8-27-2013

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Journal:	*Biochemistry*
Manuscript ID:	bi-2013-00804z
Manuscript Type:	Current Topic/Perspective
Date Submitted by the Author:	21-Jun-2013
Complete List of Authors:	Wilson, Margaret; Washington University in St. Louis, Maksaev, Grigory; Washington University in St. Louis, Haswell, Elizabeth; Washington University, Biology

MSCS-LIKE MECHANOSENSITIVE CHANNELS IN PLANTS AND MICROBES

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KEYWORDS

Mechanosensitive, MscS, MSL, ion channel

ABBREVIATIONS

MS, mechanosensitive

MscS, mechanosensitive channel of small conductance

MscL, mechanosensitive channel of large conductance

MscM, mechanosensitive channel of mini conductance

MscMJ, mechanosensitive channel of *Methanococcus jannaschii*,

MscMJLR, mechanosensitive channel of *Methanococcus jannaschii* of large conductance and rectifying

MSL, MscS-Like

Msy, MscS from yeast

MscCG, mechanosensitive channel of *Corynebacterium glutamicum*

MscSP, mechanosensitive channels of *Silicibacter pylori*

TM, transmembrane

KcsA, potassium crystallographically-sited activation channel

pS, picosiemen

nS, nanosiemen

pA, picoampere

P_{Cl^-}, preference for Cl^- ions

MSC, mechanosensitive channel

EPR, electron paramagnetic resonance

MD, molecular dynamics

ABSTRACT

The challenge of osmotic stress is something all living organisms must face as a result of environmental dynamics. Over the past three decades, innovative research and cooperation across disciplines has irrefutably established that cells utilize mechanically gated ion channels to release osmolytes and prevent cell lysis during hypoosmotic stress. Early electrophysiological analysis of the inner membrane of *Escherichia coli* identified the presence of three distinct mechanosensitive activities. The subsequent discoveries of the genes responsible for two of these activities, the mechanosensitive channels of large (MscL) and small (MscS) conductance, led to the identification of two diverse families of mechanosensitive channels. The latter of these two families, the MscS family, is made up of members from bacteria, archaea, fungi, and plants. Genetic and electrophysiological analysis of these family members has provided insight into how organisms use mechanosensitive channels for osmotic regulation in response to changing environmental and developmental circumstances. Furthermore, solving the crystal structure of *E. coli* MscS and several homologs in several conformational states has contributed to the understanding of the gating mechanisms of these channels. Here we summarize our current knowledge of MscS homologs from all three domains of life, and address their structure, proposed physiological functions, electrophysiological behaviors, and topological diversity.

INTRODUCTION

I. Ion Channels

Ion channels are membrane-spanning protein complexes that form a gated macromolecular pore. An open channel can facilitate the passive diffusion of tens of millions of ions per second from one side of the membrane to the other, down their electrochemical gradient [1, 2]. The role played by ions in the excitable membranes of muscle and nerve cells has been studied for over a hundred years [3] and the importance of ion channels as mediators of the nervous system and their role in human disease is now well established (several recent reviews include [4-6]). However, plant and microbial ion channels have also been important subjects of study [7, 8]. It is often forgotten that single-cell action potentials were first described in the giant cells of characean algae and that during the 1930s, the excitation of squid axons and algal membranes was studied side-by-side (reviewed in [9-11]. The bacterial potassium crystallographically-sited activation channel (KcsA) was the first ion channel to be characterized by X-ray crystallography [12], and it is now understood that bacteria have a wide array of ion-specific, mechanosensitive, and water channels [13]. Investigations into plant and microbial ion channels not only inform our understanding of basic cellular physiology, but may also be instrumental in engineering defenses against microbial pathogens and in crop improvement [14, 15].

Ion channels can be classified according to homology-based family groupings or behavioral characteristics such as ion selectivity or gating stimulus (in addition to other more subtle behaviors such as conductance, adaptation and opening or closing kinetics). Many channels are specific to the ion or small molecule that they allow to pass (KcsA has a 1000-fold preference for K^+ over Na^+ ions [16]), while others are not (the bacterial mechanosensitive ion channel of large conductance (MscL) has no ionic preference at all [17]). Channel conductance, the ease with which current passes from once face of the channel pore to the other, can range over several orders of magnitude in different channel types and organisms. For example, the aforementioned MscL has one of the largest conductances measured, up to 3

nS [17], while the small potassium (SK) channels associated with Parkinson's disease have a conductance of only 10 pS [18]. The burst of ion flux that results from the rapid opening of an ion channel (occurring on the order of milliseconds) can have several downstream effects: a change in membrane potential, which can serve as a signal itself by exciting other channels; a burst of intracellular Ca^{2+}; or the normalization of ion concentrations across a membrane to control cell volume. Ion channels open (or "gate") only under certain conditions, such as altered transmembrane voltage, binding of a small ligand, or mechanical force. It is one family of channels that respond to the latter stimulus, called mechanosensitive (MS) channels, which we consider in this review.

II. Mechanosensitive Ion Channels

A. Gating Models

How force administered to a cell is delivered to a mechanosensitive channel, and how the channel subsequently converts that force into ion flux are important questions requiring the purposeful integration of genetic, biochemical, structural, and biophysical approaches. Three simplified models have been proposed for the gating of channels that act directly as mechanoreceptors (that is, there is not an intermediary between the force perception and the channel) [19-21]. These models are described below and illustrated in Figure 1.

Intrinsic. In the intrinsic bilayer model (Fig. 1A), force is conveyed to the channel directly through the planar membrane in which it is embedded, and lipid-protein interactions are the primary determinants of the favored state. Biophysical modeling approaches have indicated that the closed state of the channel is favored under low membrane tensions due to the cost of membrane deformation at the perimeter of the channel. A channel can deform the surrounding membrane due to mismatch between the thickness of the membrane and the thickness of the hydrophobic domain of the channel. In addition, the membrane (which has a lower compressibility modulus than the channel [22]) can be locally distorted or bent as it conforms to the shape, or profile, of the embedded channel [20, 23, 24]. The energy cost associated with these membrane

deformations increases upon channel opening, as the cross-sectional area—and therefore the perimeter—of the channel expands. However, loading the membrane with tension through a patch pipette or osmotic pressure can offset this energy cost; under these conditions the open state is favored. Importantly, membranes are active participants in the gating of MS channels and the pressure exerted by the lipid on the channel is a critical component of the intrinsic bilayer model [25]. This model is supported by experimental evidence showing that the fluidity, thickness and curvature of the membrane influence the gating characteristics of MS channels [26-28].

Tethered. It has long been speculated that mechanotransduction by hair cells of the vertebrate inner ear is mediated by the action of tethers (called "tip links") on transducer channels located in the hair cell plasma membrane (reviewed in [29]). In the tethered trapdoor model (Fig. 1B), force is conveyed to the channel through tension applied to other cellular components, such as the actin or microtubule cytoskeleton and/or the extracellular matrix. Displacement of the cellular component pulls on the channel through the tether, thereby triggering its opening. Alternatively, it has been proposed that rather than opening a trapdoor, pulling a tether leads to reorientation of the channel within the lipid bilayer, which results in channel gating in response to the membrane deformation and tension forces described above (Fig. 1C) [21, 30, 31]. In this "unified" model, as with the intrinsic bilayer model, the biophysical properties of the membrane are an important contributor to the lowest energy conformation of a MS channel, and can either restrict or facilitate changes in state.

B. Electrophysiology and Model Systems

The first observations of ion flux in response to mechanical stimuli quickly followed the development of the patch-clamp technique in the mid-1980s. This technique allows one to record the current passing across a small patch of membrane tightly sealed to the tip of a thin glass capillary pipette (reviewed in [32]). A key aspect of this technique is the formation of a high resistance "gigaseal" between the membrane and the glass (on the order of 1GOhm or higher). When positive or negative pressure is applied to the

membrane patch through this glass recording pipette, the membrane (and any associated cytoskeletal components) is deformed. The opening and closing of individual mechanically gated ion channels can then be observed over time [33, 34]. Early patch-clamping experiments resulted in the identification of stretch-activated ion channels in animal cells known to be specialized for mechanical perception [35-38]. Similar activities were soon identified in non-specialized cells [36, 39], leading to the proposal that sensitivity to mechanical stimuli might be a basic cellular feature [22, 40]. In the decades since these first studies, many families of MS channels have been identified and characterized in bacteria, plants, animals, and archaea (reviewed in [41-43]). MS channels can be activated by membrane tension introduced through the patch pipette as described above, by the swelling associated with hypo-osmotic shock, or by treatment of cells with membrane-bending amphipaths. Their function has been investigated in endogenous membranes, in a variety of heterologous systems, and even reconstituted into artificial membranes. Leading the way in many of these studies is a suite of bacterial channels, arguably the best studied and best-understood mechanoperceptive proteins at the functional, structural, and biophysical levels.

III. *E. coli* MscL, MscS, and MscM

A. Identification

Identifying MS channels in bacteria by electrophysiological analysis at first presented several challenges as an *E. coli* cell is smaller than the diameter of a typical patch pipette tip, and has a peptidoglycan layer between the inner and outer membranes [44, 45]. This problem was solved by treating cultures with an inhibitor of cell division and then enzymatically digesting the peptidoglycan layer. These treatments result in the production of "giant *E. coli* protoplasts" amenable to patch clamp electrophysiology [46]. Using this approach, the Kung group measured current induced in response to membrane stretch in *E. coli* and observed a robust tension-sensitive channel activity [44]. Subsequent studies established that at least three distinct channel activities are detectable in the inner membrane of *E. coli*—the mechanosensitive channels of large, small, and mini conductances. MscL, MscS, MscM

activities each have different conductances (3 nS, 1 nS and 0.3 nS, respectively) and are activated at decreasing thresholds of pressure [17, 47-49].

B. Cloning

It is now established that the three classic activities of the *E. coli* membrane, MscL, MscS and MscM, represent a complex combination of activities provided by two distinct families of MS channels. The *E. coli mscL* gene was cloned through a fractionation/reconstitution and microsequencing strategy [50] and found to be essential for MscL activity. The mscS/*yggB* gene was identified through a combination of forward and reverse genetic approaches, and along with *mscL* is underlies the primary response of an *E. coli* cell to rapid increases in membrane tension [51]. While the MscS and MscL proteins are structurally and evolutionarily unrelated, at least part of the originally observed MscS activity can now be attributed to the action of another channel with homology to MscS, now referred to as kefA/MscK [52] (for more on MscK, see below). When either MscL [50] or MscS [53] monomers are purified, assembled into channels, and reconstituted into artificial liposomes, both show characteristics indistinguishable from that in native *E. coli* membranes, indicating that neither requires additional cellular structures for mechanosensitivity. Thus, both MscS and MscL are gated in direct response to lipid bilayer deformation, as in the intrinsic bilayer model (Fig. 1A). Relatively less is known about MscM, though recent reports have demonstrated that YjeP and YbdG, two more homologs of MscS, are likely to underlie this elusive activity [54, 55].

C. Physiological Function

Bacterial cells are found in a variety of dynamic environments, frequently requiring them to adapt to changing osmotic conditions. In order to maintain turgor pressure during exposure to hyperosmotic stress, bacterial cells accumulate osmolytes that are compatible with cellular metabolism [56]. On the other hand, a sudden shift to hypoosmotic conditions will cause a rapid influx of water across the lipid bilayer, leading to increased membrane tension (reviewed in [34, 57]). It has been estimated that a mere 20 mM drop in external osmolarity can result in membrane tensions that approach lytic levels if unrelieved [34]. A

hypoosmotic shock of this type might occur when soil bacteria are caught in the rain, when marine bacteria migrate to freshwater or during the transmission of pathogenic bacteria through excrement. Without a rapid response, these shocks would lead to a compromised cell wall, leaving the cell vulnerable to lysis [58].

It had long been proposed that bacterial cells were capable of relieving this type of environmental hypoosmotic stress by facilitating the exit of osmolytes from the cell, thus ensuring the physical integrity of the cell under increased turgor [45, 56, 59]. We now know that the primary mechanism for hypoosmotic shock survival is the activation of MS channels, which allows the passive diffusion of nonspecific osmolytes out of the cell, relieving membrane tension and preventing cellular lysis. *E. coli* strains with lesions in both *mscL* and *mscS* show reduced survival of hypoosmotic shock though single mutations have no discernable effect [50, 51]. Mutants lacking YbdG also show a small defect in osmotic shock survival [54] and the overexpression of YjeP promotes survival in the absence of all other MS channels [55]. Thus, these bacterial MS channels are often referred to as osmotic "safety valves" [60] and have been proposed to provide a graded series of responses allowing the bacteria to tune its response to different osmotic challenges under different environmental or developmental conditions [13, 45, 48, 52, 61].

MSCS and MSCS-LIKE CHANNELS: CONSERVATION AND DIVERSITY

These classic mechanosensitive channels from *E. coli* described above not only serve important biological functions, but MscL and MscS have also become leading model systems for the study of MS channel structure and function. Here we focus on the structure and function of the bacterial mechanosensitive channel MscS and its homologs in *E. coli*, other microbes, and in eukaryotes. Several excellent reviews on MscL have recently been published [57, 62, 63].

I. Structure

Crystallographic studies of MscS structure are beginning to answer the fundamental question of how mechanosensitivity is accomplished in MscS-type channels (recently reviewed in [64]). At present, five structures of prokaryotic MscS homologs have been solved: wild type *E. coli* MscS (*Ec*MscS) in both open and nonconducting (not necessarily closed, see below) conformations [65-67], a point mutation of *Ec*MscS that likely represents the open conformation, and MscS homologs from *Thermoanaerobacter tengcongensis* (*Tt*MscS)[68] and *Helicobacter pylori* (*Hp*MscS)[65] in nonconducting conformations. Four of these structures are shown in Figure 2. A cartoon representation of each is shown from the side (left panel), and both cartoon and space-filling models are shown from the periplasmic surface (middle and right panels). A fragment containing the three TM domains and the upper vestibule from a single monomer of each of these structures (including amino acids 27-175 for *Ec*MscS) is shown in Figure 3. Despite the inevitable possibility of artifacts associated with packing contacts and protein-detergent interactions [21, 69, 70], these structures provide an invaluable source of information about the molecular mechanism of gating and the relationship between channel structure and electrophysiological behavior.

A. Nonconducting and Open Conformations of EcMscS and Homologs

Nonconducting Conformations. The first crystal structure of *Ec*MscS was solved by the Rees group at 3.7 Å resolution [66, 67] (Fig 2A) and revealed a homoheptameric channel with three transmembrane alpha helices per monomer and a large, soluble C-terminal domain. This oligomeric state and topology were subsequently verified experimentally [71-73]. As shown in Figure 3, each monomer contributes three tightly packed N-terminal transmembrane (TM) alpha helices to the transmembrane region. TM1 (residues 28 - 60) and TM2 (residues 63 - 90) face the membrane, while TM3 (residues 93 - 128) lines the channel pore. (The residues assigned to each helix are as in [64]). One striking feature of the structure is a sharp kink at Q112/G113, which divides TM3 into TM3a, which is roughly perpendicular to the membrane, and TM3b, which is almost parallel to the membrane (Fig. 3A). The narrowest constriction of the pore has a diameter of 4.8 Å, and is created by two rings of Leucine residues (L105 and L109) with inward facing side chains.

These hydrophobic rings prevent the wetting of the pore and thereby serve as a "vapor lock" to the movement of ions through the channel [74, 75]. Mutational analysis of L105 confirmed its importance in maintaining the closed state [71]. The C-terminal region of each monomer contributes to a large hollow structure referred to here as the "vestibule". The vestibule comprises seven side portals and one axial portal located at the base of the vestibule, formed by a seven-stranded β-barrel.

Originally thought to be the open conformation, this structure it is now generally agreed to represent a nonconducting state. It is unlikely to represent the normal closed conformation, because TM1 and TM2 are not in contact with TM3, an expected requirement for tension-sensitive gating (see the section on "force-sensing" below)[34, 76]. A number of molecular dynamics (MD) simulations further support this conclusion [74, 77, 78]. The recently reported structures of *Tt*MscS (Fig 2B) and *Hp*MscS (not shown) exhibit similar transmembrane helix organization and pore size as the original *Ec*MscS structure, and therefore are also considered to represent nonconducting states [65, 68]. The C-terminal vestibule of *Tt*MscS has several differences in structure from that of *Ec*MscS, which are shown to modulate the conducting properties of the channel and are discussed below.

Open Conformations. Though invaluable for establishing the basic structure of MscS, nonconducting structures give limited insight into the channel's gating mechanism. In a directed attempt to solve the structure of MscS in an alternate conformation, the Booth and Naismith groups crystalized the A106V point mutation of *Ec*MscS at 3.45 Å resolution [79], Fig. 2C. The resulting structure has a substantially increased pore size (approximately 13 Å in diameter) due to a rearrangement of transmembrane helices. TM1 and TM2 are angled away from TM3b and the channel core, while TM3a is tilted away from the plane of the membrane and rotated slightly away from the pore (compare Fig 3A and C). TM3b and the upper vestibule are mostly unchanged compared to the nonconducting structures. These rearrangements place the vapor-lock residues out of the pore, as previously predicted based on experimental and modeling data [80-82]. A pulsed electron-electron double resonance (PELDOR) approach [83]

revealed that two *Ec*MscS mutants, spin-labeled at D67C (PDB 4AGE) or L124C (4AGF), took a similar conformation in solution, indicating that it is not an artifact of crystal packing nor of the particular A10V mutation [84]. Further confirmation that the A106V structure properly resembles the open state comes from a recent report describing wild type *Ec*MscS solubilized in a different detergent (β-dodecylmaltoside instead of fos-choline-14), at a resolution of 4.4 Å ([65] Fig. 3D). This structure closely resembles the A106V *Ec*MscS structure, establishing a solid consensus regarding the open state structure of *Ec*MscS.

B. Gating Mechanism

Despite having multiple crystal structures attributed to different states of MscS, as well as an array of mutational and functional data that have determined functionally important residues, the actual mechanism of transition between closed and open states is still not completely clear. While several models have been proposed based on MD simulations [81, 85] and electron paramagnetic resonance (EPR) spin labeling [82], the model which is currently favored is one wherein membrane tension induces the rotation and tilting of TM1 and TM2 as a whole, immersing them more deeply into the surrounding lipid bilayer. This movement pulls TM3a away from the pore until it's oriented almost normal to the membrane plane, effectively removing the L105 and L109 vapor lock side chains and opening the channel to ion flux [64, 79]. In all of the crystal structures described above, the positioning of TM1 and TM2 with respect to each other is the same, as if they act like a rigid lever (compare Fig 3.A, B to Fig. 3C and D). Assuming that the newly obtained crystal structures described above indeed represent nonconducting and open states, the "rigid-body" movement model of transition into the open state may be considered the most probable.

Lipid-protein interactions must occur at the periphery of the channel, which in MscS is likely to be comprised of TM1 and TM2. Hydrophobic residues in the protein-lipid interface of TM1 and TM2 were shown in several site-directed mutagenesis studies to affect tension sensitivity and osmotic shock protection [86, 87]. In addition, an interaction between F68 in TM2 and L111 in TM3 was shown by

electrophysiology and mutational analysis to be of critical importance for force transmission from lipid-facing helices to the pore region; disruption of this inter-helical contact results in channel inactivation [76]. These data are consistent with a model wherein TM1 and TM2 serve as a tension sensor, transmitting force from the membrane to TM3; subsequent rearrangement of TM3 helices results in channel gating. It is intriguing to consider MscS homologs that possess additional N-terminal transmembrane helices (for several examples, see Figure 4). Additional helices may shield TM2 and TM3 from lipid environment of membrane or serve as tension sensors themselves, transmitting force to the pore-lining helix through a different (yet unknown) mechanism [88].

C. Contributions by the C-terminus

Though the structure of the C-terminal vestibule is virtually unchanged in all the crystal structures assigned to open and nonconducting states of *Ec*MscS, other evidence indicates that this portion of the channel may be subject to conformational changes during opening, closing and inactivation transitions. Analyses of multiple deletion and substitution mutants have established that the vestibule is important for channel function and stability [71, 89, 90], and that interactions between the upper surface of the vestibule and the TM domain can affect gating as well as inactivation behavior [91, 92]. Co-solvents that induce compaction of the C-terminal domain have been shown to facilitate MscS inactivation [93], while experiments utilizing FRET to quantify the diameter of the cytoplasmic domain showed that it swells during gating [94]. Taken together, these data indicate that gross structural remodeling of the vestibule and its interactions with the transmembrane domain likely accompanies inactivation and gating cycles.

In addition, recent reports support a role for the C-terminus as an ion selectivity filter. In *Ec*MscS, ions likely do not enter the vestibule through the axial β-barrel, as the portal that it forms is too narrow (1.75 Å in its narrowest part); rather, they probably travel through the seven side portals into the vestibule and then cross the pore. MS simulations suggest the vestibule serves to filter and balance charged osmolytes prior to their release from the cell, keeping ion efflux largely neutral in charge and thereby

preventing membrane depolarization [95]. Another correlation between vestibule structure and ion selectivity comes from recent studies of *Tt*MscS [68]. Compared to *Ec*MscS, *Tt*MscS has smaller side portals but a much wider axial portal; at the same time it has a much higher selectivity for anions (see below for a discussion of ion selectivity). A version of *Tt*MscS where the axial β-barrel sequence (amino acids 271 to 282) was replaced with the corresponding portion of *Ec*MscS lost this preference for anions, indicating that this small portion of the C-terminus can strongly influence overall channel behavior.

D. Summary

The five independently derived crystal structures of bacterial MscS homologs available to date have revolutionized our understanding of the overall architecture of bacterial MscS homologs, provided context for the interpretation of mutagenic data and MD simulations, and established a sophisticated foundation for furthering our understanding of the gating cycle. We note that no crystal structures have yet been reported for archaeal or eukaryotic MscS homologs; such a structure would be a major step forward for those interested in the evolutionary diversification of this family of proteins.

II. Evolutionary History

The MscS protein superfamily is vast and diverse, with members found in most bacterial, archeal, some fungal, and all plant genomes so far analyzed [96-101]. However, MscS family members have not yet been found in animals. It has been suggested that MS channels first evolved in an ancestor common to all cell-walled organisms and have been maintained throughout these lineages as a solution to osmotic stress and regulation of turgor pressure [96, 97, 102]. Another explanation is that the membrane reservoirs of animal cells allow hypoosmotic swelling without producing membrane tension, or that mammalian membranes do not stretch due to their close association with the cytoskeleton [103, 104]. Alternatively, MscS homologs could simply be unrecognizable in animal genomes by current homology-based searches.

Mapped onto the MscS structure, the conserved domain comprises the pore-lining helix (in MscS, this is TM3) and the upper part of the cytoplasmic vestibule. Outside of this domain MscS family members

vary greatly in sequence and topology. The number of predicted TM helices for MscS family members ranges from 3 to 12 and a variety of conserved domains, including those associated with the binding of Ca^{2+} and cyclic nucleotides, have been identified in some subfamilies [51, 96, 105, 106]. Furthermore, multiple MscS homologs are frequently identified within a single organism (including many bacterial and all plant genomes analyzed to date), suggesting that functional specialization of MscS homologs has evolved both and within a single organism. Our current understanding of the physiological function of MscS homologs from bacteria, fungi, plant cells and plant organelles is described below and summarized in Table 1.

III. Physiological Function

While it has been clearly established that MscL and MscS serve to protect cells from extreme environmental hypoosmotic shock, it is becoming evident that the functions of the members of this family may be more complex. An emerging theme is that MscS homologs have evolved specific functions tailored to the needs of the organism, including the release of specific cellular osmolytes in response to specific environmental or developmental osmotic triggers.

A. Prokaryotes

E. coli. We know by far the most about the six MscS family members encoded in the *E. coli* genome (MscS, MscK, YjeP, YbdG, YbiO, and YnaI) [51]. Research into their physiological roles suggests that they all serve to release osmolytes from the cell under hypoosmotic stress but that their function is only required for cell viability under specific conditions. Even MscS may serve specialized roles, as MscS protein levels fluctuate. MscS levels are elevated during growth at high osmolarity, possibly a preemptive method of dealing with an impending downshock, and during stationary phase, perhaps to deal with the osmotically vulnerable state of cell wall remodeling [107, 108]. MscK contributes modestly to cell survival during standard osmotic shock assays (Levina 1999; McLaggan et al., 2002 *Molecular Microbiology* and Li et al., 2002 *EMBO*) and its mechanosensitive channel activity requires the presence of K^+ ions in the extracellular solution. It has been proposed that binding of K^+ primes the channel for gating. Such an

activity may be required for survival in soils with high concentrations of animal urine or within the kidneys during host infection [109]. The remaining *E. coli* MscS family members (YbdG, YjeP, YbiO and YnaI) can provide osmotic shock protection when overexpressed in *E coli* [54, 55], and the latter three activities may simply be expressed at too low levels to contribute under normal laboratory assay conditions. Indeed, the occurrence of the previously uncharacterized 20 picoampere (pA) mechanosensitive channel activity attributed to YbiO increased dramatically when cells were treated with NaCl prior to patching [55].

Other species. The 3 MscS homologs (*yhdy*, *yfkC*, and *yukT*) of the gram-positive bacterium *B. subtlilis* are dispensable for osmotic shock survival in the laboratory, though the *mscL yukT* double mutant strain exhibits enhanced osmotic sensitivity compared to the *mscL* single deletion strain [110-112]. As *B. subtlilis* is found in both the soil and the human gut, there may be specific growth conditions wherein these MscS homologs contribute to osmotic homeostasis that are not replicated in the laboratory environment. Other prokaryotic MscS homologs have been identified that provide tantalizing ideas about the variety of ways in which this family of channels may have evolved to provide osmotic adjustment in response to different environmental and developmental stimuli. The gram-positive bacterium *Corynebacterium glutanicum* is used in the industrial production of glutamate and other amino acids [113]. Its genome encodes homologs of both MscL and MscS (MscCG/NCgl1221), but neither is required for cell survival in laboratory-based osmotic downshock assays [114, 116]. Instead, MscCG is involved in regulating the steady state concentration of glycine betaine (the preferred compatible osmolyte of *C. glutanicum*) in response to both hypo- and hyperosmotic stress [115]. MscCG is also essential for glutamate efflux in response to biotin limitation and penicillin treatment, notably in the absence of hypoosmotic stress [116, 118]. Several lines of evidence, including the analysis of loss-of-function and gain-of-function lesions in the predicted pore-lining helix, support the model that MscCG directly mediates the efflux of glutamate and that this efflux is dependent on mechanosensitive channel gating [116-119]. Thus, MscCG is

likely a mechanically gated MscS homolog that is involved in osmotic adjustment of specific compatible solutes in response to multiple stimuli.

Finally, there are indications that MscS family members are important for pathogenesis and metabolism, perhaps indicating the importance of osmotic adjustment in these processes. Two MscS homologs from the food-borne pathogen *Campylobacter jejuni, Cjj0263* and *Cjj1025*, were recently found to be required for colonization of the digestive tract of chicks [120], and a *Pseudomonas aeruginosa* MscK ortholog has been associated with virulence [121]. PamA, a MscS homolog from the photosynthetic cyanobacterium *Synechocystis* sp.PCC6803 was reported to interact in vitro and in vivo with PII, a highly conserved carbon/nitrogen sensor [122, 123]. Furthermore, nitrogen response and sugar metabolic genes show altered expression in the absence of *PamA,* suggesting that it may serve to integrate carbon and nitrogen metabolism with osmotic conditions. Taken together, these preliminary studies illustrate how much more has yet to be revealed regarding MscS homolog function in the prokaryotic world.

B. Eukaryotes

While less studied than their prokaryotic counterparts, recent research offers a few glimpses into the important functions and novel characteristics of the eukaryotic members of the MscS family. Sequence similarities place them into two major classes (described in [98]). Class II members are predicted to localize to the plasma membrane or intracellular membranes of both plants and fungi. Class I channels, which show slightly more sequence conservation to MscS than those in class II, are predicted to localize to endosymbiotic organelles (mitochondria and plastids such as chloroplasts), and are found only in plant genomes.

Class I. Considering the origin of endosymbiotic organelles (the engulfment of a primitive bacterium), the MscS homologs found in their envelopes are likely to have a conserved function as osmotic safety valves, but in this case protecting mitochondria and plastids from fluctuations in intracellular rather than extracellular osmotic concentrations [15]. The Mechanosensitive Channel (MSC)1, from *Chlamydomonas*

reinhardtii localizes to punctate spots associated with the single plastid found in these cells, and plastid integrity is lost when the *MSC1* gene is silenced by RNAi (Nakayama 2007). To date, MSC1 is the only Class I MscS homolog to be successfully characterized by electrophysiology (see below). Like MSC1, MscS-Like (MSL)2 and MSL3 of *Arabidopsis thaliana* localize to distinct foci in the plastid envelope. These two land plant Class I homologs are required for normal plastid shape and size and for proper placement of the plastid division ring [124, 125]. Proteins involved in the regulation of division site placement are often conserved between plastids and bacteria, and abnormal division ring placement in an *E. coli* strain lacking MscL, MscS, and MscK suggest that this may hold true for MS channels as well [125, 126]. The large, round plastid phenotype of the *msl2 msl3* mutant can be suppressed by a variety of genetic and environmental treatments that increase cytoplasmic osmolyte levels, indicating that plastids are under hypoosmotic stress from within the cytoplasm and that MSL2 and MSL3 are required to relieve that stress [127]. Several Class I MscS homologs from land plants are predicted to localize to the mitochondria [98, 101], but their study has not yet been reported.

Class II. The identification of MscS homologs in plant genomes [96, 97] was exciting for plant biologists because it provided candidate genes for the MS channel activities already known to be widespread in plant membranes [98]. However, while the *Arabidopsis* genome contains seven MSL proteins that are predicated to localize to the plasma membrane, and they exhibit distinct tissue-specific expression patterns [96, 98], a clear physiological function has yet to be assigned to any (though MSL10 has been characterized by patch-clamp electrophysiology, see below). The recent characterization of two endoplasmic reticulum-localized MscS homologs from *Schizosaccharomyces pombe*, Msy1 and Msy2, suggests that these channels may serve as hypoosmotic stress signaling molecules as much as osmotic safety valves [105]. *msy1- msy2-* mutant cells exhibit greater swelling and higher Ca^{2+} influx upon hypoosmotic shock, and are more likely to subsequently undergo cell death. Consistent with this idea, we

have proposed that MSL10 could play a role in hypoosmotic stress signal transduction through membrane depolarization [128].

C. Summary

To conclude, current evidence indicates that members of the MscS superfamily exhibit unique forms of regulation and variations of function. While all are variations on a common theme—action as an osmotic conduit in response to membrane tension—the proteins within this family may have become as diverse as the organism in which they reside. We anticipate that more precise analyses, under diverse growth conditions and at the single cell or organellar level, will reveal the role played by these channels in the osmotic homeostasis of cells and organelles.

IV. Electrophysiological Behavior

Besides *Ec*MscS, many MscS superfamily members have been shown to be mechanosensitive, including five others from *E. coli* (MscK, YbdG, YnaI, YjeP, and YbiO)[54, 55, 109] and three from other bacterial species (*Tt*MscS from *Thermobacter tengcongensis* [68], MscSP from *Silicibacter pomeroyi* [129], and MscCG from *Corynebacterium glutamicum* [115]. Two MscS homologs from the archaea *Methanococcus jannaschii*, MscMJ, and MscMJR have been characterized [102, 130], as have two channels from photosynthetic eukaryotes (MSC1 from *Chlamydomonas reinhardtii* and MSL10 from *Arabidopsis thaliana* [128, 131]. Despite striking differences in topology and sometimes very low sequence identity, these channels demonstrate surprisingly conserved behavior in many aspects. Their major characteristics are shown in Table 2 and discussed in further detail below. Not included here are possible MscS-like channels from *B. subtlilis* [132], *S. faecalis* [133], and the bCNG family [106].

A. Conductance and Ion Selectivity

While MscL forms a large, completely nonselective pore, MscS is slightly anion-selective, preferring Cl^- ions over K^+ ions by a factor of as much as 3 ($P_{Cl^-} : P_{K^+} = 1.2 – 3$ [53, 109, 134, 135]). MscSP closely resembles *Ec*MscS in sequence and in channel characteristics with a 1 nS single-channel conductance and

PCl- : PK+ = 1.4 [129]. MscK also has a conductance close to that of MscS [51, 109]. However, some variation is observed among the bacterial channels, with a smaller conductance typically associated with more selectivity. MscCG has a single-channel conductance of 0.3 nS, about one-third the size of that provided by *Ec*MscS, and prefers cations (P_{Cl-} : P_{K+} = 0.3) [115]. YjeP has a similar conductance, and is also likely to have a preference for cations, as this was the early characterization of MscM activity (P_{Cl-} : P_{K+} = 0.4). However, the ion selectivity of YjeP has not yet been assessed directly [48, 55]. As described above, *Tt*MscS has a single-channel conductance approximately half that of *Ec*MscS and is more strongly anion-selective (P_{Cl-} : P_{K+} = 8.7) [68].

Though few archaeal or eukaryotic channels have yet been studied, what we know so far indicates a range of conductances and selectivities similar to those described for bacterial channels. MscMJ (270 pS) and MscMJLR (2 nS) vary considerably in conductance, but both exhibit a similar preference for cations (P_{Cl-} : P_{K+} = 0.16 and 0.2, respectively) [102, 130]. MSC1 and MSL10 are quite similar: both have conductances around a third of that of MscS under similar conditions and both show a preference for anions (P_{Cl-} : P_{K+} = 7 and 6, respectively) [128, 131, 136]. Once additional homologs are characterized, it can be determined if these particular examples are characteristic of archaeal and eukaryotic channels. Given the wide range of sequence similarity in the pore region it is perhaps surprising how similar the MscS homologs described are: all of them have weak to moderate ionic preferences and a single-channel conductance which falls approximately into a 4-fold range (under similar conditions, see Table 1 for details).

B. Gating tension

MscL is gated by tensions that are close to lytic, and is often used as an internal reference for other mechanosensitive channels. The threshold tension for activation of MscS, MscK, YjeP and MscCG activity is approximately one half of that of MscL (MscL : MscS = 1.6, MscL : MscK = 1.9 - 2.2, MscL : YjeP = 1.6) [51, 55, 109, 118, 137]. Unexpectedly, YnaI and YbiO are gated by tensions almost as high as for

MscL [55]. MscSP is less tension-sensitive than MscS and its threshold activation ratio MscL : MscSP was reported to be 1.28 [129]. For the archaeal channels it was found that MscMJ is gated at intermediate tensions (MscL : MscMJ = 1.3) and MscMJLR at lower tensions (MscL : MscMJLR = 2.5) [102, 130]. If the tension at which a MS channel gates can be considered an indication of the stimuli to which the channel has evolved to respond, it seems likely that MscS homologs from different species respond to the same type of stimulus, as in general they share similar gating thresholds.

C. Inactivation and desensitization

Models of the MscS activation cycle typically include four distinct states: open, closed, inactive and desensitized [51, 93, 137, 138]. The latter three states are distinct: in the closed state the channel can easily be gated by threshold tension. In the inactive state, the channel cannot make a transition to the open state under any tension, while a desensitized channel could be gated by the application of increased tension. However, for a channel subjected to a fixed membrane tension, the effects of inactivation and desensitization are indistinguishable and manifest themselves as sharp or gradual current decay in patch-clamp recordings. In this case, the terms "inactivation" and "desensitization" are often used interchangeably. While inactivation and/or desensitization under sustained membrane tension have been reported for MscS expressed in several systems [85, 136, 137], MscSP, MscCG, MscK, MscMJ and MscMJLR do not desensitize [51, 102, 109, 115, 129, 130]. MSL10 does not show any significant signs of inactivation [128], while MSC1 inactivates at positive membrane potentials, but not at negative [131]. These results leave unclear the physiological relevance of inactivation [57].

D. Hysteresis

Another feature of mechanosensitive channel behavior is hysteresis, or a difference between the tensions required for opening and closing. In the case of MscS, which is routinely observed to close at higher tensions than at which it opened (summarized in [52]), this phenomenon was at least partially attributed to the artifacts of membrane patch structure [139]. The eukaryotic channels MSC1 and MSL10

also show hysteresis, but of a different type. These channels typically close at a lower tension than at which they opened. Strikingly, a subpopulation of both types of channels often is observed to stay open even after all membrane tension has been released [128, 131]. There are no reports of any functional importance attributed to this phenomenon, but the continuous slow depolarization of the membrane due to channels staying open after membrane tension is relieved could result in the gating of depolarization-activated channels and/or the propagation of a systemic signal.

E. Summary

Despite limited sequence identity, the MscS family members so far characterized share similar basic channel characteristics such as conductance and ion selectivity. Other behaviors observed under patch clamp, such as hysteresis and inactivation/desensitization, are more variable and unclear physiological relevance. One could speculate that the conserved features of these channels reflect their common function (rapid release of osmolytes in response to membrane tension) while their characteristic differences reflect the specific natures of their ecological niches [55]. Additional examples may help to determine the functional range of properties that have been selected by evolution.

V. Topological Diversity in the MscS Superfamily

The increased topological complexity of MscS family members (as described above and illustrated in Figure 4) has been taken to imply regulatory complexity [21, 100], and data is accumulating that suggest this may indeed be the case. Many members of the MscS family contain N- and C-terminal domains dramatically larger than that of MscS, presenting the possibility of additional functions and regulation sites. For example, the unusually large periplasmic N-terminal region of MscK could regulate channel activity by preventing gating in the absence of high K^+ [109, 140]. Removal of the N-terminal region of MscK, including TM helices1-9, abolishes K^+-dependent gating and promotes its ability to provide protection from hypoosmotic shock [71]. Similarly, the presence of an extra TM helix C-terminal to the pore-forming helix is unique to MscCG, and can confer the ability to facilitate glutamate efflux when fused to *Ec*MscS

[118]. Proteins comprising the bCNG family all encode a large soluble C-terminal domain containing a cyclic nucleotide-binding domain. This domain has been shown to negatively regulate the mechanosensitive channel activity of one of the family members [106, 141].

The eukaryotic family members show topology that is just as diverse. A variety of physiological functions have been attributed to the chloroplast channels MSL2 and MSL3, which contain a C-terminal cytoplasmic domain three times the size of the MscS soluble domain [125, 142]. Although the regulatory and functional importance of this domain has yet to be confirmed, preliminary evidence suggests that a highly conserved domain within this region is required for proper subcellular localization and channel function *in vivo* (E. S. Haswell, unpublished). Class II (plasma membrane- and ER-localized) eukaryotic homologs of MscS, such as MSL10, typically share a common topology of 6 TM regions, large soluble N- and C-termini, and a large cytoplasmic loop between TM helix 4 and 5 [98, 105], suggesting that their conserved structure serves a eukaryote-specific function. The large cytoplasmic regions of many Class II proteins suggest a number of possible regulatory mechanisms. For example, Mys1 and Mys2 contain an EF-hand Ca^{2+}-binding motif [143] in the large cytoplasmic loop between TM4 and TM5. Genetic analyses suggest that this region is important for sensing and/or controlling Ca^{2+} influx as well as contributing to channel function in response to hypoosmotic stress [105].

FUTURE DIRECTIONS

As we hope we have demonstrated above, these are exciting times for scientists who study mechanosensitive ion channels. Every new detail regarding the structure, the physiological function, and the biophysical parameters that govern the gating mechanism of *Ec*MscS adds to our understanding of *E. coli* biology, and helps elaborate an important model system for the study of mechanosensitivity. Prokaryotic homologs of MscS provide additional examples of the ways in which various bacteria might exploit the membrane tension sensor and osmotic safety valve provided by a MscS family member. The

suggestion that more diverged MscS families may have additional regulatory mechanisms overlaid onto a conserved mechanosensitive channel core is particularly interesting in this regard [106]. The coming years should also bring a greater understanding of the role played by the diverse eukaryotic family of MscS homologs. Eukaryotic cells respond to osmotic stress differently than bacteria, inducing cell signaling pathways in addition to releasing osmolytes [19] Studies of the yeast Msy1 and Msy2 suggest that they might play a role in both of these responses [105]; further investigation will establish this point. New discoveries are also likely as some of the technical challenges associated with the study of mechanosensitive channels are overcome. Approaches to investigate osmoregulation and osmotic stress response in single cells and organelles may reveal more subtle phenotypes than can be detected in a bacterial culture or from a whole-plant phenotype. The development of fluorescent biosensors that report on ion flux, pH, transmembrane voltage, and membrane tension could produce unexpected insights into the function of MscS-Like mechanosensitive channels in their endogenous cellular context.

ACKNOWLEDGEMENTS

Our current work on MscS-Like channels in plants and microbes is supported by grants from the National Science Foundation (MCB-1253103) and the National Institutes of Health (R01 GM084211-01). We gratefully acknowledge the discoveries and insights of our colleagues and apologize to those whose work we could not include due to size limits.

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TABLES

Table 1. Single-channel properties of MscS family members.

	Organism	Gene Name	Amino Acids	Physiological Function	Mutant Phenotype	Subcellular Localization	References
Prokaryotes	*Escherichia Coli*	*YggB (MscS)*	286	Release of ions during hypoosmotic shock	*mscS mscL* mutant exhibits loss of viability during osmotic down-shock; *mscS mscK* mutant has complete loss of MscS channel activity	Plasma membrane	Levina *et al.*, 1999
		MscK (KefA)	1120*	Release of ions in high K^+ environments	*mscS mscK* mutant has complete loss of MscS channel activity	Plasma membrane	Levina *et al.*, 1999; Mclaggan *et al.*, 2002
		YbiO	741	Release of osmolytes in high NaCl environments	*ybiO* mutant has loss of 20 pA channel activity	Plasma membrane	Edwards *et al.*, 2012
		YjeP	1107	Release of ions during hypoosmotic shock	*yjeP* mutant has loss of 7.5-13pA channel activity	Plasma membrane	Edwards *et al.*, 2012
		YnaI	343	NR	*ynaI* mutant has loss of 2 pA channel activity	Plasma membrane	Edwards *et al.*, 2012
	Campylobacter jejuni	*Cjj0263*	627	Osmotic protection and host colonization	*cjj0263* has decreased viability after osmotic down-shock; *cjj0263 cjj1025* mutant exhibits impaired chick ceca colonization	Plasma membrane	Kakuda *et al.* 2012
		Cjj1025	523	Host colonization	*cjj0263 cjj1025* mutant exhibits impaired chick ceca colonization	Plasma membrane	Kakuda *et al.* 2012
	Bacillus subtilis	*YkuT*	267	Osmotic protection	*mscL ykuT* mutant strain has increased sensitivity to osmotic down-shock	Plasma membrane	Hoffmann *et al.*, 2008; Wahome and Setlow, 2008; Wahome *et al.*, 2009
	Corynebacterium glutanicum	*MscCG*	533	Involved in betaine and glutamate efflux	*mscCG* mutant is impaired in betaine efflux during hyper and hypoosmotic shock and exhibits a 70% decreases in glutamate export	Plasma membrane	Yao *et al.*, 2009; Börngen *et al.*, 2010; Nottebrock *et al.*, 2003; Nakamura *et al.*, 2007; Becker *et al.*, 2013
	Synechocystis sp. PCC 6803	*PamA*	680	Involved in the transcriptional control of sugar and nitrogen metabolism genes	*pamA* mutant is glucose sensitive; shows decreased levels of nitrogen-response genes and the stress sigma factor SigE	NR	Osanai *et al.*, 2005

Eukaryotes	*Arabidopsis thaliana*	*MSL2*	673*	Plastid osmotic stress response; division ring placement	*msl2* null mutants show defective leaf shape; *msl2 msl3* mutant has enlarged, round non-green plastids and enlarged chloroplast exhibiting multiple division rings	Plastid envelope	Haswell and Meyerowitz, 2006; Jensen and Haswell, 2011; Wilson *et al.*, 2011; Veley *et al.*, 2012
		MSL3	678*	Plastid osmotic stress response; division ring placement	*msl2 msl3* mutant has enlarged, round non-green plastids and enlarged chloroplast exhibiting multiple division rings	Plastid envelope	Haswell and Meyerowitz, 2006; Wilson *et al.*, 2011; Veley *et al.*, 2012
		MSL4	881	NR	Loss of predominant MS channel activity in root of the *msl4 msl5 msl6 msl9 msl10* quintuple mutant	NR	Haswell, Peyronnet *et al.*, 2008
		MSL5	881	NR	Refer to *MSL4*	NR	Haswell, Peyronnet *et al.*, 2008
		MSL6	856	NR	Refer to *MSL4*	NR	Haswell, Peyronnet *et al.*, 2008
		MSL9	742	NR	*msl9* null mutant is associated with a loss of 45 pS activity in root protoplast	Plasma membrane	Haswell, Peyronnet *et al.*, 2008
		MSL10	734	NR	*msl10* null mutant is associated with a loss of 137 pS activity in root protoplast	Plasma membrane	Haswell, Peyronnet *et al.*, 2008
	Chamydomonas reinhardtii	*MSC1*	522	Chloroplast organization	RNAi-mediated knockdown lines show reduced chlorophyll autofluorescence and loss of chloroplast integrity	Chloroplast envelope	Nakayama *et al.*, 2007
	Schizosaccharomyces pombe	*Mys1*	1011	Involved in regulating intracellular Ca^{2+} and cell volume during hypoosmotic stress	*mys1*$^-$ *mys2*$^-$ mutants show decreased viability during osmotic down-shock and treatment with $CaCl_2$	Perinucler ER	Nakayama *et al.*, 2012
		Mys2	840	Involved in regulating intracellular Ca^{2+} and cell volume during hypoosmotic stress	*mys2*$^-$ and *mys1*$^-$ *mys2*$^-$ mutants show decreased viability during osmotic down-shock and treatment with $CaCl_2$	Cortical ER	Nakayama *et al.*, 2012

NR = Not Reported

* = Unprocessed protein

Table 2. Physiological function of MscS family members.

	Species	Name	Unitary conductance	Ion selectivity (P_{Cl} : P_K)	Number of TMHs[c]	Identity in the pore-lining domain + upper vestibule to *Ec*MscS, %[d]	References
Prokaryotes	*E. coli*	*Ec*MscL[a]	3 nS [1]	Non-selective	2	-	Sukharev, 1994; Häse, 1995
		*Ec*MscS	1.2 nS [1] / 350 pS [5]	1.2 - 3	3	100	Levina, 1999; Sukharev, 2002
		*Ec*MscK	1 nS [1]	< *Ec*MscS	11*	32	Martinac, 1987; Li, 2002
		YjeP	250-400 pS [1]	NR	11*	27	Edwards, 2012
		YbdG[b]	350-400 pS [1]	NR	5*	21	Schumann, 2010
		YnaI	~ 100 pS [1]	NR	5*	30	Edwards, 2012
		YbiO	~ 850 pS [1]	NR	12*	24	Edwards, 2012
	S. pomeroyi	MscSP	1.04 nS [2]	1.4	3*	49	Petrov, 2013
	T. tengcongensis	*Tt*MscS	134 pS [1]	8.7	3	29	Zhang, 2013
	C. glutamicum	MscCG	328 pS [2]	0.3	4	29	Börngen, 2010; Becker, 2012
	M. jannashii	MscMJLR	2 nS [3]	0.2	5*	41	Kloda, 2001a, b
		MscMJ	270 pS [3]	0.16	5*	36	Kloda, 2001a
Eukaryotes	*C. reinhardtii*	MSC1	390 pS [4]	7	5*	32	Nakayama, 2007
	A. thaliana	MSL10	103 pS [5]	5.9 (P_{Cl} : P_{Na})	6*	18	Haswell, 2008; Maksaev, 2012

[1] 200 mM KCl, 90 mM $MgCl_2$, 10 mM $CaCl_2$, and 5 mM HEPES (pH 7.0)
[2] 250 mM KCl, 90 mM $MgCl_2$, and 5 mM HEPES (pH 7.2)
[3] 200 mM KCl, 5 mM $MgCl_2$, and 5 mM HEPES (pH 7.2)
[4] 200 mM KCl, 40 mM $MgCl_2$, 10 mM $CaCl_2$, 0.1 mM EDTA, and 5 mM HEPES-KOH (pH 7.2)
[5] 96 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES (pH 7.38)
[6] 100 mM KCl, 10 mM $MgCl_2$, 10 mM HEPES-KOH (pH 7.4)
[a] MscL is not a MscS homolog, added for reference
[b] Channel activity was only shown for a V229A mutant of YbdG-encoded protein
[c] number of transmembrane helices were predicted via TMHMM 2.0 server.
[d] Alignments were made using Kalign algorithm in Unipro UGENE software
* = Predicted
NR = not reported

FIGURE LEGENDS

Figure 1. Schematic representation of models for mechanosensitive channel gating. (A) The intrinsic bilayer model, wherein lateral membrane tension favors the open state of the channel. (B) The

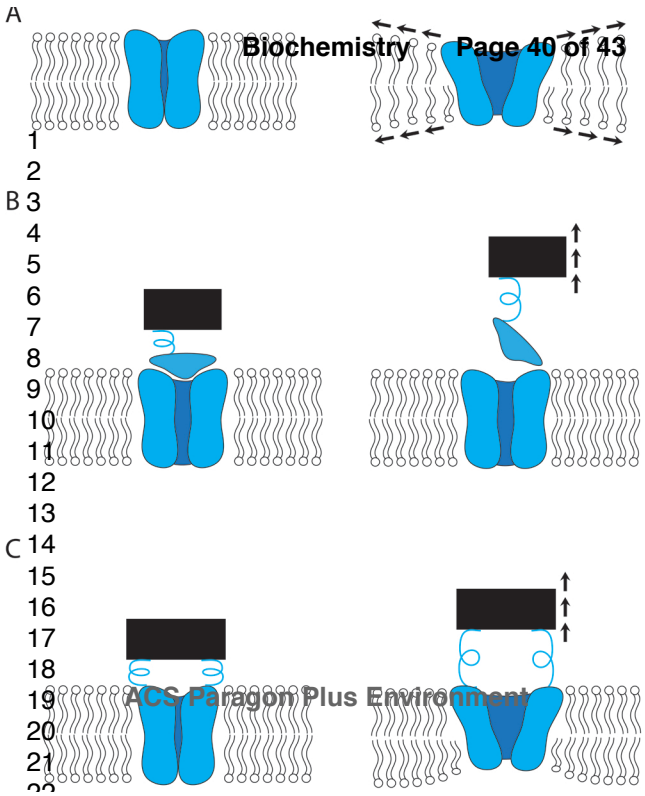
tethered trapdoor model, wherein a tether to an extracellular (in this case) component exerts force on the channel, leading to its gating. (C) The unified model, wherein a tether to an extracellular component leads to reorientation of the channel within the membrane bilayer, thereby gating it.

Figure 2. Crystal structures of *E. coli* MscS and homologs. (A) *Ec*MscS in "inactive/non-conductive state" (2OAU, Steinbacher, 2007); (B) *Tt*MscS from *T. tengcongensis* in "closed" state (3UDC, Zhang, 2012); (C) A106V *Ec*MscS mutant in "open" state (2VV5, Wang, 2008); (D) *Ec*MscS in "open" state (4HWA, Lai, 2013). The monomers in a heptamer labeled by color; surface map, viewed from the periplasm is truncated at I175 in order to display unobstructed channel pore. Left panel: side view of the heptametic channel; middle panel: view of the channel from the periplasmic side; right panel: space-filling representation of the channel with a pore, view from the periplasmic side. Basic residues are blue, acidic residues are red, polar residues are green, non-polar residues are white. The images were generated with VMD software (University of Illinois).

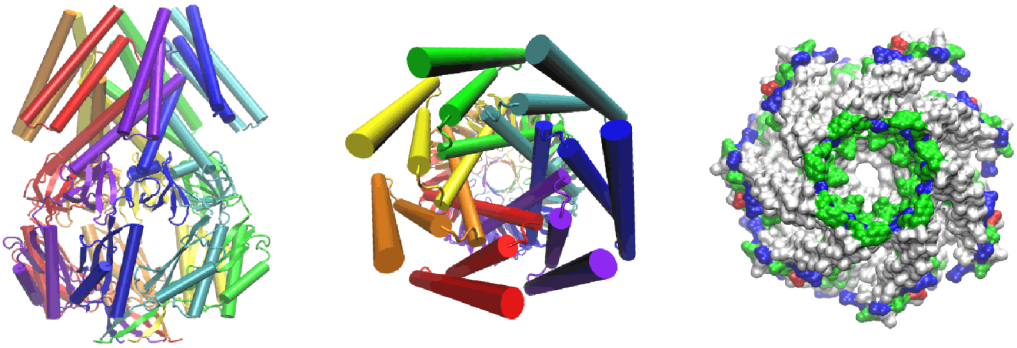
Figure 3. The conserved region of *Ec*MscS and *Tt*MscS monomers in different conformations. A single monomer of (A) *Ec*MscS (aa 27-175) in a nonconducting state (2OAU, Steinbacher, 2007); (B) *Tt*MscS (aa 13-175) in a nonconducting state (3UDC, Zhang, 2012); (C) *Ec*MscS A106V (aa 25-175) in an open state (2VV5, Wang, 2008). (D) Superposition of panel A (silver) with a single monomer of *Ec*MscS (27-175) in an open state (4HWA, Lai, 2013, cyan). The kink-forming residues G113 (*Ec*MscS) and G109 (*Tt*MscS) are represented as blue spheres and the A106V mutation as a red sphere. The vapor-lock residues L105 and L109 (*Ec*MscS) and L104 and F108 (*Tt*MscS) are labeled in yellow. F68 and L111, residues proposed to mediate the TM2-TM3 interaction in *Ec*MscS (Belyy, 2010) are labeled magenta. Images were generated with VMD software (University of Illinois).

Figure 4. Monomer topologies of representative MscS family members. MscS monomer topology was rendered based on Naismith, 2012. For the purpose of clarity TM3b of MscS is represented outside the lipid bilayer. MscK and MscCG topologies were predicted with TOPCONS (http://topcons.net/) and

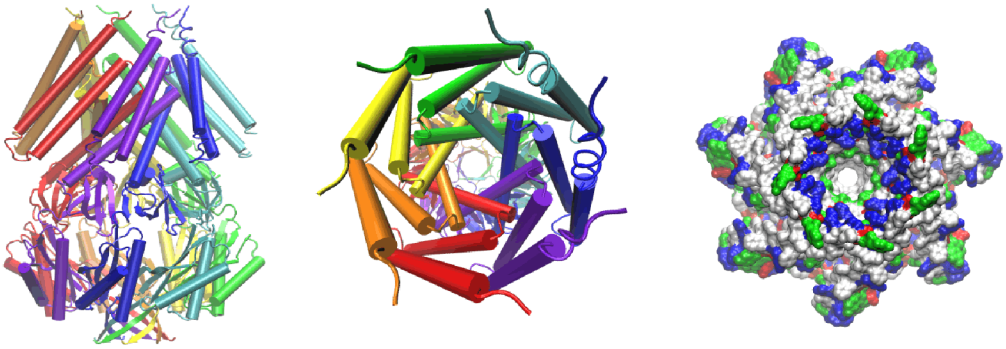
ARAMEMNON (http://aramemnon.botanik.uni-koeln.de/) for MSL2 and MSL10. Processed versions of MscK and MSL2 are presented.



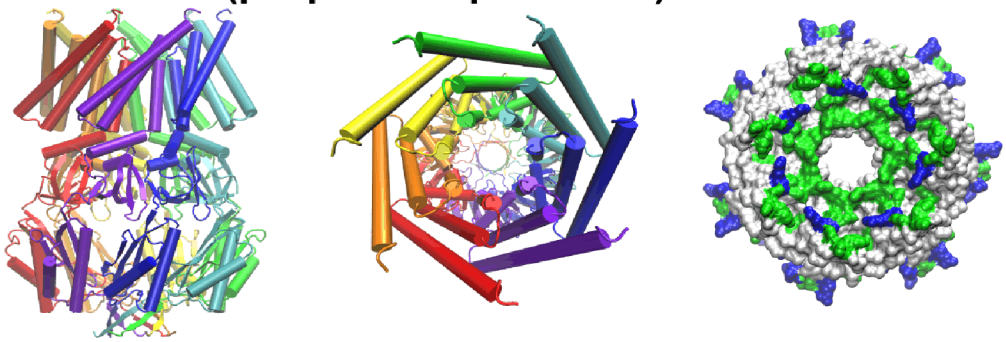
A) *Ec*MscS (proposed inactive state)



B) *Tt*MscS (proposed closed state)



C) *Ec*MscS A106V (proposed open state)



D) *Ec*MscS (proposed open state)