Mechanosensitive channels in bacteria: signs of closure?

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Abstract | Bacterial mechanosensitive channels are activated by increases in tension in the lipid bilayer of the cytoplasmic membrane, where they transiently create large pores in a controlled manner. Mechanosensitive channel research has benefited from advances in electrophysiology, genomics and molecular genetics as well as from the application of biophysical techniques. Most recently, new analytical methods have been used to complement existing knowledge and generate insights into the molecular interactions that take place between mechanosensitive channel proteins and the surrounding membrane lipids. This article reviews the latest developments.

Mechanosensitive (MS) channels have a major role in cellular homeostasis and in maintaining the physical integrity of bacterial cells[1]. These channels are sensor transducers that sense the physical perturbation of the membrane and gate in response to this signal. It is thought that no other proteins are required to sense a change in membrane tension, as purified MS channel proteins reconstituted in vesicles have the same intrinsic ability to sense tension, leading to a transition in the channel from the closed to the open state[2–4]. Under physiological conditions in cells, increases in membrane tension arise from the rapid influx of water, which is associated with the transition of the bacterial cell from a high osmolarity environment to a low osmolarity environment, a condition known as hypoosmotic shock[1,5,6].

Bacteria maintain a positive turgor pressure, which pushes the cytoplasmic membrane against the peptidoglycan in the cell wall. Calculations based on the total weight of intracellular solutes have indicated that the turgor pressure in *Escherichia coli* lies in the range of 2–6 atm, compared with up to 30 atm for *Staphylococcus aureus*[7]. When bacteria grow at high osmolarity, they retain their turgor pressure by accumulating solutes to proportionately high levels. So, in a basic glucose-salts minimal medium (~250 mosM) the concentration of cytoplasmic K^+ will be ~300 mM, but with an external increase in osmolarity by 600 mosM (achieved for example by the addition of 0.3 M NaCl) this can rise to ~600 mM. The predominant anion becomes glutamate in conditions of high osmolarity. Adaptation to high osmolarity is multiphasic: an initial loss of water is followed by the rapid accumulation of K^+ and glutamate. In some microorganisms, this phase is immediately followed by the synthesis of compatible solutes (such as betaine, proline, trehalose and related compounds) and/or the accumulation of these solutes from the environment[8,9]. Consequently, the composition of the bacterial cytoplasm is dynamic and requires interactions between various uptake and efflux systems for ions and solutes, including MS channels.

The transfer of bacterial cells adapted to high osmolarity medium into low osmolarity medium causes a rapid influx of water into the cells down the osmotic gradient. This can increase the turgor pressure by ~10 atm in a few milliseconds, which places considerable strain on the cytoplasmic membrane and cell wall (FIG. 1). Peptidoglycan comprises alternating residues of *N*-acetylglucosamine and *N*-acetylmuramic acid that are crosslinked by peptides, with the sugar chains arranged circumferentially and the peptides aligned with the long axis of the cell. However, this is not thought to form a continuous structure because in *E. coli* it has been shown that up to 400,000 short chains are irregularly crosslinked[10]. The variable length of the sugar chains, coupled with the incomplete crosslinking, creates a heterogeneous mesh structure of pores that are bounded by polysaccharide and peptide chains. Bacterial cells can undergo significant remodelling of their cell wall during entry to — and exit from — stationary phase[11], in response to osmotic stress, during phage infection[12] and during attachment to surfaces and entry into host cells[13]. In addition, the composition of the cell envelope can be modulated by environmental stresses, such as changes in external pH and temperature[14]. This makes

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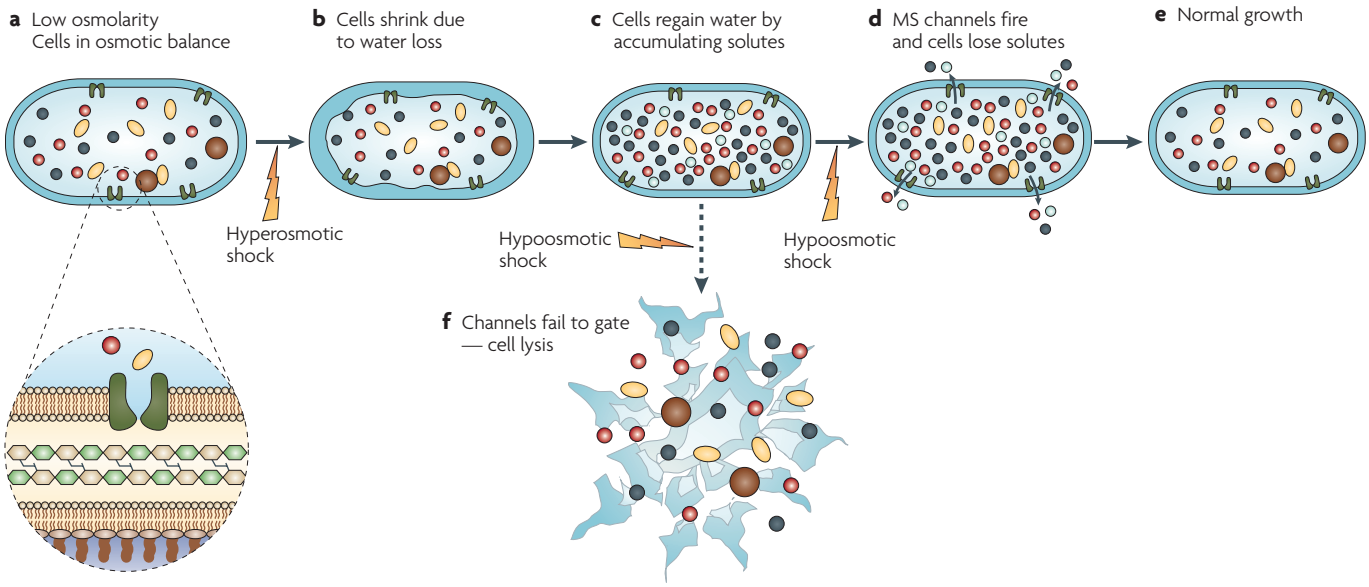

Figure 1 | **Physiological function of mechanosensitive channels in bacteria. a** | During growth at low osmolarity, cells accumulate sufficient K^+ (red circles) and glutamate (black circles) to achieve an outwardly directed turgor pressure of ~4 atm, which is balanced by the resistance of the cell wall and outer membrane. The inset shows a mechanosensitive (MS) channel in a Gram-negative bacterium. **b** | Upon hyperosmotic shock, cells lose water and shrink. **c** | They recover full size by the accumulation of more K^+ and glutamate as well as other compatible solutes (light blue circles). Under these conditions the MS channels remain closed. **d**,**e** | Dilution of the cells into a low osmolarity environment (hypoosmotic shock, panel **d**) leads to the rapid entry of water accompanied by the immediate activation of the MS channels, which results in the loss of low molecular mass solutes (K^+, glutamate and compatible solutes) but the retention of large proteins and solutes (brown circles and yellow ovals) (panel **e**). **f** | If the channels are absent or fail to open, the influx of water generates high turgor pressure, which leads to the lysis of the cell when the pressure exceeds the mechanical strength of the wall[1].

the cell envelope a dynamic structure in which MS channels can have other important, but as yet not fully recognized, roles.

The strongest phenotype currently associated with MS channel activity is due to the role of these proteins in survival from hypoosmotic shock. In turgid cells, the cytoplasmic membrane is pushed hard against the cell wall, which, on this short (millisecond to second) timescale, has limited capacity to stretch. The rise in turgor pressure associated with entry into dilute medium results in deformation of the cytoplasmic membrane, and consequently the strain on the cell wall is increased. If this strain is not relieved it can lead to cell lysis[1] (FIG. 1). When MS channels open, they create large pores that allow hydrated solutes to pass through freely, essentially at the rate of diffusion in free solution[5,6]. This enables a rapid decrease in the cytoplasmic concentrations of osmotically active solutes and so lowers the osmotic driving force for water entry. Early analyses of amino-acid pools in *E. coli* cells indicated their rapid release in response to hypoosmotic shock[15]. Although at that time channels were unheard of in bacterial cells, these data were consistent with the large changes in membrane permeability that have subsequently been partially explained by the discovery of MS channels. The application of patch-clamp techniques to bacterial protoplasts[16] and membrane proteins reconstituted into liposomes[17] led to the discovery of different types of MS channel proteins, each of which is associated with a defined conductance, open dwell time and pressure sensitivity.

With our current knowledge on MS channels, we can pose a set of questions concerning their characteristics and roles in bacterial physiology (BOX 1). This Review describes our current understanding of the answers to these questions, highlighting recent developments in the strategies that are used to drive the analysis of these key bacterial membrane proteins to deeper levels.

Patch clamp
A technique whereby a small glass electrode tip is tightly sealed onto a patch of cell membrane, thereby making it possible to record the flow of current through individual ion channels or pores in the patch.

Conductance
Calculated from the increase in current when a single channel is fully open, under known conditions of applied transmembrane voltage.

Open dwell time
The average time a single channel remains in the fully open state under conditions of constant transmembrane pressure and voltage; this parameter can only be determined statistically based on the analysis of many single openings of channels that occur over several minutes in a patch-clamp recording.

Box 1 | **Key questions regarding microbial mechanosensitive channels**

- Which gene product correlates with the measured activity?
- What are the structures of the mechanosensitive (MS) channels in both the closed and open states?
- How do the channels interact with membrane lipids to sense tension?
- What is the significance of the diversity of structural types of MS channels?
- What are the roles of the MS channels in bacteria, archaea and organelles?

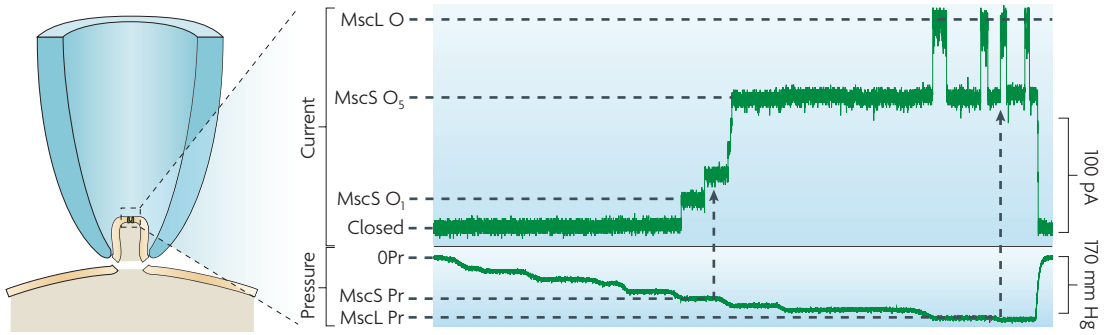

Figure 2 | **Measuring the conductance and pressure sensitivity of MscS and MscL.** A representative trace from a patch-clamp experiment is depicted, showing current activity against the pressure applied. Protoplasts were prepared from *Escherichia coli* cells expressing wild-type MscS (from a plasmid) and native MscL channels. Currents were recorded from an inside-out excised membrane patch at a membrane holding potential of +20 mV. Baseline (closed), a single opening of MscS (MscS O_1), the five fully open MscS channels present in the patch (MscS O_5) and the single-channel open level of MscL (MscL O) are indicated on the current trace. Measurement of the current amplitude of a single channel opening allows the calculation of channel conductance using Ohm's law. To establish pressure sensitivity, the levels of pressure required to open at least two MscS channels and generate frequent openings of MscL (highlighted by arrows) are determined and the appropriate pressure ratio is calculated.

MS channels

Three structural classes of MS channels have been detected in bacterial and archaeal cells by electrophysiology: MscL, MscS and MscK (FIG. 2). A fourth activity, which has been detected reproducibly in *E. coli*[18] and is called MscM, has not been genetically characterized but might belong to the same structural class as MscS. MscL and MscS have been studied the most extensively, aided by the crystal structures of these proteins[19,20]. These two channel proteins are generally not found in animals, but homologues are present in plants and in some fungi and oomycetes. Other structural classes of MS channels are known to exist in higher organisms, from yeast to humans[21].

Cellular homeostasis and energy transduction through the protonmotive force are dependent on the relative impermeability of the cytoplasmic membrane to ions (H^+, K^+ and Na^+)[14]. So, the presence of MS channels in the same membranes requires that these channels must be impermeable to even the smallest ions in their closed states. When open, MS channels create pores estimated to be ~16 and ~40 Å in diameter for *E. coli* MscS and MscL (MscS-Ec and MscL-Ec), respectively[4,22] (note that in this article the channel proteins will be designated by the channel type followed by an abbreviation for the relevant microorganism). The open channels are freely permeable to solutes that are <400–500 Da, such as disaccharides, amino and organic acids, polyamines, aminosugars and, possibly, ATP[5,6,23]. Early studies with mutants that lack MscL suggested that small proteins could pass through the open channel[24–27]. However, the mechanistic basis of these observations has been questioned[26,27]. More recent experiments, with reconstituted channels and fluorescently labelled compounds, have established that small proteins of ≤6.5 kDa can pass through open MscL channels[28], but the proteins that were previously reported to pass through MscL were much larger. Biotechnological applications based on protein and small-molecule translocation have been proposed; for example targeted or slow drug release via MS channels[26,28,29].

Channel gating leads to the release of solutes from the cytoplasm, but at the same time renders the cytoplasmic membrane transiently permeable to the ions that are present in the environment, thereby perturbing cellular homeostasis[6]. Clearly, these channels will therefore only open when extreme remedies are required to counter the stress to which the cell is exposed. Different channel classes, and members of the same class from different organisms, display subtle differences in gating pressure, selectivity and structure[21,30]. Electrophysiological analysis has yielded four major classes of MS channels in *E. coli*[18,23]. However, it is well documented from mutant studies that the correlation between electrophysiological signal and physiological activity is not perfect[31]. Extensive studies of both MscL-Ec and MscS-Ec mutants have shown that channel proteins that confer phenotypes in physiological assays do not always give rise to measurable channels in patch-clamp assays. To understand the anomalous behaviour of these channel proteins one needs some insight into the assay systems that have been developed to detect MS channel activity (BOX 2). For example, analysis of the *Lactococcus lactis* MscS homologue YncB (MscS-Ll) confirmed that the protein could be expressed in *E. coli* and displayed typical MscS-like activity that was not evident in membranes derived from *L. lactis* cells[32]. Similarly, *E. coli* has multiple homologues of MscS that are expressed in growing cells, are regulated in their expression and that protect cells against hypoosmotic shock when overexpressed (I.R.B., unpublished data). However, they appear to be electrically silent in patch-clamp analysis.

The closed and open states of MscL and MscS

The crystal structures of the *Mycobacterium tuberculosis* MscL (MscL-Mt) and MscS-Ec channels have been solved[19,20] (FIG. 3). MscL-Mt is a homopentamer and MscS-Ec is a homoheptamer[19,33,34]. One of the major

Pressure sensitivity
The pressure required to open channels is most often quoted relative to another channel. For example, the pressure required to open MscL-Ec is often quoted as a ratio by reference to the pressure required to achieve the first openings of MscS-Ec in the same membrane patch. Absolute measures of sensitivity to membrane tension can be achieved only by measuring the curvature of the patch under pressure using video microscopy and by applying Laplace's law, which relates the tension in the bilayer to the transmembrane pressure through the radius of the curvature of the patch.

Protonmotive force
The protonmotive force is created when protons are expelled from the cell during respiratory and photosynthetic electron flow or by the action of an ATPase. The protonmotive force consists of the proton gradient (ΔpH) and a gradient of charge (ΔΨ). Proton (and Na^+) ions enter the cell, driven by the protonmotive force, to do useful work such as ATP synthesis, flagellar rotation and membrane transport.

structural differences between MscL and MscS is the complexity of MscS. Whereas each MscL subunit contains ~136–156 residues, MscS-Ec contains 286 residues and is representative of a family that also includes proteins as large as MscK at 1,120 amino acids. Many MscS homologues are predicted to have more than the three transmembrane (TM) spans observed in MscS-Ec.

The simpler contrast between MscL and MscS lies in the complexity of their carboxy-terminal domains and in their mechanisms of pore formation. MscL proteins generally have a short C-terminal α-helix that forms a bundle below the pore. Much of this helix can be deleted without severely compromising channel activity; however, a semiconserved cluster of basic amino acids is essential for activity[35]. Recent work suggests that this cluster of basic residues might interact with acidic phospholipids, and the expression of a mutant channel in which these residues are substituted with neutral amino acids leads to growth inhibition, consistent with the channel gating at low pressures[36]. By contrast, MscS has a large C-terminal domain that contains several subdomains that come together to define a large vestibule at the cytoplasmic surface of the membrane. The channel pore is accessed from the cytoplasm by lateral portals at the interfaces between the subunits, and so all solutes must pass through the vestibule as they exit from the cell.

The C-terminal domain of MscS-Ec is flexible, allowing the formation of intersubunit disulphide bridges between Cys residues inserted at positions that are ~20 Å apart in the crystal structure[34]. Deletions that disrupt the base of the vestibule do not prevent the assembly or gating of the channels, although they do impair stability[37]. Mutant channel proteins containing larger deletions that would remove subdomains from the C-terminal domain are not sufficiently stable to assemble in the membrane[37]. The base of the C-terminal domain is a seven-stranded β-barrel (one strand from each subunit). Extensions of this sequence are found in homologues in other bacteria, and even MscS-Ec can be lengthened by the addition of either alkaline phosphatase[38] or green fluorescent protein (I.R.B., unpublished data) without significant impairment of gating. Similar observations have been made for MscL-Ec[39].

There is still a debate about whether the crystal structures of MscL-Mt and MscS-Ec represent closed or open states, or intermediates in the transition from closed to open that have been stabilized by the crystallization conditions[34,40–42]. In the MscL-Mt structure, the pore-forming TM1 helices approach each other sufficiently closely that a seal is created by a ring of hydrophobic residues (Val21). Indeed, the whole pore-lining surface is predominantly hydrophobic, particularly around Val21 (REF. 19). Similarly, the pore-lining surface is hydrophobic in MscS-Ec[20,40]. The parallel with K^+ channels and the acetylcholine receptor (AchR), which are also sealed by rings or clusters of hydrophobic amino acids, has led to the view that even an apparently open pore observed in the crystal structure might be operationally closed because the hydrophobicity of the pore-lining surface prevents a column of water from forming[40,43,44] (see BOX 3 for more information on this vapour-lock mechanism). In the absence of a water column, ion movement will be blocked, even though the hydrated ion can theoretically pass through the pore[43]. Sansom and colleagues have defined the ability of an 'open pore' to block ion movement by the relationship between the pore diameter and character and the size of the ion with its first hydration shell[43]. For water alone, the lumen of the pore can be blocked even when the diameter is as wide as 9 Å, and for cations, such as K^+, this is increased further to 13 Å[43]. The double rings of hydrophobic residues frequently encountered in ion channels and MS channels might create a long enough hydrophobic pore to block the channel. In terms of evolution, this structure would ensure that mutations that introduce hydrophilic residues to these

Box 2 | **Measuring mechanosensitive channel activities**

The available assay systems for mechanosensitive (MS) channels can be broadly broken down into *in vitro* and *in vivo* assays, each of which provides unique insights into channel function.

***In vitro* assays**

Electrophysiology[16,77] is the gold standard for the detection and analysis of MS channel activities, and it allows the measurement of the intrinsic parameters that describe channel activity. Bacterial cells are too small to be analysed directly by patch-clamp methods. This has been overcome by generating giant protoplasts from cells[16], by fusing membranes with liposomes[18] or by inserting the purified channel into liposomes[78]. The formation of a GΩ seal between a fine glass pipette (diameter 3–10 μm) and the lipid of the membrane ensures that the ion movement measured takes place through the channel. Imposing a membrane potential across the membrane patch drives ion movement through the open channel. Individual channel openings and closures are observed as 'step changes' in current (FIG. 2). Controlled application of a pressure differential across the membrane patch leads to channel gating. One can then measure the key parameters of pressure sensitivity, open dwell time and conductance, as well as ion selectivity[16,79], the effects of chemicals[23] and the influence of compounds that intercalate into the membrane[66].

Electrophysiological data can be correlated with other biochemical measurements. Significant insights have been obtained using the intrinsic fluorescence of Trp residues[36,65,80] and electron paramagnetic resonance (EPR) spectroscopy[56,57,81]. Both studies require the mutagenesis of the channel gene to introduce single Trp or Cys residues. Chemical modification combined with single introduced Cys residues has also proved to be informative[33,59,82,83] in the analysis of the *Escherichia coli* MscL channel.

***In vivo* assays**

In vivo methodologies provide both complementary and novel insights into channel properties, but they are subject to greater caveats in interpretation. Analyses can be conducted with the native channel, often expressed from a plasmid. Such measurements can be restricted to simple determination of function in relieving the osmotic gradient across the cell membrane (FIG. 1). The presence of the cell wall means that the observed pressure sensitivity bears little relationship to that measured by electrophysiology — however, channels with different gating characteristics retain these in *in vivo* assays. Channel mutants can be assessed for their gain or loss of function. Gain-of-function mutations cause the channel to gate at low pressures and are frequently detected by their inhibition of growth when the mutant channel is overexpressed[38,58,73]. This assay can miss subtle phenotypes, but this difficulty can be overcome by modifying either the growth environment or the genetic background of the strain[84–86]. Gain-of-function mutations often fail to protect cells against hypoosmotic shock owing to the general perturbation of homeostasis. Loss-of-function mutations are detected by the loss of the ability of the channel to protect a strain against hypoosmotic shock, leading to cell lysis[1,38].

These assays are qualitative rather than quantitative. Only low numbers of active channels are required for high levels (30–100%) of survival[1,11]. Consequently, 100% survival of cells after shock can be obtained with a small number of channels that are highly active or a large number of channels that possess only a small tendency to gate in the pressure range[37]. A clearer indication of channel function is often gained by studying cells in which the channels are expressed at a very low level[38].

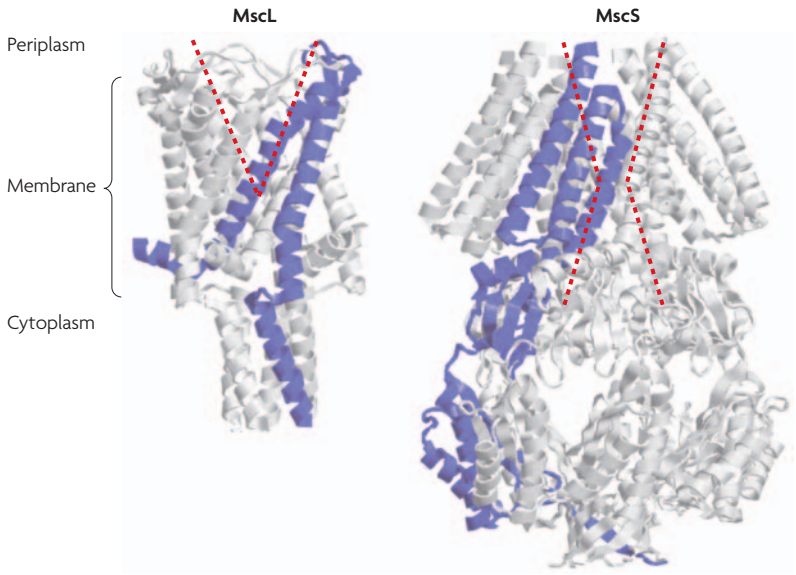

Figure 3 | **Structures of MscL-Mt and MscS-Ec.** A single subunit in the mechanosensitive channels MscL from *Mycobacterium tuberculosis* (MscL-Mt)[19] and MscS from *Escherichia coli* (MscS-Ec)[20] is highlighted (blue). Note that in MscS-Ec, the pore-lining transmembrane-3 (TM3) helix is considered to be composed of two parts: TM3A, the actual pore-lining helix, and TM3B, a continuation of the helix that lies along the membrane surface after the helix breaks around Gly113. The broken red lines indicate the general shape of the vestibule and the pore, as created by the helix packing in the crystal structures.

rings do not generate channels that are permanently open[38,45]. Indeed, gating and the barrier function are separate — mutant channels with hydrophilic substitutions in the pore-sealing ring, whether AchR, MscL or MscS, still require an additional conformational change to achieve the open state, although this is achieved at either lower ligand concentrations (for AchR[46]) or lower pressures (for MscS-Ec and MscL-Ec[45,47]).

On the basis of the crystal structures, the challenge of maintaining a barrier to proton and ion entry appears less severe for MscL-Mt than for MscS-Ec, in which an 'open pore' is evident at the constriction zone created by rings of Leu105 and Leu109 (REF. 20). Recent revisions to the crystal structure of MscS-Ec[48] and Brownian dynamics simulations[49] have suggested that the pore constriction around Leu105 and Leu109 is ~5–6 Å in diameter, which places the pore well within the range for the vapour-lock mechanism[40,43] (BOX 3). Rees's group and others have determined that the packing of the TM3 pore-lining helices of MscS-Ec is sufficiently tight that it would be difficult to further narrow the pore without collapsing the structure[20,50,51]. The open pore is predicted, based on the electrophysiology[4] of the reconstituted protein, to be ~16 Å in diameter, and consequently the crystal structure is unlikely to represent the fully open state.

The situation is more complicated for MscS-Ec. Under sustained pressure in patch-clamp experiments, MscS-Ec is inactivated and can only be resuscitated by allowing the patch to relax by removing the pressure[1,52]. So, for this protein there are a minimum of three stable states: closed, open and inactivated. Little is known about the inactivated state, but it is clear that the crystal structure might represent either the non-conducting form or the closed state[53]. The emerging consensus is that the crystal structure of MscS-Ec is unlikely to represent the open state.

The closed–open transition. High-resolution analysis of the opening of MscL-Ec and MscS-Ec has shown that the first structural transitions take place on the microsecond timescale[54]. MscL-Ec opens through several small conductance substates. Sukharev and colleagues have carried out detailed analyses of the substates exhibited by mutant MscL-Ec channels that gate more readily than the wild-type channel, and have shown that they exhibit stable occupancy of the lower conductance substates[55]. High-resolution measurements of the MscS-Ec gating transition show that the channel opens to around two-thirds conductance and then opens fully on a slower timescale[55]. Mutant analysis has shown that MscS-Ec channels, particularly those in which Val is substituted into the pore-lining surface, can adopt stable substates[45]. These studies point to the closed–open transition taking place rapidly, but through several (>2 for MscL-Ec) intermediate conformations.

The MscL gating transition. The elegant electron paramagnetic resonance (EPR) studies by Perozo's group[56,57], supported by genetic[33,35,42,58–60] and biophysical[17,55,61,62]

Inactivation
(also known as desensitization). MscS-Ec has been observed to undergo spontaneous loss of channel activity when held under constant pressure; activity can be restored to the majority of channels in a patch by resting the membrane (re-setting the pressure to zero) for a short period before re-imposing pressure.

Electron paramagnetic resonance
Observation of the transitions between spin states of an unpaired electron in a magnetic field.

Box 3 | **The vapour-lock mechanism of channel occlusion**

The crystal structure of the *Escherichia coli* mechanosensitive channel MscS revealed a central pore that has a diameter of ~8 Å[20]. This led to the reasonable speculation that the crystal form might be in the open conformation. However, the cross-sectional area of the pore did not appear to be sufficient to allow for rapid conduction of hydrated solutes, as would be required to relieve osmotic stress[4,40]. A vapour lock had been proposed as a mechanism to block channels that appear, by structural criteria, to be perpetually open but that achieve non-conducting states[40,87]. By analogy with carbon nanotubes, it was proposed that channel pores lined with strongly hydrophobic residues would be unable to be wetted by water and so would be blocked by the formation of a vacuum or vapour lock[43,44]. The formation of a vapour lock is strongly dependent on the diameter of the pore. A narrow hydrophobic pore favours the formation of a vapour lock. So, channel opening would involve the movement of transmembrane helices away from the central axis of the pore, such that the pore diameter is increased, leading to wetting and consequent ion conduction.

MscS fulfils all the criteria for a vapour-locked channel. Despite close packing of the pore-lining helices in the crystallized state, the constriction at Leu105 and Leu109 is insufficient to completely close the channel[40]. The pore lining is extremely hydrophobic along its length, particularly above Leu105 (REF. 40). The model proposed for the gating of the channel involves turn and tilt of the pore-lining helices, which would create a pore of sufficient diameter to allow ion conduction[45].

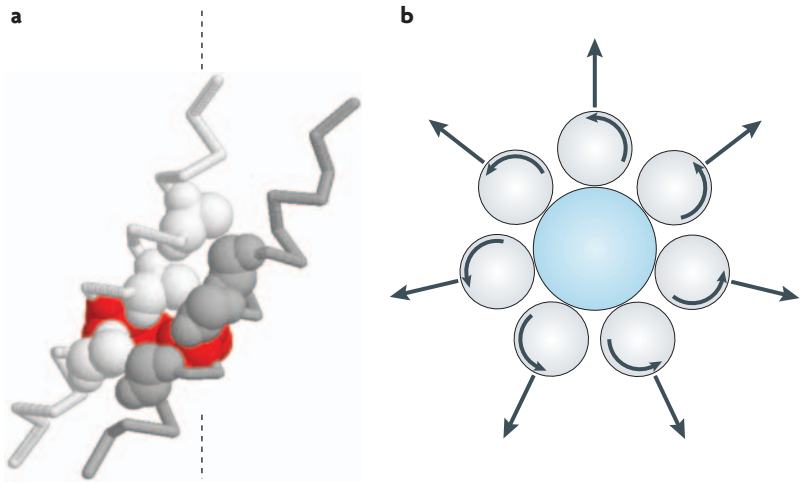

Figure 4 | **Helix packing and proposed gating movement in MscS-Ec. a** | The pore-lining regions (residues 93–113) of transmembrane-3 (TM3) helix from two subunits are depicted, showing the upper seal residue (Leu105; red), the three Ala residues (white) and the three Gly residues (grey) that form the helix interface. The broken line depicts the axis of the pore. The periplasmic side is placed at the top of the diagram. **b** | The seven TM3 helices visualized from the periplasmic face; each curved arrow indicates that during gating the helix rotates, and the straight arrows indicate that the helices adopt a more marked tilt away from the pore axis[45]. The blue circle represents the pore. The energy barrier for rotation is low owing to the complementary surfaces created by the Ala and Gly residues. Introducing bulky residues on either surface increases the pressure required for the transition to the open state[45].

analyses, have elucidated the principal structural alteration that underlies the MscL gating transition. MscL is a homopentamer, in which each subunit has two TM helices, TM1 and TM2, separated by a periplasmic loop. The TM helices are flanked by short cytoplasmic helices; recent refinement of the analysis of the crystallographic data revealed that the amino-terminal helix (residues 1–12) adopts an α-helical structure and is probably positioned in the plane of the membrane, threaded between the TM1 and TM2 helices of an adjacent MscL subunit[48] (FIG. 3). In the revised structure, the periplasmic loop is thought to form two anti-parallel β-strands at the surface of the protein. These changes have led to a convergence between the structures arrived at by crystallography and those predicted on the basis of biochemical analysis[62,63].

The relative importance of TM1 compared with TM2 was shown not just by genetic analysis[47], but also by studies in which TM1 was expressed independently of TM2, purified and reconstituted. Ion channels of variable conductivity resulted from the reconstitution of purified TM1 helices, but with no pressure-sensitive gating[64]. Co-reconstitution with purified TM2 helices, which had no channel-forming capacity alone, regenerated MS channel activities similar to those of MscL. The resultant channel gated at lower pressures than intact MscL. These data point to the crucial importance of TM2 in forming a gated structure, and also indicate a role for the periplasmic loop in setting the energy levels needed to gate MscL[64].

In the closed state, five TM1 helices cross close to the cytoplasmic side of the membrane to create the hydrophobic seal (Val23 and Val21 in MscL-Ec and MscL-Mt, respectively) while the TM2 helices interact predominantly with TM1 and with the lipid bilayer. Hydrophilic residues from both TM1 and TM2 contribute to the vestibule on the periplasmic side of the channel, creating a funnel-like appearance[48]. However, the channel provides a pathway for solute movement from the cytoplasm to the periplasm, and consequently this structure, like that of MscS, is probably not significant for solute movement. Attainment of the open state was analysed by trapping an intermediate and the fully open state (by modification of the lipid composition of the membrane and by perturbation of membrane tension with lysophosphatidylcholine[56,57]). By analysing the EPR signals from a nitroxide spin label attached to individual Cys residues inserted at different positions in TM1 and TM2, a model was derived for the intermediate and fully open states. From these analyses, opening the channel is considered to be achieved by rotation and tilting of the helices in the membrane plane such that they adopt a more acute crossing angle relative to the crystal structure. This movement is accompanied by a shift in TM1 away from the axis of symmetry to a more peripheral position, thereby breaking the seal[56,62,63].

Structural transitions in MscS. The closed–open transition in MscS is proposed to arise from the sliding of the TM3 helices across each other such that the pore is widened at the crucial Leu105 and Leu109 dual ring[45]. As indicated above, the TM3 pore-lining helices are packed tightly against each other in the crystal structure. This arises from the complementary surfaces created by matching Gly-rich and Ala-rich helix faces (FIG. 4). The TM3 of MscS-Ec and its most closely conserved homologues exhibit a repeating pattern of Gly and Ala residues spaced such that they create a series of 'knobs' (Ala) and 'grooves' (Gly). In the solved structure, Ala106 on one helix interacts with Gly108, but the other matched pairs (Ala98–Gly101 and Ala102–Gly104) are slightly more widely spaced. To bring these pairs of residues into close contact would involve helix rotation and an increase in the tilt of the helices relative to the axis of symmetry. Rotation of the helix could move the bulkier residues (Leu, Val and Ile) into the helix interface, thereby separating them and widening the channel. Support for this model was generated by mutant creation and analysis[45]. Substituting conserved Gly and Ala residues with bulkier residues (Ala and Val, respectively) created channels that were more difficult to open. This is the predicted outcome, as the bulkier residues would require greater energy for them to pass by each other during helix rotation. Correspondingly, converting Ala106 to Gly106 created a channel that gated more easily as the resistance provided by the knob was removed. Normal function could be restored to the Ala106Gly mutant channel by simultaneously converting Gly108 to Ala108, thereby creating complementary surfaces but with the knob coming from the opposite face of the helix compared with the wild-type channel[45]. More detailed analysis of this type has revealed that Ala102 is crucial for the stability of the open state of MscS-Ec; Ala102Gly mutant

channels exhibit extremely short open dwell times that can be restored by introducing Ala residues at either Gly101 or Gly104 (I.R.B., unpublished data). So, the TM3 helices exhibit strong summation properties — mutations at one point in the helix can provide some compensation for changes at other positions. However, this property is least evident in the region of the hydrophobic seal, emphasizing the importance of specific residues in this region.

Interactions with the lipid environment

The pore is essentially sheltered from the lipid environment of the membrane and so changes in the lipid phase that are caused by increased membrane tension must be transmitted through the 'wall' of the channel. For MscL, each subunit has been calculated to be exposed to approximately 3 phospholipids in each leaflet of the bilayer, giving an annulus of 30 lipid molecules[65]. Similarly MscS — assuming a diameter of ~50–60 Å, which requires that in the membrane the TM1–TM2 domain is packed against the TM3 pore helix — would be exposed to an annulus of ~35–40 phospholipids.

Gating of both MscL and MscS can be achieved by generating membrane deformation through the introduction of local anaesthetics[66] or lysophosphatidylcholine[57] into one leaflet of the membrane (I.R.B., unpublished data). This is consistent with the two channels possessing similar sensing mechanisms but different thresholds, leading to differential activation of the channels. Understanding how the change in the membrane is sensed is a major challenge that shifts the emphasis from the simple structural prerequisites for forming a channel to the interaction of the channel protein with the lipid bilayer. Although all integral multispan membrane proteins must experience the membrane perturbation, only a small class — those eventually defined as mechanosensing proteins — respond to the change with altered activity.

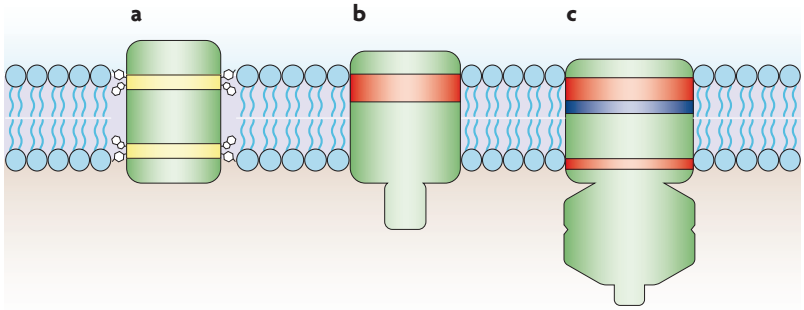

Figure 5 | **Defining the interactions that might prevent mechanosensation. a** | In the majority of membrane proteins, there is an enrichment of Tyr and Trp residues close to the periphery of the membrane helices (yellow bands), where these residues can interact with the glycerol groups of the phospholipids[71]. **b** | Cartoon of MscL. In the red region, the introduction of residues (Trp, Tyr, Asn or Asp) that can interact with glycerol from phospholipids leads to a reduced capacity for mechanotransduction[60,68]. **c** | Cartoon of MscS showing similar regions that are associated with loss of function upon the introduction of Asn residues (red). In addition, the region of MscS that is associated with gain-of-function mutations that can arise by modified interactions with lipid headgroups is indicated (blue)[67,73].

Can one define essential differences between sensitive and insensitive proteins? In essence, studies so far have defined what is absent that would normally 'prevent' mechanotransduction[60,67,68]. In art, the term 'negative space' is used to describe the space around an object; negative space can be at its most interesting when it defines a fascinating relevant shape. In a sense, we can define what we understand about the interaction between MS channels and the lipid environment as negative space — it is what is absent that is most informative. Multispan membrane-protein topology is normally determined by the presence of basic amino acids at the cytoplasmic end of some of the helices (the 'positive-inside' rule of von Heijne[69,70]). MscL and MscS appear to follow this rule, although it is less clear for MscL-Mt than for MscS-Ec. Many multispan proteins for which the crystal structure has been determined display peripheral rings of aromatic amino acids close to the interface with the phospholipid headgroups. Frequently, it is observed that Trp residues burrow down into the lipid, but Tyr residues are orientated such that their hydroxyl groups point to the glycerol molecules at the aqueous interface of the membrane[71] (FIG. 5). Tyr and Trp residues are infrequent in the membrane domains of both MscS and MscL[62]. The introduction of Tyr or Trp into MscL-Ec slowed the kinetics of gating and decreased the sensitivity to pressure. The severity of the mutations for channel function was dependent on the distance between the 'capping' aromatic residues. Similarly, introducing an acidic residue into TM2, in close proximity to the end of the helix, impaired channel gating[60].

Complementary data were obtained from a suppressor screen of a MscL-Ec Gly22Asp mutation that exhibited an extreme gain-of-function phenotype[68]. A systematic analysis, using Asn as a substitute for hydrophobic residues in both TM1 and TM2, confirmed that only amino-acid changes at the periplasmic ends of the helices were strong blockers of channel gating[68]. Much weaker effects were observed when changes were introduced to the cytoplasmic end of the helices. It has been proposed that constraints on the cytoplasmic end of TM2 are imposed by a cluster of basic residues that interact with lipids[36]. Mutation of these residues renders the channel easier to gate. These data are consistent with the proposed change in the structure of MscL during gating — the tilt and rotation of the helices would be constrained if there were strong interactions with lipid headgroups.

Similarly, Asn-scanning mutagenesis[67] also provided new insights into the gating movement in MscS. It was recognized from the determination of the crystal structure that TM1 and TM2 could behave as an almost independent entity, in the manner of the 'paddle' of the voltage-gated K^+ channel KvAp[72]. Indeed, it has been demonstrated that this 'domain' (residues 1–94) could be stably expressed as an alkaline phosphatase fusion protein[38]. From crystallographic data it was predicted that gating would arise from the movement of this domain under the influence of membrane tension. The change in position would be transmitted to

the pore domain through an extended linker (residues 90–94) at the periplasmic end of the pore. Consistent with the crucial role of this sequence, gain-of-function mutations have been identified in this linker (specifically, Thr93Arg[38]). Recent studies in which Asn residues were substituted for hydrophobic residues at the ends of TM1 and TM2 of MscS have yielded data similar to those acquired for MscL[67,68]. Substitutions at the periplasmic ends of the helices had the most dramatic effects and rendered MscS harder to gate. Asn substitutions that made the channel easier to open were also identified. The affected residues lie on TM1 towards the middle of the membrane. It seems that to satisfy the hydrogen-bonding potential of the introduced Asn residue, the TM1–TM2 domain must be pulled away from the pore, poising the channel towards the open state. This observation parallels the discovery of an MscS gain-of-function mutation, Val40Asp, in a screen of randomly introduced mutations[73]. It can be inferred from these data that increasing the hydrogen-bonding potential at the end of the helices decreases sensitivity to membrane tension, but introducing similar residues deeper into the helix can cause the channel to gate more easily.

Correlating diversity with physiological function

Mechanotransduction is almost ubiquitous, but there are many molecular solutions rather than a single channel type that evolved in bacteria and is retained by higher organisms[21]. A partial explanation for this might be related to the specific functions of the channels in different organisms. In animal cells, K^+, Na^+ and Cl^- gradients are central to sensory processes and to whole-body homeostasis. Here, the adoption of the bacterial nonspecific channels to relieve osmotic imbalance could be too great a price to pay. Plants (and some fungi), however, use nonspecific channels to great effect in their organelles. In bacteria, the major role most frequently associated with MS channels arises from their action as emergency safety valves during hypoosmotic shock. However, this does not readily explain the multiplicity of the channels or their presence in higher organisms. Channel diversity in bacteria has been linked to preferred environmental niche[74]. An expansion in the numbers of both MscL and MscS homologues was observed in a comparison of genomes of *Synechococcus* species isolated from coastal and oceanic niches. Species from coastal niches might be expected to be subjected to the greatest environmental variation, and this was proposed to correlate with the larger number of channel homologues.

In plants it could be the diversity of organelle structures, which are thought to derive from bacterial endosymbionts, that requires the multitude of MS channel homologues. Genomic data from *Arabidopsis thaliana*, which has ten MscS homologues, indicates that *mscS* genes have been acquired on at least two separate occasions[75]. Sequence analysis shows that the genes fall into two clusters, one defined as plant-like (that is, sequences that are evolutionarily remote from bacterial channels but that are closely related to homologues found in other plants and green algae) and the other as bacterial. Of three of the bacterial type, two are located in the chloroplast membrane. Deletion of both channels caused defects in chloroplast structure, leading to a mild variegation phenotype[75]. Similar observations have been made in *Chlamydomonas reinhardtii*[76]. These are exciting findings that implicate MscS-type channels in the structural integrity of organelles. Chloroplasts might be expected to undergo diurnal changes in stromal osmolarity as a consequence of photosynthesis. Potentially, MscS, which is activated at ~70–150 mm Hg pressure (corresponding to a change in solute concentration of 5–10 mosM), would be activated during such transitions. In the absence of a cell wall, the chloroplast will need to make subtle adjustments.

Bacterial cells are protected by the peptidoglycan cell wall and, in Gram-negative bacteria, by the lipopolysaccharide (LPS) of the outer membrane. *E. coli* MS channel activation (considering all measurable channels: MscM, MscK, MscS and MscL) in membrane patches occurs over the range of ~70–250 mm Hg. Turgor pressure in *E. coli* cells is ~3,040 mm Hg (4 atm), which means that if the MS channels are to remain closed the turgor must be balanced by the resistance of the cell wall (peptidoglycan and LPS) (that is, the net pressure must be <50 mm Hg, the lowest recorded pressure for the activation of *E. coli* MS channels). Experiments conducted to determine the degree of hypoosmotic shock required to activate MS channels in *E. coli* K12 cells suggested that a change of ~300 mosM (150 mM NaCl, ~2 atm) was essential. This pressure lies immediately below the lysis pressure of cells that lack MscS, MscK and MscL[1]. More needs to be understood about the interplay between the membrane and the wall as this interplay ensures that, in bacteria, small changes in osmolarity do not activate MS channels and thereby disrupt cellular homeostasis.

Conclusions

Without the crystal structures for MscS and MscL, we could not have made the significant progress towards understanding mechanotransduction that has been achieved over the past decade. These structures have allowed the hypothesis construction and testing that has enabled researchers to progress towards a consensus on the closed–open transition and on crucial determinants of channel function. Molecular genetics is beginning to contribute to our understanding of the interactions that take place at the protein–membrane lipid interface, at least in defining negative space. However, it is often overlooked that the analysis of a mutant channel is mainly informative about the mutant, and considerable care must be taken in extrapolating observations from mutant studies to the wild-type protein. We look ahead to further contributions from biophysical and biochemical analyses to ascertain the working relationship between MS channel proteins and membrane lipids, such that we might then be able to more fully define the functions of the multiple MS channel homologues that are present in microbial systems.

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Competing interests statement
The authors declare no competing financial interests.

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