographs in panels *b* and *c* courtesy of Tamara Hoffmann.

5. CONCLUDING REMARKS

Great strides have been made to understand how microorganisms cope genetically, physiologically, and biochemically with both sustained and fluctuating osmotic stress, an issue that is fundamental for an in-depth understanding not only of the ecophysiology of microorganisms in their varied natural habitats but also of food safety, virulence of pathogens, and the performance of microbial cell factories in industrial-scale fermenters. As the field progresses, old questions persist and new ones emerge.

FUTURE ISSUES

1. What is the precise nature of the physical stimuli used by the cell as a proxy for osmotic challenge? Are the two known ways of stimulus generation, in the cytoplasm (solute concentration, hydration state) and in the membrane (lateral forces), completely separate principles? Is the cell capable of measuring the concentration of particular solutes in the cytoplasm, e.g., ions or compatible solutes? How are internal forces in the phospholipid membrane transferred to membrane proteins? If, besides bulk-phase interaction, specific lipid molecules are involved, what is their function at the protein surface?
2. How can we translate the events occurring during signal transduction induced by osmotic stress into protein action? How does this happen in terms of dynamic structural events in sensor proteins and target systems (soluble or membrane bound)? Do common structural principles for sensor mechanisms exist, e.g., using molecular devices of similar or functionally comparable construction (sensory domains, sensory surfaces)?

3. What are the molecular and biochemical mechanisms that allow a high-osmolality stressed cell to precisely tune cytoplasmic compatible-solute pools to the degree of the stress it perceives? How are activated enzymes or transporters deactivated upon osmotic adaptation in spite of the fact that at least some of the stimuli persist, e.g., an increased solute concentration in the cytoplasm? Do dedicated efflux systems (substrate-specific channels and/or carriers) for compatible solutes exist, and what might be their precise physiological role?
4. How do osmotic forces shape the spatial structure of microbial communities assembled in biofilms, and how do they affect matrix production and the communication and social behavior of the resident bacteria in these assemblies? Do compatible solutes released by producer bacteria serve as public goods for those microorganisms that can acquire them via transport?

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The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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152. Expert insight into the complex issue of macromolecular crowding in the cell.

153. In-depth analysis of structural and functional aspects of a major class of osmoregulated transporters.

Annual Review of Microbiology

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Contents

Indexes

Errata

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