OPINION

Microorganisms maintain crowding homeostasis

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Abstract | Macromolecular crowding affects the mobility of biomolecules, protein folding and stability, and the association of macromolecules with each other. Local differences in crowding that arise as a result of subcellular components and supramolecular assemblies contribute to the structural organization of the cytoplasm. In this Opinion article we discuss how macromolecular crowding affects the physicochemistry of the cytoplasm and how this, in turn, affects microbial physiology. We propose that cells maintain the overall concentration of macromolecules within a narrow range and discuss possible mechanisms for achieving crowding homeostasis. In addition, we propose that the term 'homeocrowding' is used to describe the process by which cells maintain relatively constant levels of macromolecules.

The biochemical processes that characterize all living cells, such as the provision of energy, gene expression and cell division, take place in a confined and highly crowded space[1]. High concentrations of macromolecules give rise to the phenomenon of macromolecular crowding (BOX 1), which affects individual proteins, the formation of protein complexes and the structure of the cytoplasm. The physicochemical properties of the cytoplasm are usually determined by internal pH, ionic strength, water activity, viscosity and osmotic pressure; however, to fully understand bacterial physiology, macromolecular crowding must also be considered. There is evidence that the bacterial cytosol is more crowded than the cytosol of mammalian cells[2], which emphasizes the need for microorganisms to control this parameter.

In *Escherichia coli*, proteins constitute the majority of cellular macromolecules (~55% of the total dry weight), and, together with ribosomal RNA (rRNA; ~15% of the total dry weight), they are the most space-consuming molecules[3]. DNA (which in *E. coli* is 4.6 Mbp in length) constitutes ~3% of the cellular mass of fast growing cells[3], although the compacted nucleoid spans across ~75% of the cytoplasmic space[4]. mRNA (~1%) and tRNA (~3%) are less abundant but also contribute to macromolecular crowding[3]. In the cytoplasm of *E. coli*, the macromolecules occupy 15–20% of the total available space and a macromolecular volume fraction (Φ) in the range of 0.13 to 0.24 has been reported[5–8]; note that in REF. 5, and in many papers that cite this work, Φ values up to 0.44 are presented, but these numbers report the excluded volume rather than volume fractions (see below). At $\Phi = 0.2$, if particles with a diameter of 5 nm are homogeneously distributed, the surface-to-surface distance between macromolecules is <2 nm on average, which emphasizes how crowded the bacterial cell is. The highest possible volume fraction of a cell is $\Phi = \sim 0.5$, on the basis of the maximal packing of spherical particles.

The bacterial cytoplasm, which has a dynamic structure (see Supplementary information S1 (box)), lacks membrane-bound organelles but the DNA has a nucleus-like organization (nucleoid); certain macromolecules are enriched in, and others are excluded from, the nucleoid. Furthermore, Gram-negative bacteria have two membranes between which the periplasm forms; this can be as crowded as the cytoplasm itself[9,10]. The macromolecules in the periplasm may be more confined than those in the cytoplasm (BOX 1), because the space between the two membranes along the long axis of the cell is only ~30 nm. In Gram-positive bacteria, which have a single membrane, the extracytoplasmic proteins are tethered to the outer surface of the cytoplasmic membrane by a lipid moiety or are covalently linked to a membrane protein[11].

In the highly crowded environment of the bacterial cytoplasm or periplasm, macromolecules experience less available volume than when they are in a dilute buffer, which means they have a larger 'excluded volume' at these locations[12] (BOX 1; FIG. 1a). The macromolecule and the surrounding crowding macromolecules cannot overlap, which gives rise to steric repulsion and decreased translational degrees of freedom (that is, a decrease in the ability to move around the cell). Hence, placing a macromolecule in a crowded environment produces a configurational entropic penalty. Any decrease in the volume of the macromolecule would increase the entropy and thus decrease its free energy[12,13]. In practice, this means that excluded volume enhances the folding of a macromolecule, the oligomerization of proteins and the condensing of DNA[14,15]. These effects of excluded volume have been explained through various theories[12,16,17], a selection of which is highlighted in BOX 1. All of these theories propose that high volume occupancy favours a more compact macromolecule owing to an entropic gain[18,19]. Furthermore, the excluded volume is dependent on the size and shape of the molecule; for example, a small molecule can approach the crowders more closely than a large one (FIG. 1a). Specifically, larger molecules experience more excluded volume than smaller ones; hence, self-association of the crowders can decrease the effects of excluded volume. These steric repulsion effects are the most universal nonspecific interactions that a macromolecule experiences in a crowded solution, such as the bacterial cytoplasm or periplasm, but they will certainly not be the only type of interaction: a protein will experience nonspecific (that is, soft) interactions that can either be repulsive or attractive depending on the shape

and surface properties of the protein and the surrounding crowders (BOX 1). The consequences of both excluded volume and soft interactions on macromolecular reactions were recently reviewed (see REF. 20).

In this Opinion article, we first describe how crowding is studied and how it affects biological processes. We then present the current thinking on the structure of the bacterial cytoplasm, including its spatial heterogeneity and the dynamic reorganization of proteins into intracellular bodies. Last, we discuss whether optimal crowding exists in bacteria and present mechanisms that might maintain crowding within a defined range, through a process that we refer to as 'homeocrowding'. We focus on crowding in microorganisms but present a few case studies in higher eukaryotes to highlight key earlier work.

Box 1 | **Crowding-related terms**

Macromolecular volume fraction (Φ)
The volume fraction (Φ) is the volume that is occupied by macromolecules relative to the volume of the compartment (for example, the cytoplasm) and is expressed as volume per volume (v/v)[5].

Excluded volume
The volume that is accessible to a tracer molecule is decreased in the presence of crowding molecules. The volume excluded is the apparent volume of the crowder molecules, which is given by the distance between the centre of a tracer particle and the centre of the crowding molecule (see FIG. 1a).

A selection of theories that are relevant to macromolecular crowding

- The Asakura–Oosawa depletion theory[16] describes the effect of the attraction of two particles due to the depletion of solutes in between those particles. This local solute depletion induces an osmotic pressure difference within the bulk solution that pushes the particles together. The depletion force occurs in crowded solutions and might be applicable to protein crowding, albeit only on a qualitative level. The depletion force explains, for example, the compression of tracer molecules and the size-dependent sorting of crowder molecules in dense solutions.
- The Flory Huggins theory[17] describes the thermodynamics of polymer solutions but has been adapted to explain the effects of crowding on intrinsically disordered proteins. Besides excluded volume, this theory contains an interaction parameter to account for the miscibility between disordered proteins and crowders.
- The scaled-particle theory[12] has been adapted to quantify the effects of macromolecular crowding. The theory treats proteins and crowders as hard convex particles that cannot overlap, inducing an entropic cost when placing the tracer molecule in a crowded solution compared with a dilute solution. The entropic cost increases (that is, entropy decreases) with larger overlap volume between the crowder and tracer molecule, and the concentration of the crowder (see FIG. 1a).

Soft interactions, weak chemical interactions
Soft interactions affect both entropy and enthalpy, and include non-covalent interactions, such as electrostatic, hydrogen bonding, van der Waals and hydrophobic interactions. Unlike steric interactions, they are caused by the chemical nature of the molecules. Soft interactions can either counteract or enhance the effects of the excluded volume.

Macromolecular confinement
Confinement refers to the phenomenon of volume exclusion due to a fixed (that is, confining) boundary to a macromolecule[26] — for example, the membranes that confine the narrow space of the periplasm. Contrary to macromolecular crowding, the free energy cost of confinement is not necessarily minimal when a molecule is in its most compact conformation, and whether the molecule has a complementary shape to the confining boundary becomes a factor in the free energy term.

Phase separation
Attractions between macromolecules can lead to macromolecule-enriched and macromolecular-depleted phases, or phases that are enriched in a certain type of macromolecule, such as in the eukaryotic nucleolus and in germ cell granules (P-granules) of *Caenorhabditis elegans*. Attraction can be induced by the depletion force (Asakura–Oosawa depletion theory) or chemical interaction (similar to the Flory Huggins theory). The phases need to be in osmotic equilibrium and thus the presence of osmolytes enhances the possibility of forming phases, as high local concentrations of macromolecules need to be osmotically balanced. In the bacterial cell, this may lead to spatial heterogeneity and vectorial chemistry.

Homeocrowding
We use homeocrowding as an acronym of 'macromolecular crowding homeostasis'; it refers to the ability of the cell to maintain relatively constant levels of macromolecules, similarly to the ability of a system to regulate its internal pH and ion concentration (that is, pH and ion homeostasis or ionic strength homeostasis).

Donnan effect
The Donnan effect (also known as the Gibbs–Donnan effect or Donnan equilibrium) occurs when large particles (for example, anionic proteins) are present on one side of a membrane and create an uneven electrical charge. This will attract small cations, such as K^+, the accumulation of which will increase internal osmotic pressure.

Synthetic and natural crowders

The effect of crowding is hard to measure *in vivo* because of the complexity of cells and the heterogeneity of the cytoplasm. Synthetic crowders, such as Ficoll, dextran or polyethylene glycol (PEG), are often used to mimic *in vivo* crowding effects. At high concentrations, synthetic crowders exert excluded volume effects and stabilize proteins[21], promote protein aggregation[22] and influence protein–protein interactions[23] (further examples are reviewed in REF. 24). Theoretical approaches are required to completely understand the effects of synthetic and natural crowders on these protein properties. In several studies, the agreement between theoretical and experimental results is qualitative, but the models do not predict biological outcomes[25]. Most models consider the crowders as hard-sphere and hard-rod crowding agents, which ignores the attractive interactions between the molecules and their polymeric nature. Indeed, synthetic crowders can enter a semi-dilute state at 5–20% weight per weight (w/w), in which their radii overlap and their chains interpenetrate, and the molecules can no longer be treated as hard spheres. Furthermore, PEG interacts with proteins[26] and can form coacervates (that is, it can undergo phase separation) at high concentrations and in the presence of inorganic salts, and these crowders compress at high concentrations owing to self-crowding.

High concentrations of bacterial cell lysates, such as those of *E. coli,* are physiologically more relevant, provide more heterogeneous surfaces and more closely mimic the soft interactions of the cytosol than synthetic crowders (BOX 1), but they still do not produce crowding levels comparable to those found *in vivo*. A fluorescence resonance energy transfer (FRET)-based probe was compressed by synthetic crowders but not by the presence of concentrated *E. coli* cell lysate. Thus, different crowders at similar macromolecular volume fraction exerted different effects on the probe, which can be explained by a compensation of the

depletion force (excluded volume effect) with attractive soft interactions between the probe and cell lysate[27]. In addition to non-covalent chemical interactions, crowding agents can also cause osmolyte effects, influencing protein hydration through changes in water polarity and activity[28]. Thus, the thermodynamic consequences of macromolecular crowding involve excluded volume effects and long-range soft interactions between molecules that are influenced by small molecules. Furthermore, the behaviour of individual proteins in the cell or a cell-like environment might be influenced by interactions with chaperones or the membrane.

Crowding effects in living cells

The interior of a cell is a complex and dense network of proteins, nucleic acids and small molecules that act in concert to sustain all essential processes of life. Under normal physiological conditions of ionic strength and macromolecular crowding, hydrogen bonding, screened electrostatic forces and excluded volume repulsions act over a commensurate distance of ~1 nm (see Supplementary information S1 (box)); this intermolecular distance enables macromolecular surfaces, such as the surfaces of nucleic acids, proteins and membranes, to co-evolve and bacterial cells to maintain the dynamic structure of the cytoplasm.

Crowding effects on biochemical reactions. Macromolecular crowding affects metabolic networks and information processing in bacterial cells at various magnitudes. Relatively small changes in available cytoplasmic volume can have a major effect on the chemical equilibrium of a process (for example, the self-association of a protein) and enzyme kinetics[13,19]. The rate of enzyme-catalysed reactions in a crowded environment is affected if the conformational change that is imposed on the enzyme by substrate binding decreases or increases the volume of the entire complex[29]. Usually, the change in the volume of an enzyme and its intermediate enzyme–substrate complex is small. The effect of macromolecular crowding on the activity of individual metabolic enzymes is therefore dependent on the specific biochemical process. The Michaelis–Menten constant (K_m) and the turnover number (k_{cat}) of enzymes in crowded solutions are typically less than twofold different to those observed in dilute solutions[30]. However, even small changes in K_m or k_{cat} can affect the metabolic flux of cells when several enzymes are affected, because metabolic pathways form elaborate networks of interconnected enzymes and metabolites.

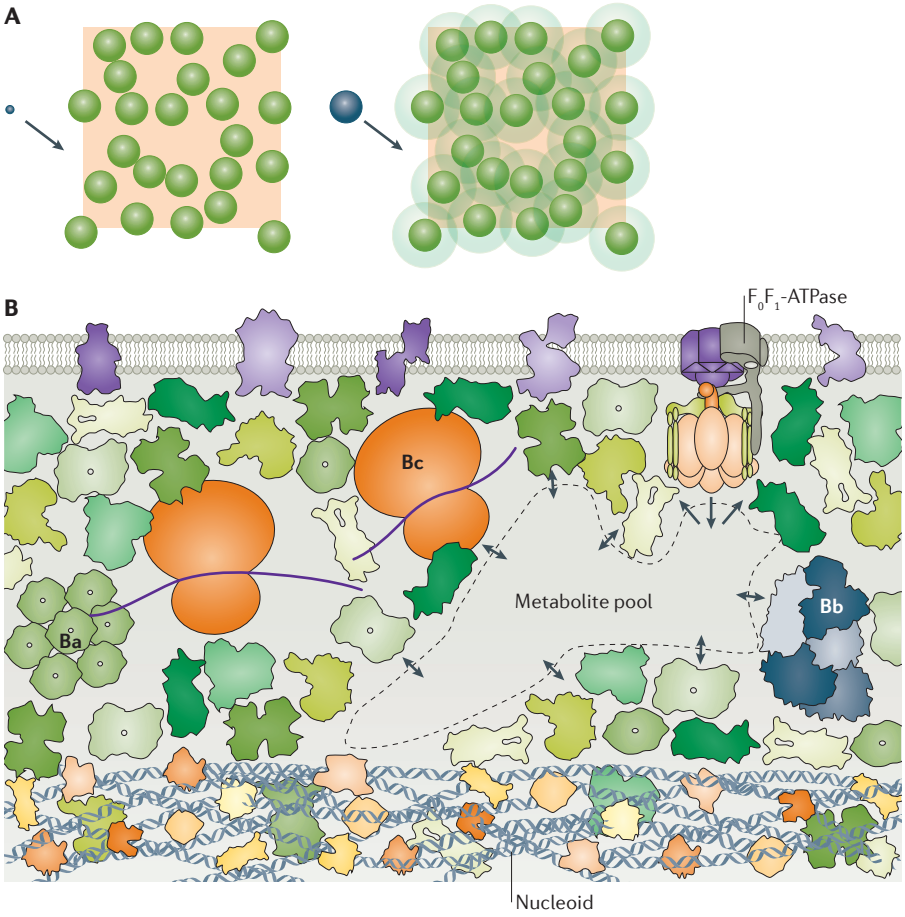

Figure 1 | **General principles of macromolecular crowding. A** | Schematic of the effect of excluded volume on macromolecular crowding. The volume of solvent (orange) available for the centre of mass of a small tracer molecule (left; dark blue) and a large tracer molecule (right; dark blue) when added to a crowded medium (Φ = ~0.2) of macromolecules (green) is depicted. The rings around the macromolecules mark the excluded volume for the added particle. **B** | Cartoon showing spatiotemporally 'supercrowded' regions of biomacromolecules that are joined by attractive non-covalent forces at specific points, which can lead to the formation of intracellular bodies (part **Ba**) or metabolons (part **Bb**); note that ribosomes (part **Bc**) are excluded from the nucleoid. Areas of higher-than-average levels of crowding cause the formation of metabolite and electrolyte pools that have low concentrations of macromolecules. In the upper right corner an F_0F_1-ATPase is shown; this connects the vectorial reactions at the plasma membrane to the ATP-consuming and ATP-producing protein complexes at the reactive periphery of both the undercrowded and supercrowded phases. The arrows indicate that small molecules can move into and out of the metabolite pool. Part **a** is adapted with permission from REF. 19. © (2001) The AmericanSociety for Biochemistry and Molecular Biology. Part **b** is from *Microbiol. Mol. Biol. Rev.*, (2009), **73**, 371–388, http://dx.doi.org/10.1128/MMBR.00010-09 and amended with permission from American Society for Microbiology.

However, DNA replication and transcription are strongly dependent on crowding. In an *in vitro* replication system, DNA could only be replicated in the presence of high concentrations of PEG or polyvinyl alcohol (PVA)[31]. The rate constant for transcription in a cell-free system was five times higher at high crowding, and the values under crowded conditions were similar to those observed *in vivo*[32]. By contrast, protein synthesis (that is, translation) was inhibited by synthetic crowders[33]; this was attributed to the binding of translation factors to Ficoll or to PEG-induced protein precipitation.

The association between the *lac* repressor and the *lac* operator, and that of RNA polymerase and the bacteriophage λ P_R promoter, decreases with increasing concentrations of salt *in vitro*[34,35]. However, this salt dependence was not observed

Box 2 | **Quantification of macromolecular crowding**

Water-accessible volumes
The water-accessible volumes of the cell, cytoplasm and periplasm can be measured by comparing the volumes that are accessible to $^{3}H_{2}O$ (all compartments) with those that are accessible to ^{14}C-inulin (a probe that is excluded from the cell) or ^{14}C-sucrose (a probe that is excluded from the cytoplasm but not from the periplasm)[102]. The specific volume is obtained by relating the volume to the total protein or dry weight of the suspension (which is expressed as volume per mg of protein or dry weight). For *Escherichia coli* grown in a 3-(*N*-morpholino)propanesulfonic acid (MOPS)-based glucose minimal medium (MBM; analysed at an external osmolarity of 0.28 osmol), the specific volume of the cytoplasm and the periplasm are ~2.1 ml mg^{-1} dry weight and ~0.4 ml mg^{-1} dry weight, respectively.

Crowding levels
Macromolecular crowding has been quantified using genetically encoded or synthetic crowding sensors[8,103]. Our genetically encoded crowding sensor consists of two fluorescent proteins that constitute a fluorescence resonance energy transfer (FRET) pair and that are linked by two α-helices and three flexible linkers. In more crowded environments, the sensor is compressed, which leads to an increase in FRET efficiency[8]. By comparing intracellular FRET readouts with a calibration solution, such as Ficoll, the crowding level in cells is estimated.

Proteome analyses
Quantitative proteome analysis (for example, by liquid chromatography–mass spectrometry (LC-MS) quantification[76] or ribosomal profiling[104]) can be used to relate the protein mass to cell volume and obtain specific information about macromolecular volume fractions.

Lateral diffusion coefficients
Lateral diffusion refers to the lateral movement of molecules. The lateral diffusion coefficient (*D*) is related to the temperature (*T*) and viscosity (μ) of the medium and the hydrodynamic radius (R_s) of the particle, as described by the Stokes–Einstein relationship:

$$D = k_B T / 6\pi R_s \mu$$

where k_B is the Boltzmann constant. As the lateral diffusion coefficient is also dependent on macromolecular crowding, diffusion measurements have been used to probe crowding in the cell[43,48,64]. Fluorescence recovery after photobleaching (FRAP) of fluorophore-tagged macromolecules is typically used when diffusion is fast; single-particle tracking is used if diffusion is slow and can distinguish Brownian dynamics from subdiffusion or superdiffusion[46]. Single-particle tracking can also reveal the confinement of a molecule to a small area of the cell or its transient binding to other components.

Buoyant densities
The buoyant density refers to the cellular mass (which comprises biopolymers, salts and water) per cell volume; that is, the summation of the density of the components of a cell multiplied by their relative abundance. Buoyant densities are usually measured by applying cells to a concentrated medium (often containing polysaccharides such as Ficoll or dextrans) that form a linear density gradient following ultracentrifugation.

in vivo in *E. coli*[36], probably owing to compensation from crowding. Furthermore, in another system in the bacterium *Lactococcus lactis*, ionic strength and macromolecular crowding may act synergistically. Specifically, the gating of the osmoregulatory ATP-binding cassette (ABC) transporter OpuA is less dependent on ionic strength in the presence of synthetic crowders[37]. Thus, these examples indicate that the effects of macromolecular crowding may be linked to the electrostatic stabilization of the surfaces of macromolecules in the cytoplasm.

The effect of soft interactions on protein stability was shown by several NMR experiments in *E. coli*. In these studies, *in vivo* macromolecular crowding conditions destabilized chymotrypsin inhibitor 2 and protein L more than aqueous solutions with synthetic crowders[38,39]. Although the surface chemistry of these small proteins may not be representative of native proteins, these findings indicate that soft attractive interactions can outbalance the excluded volume effect to decrease protein stability[40]. The ability of high crowding to destabilize proteins can be mitigated by the compatible solute glycine betaine[41], which also counteracts protein aggregation *in vivo*[42]. Thus, the effects of soft interactions can be less when cells have accumulated large amounts of glycine betaine (see below).

Crowding effects on lateral diffusion. Macromolecular crowding decreases the mobility of biomacromolecules more than dilute solutions, because the volume of the hydrodynamic fluid (that is, the solvent) is decreased. In terms of diffusion, the 'crowder' provides a barrier to solute movement, which can give rise to anomalous diffusion[43]; in contrast to normal diffusion, in anomalous diffusion the mean squared displacement of a particle is nonlinear with time. The viscosity of the bacterial cytoplasm has been reported to be up to 10 times higher than that of water[44], which affects diffusion according to the Stokes–Einstein relationship (BOX 2). However, the slowed diffusion in the cytoplasm of *E. coli* cannot be explained by the increase in viscosity alone. In fact, the lateral diffusion coefficient (*D*) as a function of molecular weight (Mw) scales with a power law of $\alpha \bullet Mw^{-0.7}$, whereas $D = \alpha \bullet Mw^{-0.33}$ is predicted by the Stokes–Einstein relationship (α includes temperature and viscosity, see BOX 2). The observed dependence on power law indicates that, in the bacterial cytoplasm, large macromolecules are slowed more than small macromolecules[7,45–47].

Furthermore, the crowded environment provides barriers for long-range solute diffusion (that is, it causes subdiffusive behaviour), and leads to confined protein mobility[48,49]. As bacteria rely on long-range diffusion for the transport of molecules and cytoplasmic mixing, diffusion can become a limiting factor in the success of their cellular processes. For example, the lateral diffusion of bulky tRNA complexes in the crowded cytoplasm of *E. coli* imposes a physical limit on the speed of translation[50].

In summary, the excluded volume effects of crowding in living cells are the strongest for reactions in which the free volume gain is high, such as for protein–protein and protein–nucleic acid interactions; individual metabolic enzymes are typically not markedly affected. The excluded volume effects of crowding on protein stability can be mitigated by soft interactions, as shown by *in vivo* NMR for small tracer proteins. The effects of crowding also come into play when reactions are limited by diffusion (see below).

Structure of a crowded cytoplasm

In living cells, biopolymers are always exposed to soft interactions between themselves and surrounding molecules. The short distances between the biomolecules are in the range of screened electrostatic forces, hydrogen bonding and excluded volume (see Supplementary information S1 (box)). These forces stabilize the cytoplasm against random collapse (that is, against the nonspecific aggregation of macromolecules), and enable unequal

crowding[51]. The non-covalent repulsive and attractive forces that are caused by the uneven distribution of hydrophilic, hydrophobic and charged surface areas of macromolecules, enable the clustering of proteins and nucleic acids[51,52]; this can result in the formation of subcellular structures[53,54] such as metabolons[55], hyperstructures[56], intracellular bodies[57] and filaments[58]. These structures are comprised of temporary complexes of sequential metabolic enzymes or of proteinaceous bodies of a single type of enzyme, which are held together by non-covalent interactions. In addition, macromolecules can undergo phase transitions (that is, they can form skeletal structures or droplet phases)[59], which contribute to the structuring and the heterogeneity of the cell. Protein clustering or phase separation can locally increase crowding to an above-average level, leading to the formation of 'uncrowded' areas elsewhere in the cytoplasm (FIG. 1b). A recent study on probing short-range protein diffusion in Chinese hamster ovary cells unveiled unobstructed Brownian motion from 25 nm to 100 nm (REF. 60), which is only possible if the cell contains undercrowded and supercrowded areas. In bacteria, the undercrowded areas may be smaller and more dynamic.

We recently proposed that the bacterial and archaeal cytoplasms might form multiphase systems of supercrowded cytogel and dilute cytosol[61] (see Supplementary information S1 (box)), in which the size of a molecule affects the cytoplasmic space it can visit. This is different to when the molecule is in a homogenous crowded solution. Large particles become caged in the supercrowded areas and only move long distances if the pools rearrange. In addition to supercrowding and undercrowding, macromolecular confinement restricts the lateral diffusion of macromolecules. For example, the majority of ribosomes are confined to the end-caps of *E. coli* cells and are excluded from the nucleoid[62], whereas free ribosomal subunits can reach the nucleoid and can bind to mRNA[63]. Owing to this ribosome–nucleoid segregation, mRNA (when bound to a ribosomal subunit) has to diffuse to the end-caps of the bacterial cell before translation[62]. Consistent with this view, small molecules visit a larger volume of the bacterial cytoplasm than large macromolecules[64]. This demonstrates that the effects of macromolecular crowding are diverse and might be locally amplified in regions of increased or decreased macromolecular density (FIG. 1b). The spatial heterogeneity of the bacterial cytoplasm is also evident from measurements of lateral diffusion[46] (BOX 2). Simple homogeneous crowding and the scaled-particle theory do not account for diffusion in the cytoplasm, nor do they account for the variability in lateral diffusion coefficients of GFP (D_{GFP}) among cells[65,66] or the progressive size exclusion of the diffusing particle with increasing biopolymer fraction[7,64].

A recent series of papers[67–69] suggest that the cytoplasmic events can be viewed as complex vectorial chemistry (see Supplementary information S1 (box)). For example, single-particle tracking in *E. coli* and *Caulobacter crescentus*, and also in lower eukaryotes (namely *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Dictyostelium discoideum*), indicates that the cytoplasm changes from a fluid to a more solid-like 'colloidal glassy' state when the energy metabolism of the cell is shut down. One idea is that the solid-like state is caused by the acidification of the cytoplasm, which leads to the decreased diffusion of large probe particles, the widespread assembly of macromolecules and an increase in the mechanical stability of the cell[68]. The same group concluded that acidification and osmotic stress result in different states of the cytoplasm; osmotic upshift increases crowding but does not lead to the same solid-like state as acidification. In our opinion, the two stresses may lead to a different degree of confinement of the large particles, owing to the formation of biopolymer networks with a different mesh size[64], rather than to the formation of a solid-like state in the cytoplasm. Regardless of the molecular basis, the transition in the state of the cytoplasm is important for cell survival under conditions of energy starvation. Another study proposed that the transition is due to the decrease in cell volume and the accompanying increase in macromolecular crowding that result from glucose starvation[69]. In agreement with this idea, glucose starvation and an osmotic upshift in media lead to a similar cytoplasmic state. Thus, even though the molecular mechanism that underlies the dynamic heterogeneity of the bacterial cell and that of lower eukaryotes is still elusive, it is evident that the cytoplasm is more than a bag of randomly organized enzymes and that the formation of phase-separated macromolecular assemblies is at the heart of localized biochemistry.

Optimal levels of crowding

Biochemical reactions are optimal in their natural crowded environment because proteins and nucleic acids have co-evolved together. Similar to pH and ion homeostasis, we propose that bacterial cells maintain homeostasis of macromolecule density (that is, of macromolecule crowding) to optimize the collective reaction and interaction rates of these molecules. What is the optimal level of crowding, given an internal pH of ~7.5 and an ionic strength of ~0.25 M, for bacteria such as *E. coli*?

With increasing macromolecular crowding the effective concentration of enzymes and biomacromolecules is increased, which accelerates biochemical reactions but slows down their lateral diffusion; the latter can become limiting. A flux balance model simulates the highest macromolecular density at which cells still benefit from increased enzyme concentrations[70]. The optimal level of crowding thus depends on the number of diffusion-limited reactions. Assuming all reactions are limited by diffusion, the model predicts an optimal macromolecular volume fraction of $\Phi = 0.22$. If none of the biochemical reactions was diffusion limited, the macromolecular volume fraction could theoretically reach a maximum of $\Phi = 0.8$. To estimate the number of diffusion-limited reactions in the cell, the authors used data on the concentration of more than 100 metabolites in *E. coli*[71]. If the concentration of a metabolite exceeds the K_m of the corresponding enzyme–substrate pair, the reaction is assumed to be saturated and therefore not limited by diffusion. There are about three times more reactions at saturation compared with diffusion-limited reactions, which would correspond to an optimal volume fraction $\Phi = 0.37$.

Another modelling approach is based on the idea that cells have an optimal protein density that maximizes their biochemical reaction rates[72]. The hypothesis is that a cell can regulate its density (and consequently crowding) by adjusting its volume. The model also assumes that the rate of diffusion-limited reactions is proportional to the concentration of reactants and the lateral diffusion constant D. They found $\Phi = 0.19$ to be the optimal density for maximal reaction rates. Both studies made several assumptions on what limits reaction rates and diffusion that need to be validated. However, the general idea that there are macromolecular densities that maximize reaction rates without substantially inhibiting diffusion is probably valid.

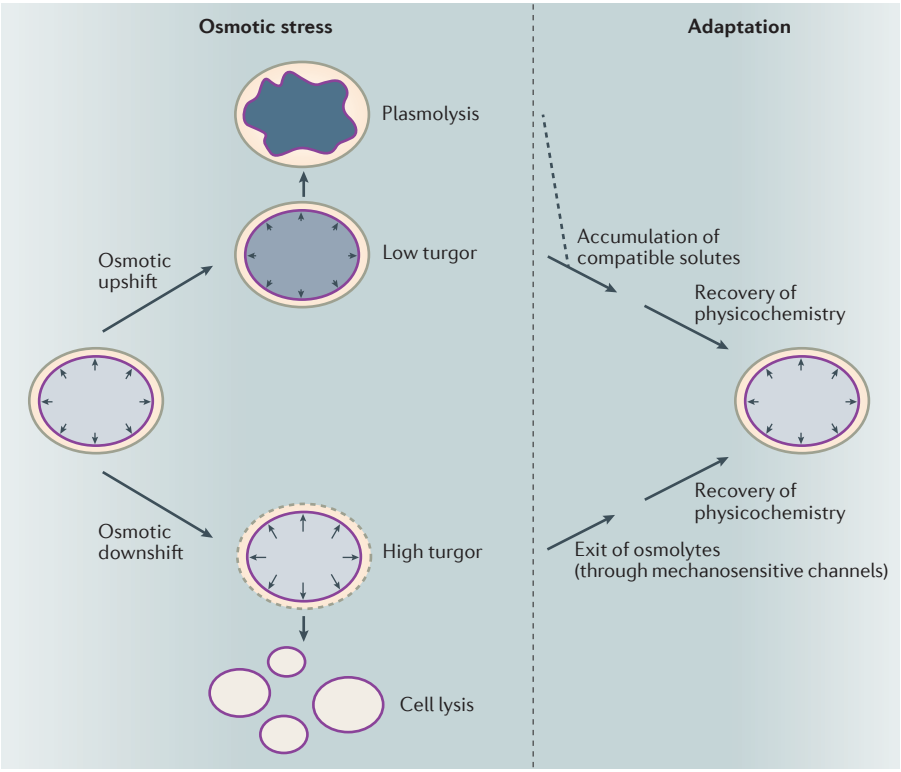

Figure 2 | **Schematic showing the effect of osmotic stress on the volume of a bacterial cell.** The magnitude of the turgor pressure, which is the difference in hydrostatic pressure that balances the difference in internal and external osmolyte concentration, is indicated in cells by arrows. Cells can adapt to an osmotic upshift through the accumulation of compatible solutes by import or synthesis, and they can adapt to an osmotic downshift by removing osmolytes through mechanosensitive channels. Plasmolysis and cell lysis occur when cells cannot adapt to osmotic upshift and downshift, respectively. Cells can recover from plasmolysis if the contraction of the cytoplasm (loss of water) does not last too long, whereas lysis is irreversible. The right panel (adaptation) shows what is required to return to the correct physicochemical state; for example, the accumulation of compatible solutes by uptake or synthesis for osmotic upshift and the rapid exit of osmolytes for osmotic downshift.

The Φ reported in the literature varies; the lowest and highest reported Φ values of *E. coli* are 0.13 and 0.44, respectively[5–8]. This range is caused by the physical meaning of the reported volume fractions; the high-end numbers of 0.33–0.44 represent the excluded volume fraction[5] rather than the volume fraction. To calculate the volume fraction, the partial specific volumes of the biomacromolecules must be known. Thus, using a partial specific volume of 0.7 ml g^{-1} for proteins and 0.58 ml g^{-1} for RNA, for *E. coli* grown in 3-(*N*-morpholino) propanesulfonic acid (MOPS)-buffered medium (MBM; with ~0.28 osmol) Φ = 0.16 (REFS 7,73). Using a genetically encoded FRET-based sensor we found *E. coli* to have an Φ of 0.13 (REF. 8). The values of 0.33–0.44 were calculated using an effective partial specific volume for excluded volume interactions of 1.0–1.3 ml g^{-1}, with the assumption that all macromolecules are equivalent hard spheres that are homogeneously distributed in the cell; this does not take into account the heterogeneity and soft interactions in the cell. When a partial specific volume of 0.7 ml g^{-1} was applied to this dataset a Φ value of 0.24 was obtained. Thus, the range of volume fractions in the literature is smaller when they are based on the same parameters, with Φ values ranging between 0.13 and 0.24. If optimal crowding exists, as we propose in this Opinion article, then bacterial cells probably control crowding when perturbed by changes in environmental conditions. Below, we describe how bacterial cells adapt to changes in nutrient availability and to changes in the osmolarity of media.

Macromolecular crowding as a function of growth rate. In 1958, it was reported that cell size correlates with specific growth rate or nutrient availability[74]. In a recent ribosome profiling study, the mass of proteins in *E. coli* cells that were grown at a specific growth rate of 0.74 h^{-1} (240 fg per cell) was shown to be ~3 times less than that of cells grown at a specific growth rate of 1.9 h^{-1} (680 fg per cell). As fast-growing cells have a larger volume, the overall crowding may be relatively constant. In accordance, the buoyant density (BOX 2) of *E. coli* cells that are grown at different growth rates is constant[75]. Furthermore, quantifying the majority of *E. coli* proteins in the cell and examining their location showed that the mass of proteins in the cytoplasm and periplasm increases and decreases, respectively, with increasing growth rate[76]. As the cytoplasmic and periplasmic volumes change accordingly, the crowding of the cytoplasm and periplasm may remain constant[10].

Perturbation of macromolecular crowding. Osmotic stress leads to changes in cytoplasmic volume and, consequently, to changes in the magnitude of macromolecular crowding. When bacterial cells face an osmotic downshift, they release solutes through mechanosensitive channels, which enables a rapid decrease in intracellular osmolyte concentration and, consequently, reduces the osmotic driving force for water entry to prevent cell lysis[77] (FIG. 2). The dynamics of an osmotic downshift in *E. coli* have been studied[78], but there is no information on the macromolecular volume fraction or the structure of the cytoplasm immediately following an osmotic downshift. However, the assembly of biomolecules or the formation of supramolecular complexes is likely to be affected after the majority of electrolytes and metabolites have exited the cell and the volume of the cytoplasm has decreased.

The response of *E. coli* to an osmotic upshift in media depends on the magnitude of the perturbation[79] (FIG. 2). Modest osmotic upshifts decrease turgor but do not affect cell function, even though the volume of the cytoplasm decreases[80] and, thus, crowding probably increases. In fact, D_{GFP} in cells that were stressed by up to ~0.3 osmol hardly changed[48] (see Supplementary information S2 (figure)). However, larger osmotic upshifts disturb *E. coli* cell function and lead to growth arrest. The efflux of water following severe upshifts leads to instantaneous cell shrinkage, with a decrease in cytoplasmic volume of up to ~50%[65,79]. After the internal and external osmolarities become equal, the cytoplasm shrinks and plasmolysis spaces become visible (FIG. 2). The effect on the cell is evident from the decrease in D_{GFP}, which

decreases proportionally to the magnitude of the osmotic upshift, and under extreme conditions crowding is so high that the macromolecules are essentially frozen[48,64] (see Supplementary information S2 (figure)). Plasmolysis leads to barriers to the diffusion of molecules such as GFP but not to small metabolites[66]. Thus, the cytoplasm of osmotically stressed cells may form a meshwork of biopolymers, which enables the free passage of small molecules while restricting the diffusion of larger ones[64]. Intriguingly, in the Gram-positive bacterium *L. lactis*, D_{GFP} decreases more rapidly with a decrease in cytoplasmic volume than in *E. coli*[65] (see Supplementary information S2 (figure)); this requires even stricter control of crowding levels.

When *E. coli* undergoes a sudden osmotic upshift from 0.1 to 1.45 osmol, Φ increases from 0.15 to 0.33 and D_{GFP} decreases by more than 100-fold[7,73]. However, when cells are allowed to adapt to 1.45 osmol, Φ increases to 0.36 and D_{GFP} is similar to that of unstressed cells. Hence, the mobility of proteins in adapted cells seems weakly dependent on Φ, which is in contrast to the diffusivity of proteins in cells shortly after osmotic upshift. To adapt to an osmotic upshift, cells accumulate potassium ions and compatible solutes[46,81,82], which restores the cytoplasmic volume (FIG. 2). Compatible solutes, such as glycine betaine, accumulate at submolar-to-molar levels, depending on the magnitude of the osmotic upshift[82]. The increase in osmolyte concentration draws water into the cell and restores the cytoplasmic volume. As *E. coli* cells regain their volume within a few minutes after shrinkage, it is likely that macromolecular crowding has a low dependence on external osmolarity. However, the observation that diffusion does not follow the macromolecular volume fraction[7] is unexpected and warrants further investigation.

In summary, bacteria may operate homeocrowding at Φ of 0.15–0.20 to achieve optimal growth rates. Osmotic downshifts decrease Φ and osmotic upshifts increase Φ, but bacterial cells can rapidly restore their cytoplasmic volume by activating mechanosensitive channels or osmoregulatory transporters.

How is homeocrowding achieved?

Macromolecular crowding that is too high or too low probably hampers cell function. Throughout the bacterial cell cycle, DNA replication and cell division must be coupled to growth to ensure that average cell size and crowding are maintained under a given growth condition[83]. Cells that divide before they double their mass of proteins would, after several generations, become unsustainably small. Conversely, delayed division yields cells that grow into filaments. As the bacterial cell cycle is robust and reproducible it is likely that cells actively regulate their size and macromolecule concentration to keep crowding within a certain range. If, as we propose, optimal crowding levels do exist, there must be mechanisms for achieving crowding homeostasis (see below).

Nutrient-dependent regulation of cell size. Using a flux balance analysis that includes the spatial constraints that occur in a densely packed environment[84], one group proposed that carbon catabolite repression (CCR) maintains constant crowding at varying metabolic fluxes and thus enzyme concentrations. CCR enables many bacteria to quickly adapt to a preferred carbon and energy source, and it also controls the metabolic switch that *E. coli* undergoes in its transition from a low growth rate to a high growth rate. Slow-growing cells oxidize carbohydrates to carbon dioxide, whereas fast-growing cells produce fermentation products in a process termed 'overflow metabolism'. To transition from slow growth to faster growth, *E. coli* requires more enzymes to keep up with its biosynthetic requirements and cells become larger[74]. However, the biomass density may stay within the optimal range of crowding (see FIG. 3a, step 1). At even higher growth rates, the number of enzymes required to produce carbon dioxide exceeds the available space in the cell; instead of increasing macromolecular crowding (FIG. 3a, step 3), which would lead to suboptimal growth, the cells start to produce fermentation products (FIG. 3a, step 2). The model takes into account that, per unit of metabolic energy that is generated, cells require twice as much space for the enzymes that oxidize carbohydrates to carbon dioxide than for fermentative glycolysis[85]. Thus, CCR would prevent overcrowding of the cytoplasm at high growth rates and is a potential mechanism of homeocrowding under these conditions. However, CCR does not operate under all growth conditions, and other nutrient-dependent mechanisms that regulate cell size could have a role in homeocrowding. In *E. coli* and *Bacillus subtilis*, uridine diphosphate (UDP)-glucose senses carbon availability and growth rate, and UDP levels are transmitted to the cell division machinery[86], possibly linking cell size and crowding to the cell cycle.

Osmolyte transport to control cell volume. Crowding levels do not change substantially when *E. coli* cells are inflated by the expression of large amounts of a 'useless' protein, such as β-galactosidase (LacZ)[87]. Bacterial cells accumulate counterions when biopolymer synthesis is increased to maintain electroneutrality and to compensate for the Donnan effect[87] (BOX 1). The accumulation of counterions results in water influx, which increases the volume of the cell (FIG. 3b). This mechanism could coordinate the production of biomass with cell volume and achieve homeocrowding and ion (and ionic strength) homeostasis. The required adjustments in volume may be modulated through the synthesis of lipids or by the rate of insertion of peptidoglycan monomers into the existing cell wall when cells elongate[80].

Furthermore, cells control their volume through osmoregulatory transporters and the gating of mechanosensitive channels[77,82] (FIG. 2). Following an osmotic upshift, the volume of the cell decreases and the level of macromolecular crowding and internal ionic strength increase instantaneously. Ionic strength or intracellular potassium ions activate osmoregulatory transporters in *E. coli, L. lactis* and *Corynebacterium glutamicum*[82,88,89], and the accumulation of compatible solutes and the accompanying water influx restore the volume of the cell over time. Following osmotic downshift, cells rapidly release osmolytes through membrane-stretch-activated mechanosensitive channels and thereby mitigate cell expansion due to water influx and prevent cell lysis[77].

We consider it likely that the osmoregulatory transporters and some of the more sensitive mechanosensitive channels have a role not only in osmotic regulation but also in the regulation of cell volume and thus the fine-tuning of macromolecular crowding (FIG. 3b). This hypothesis is strengthened by the observation that the activity of the osmoregulatory transporters OpuA and ProP, which are gated by internal ionic strength[90] or cation concentration[91], is tuned by macromolecular crowding, at least *in vitro* through the use of synthetic crowders[37,92]. Similarly, the cytoplasmic cage of the mechanosensitive channel MscS

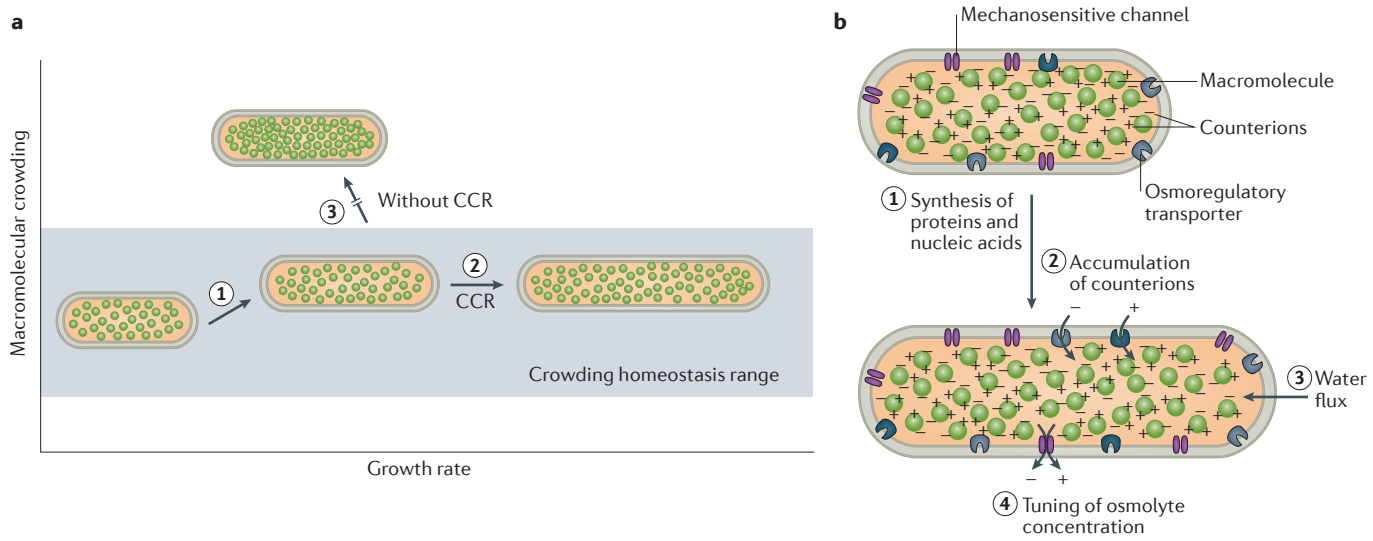

Figure 3 | **Possible mechanisms of crowding homeostasis. a** | Carbon catabolite repression (CCR). Following an increase in growth rate, cells expand their volume to accommodate the increased biomass (step 1). At even faster growth rates, cells activate CCR and switch to a metabolism (specifically, glycolysis) that requires less biomass per unit of metabolic energy (step 2). This switch changes the composition of the proteome and enables the fine-tuning of macromolecular crowding. Without CCR, macromolecular crowding would become too high and would slow down growth (step 3). **b** | Regulation by ion sensing. An increase in the synthesis of macromolecules, such as proteins and nucleic acids (step 1), leads to the accumulation of counterions (postively charged (+) and negatively charged (–); step 2) to achieve electroneutrality. This increases the internal osmolarity and the accompanying influx of water (step 3) increases the cytoplasmic volume. In addition, osmoregulatory transporters sense the internal ion concentration and mechanosensitive channels sense membrane tension to adjust the volume through osmolyte import or export (step 4). The figure in part **a** is modified with permission from REF. 84, BioMed Central

senses macromolecular crowding and enhanced crowding increases the rate of adaptive inactivation of the channnel[93]. In the early 1990s, it was shown in erythrocytes that levels of chloride-dependent K^+ transport and Na^+/H^+ exchange positively correlated with macromolecular crowding, that is, with the total intracellular protein concentration[94,95]. A model was proposed in which crowding-dependent phosphorylation and dephosphorylation control the activity of K^+ and Na^+/H^+ transporters and thereby the volume of the cell[96]. The changes in reaction rates (for example, as a result of kinase inhibition), owing to alterations in crowding, enable erythrocytes to sense and control their volume through ion transport[97].

Changes in crowding parallel changes in ionic strength; both of these parameters are linked to the physicochemical control of cellular processes. To date, the most abundant cation and anion studied in bacterial cells are K^+ and glutamate, respectively, and this ion pair has specific effects on protein–nucleic acid interactions, protein folding and assembly[98,99]. For example, potassium glutamate (K-glutamate) stabilizes protein–nucleic acid complexes more than the same concentration of KCl. This stabilizing effect originates from the unfavourable interactions between K-glutamate and hydrocarbon groups and amide oxygens that become exposed when a protein is unfolded[98]. The special role of K-glutamate in bacterial physiology is further highlighted by its transient accumulation in *E. coli* (and other bacteria) cells that are exposed to an osmotic upshift[100]. However, when cells adapt to hyperosmotic stress, K-glutamate is replaced by trehalose and other neutral compatible solutes, which restores ion homeostasis. The transient increase in K-glutamate (and thus in ionic strength) derepresses the expression of genes that encode osmoregulatory transporters (for example, ProP in *E. coli* and OpuA in *L. lactis*) and gates their activity; this controls cell volume and maintains the homeostasis of crowding and ionic strength.

Also, *E. coli* has seven mechanosensitive channels, some of which may have roles in addition to osmoregulation — for example, YjeP has a role in lipid biosynthesis[101] — and may control cell volume and crowding during normal growth. In summary, we propose that some of the mechanisms that enable bacteria to adjust their volume by osmolyte import (or osmolyte synthesis) and release have a role in homeocrowding under diverse growth conditions. These fast-operating mechanisms for switching proteins on and off may function in addition to slow-operating gene-regulatory mechanisms, such as CCR.

Concluding remarks

A high level of macromolecular crowding is a universal property of all living cells. Crowding influences the properties of macromolecules and increases their chemical potential, as well as the rate at which they encounter and interact with other cellular components. The strongest effect of crowding is observed for reactions in which the free volume gain is high, such as the interaction of proteins with nucleic acids or the formation of fibrils. At the macroscopic level, crowding may affect the physiology of the cell by organizing the cytoplasm into dynamic compartments[52] and inducing phase transitions[67]. Hence, crowding is an important component in the life of a microorganism. In view of this, we propose that bacterial cells operate crowding homeostasis. Whether this is through the nutrient-dependent regulation of cell size or osmolyte transport to control volume, a cell is not able to adjust its macromolecular crowding instantaneously and thus it should be able to tolerate transient crowding changes or to control the spatial heterogeneity of macromolecular crowding. On longer timescales, it will be very beneficial to the cell if it can work at optimum levels of crowding to maximize reaction rates and to enable protein surfaces to co-evolve and maintain the structure of the cell.

The challenges that remain are to determine the precise mechanisms that underlie homeocrowding and to put these mechanisms into the context of other factors that influence crowding effects, such as spatial heterogeneity, soft interactions and dynamic compartmentalization. It will be important to find out how cells cope with transient changes in crowding and to determine how the magnitude and timing of crowding have roles in cell physiology. We believe that controlling regulatory proteins using optogenetics, directly visualizing crowding effects in space and time using targeted probes, and determining the response of cells to environmental changes, together with theoretical experiments and simulations, will reveal the mechanisms that govern the homeocrowding of bacterial cells.

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Competing interests statement

The authors declare no competing interests.

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