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Phosphatidylinositol Is Crucial for the Mechanosensitivity of *Mycobacterium tuberculosis* MscL

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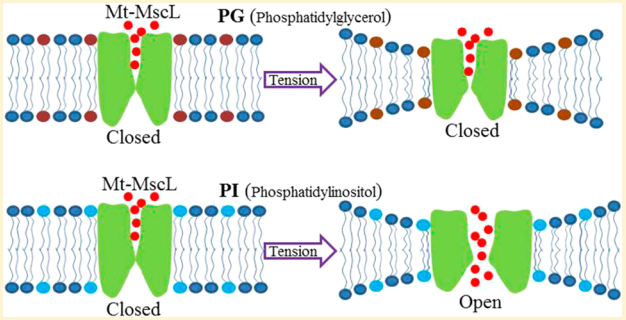
ABSTRACT: The bacterial mechanosensitive channel of large conductance (MscL) directly senses and responds to membrane tension. It serves as an "emergency release valve" upon acute decreases in the osmotic environment, thus preventing cell lysis. It is one of the best studied mechanosensitive channels and serves as a paradigm of how a channel senses and responds to its membrane environment. The MscL protein is highly conserved, found throughout the bacterial kingdom, and has been shown to encode a functional mechanosensitive channel in all species where it has been studied. However, channels from different species have shown some functional variance; an extreme example is the *Mycobacterium tuberculosis* MscL, which when heterologously expressed in *Escherichia coli* requires significantly more membrane tension for gating than the endogenous *E. coli* MscL. We previously speculated that the membrane environment or factors not found in *E. coli* promoted the proper gating of the *M. tuberculosis* MscL channel in its native environment. Here, by reconstituting the *M. tuberculosis* and *E. coli* MscL channels in various lipids, we demonstrate that inclusion of phosphatidylinositol, a lipid found in *M. tuberculosis* but not *E. coli*, is sufficient for gating of the *M. tuberculosis* MscL channel within a physiological range of membrane tension.



MscL is one of the best studied mechanosensitive channels from any species.[1−4] It was initially isolated from *E. coli*, where many of the initial studies were performed. However, it was a homologue, *Mycobacterium tuberculosis* MscL (Mt-MscL), which was found to be amenable to crystallization. The crystal structure of Mt-MscL, solved at 3.6 Å, revealed that the protein forms a homopentameric complex (Figure 1 A, right two panels);[5] although there have been controversies on the oligomeric structure, specifically for a *Staphylococcus. aureus* orthologue,[6] the pentameric nature of the complex *in vivo* has been recently confirmed.[7,8] Each subunit of the complex contains an N-terminal helix that runs along the membrane, two transmembrane domains, TM1 and TM2, which are connected by a periplasmic loop, and a C-terminal helix that forms a helical bundle from the five subunits[5,9,10] (Figure 1A, left panel). Several studies have led to proposals for the function of each domain, as well as a cohesive model for conformational changes that occur upon channel gating.[2,4,11] The function of the protein appears to be preserved among many bacterial species; a previous study had found that the *mscL* genes from a variety of bacterial species encoded a mechanosensitive channel activity when expressed in *E. coli*; however, Mt-MscL was not among the initial orthologues studied.[12] Thus, after the crystal structure had been published, we sought to determine if the Mt-MscL was functional; that is, could we definitively state that Mt-MscL was an orthologue? We found that although MscL-like channel activities could be recorded, significantly more membrane tension was required to gate the channel when expressed heterologously in *E. col*.[13] In addition, unlike other orthologues, wild type Mt-MscL was unable to rescue MscS/MscL double null *E. coli* cells from an acute osmotic down shock.[13] These properties of the Mt-MscL channel make the acquisition of recordings extremely difficult; thus, in spite of the advantage of an available crystal structure, most MscL studies have continued to be performed on the *E. coli* MscL (Ec-MscL) orthologue.

It is well accepted that specific lipids can play an important role in the function of membrane proteins, including ion channels. For example, anionic lipid components are crucial to the channel function of the bacterial inward rectifier potassium channel KcsA.[14] In the original functional characterization of Mt-MscL, we postulated that there was an environmental factor missing from *E. coli* that would allow normal gating in its native environment.[13] It seems reasonable that a membrane lipid component could be this factor. The inner membrane of *E. coli* is composed of 75−85% of phosphatidylethanolamine (PE), 10−20% of the anionic phosphatidylglycerol (PG), and about 5−15% of cardiolipin (CL).[15] However, while the *M. tuberculosis* membrane is also dominated by PE lipids and contains a small percentage of CL, the second major component is the anionic lipid phosphatidylinositol (PI),[16,17] which can account for up to a quarter of total phospholipids in *Mycobacteria* membranes.[16] Hence, the major difference in the lipid composition is the primary anionic lipid. Here we have

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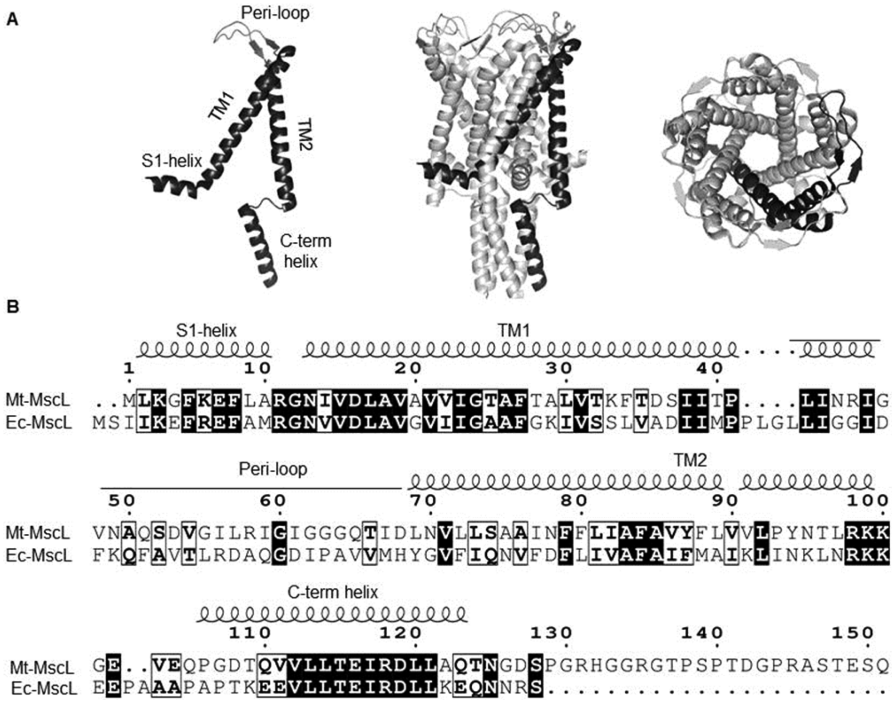

Figure 1. Crystal structure of Mt-MscL and alignment of MscL homologues. (A) Crystal structure of Mt-MscL, a homopentamer with each subunit containing two transmembrane domains (TM1 and TM2), an N-terminal S1-helix, a C-terminal helix, and linkers between helices. (B) Amino acid sequence alignment of MscL homologues from *M tuberculosis* (Mt-MscL) and *E. coli* (Ec-MscL). Identical and similar residues are shown in boxes. The functional regions of MscL protein, the S1-helix, TM1, the periplasmic loop, TM2, and the C-terminal helix are shown above the sequences.

studied the effects of different lipid components on the Ec- and Mt-MscL channels and found that inclusion of PI is sufficient for normal Mt-channel function.

MATERIALS AND METHODS

Strains and Protein Expression. Wild type *E. coli MscL* and *M. tuberculosis MscL* with C-terminal six-His-tags were inserted into pET21a expression construct and transformed into *E. coli* strain PB116 cells for protein expression.[18,19] Wild type *E. coli MscS* with a six-His-tag was subcloned into pB10b expression vector and expressed in PB104 (Δ mscL:Cm) cells.[18,19] Cultures were grown routinely in Lennox broth medium (Fisher Scientific, Pittsburgh, PA, USA) containing 100 ug/mL ampicillin. Expression was induced by addition of 1 mM IPTG at 37 °C. Cells were harvested after 3 h of induction.

Protein Purification. Protein purification was performed as previously described.[20] Briefly, cells were resuspended in base buffer containing 10 mM potassium phosphate buffer at pH 8.0 (KPi), 300 mM NaCl, plus 0.5 μg/mL Dnase, 1 mg/mL Lysozyme, 1 tablet protease inhibitor cocktail (Roche, Indianpolis, IN, USA), and they were subjected to two passages through a French pressure cell at 16,000 psi to complete the lysis at 4 °C. The lysate was subjected to extraction by addition of 15 mM imidazole and 2% Triton. MscLH6 or MscSH6 was purified from the membrane by using Ni-NTA resin (Thermo Scientific, Rockford, IL, USA). The Ni-NTA was added to the cleared lysate and incubated at room temperature for 1 h. Then the resin was washed once with extraction buffer (10 mM KPi, pH 8.0, 300 mM NaCl, 15 mM imidazole, and 2% Triton), washed twice with wash buffer (10 mM KPi, pH 8.0, 300 mM NaCl, 20 mM imidazole, and 1% Triton), and transferred to a column. The column was washed with 30 mL of wash buffer and eluted with elution buffer (10 mM KPi, pH 8.0, 300 mM NaCl, 250 mM imidazole, and 0.2% Triton). The elution fractions were subjected to SDS-PAGE, and protein was pooled for concentrating and BCA assay (Pierce, Rockford, IL, USA).

Co-reconstitution. Lipid vesicles for reconstitution were prepared as previously described.[20] Lipids, Azolectin, also known as soy total lipid extract (Soy PC 20% Cat# 541601), cardiolipin (CL), 1,2-dioleoyl-*sn*-glycero-3-XX (DOXX), where XX is one of the following headgroups, phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylinositol (PI), or phosphatidylethanolmine (PE), were utilized (Avanti polar lipids, Inc., Alabaster, AL, USA). Briefly, lipids were dissolved in chloroform and the proper amounts of different lipids were added to a glass tube, mixed, and dried under argon. The lipid film was further dried under vacuum for another 2 h and resuspended to 20 mg/mL buffer (10 mM Tris, pH 7.5, 1 mM EDTA, and 1 mM EGTA) and rehydrated at 45 °C for at least 2 h. The lipid vesicles were formed by bath sonication. MscLH6 (Mt-MscLH6 or Ec-MscLH6) and MscSH6 proteins were combined with lipid vesicles to a 1:500 protein-to-lipid mass ratio. Detergent was removed by dialysis with 3 × 1000 mL of buffer (5 mM Tris, pH 7.5, 100 mM NaCl, 0.2 mM EDTA, and 0.02% NaN_3) containing Biobeads (Bio-Rad, Hercules, CA, USA). The resultant liposomes were collected by a 20-min centrifugation at 30 psi in an airfuge. The pellet was resuspended to 1 mg/5 μL in buffer (10 mM MOPS, pH 7.5, 5% ethylene glycerol) and desiccated overnight under vacuum at 4 °C.

Electrophysiology. Desiccated liposomes were rehydrated in buffer (0.25 mM Hepes, pH 7.5, 10 mM KCl, and 2 mM $MgCl_2$ plus 320 mM sucrose) at a lipid concentration of 90 mg/mL as described previously.[21] Excised patches were studied at room temperature under symmetrical patch buffer conditions (5 mM Hepes, pH 7.5, 200 mM KCl, 40 mM MgCl2). Data

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were acquired at −20 mV at a sampling rate of 20 kHz filter, using an AxoPatch 200B amplifier in conjunction with Axoscope software (Axon Instruments, Union City, CA, USA). A piezoelectric pressure transducer (World Precision Instruments, Sarasota, FL, USA) was used to monitor the pressure introduced to the membrane by suction throughout the experiments. For neutral liposomes, the presence of 200 mM KCl inhibited the formation of unilamellar blisters; thus, patch buffer containing no KCl was used in the chamber, and KCl was added to a final concentration to 200 mM after the seal formation. The channels were gated by applying negative pressure to the patch. Trace acquisition and analyses were performed using Clamfit10 from pCLAMP10 (Axon instruments).

Mechanosensitivity. Mechanosensitivity was determined as previously described.[18,22] Briefly, MscS is used as an internal standard within the patch, and the ratio of gating pressure for MscL divided by the pressure of MscS, expressed as pL/pS ratio, is determined. For neutral liposomes, the channel threshold pressure was used instead of pL/pS ratio, due to the reversal of MscS/MscL sensitivities, which made analysis impractical. All data were obtained from at least three independent recordings performed in at least two independent protein purifications and reconstitutions.

Statistics. Data were analyzed for statistical significance using the Student's *t* test.

RESULTS

We sought to determine the functional properties of the Ec- and Mt-MscL channels in different anionic lipid environments. We have previously utilized another mechanosensitive channel endogenously expressed in *E. coli*, MscS (mechanosensitive channel of small conductance), as an internal standard control.[18,22] This allows for a more accurate determination of differences in sensitivity to membrane tension, or for simplicity what we will call mechanosensitivity. This approach is easily performed when the channels are expressed in giant cells derived from *E. coli*. The mechanosensitivities of the channels are then expressed as the pressure threshold of the MscL gating divided by the pressure threshold of MscS (pL/pS). This convention was utilized in our previous study, where we found that the Mt-MscL required a much greater pL/pS ratio (2.8) relative to the Ec-MscL channel (pL/pS of 1.4) when heterologously expressed in giant *E. coli* cells;[13] or put another way, the Mt-MscL channel was far less sensitive to membrane tension when expressed in *E. coli* native membranes.

We wanted to use MscS as a standard in reconstitution studies, as reported by Battle and co-workers,[23] where we tested the effect of different lipid compositions on channel function. Thus, we co-reconstituted and assayed by patch clamp purified MscL and MscS proteins into liposomes composed of synthetic lipids that would largely reproduce either the *E. coli* inner membrane (85%DOPE/13%DOPG/2%CL) or that of *M. tuberculosis* (85%DOPE/13%DOPI/2%CL). Figure 2A shows typical current traces of the channels with the pressure threshold (mmHg) under each trace. Figure 2B summarizes the mechanosensitivity threshold (pL/pS) of Mt-MscL and Ec-MscL in the different lipid conditions. Consistent with our results from the giant *E. coli* cells, in the PG containing liposomes, the activation threshold of Mt-MscL was significantly higher than the threshold required for Ec-MscL channel activation. In contrast, in PI containing *M. tuberculosis*-like (Mt-like) lipids, the Mt-MscL became more mechanosensitive, with

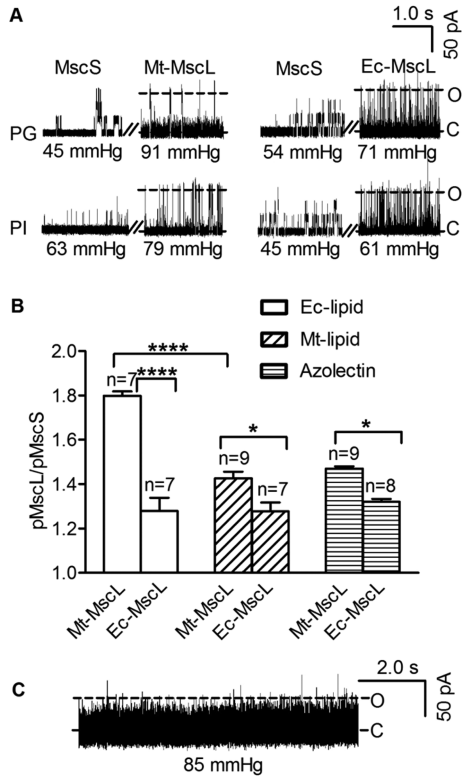

Figure 2. Mechanosensitivity of Mt-MscL and Ec-MscL in different membrane lipid conditions. (A) Patch clamp recording of Mt-MscL and Ec-MscL in *E. coli*-like (Ec-like) lipids and Mt-like lipids. Upper and lower rows show the typical current traces of Mt-MscL (left) and Ec-MscL (right) along with the Ec-MscS in PG containing Ec-like lipids (85%DOPE/13%DOPG/2%CL) and PI containing Mt-like lipids (85%DOPE/13%DOPI/2%CL), respectively. Pressure thresholds are shown under each trace. "O" and "C" refer to channel open and closed states. The scale bar is shown at the top right. (B) Pressure sensitivity analysis of Mt-MscL and Ec-MscL in Ec-like lipids, Mt-like lipids, and Azolectin. $*p < 0.05$, $****p = 0.0001$ (two tailed unpaired student *t* test). (C) A short open dwell behavior observed in a subset of Mt-MscL channels in Mt-like lipids. "O" and "C" refer to channel open and closed states. The scale bar is shown at the top right.

a ratio approaching that observed for the Ec-MscL, thus indicating the mechanosensitivity of the Mt-MscL channel is within the normal range when 13% PI is present.

To further verify this finding, we coreconstituted MscS and Mt-MscL in azolectin lipids, which contain approximately 12% PI. Consistent with the results from lipids containing PI, we found that Mt-MscL was more mechanosensitive in azolectin when compared to PG containing lipids. These results support the observation that the presence of PI is sufficient for an increased mechanosensitivity of Mt-MscL. While it appears that, in both cases PI greatly enhances the mechanosensitivity of Mt-MscL, it does not quite fully achieve the mechanosensitivity of Ec-MscL; this could indicate yet another subtle factor within the native environment of *M. tuberculosis* that is missing, or simply be a slight species variance.

Even though a fully open state of Mt-MscL was observed on many occasions when reconstituted in synthetic lipid membranes containing anionic lipids, the channel activities appeared heterogeneous. In a subset of patches, a channel showed a behavior in which it obtained very short open dwell times, beyond equipment and computational resolution. Figure 2C shows a typical trace with such channel behavior in

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synthetic lipids containing PI. The mechanisms underlying this heterogeneity are currently unknown. Nevertheless, channels showing different kinetic properties had no difference in their mechanosensitivity.

The data suggest that PI interacts with the Mt-MscL to allow it to function at more physiological levels of membrane tension. However, from the above data we could not formally rule out an alternative possibility: that PG inhibits Mt-MscL channel gating. To test this, we removed all negatively charged lipid headgroups and reconstituted the channels into neutral lipids containing 50% DOPE and 50% DOPC. Unfortunately, both MscL channels opened at tensions lower than MscS, making it difficult to obtain an accurate threshold ratio. Therefore, we were confined to assaying absolute pressure rather than threshold ratios. The results, however, were clear. Figure 3A shows typical traces of Mt-MscL and Ec-MscL in 50% DOPE/50% DOPC liposomes. As shown in Figure 3B, we still needed over twice the pressure to activate Mt-MscL relative to Ec-MscL. Thus, it appears that PG within the *E. coli* membrane does not inhibit Mt-MscL channel function; instead, PI increases the sensitivity of the Mt-MscL channel.

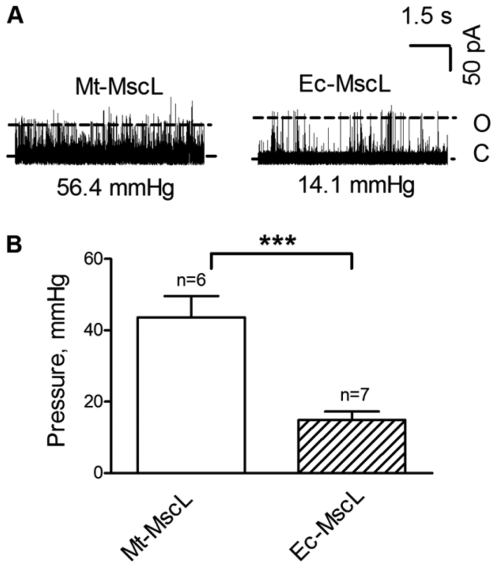

Figure 3. Pressure threshold of Mt-MscL and Ec-MscL in neutral lipids. (A) Patch clamp recording of Mt-MscL and Ec-MscL in lipids composed of 50%DOPE/50%DOPC. "O" and "C" refer to channel open and closed states. The pressures for MscL open are shown under each trace. The scale bar is shown at the top right. (B) Pressure threshold analysis of Mt-MscL and Ec-MscL in lipids composed of 50%DOPE/50%DOPC. ***p = 0.0006 (two tailed unpaired student t test).

DISCUSSION

While it has been known for a number of years that the Mt-MscL was not functional when expressed in *E. coli* cells, the underlying reasons were not clear. Here we find that it is because PI, a lipid not expressed in *E. coli*, is crucial for its normal function. Our results largely explain the data obtained in the original characterization of Mt-MscL, except for one observation. In the previous study, the Mt-MscL channel activity was characterized in several systems including azolectin lipids, which contains approximately 12% PI; in contrast to the present study, the previous results suggested that the Mt-MscL required more stimuli to gate.[13] However, there are complications to the interpretation of the previous study. The reconstituted protein was N-terminally multi-His tagged, and modification at this end of the protein has been shown to disrupt channel function;[22,24,25] here we have used a C-terminally tagged protein. In addition, the experiments could not be well controlled because at the time MscS had not yet been observed in a reconstitution system and was therefore not used as an internal control. Hence, in this study we repeated this experiment and utilized our co-reconstitution approach to allow us to better interpret the results. The results we report here support the observation that the presence of PI is crucial and sufficient for an increased mechanosensitivity of Mt-MscL.

Lipids, especially negatively charged lipids, have been shown to modulate many different channel types.[26−28] However, this has not been true for MscL. Although one study suggested anionic lipids helped in the reconstitution of functional Ec-MscL channels,[29] this was performed using flux assays; the best evidence using patch clamp as an assay suggests anionic lipids play little if any role in normal Ec-MscL function[20] (also see ref 11 for a detailed discussion). The relative lack of influence of lipid composition on Ec-MscL channel function seemed surprising, given the function of the protein: directly sensing biophysical changes in the lipid membrane. Thus, the finding that PI is sufficient for normal Mt-MscL activity demonstrates not only that lipids can influence MscL channel activity, it also indicates that lipid interactions may be dependent upon the orthologue being studied.

The mechanism for how PI influences the gating of Mt-MscL would be of prime interest. There have been mutations that influence mechanosensitivity, for instance E104Q and V15C gate at lower tension than wild type Mt-MscL;[30] however, these studies were performed in *E. coli* speroplasts and therefore do not reflect mutations that influence the protein interactions with PI. On another front, Powl and co-workers have identified the C-terminal charged cluster, specifically Arg-98, Lys-99, and Lys-100, as a hot spot for anionic lipid binding in Mt-MsccL;[31] here again, however, the studies were performed in a reconstitution system where the liposomes did not contain PI. Hence, the mechanisms of how and where the Mt-MscL channel interacts specifically with the PI headgroup remain a mystery that will require further study.

One of the surprising findings was that MscL opened at lower tension than MscS when co-reconstituted in neutral lipids. At first glance, this seems to contradict observations by Nomura and co-workers, who reported activation ratios of pL/pS in 70%PE/30%PC liposome of about 1.7, indicating that MscL opened at a higher pressure threshold than MscS.[32] However, the lipid composition used here is slightly different and is composed of less PE, having a ratio of 50% PE/50% PC. It is currently unclear why this reversal occurs; however, previous studies have shown localization of MscS at the cell poles was CL-dependent; thus, CL may influence the function of MscS.[33] Another possibility is that the increased PE plays a role; it has been shown that PE makes MscL less sensitive to membrane tension, presumably because of the small headgroup size;[20,34] thus, it is possible that this is exaggerated for MscS, causing the observed reversal in channel opening. Regardless, the pressure required for Mt-MscL to open was significantly higher than that for Ec-MscL in neutral lipids. Our results demonstrate that PG does not inhibit Mt-MscL channel gating.

Together the data strongly suggest that Mt-MscL is indeed an orthologue, serving the function of an osmotic emergency

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release valve when expressed in its native environment. The reason for its apparent inability to function at physiological membrane tensions in previous studies was because PI, which is expressed in the *M. tuberculosis* but not *E. coli* membranes, increases the sensitivity of the Mt-MscL channel so it is within a physiological range.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

MscL, mechanosensitive channel of large conductance; MscS, mechanosensitive channel of small conductance; Ec-MscL, *Escherichia coli* MscL; Mt-MscL, *Mycobacterium tuberculosis* MscL; Ec-like, *Escherichia* coli-like; Mt-like, *Mycobacterium tuberculosis*-like; CL, cardiolipin; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylglycerol; DOPI, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylinositol; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolmine

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