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Invited review

The evolution of bacterial mechanosensitive channels

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ABSTRACT

Mechanosensitive channels are ubiquitous and highly studied. However, the evolution of the bacterial channels remains enigmatic. It can be argued that mechanosensitivity might be a feature of all membrane proteins with some becoming progressively less sensitive to membrane tension over the course of evolution. Bacteria and archaea exhibit two main classes of channels, MscS and MscL. Present day channels suggest that the evolution of MscL may be highly constrained, whereas MscS has undergone elaboration via gene fusion (and potentially gene fission) events to generate a diversity of channel structures. Some of these channel variants are constrained to a small number of genera or species. Some are only found in higher organisms. Only exceptionally have these diverse channels been investigated in any detail. In this review we consider both the processes that might have led to the evolved complexity but also some of the methods exploiting the explosion of genome sequences to understand (and/or track) their distribution. The role of MscS-related channels in calcium-mediated cell biology events is considered.

1. Introduction

Mechanosensitive (MS) channels are ubiquitous and belong to many different protein families [1–5], which in itself may be indicative of multiple evolutionary origins. These channels differ widely in their structural complexity, potential functions and sensitivity to mechanical forces. Indeed it has been suggested that all channel proteins have the potential to be mechanosensitive in their gating [6]. Consequently one view would be that evolution may have selected for the loss of this property rather than mechanosensitivity being selectively acquired. This proposition would require significant constraints on the primary sequences in the initial channel families to avoid residues that lock channels in an inactive state [7,8], followed by acquisition of, and selection for, proteins of diminished mechanosensitivity during the time course of evolution. Discussion of such intrinsic mechanosensitivity has focused both on channels that exhibit a low threshold for activation [6] and on those with higher thresholds for example the well-characterised bacterial MS channels, MscS and MscL [9–11] and the eukaryotic TRAAK family [4,12–14]. The importance of the specific interactions of mechanosensitive channels with lipids for their gating has become a dominant theme in recent years and there is a critical interplay between structure and mechanism [13,15,16]. This review seeks to speculate on the evolutionary paths to current bacterial mechanosensitive channels, particularly the MscS family [16,17]. This structurally diverse family is built around a core mechanosensitive channel domain that has become decorated with additional domains often of unknown function. However, the EF-hand MscS channels provide a clear example of elaborations that are understood. The potential role of channels in Ca^{2+} signalling is also considered in the context of the creation, by mechanosensitive channels, of a non-specific spore across the lipid bilayer that might serve as a conduit for cation entry (both Ca^{2+} and Na^{+}) [18–21].

1.1. In the beginning

There are few certainties around the evolution of life from the primordial cells to current day cell complexity [22–24]. However, the absence of a rigid cell wall, even one that is dynamic, like bacterial peptidoglycan, seems almost a certainty. Equally, given the predominance of phosphate-based metabolism throughout life, Ca^{2+} exclusion may have been an essential prerequisite for evolution. A semipermeable membrane that set the boundary for the cytoplasm would have surrounded primordial cells. Such cells could have had

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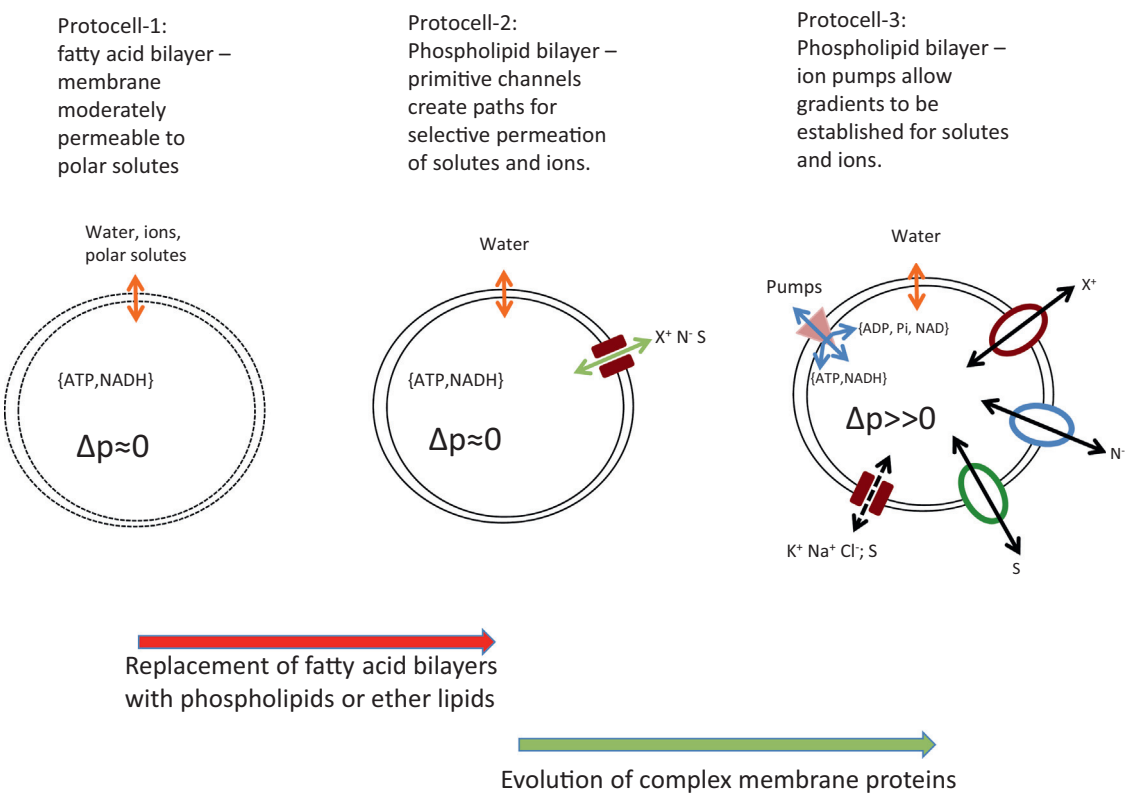

Fig. 1. Evolution of membrane processes. Potential events in the early evolution of membrane processes. The model envisages three initial stages of complexity. Protocell-1: has a permeable fatty acid bilayer membrane [23,25]; Protocell-2: the emergence of phospholipid bilayers and the necessary, simple, channels envisaged as homo-oligomers of small peptides [112]. Protocell-3: the evolution of more complex membrane proteins enabling the generation and utilisation of ion gradients. In Protocell-1 metabolism generates NAD(P)H required for reductive anabolism and ATP by substrate level phosphorylation. The intrinsic leakiness of the membrane means that there is no substantial protonmotive force (Δp) but there is likely to be a Donnan Potential due to the presence of fixed anions in the cytoplasm on DNA and RNA. Protocell-2, the formation of a phospholipid bilayer restricts transmembrane ion (X^+ and N^-) and solute (S) movement to protein-lined pathways, such as simple channels constructed from homo-oligomers of single peptides. In the absence of ion pumps Δp remains close to zero and chemical gradients drive solute influx to equilibrium. Protocell-3, elaboration of membrane proteins through gene duplication, fusion and divergence leading, ultimately, to energy transducing complexes that can either generate or utilize a protonmotive force ($\Delta p \gg 0$). The pathways of gene divergence allow transporter and channels proteins to acquire specificity and channels to acquire gating.

bilayers constructed from two leaflets of fatty acids [23,25] rather than the current structure derived from phospholipids or ether lipids (Fig. 1). Fatty acid membranes have the advantages that they self-assemble in a concentration-dependent manner, which gives an early basis for growth, and they have a higher permeability to polar solutes than is the case for phospholipids. Their great disadvantage is that assembly and stability of the bilayer is limited to a small set of potential environmental conditions and such a bilayer would certainly have constrained the range of pH value in which such protocells could have been stable [25]. Thus a major driver for the evolution of phospholipids and ether lipids would have been the greater stability of the bilayers, allowing cells to expand into a wider range of niches. Simultaneously the lower permeability of the phospholipid bilayer would have required mechanisms for solute transfer. One scenario is that the first channels arose to facilitate increased selective ion permeation to counter the permeability barrier imposed during the transition from fatty acid membranes to phospholipid bilayers. Simple homo-oligomers of single span transmembrane peptides, like Alamethicin, or the slightly more complex gramicidins [26], would allow selective ion movement (Fig. 1). Such channels as these can exclude Ca^{2+} whilst facilitating the movement of other ions e.g. K^+ and Na^+. Present day toxins, e.g. *Helicobacter* VacA, may mimic the original channels, and structural remnants may exist in mechanosensitive channels, such MscS. In the latter the pore is in essence created by a heptamer of transmembrane helices (TM3a) anchored in the bilayer by TM3b [16,27]. The VacA toxin creates anionic-selective pores via a hexameric assembly of a single transmembrane helix that is organised like the pore helices of MscS [28]. Subsequent events, such as those described below, would have led to the elaboration of complex transporters and their necessary energy coupling (Fig. 1).

1.2. The problems of osmosis

Water flows relatively freely through membranes, even in the absence of aquaporins [29]. All cells have a problem with water flow during metabolite transformations that must occur in the cytoplasm during growth. For example, a simple act, such as initiating metabolism of a sugar, generates multiple metabolites [30] leading to an increase in cytoplasmic osmolarity and a driving force for water entry. The lytic tension of *Escherichia coli* membranes equates to around 250 mmHg applied pressure across the membrane and this can be achieved by a simple rise in the concentrations of cytoplasmic metabolites of around 10 mM [3]. In addition, extant metabolism predominantly generates anions [30] that must be accompanied by either protons generated from metabolism or other cations, which today are principally potassium or sodium ions, though some contribution from magnesium and organic polycations (polyamines) are also evident in today's cytoplasm [30]. The movements of K^+ and Na^+ in exchange for protons would be required to stabilise the cytoplasmic pH [31], so channel functions might initially have also evolved for this purpose. Inward movements of K^+, and possibly Na^+, are now mediated by channel-like proteins (Trk, Kdp, Ktr, MthK and NaChBac) [32–38] although some of these proteins may have evolved different modalities (e.g. Kdp;

[39]). In contrast, ion efflux is usually via antiports that exchange the cytoplasmic cations for external protons [40] (see below).

1.3. Calcium in cells

Cells almost always have mechanisms to exclude Ca^{2+} since high concentrations of this cation in the cytoplasm are inimical, due to the insolubility of its phosphate salt at the mildly alkaline pH found in the majority of bacterial cells [31]. Maintenance of low Ca^{2+}, plus other properties of this cation, has led to its almost unique role in signalling [26]. Thus, during ion ingress through transporters and channels, organisms exhibit strong selectivity against Ca^{2+} despite its similar ionic radius to Na^+ and also have active antiport mechanisms to exclude this divalent ion from the cytoplasm [37,38,41–43]. Bacterial Ca^{2+} pools of around 90 nM have been measured in cells incubated with mM external Ca^{2+} [42]. Selective exclusion of Ca^{2+} must have arisen early in evolution, given that it is desirable not to allow its penetration into the cytoplasm.

Present day mechanosensitive channels create relatively large, transient, non-selective pores in the membrane and equilibrate ion gradients, if only for several minutes following their gating [44]. During hypoosmotic shock in the presence of high external concentrations of Ca^{2+}, this ion can penetrate to the cytoplasm through an open MscS or MscL pore [18]. This may be combined with the exquisitely sensitive Ca^{2+}-sensing systems to effect changes in gene expression [18,45,46]. There is a subtle difference between the projected early evolutionary events to exclude Ca^{2+} and the current day inadvertent flooding of the cytoplasm with this ion by the formation of a pore by a mechanosensitive channel or a toxin. Control is the essence of this distinction and can be seen in yeast [19,20] and in *Bacillus* [21] to effect specific physiological events (see Section 4).

1.4. Mechanosensitive channels and metabolism

Retention of mechanosensitivity in primordial ion-specific channels might have originally had the purpose of allowing control over of cation uptake (particularly potassium), limiting influx to that required to compensate for metabolically-generated anions. For this reason, inhibition of channel activity by bilayer tension (in distinction to activation, as in MscS, TRAAK and MscL) may have been a more critical evolutionary event. Once metabolism reaches a steady state, the cytoplasmic osmolarity would be stable but the cell might have a slight net outwardly directed turgor. Intrinsically the volume expansion associated with metabolism must be within the stability limits of the bilayer. Ion transport functions that might raise the turgor pressure to unsustainable levels need to be controlled. The lytic threshold in naked protoplasts, as measured by patch clamp technology, is quite high [3,9]. Thus primordial wall-less cells would be subject to small routine fluctuations in membrane tension as a result of transitions in metabolism. The major bacterial mechanosensitive channels, MscS and MscL, exhibit high gating thresholds [9,11] compared with known K^+ channels [6] and thus the high threshold may only have been selected for once cells evolved a cell wall. One can also argue that the high gating thresholds prevent inappropriate gating of the channels since the latter has been shown to be a lethal event [47].

The extant organisms that most resemble the primordial cells are probably the Mycoplasmas and bacterial L-forms. The latter are generated by digestion of the existing cell wall to release the protoplast and, while they are intrinsically very interesting, their mechanosensitivity should principally reflect that of their bacterial progenitors [48]. Intriguingly, genetic analysis of the requirements for growth of *Bacillus* L-forms identified enzymes that mediate the synthesis of branched chain lipids. These enzymes were critical for membrane fluidity and cell division, but not for cell expansion, which was unaffected by the genetic lesion [49–51]. In contrast to L-forms, Mycoplasmas have existed for a very long time as independent wall-less bacteria [48]. Mycoplasmas are stable dividing cells that exist without a bacterial cell wall but incorporate cholesterol to facilitate growth [48]. Cholesterol is known to stabilise membranes by stiffening the lipid bilayer [52]. Mycoplasmas are believed to have evolved by degenerate evolution from Lactobacilli and Clostridia. These two genera have 'normal' complements of mechanosensitive channels (i.e. both MscL and MscS-related channels) and most Mycoplasmas reflect their origins in this respect, having retained mechanosensitive channels during their evolution. Critically the retention of mechanosensitive channels over the 600,000 years of severe reductive genome complexity in the Mycoplasma genome [48], speaks volumes for their function in stabilizing even wall-less cells.

2. Current roles for channels in cell physiology

A great diversity of ion channels, both selective and non-selective, have been found in bacterial genomes and some of these have been characterised biochemically, often after heterologous expression in *E. coli*. The two most highly characterised families are the K channels and the mechanosensitive channels, although significant analysis of gating of the Na^+ channel has also been reported [37,38,53,54].

2.1. Potassium channels

Potassium channels are the major route for K^+ accumulation by bacterial cells. In higher organisms the roles of K^+ channels in cell physiology are more varied and complex, but in bacteria and some yeasts their core function is to provide a regulated pore for potassium movements across the membrane. In bacteria the channels are thought to be one half of the solution needed to maintain a potassium pool that is primarily set by the external osmolarity [55]. Thus, both influx and efflux must be managed and while the former, with its role for ion channels, has been moderately well-characterised, the efflux routes are poorly understood. Potassium efflux systems that play an important role in protecting cells against toxic insults are almost ubiquitous [56,57]. They share common regulatory structures with uptake channels, but they respond to specific and different signals [58]. The debate on their channel-like functions is unresolved.

For the most part bacterial potassium channels exist either as complex, multimeric, assemblies of at least two proteins – the pore domain and the regulatory KTN (RCK) domain (TrkAH, KtrAB are examples; [32,36]) or the same modules as a single polypeptides bearing two domains (MthK, GsuK, CglK; [33,34,59,60]). In some cases translation sites internal to the structural gene result in the expression of extra regulatory (KTN) domains that are critical to function [33]. The K^+ channel pore can be formed from either four identical subunits or from a single subunit that contains four similar domains – the consequence is that the symmetrical pore in the former is replaced by an asymmetrical structure in the latter (compare MthK with TrkHA; [33,36]). Variation in gene structure, and thereby the organisation of protein complexes to form functional channels, indicates multiple evolutionary pathways to similar functional solutions. It seems highly likely that the TRAAK and related mechanosensitive channels are evolutionary derivatives of the conventional potassium channels that drive electrical signalling and ion accumulation in bacteria [4,5,13]. Similar complexity in the extant structures for the bacterial multi-subunit sugar phosphotransferases also indicates a range of solutions that can be accommodated by the functional requirements of the system

[61,62]. Thus, one can argue that while function is selected for by evolution the structural solution is not necessarily conserved.

The KTN modules bind nucleotides and it is believed that the bound nucleotide may either generate a channel-gating signal [36] or may be required purely for structural reasons. This is most probably only part of the story and much remains to be discovered to account for the cellular properties of the transport complexes to which the KTN domains are coupled. Little is known in terms of the mechanosensitivity of these channels. However, analysis of K^+ movements into whole cells (using cells that have a reduced proton motive force generated by oxidation of cytoplasmic reserves) revealed complexities in relation to modulation of the K^+ pool that suggest some regulation of Trk channel activity by changes in membrane tension [63]. The simplest explanation for the observations is that the Trk channel is held relatively inactive by high cytoplasmic turgor and is stimulated by lowering of the turgor either by growth-associated volume changes or by raising the external osmolarity. While these changes in activity may reflect the regulation of Trk by nucleotides, the current literature cannot eliminate the possibility of mechanosensitivity. Unfortunately, these studies have not been developed systematically over the last 30 years.

2.2. Mechanosensitive channels

Two main families of channels, recognised for their mechanosensitivity, exist in bacteria and archaea and are also found in plants (particularly plant organelles) and some fungi [1,64,65]. Whereas the channels from bacteria, archaea and plants have received a large amount of direct analysis, the properties of many channels have not been analyzed because their existence is only inferred from genome sequences. The two principal bacterial mechanosensitive channels are MscL and MscS. Sequence conservation is quite strong among members of both families [64,66,67], but there is much greater diversity in structure among MscS homologues than is obvious among MscL channels [3,68].

The roles of mechanosensitive channels have been analyzed in a range of bacteria, and the results are least equivocal for *E. coli* [69]. MscS and MscL open in response to increases in membrane tension and transiently create large pores in the cytoplasmic membrane [9,11]. Different channels exhibit a unique conductance that should reflect a range of pore diameters ranging from the size of a hydrated potassium ion (~6 Å; *E. coli* YnaI) to MscL (~30 Å diameter). Such pores allow the rapid release of osmotically-active solutes from the cytoplasm in a manner that is limited predominantly by the diameter of the pore [70]. The pores are lined with hydrophobic residues and some of these residues also form the hydrophobic seal that maintains a non-conducting pore when the channel is 'closed' [27,71,72]. Gating, or opening, of the pore involves the movement of the pore-lining helices away from the central axis of the pore such that a continuous water column can be established, which provides the conduit for solute movement [73]. The driving force for solute movement are the chemical gradients of solutes across the membrane and thus, ions and solutes can move in both directions through the open pore [44]. This includes small ions such as protons, Ca^{2+} and Na^+ that are maintained at low concentrations in the cytoplasm but are at much higher abundance in the environment. Thus, cells must maintain the channel tightly closed until it is essential for survival, since energy conservation requires a proton impermeable membrane, pH homeostasis requires that protons are excluded from the cytoplasm and high cytoplasmic Ca^{2+} is inimical to cell biochemistry. We have previously established that gating of MscS and MscL at low external pH is a lethal event for *E. coli* [17].

The primary function of MscS and MscL is the maintenance of cellular integrity during extreme osmotic transitions [17]. Initial studies with *E. coli* mutants lacking the two major MS channels, MscL and MscS, demonstrated that the channels are essential for survival of severe hypoosmotic shock (the rapid transfer from high to low osmolarity). Under conditions of rapid osmotic transition, MscL and MscS in *E. coli*, are essentially functionally redundant – either alone can provide excellent survival. However, a subtler pattern emerges from the study of the different channel homologues in *E. coli* [74,75] (see below). The presence of multiple MS channels in any one organism has simultaneously complicated the analysis of the roles of specific channels and raised questions regarding their individual functions. *E. coli* has a single MscL channel but has six members of the MscS family (MscS, YnaI, YbdG, YbiO, MscK and YjeP) [17]. Each has been shown to be mechanosensitive by patch clamp analysis and, when over-expressed from a plasmid, each can protect cells against hypoosmotic shock [74]. Dowhan [76] has identified an essential role for YjeP when cells are blocked in their conversion of phosphatidyl serine (PS) to phosphatidyl ethanolamine (PE), the major lipid of *E. coli* cells. The *yjeP* gene is in an operon with *psd*, the gene encoding phosphatidyl serine decarboxylase, which catalyses the conversion of PS to PE. However, ribosomal profiling data suggests that the regulation is quite complex with a significantly greater expression of Psd over YjeP during exponential growth [77].

Initial analysis of the response to extreme hypoosmotic shock pointed to major roles only for MscS and MscL [17]. Thus, only the mutants lacking both MscS and MscL exhibit a severe deficiency in survival of extreme hypoosmotic shock. Removal of additional channels changes the threshold osmolarity at which death is observed [75] indicating that such channels have subtle roles in protection – the fundamental issue with these channels is not their mechanosensitivity but the regulation of their expression [69,74]. The failure of mutants lacking these channels to manifest a clear survival phenotype is a consequence both of their low level of expression and of the very rapid imposition of hypoosmotic shock in standard assays [17,74,75]. In contrast, subtle effects of different channel complements are evident in experiments designed to impose osmotic stress at slower, well-defined rates [78,79]. Mutants lacking a single channel were indistinguishable from the wild type strain, whereas a mutant lacking all seven channels exhibited a catastrophic loss of viability at even the slowest rate of exchange into low osmolarity media [78]. Intermediate rate dependencies of survival were observed for strains lacking different homologues, such that a combinatorial effect of different channels was observed. This effect was, of course, only seen in the absence of the MscL and MscS channels and the problem lies in knowing whether the minor channels still gate in the presence of the major ones. This problem has yet to be addressed in detail (although see [80]). It is not clear whether the tension at which the channels gate is a strong driver in the evolution of the channels. However, given the importance that channel abundance plays in determining the kinetics of relief from hypoosmotic shock [69] one might envisage that if expression is low, as is the case for most of the 'minor' *E. coli* channels [77], selection on the basis of gating tension would not necessarily be significant.

3. Evolutionary patterns in mechanosensitive channels

Channel evolution must have proceeded at multiple levels. An initial cohort of pore-forming, potentially mechanosensitive, channel peptides would be required to have evolved prior to all subsequent elaborations that are evident today. Modular structures are clear-cut for the MscS family [17,81] (Fig. 2), but much less so for MscL (see however, [82]). Genome sequence comparisons suggest that gene fusions to produce more complex proteins arise ~5× more frequently than fission events [83,84]. The selection pressure for such fusions might arise from advantages

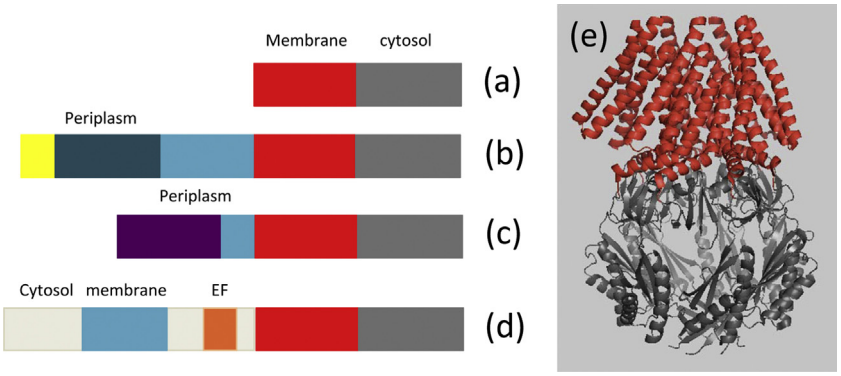

Fig. 2. Modular assembly of MscS-family channels. (a) The archetypal MscS from *E. coli* has three transmembrane segments (TM1-3; red) of which TM3a lines the pore and TM3b anchors the channel domain in the lipid bilayer [27]. In addition there is an extensive cytosolic domain (grey). Both domains have undergone extensive elaboration over evolutionary time, as exemplified by (b–d) (see also [16,17,81]). (b) Bacterial cells exhibit many variants that have additional transmembrane domains (pale blue) and a periplasmic domain of variable size (dark blue) [17], which is preceded by a signal sequence (yellow). The lengths of these proteins vary considerably with the smallest being ~500 and the largest ~1200 amino acids (total length). These channels may exhibit considerable sequence similarity across the core membrane domain (red) but can be very variable in the additional membrane domains (pale blue) and often have species-defined periplasmic domains (dark blue). (c) The PBP-1-MscS channels have a core channel domain (red-grey), with two additional transmembrane domains (pale blue) and a periplasmic domain (purple) that is structurally identifiable as a solute binding protein, such as is found in the ABC transporter superfamily. (d) The EF-hand-MscS homologues are found in fungi and have been investigated in detail in *S. pombe*. Note that here the EF domain (orange) is located in the cytoplasm and these channels have been identified as components of the endoplasmic reticulum [20]. For further examples of elaborations of MscS see [81] and pFam *MscS_TM PF12794* (http://pfam.xfam.org/family/PF12794#tabview=tab0) [113]. (e) The molecular structure of MscS (crystal structure 2oau; [27]) using red and grey in the same sense as (a) above (model created using Pymol; The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC).

in both expression and assembly of the functional complexes. In contrast, fission events must initially be disadvantageous, as the pre-existing functional protein loses expression of a structural module that was possibly required for activity. Thus, while transcription might proceed through the 'mutation' that has led to separation of the genes into two units, subsequent mutations would be required to provide translation signals allowing production of the new, separate, downstream element. Once a cohort of genes has evolved then other factors come into play that generate diversity of structure, expression and function (Fig. 3). At the protein level, where distinct modular structures are evident (and this is a strong feature of both the MscS family of mechanosensitive channels and the potassium channels), there can be module acquisition and/or rearrangement leading to structures that might exhibit different folding patterns [81] (Fig. 2).

Horizontal gene transfer can lead to gene acquisition. Once acquired, genes can be duplicated and then evolve along different pathways. Regulatory sequences can mutate and lead to acquisition of new expression patterns. Some organisms show extensive genome degradation, particularly intracellular pathogens, which might lead to loss of channel genes that are not essential to the niche. *Arabidopsis* has multiple MscS homologues but no MscL and this reflects a general paucity of MscL-like proteins in eukaryotes. Given that some of the MscS homologues are found in the chloroplasts [85], it is reasonable to speculate in this context, that either the original endosymbiont evolved to chloroplasts via loss of MscL or that *Arabidopsis* acquired MscS selectively and then subjected it to elaboration in terms of structure, subcellular location and expression control. Any consideration of channel evolution must incorporate these factors and try to evaluate what might have been the driving selective force for channel acquisition and modification.

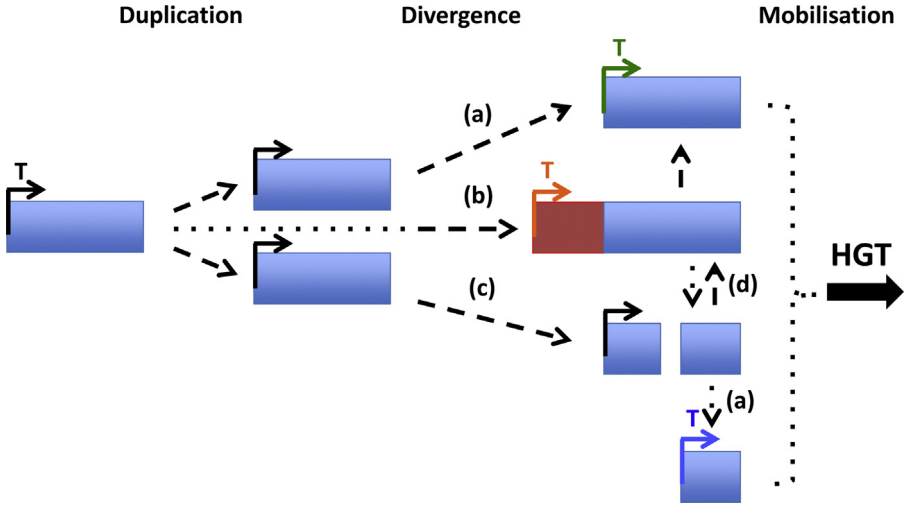

Fig. 3. Potential pathways of evolution of complexity in the MscS family. The primordial *mscS* gene transcribed and translated from an 'expression control sequence (T)' would undergo duplication followed by divergence: (a) acquisition of new transcriptional and translation control sequences (indicated by change of colour for 'T'); (b) gene fusion events where the channel not only acquires amino-terminal domains but potentially new transcription and translation signals from the fused domain; (c) by gene fission leading to loss of sequences amino-terminal to the core pore domain, followed by acquisition of new 'T' sequences (a) to give expression and/or gene fusion events (d) to create novel channel topologies. In parallel with such events genes may be mobilized across species boundaries by horizontal gene transfer (HGT).

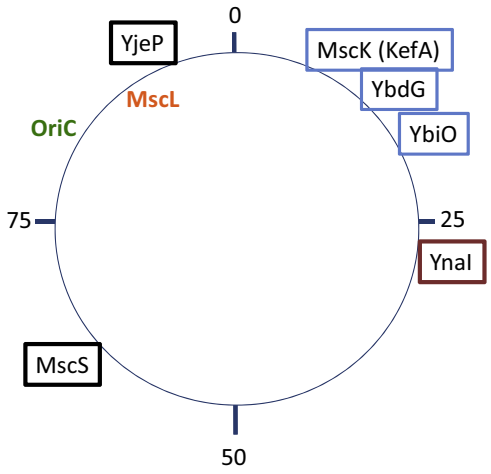

Fig. 4. Location of MS channel genes in *E. coli*. The approximate locations of the six MscS and one MscL gene on the *E. coli* chromosome are illustrated along with *oriC* the origin of replication. The chromosome has been divided into four quadrants starting at the zero clock position to reflect the proposed origin from two genome duplication events [86]. The box colours indicate the outcome of synteny analysis [114] across *E. coli*, *S. typhimurium*, *Klebsiella*, *Enterobacter*, *Erwinia* and *Pseudomonas* (see text).

3.1. Genome duplication and genome degradation

It is thought that some genomes evolved at an early stage by whole genome duplication. The *E. coli* genome is considered to have undergone at least two such major duplications [86]. Thus the current positions of mechanosensitive channel homologues on the chromosome may inform whether they were present pre-duplication or have been acquired since. Thus, *E. coli* has six MscS homologues – three (MscK, YbdG and YbiO) map in the first genome quadrant (0–25 min), and single channels are found in each of the other three quadrants (YnaI – 25–50 centisomes; YggB – 50–75 centisomes; and YjeP – 75–100 centisomes) (Fig. 4). When combined with gene synteny analysis (see below) across related species, a plausible model could place two as ancestral genes – MscS and YjeP. MscK and YjeP are highly similar at the amino acid level along their length, but MscK has a more limited distribution than YjeP in the γ-proteobacteria and the two channels have evolved very different expression controls [87] and conductance [17,88]. Synteny analysis also places YnaI, YbdG and YbiO as later arrivals, possibly having arisen from either an early gene duplication or gene acquisition. Genome reduction has been referred to above and has clearly played a role current genome structures with the most clear-cut example being in the Mycoplasma group.

Analysis of synteny, the chromosomal context within which the current channel structural gene is found, can hint at aspects of the evolution of organisms. Genomes of closely related organisms are typically characterised by a high degree of synteny, and deterioration in synteny can be indicative of genomic re-arrangements and greater evolutionary distance. Candidate genes for horizontal gene transfers can thus be identified at breakpoints of otherwise syntenic regions [89]. Interesting relationships are revealed by comparing MS gene synteny in *E. coli* K12 with *Salmonella*, *Enterobacter, Klebsiella, Erwinia* and *Pseudomonas aeruginosa*. The gene synteny for MscS is wholly conserved across these genera with the exception of *Pseudomonas*. The operon *psd-yjeP* is conserved in the five related organisms but not seen in *Pseudomonas*, which is consistent with the observation that YjeP-related channels in the latter have also evolved distinctly different periplasmic domains, possibly arising from several unique gene fusion events (see below). The *kefA* gene appears to be missing from *Erwinia*, as is *ybiO*, but other genes that are close to these two channel genes retain their synteny. Similarly, YnaI has a very narrow distribution in the γ-proteobacteria, being only found in *E. coli* and *Salmonella* and missing from *Klebsiella*, *Enterobacter*, *Photorhabdus*, *Serratia*, *Yersinia*, *Erwinia* and *Pseudomonas*. Generally the synteny for the *ynaI* gene is conserved in the γ-proteobacteria with the exception that the two most proximal genes (*insH* and *ynaJ*) are only found when *ynaI* is present. For all of the MscS homologues (and for MscL) synteny proximal to the channel gene is maintained in the γ-proteobacteria, even when the channel gene has been lost. The exception is *ybdG* in *Klebsiella*, which appears to have been transposed and now lies between *lacI* and a gene for a major facilitator membrane protein. Thus, for *E. coli* and its relatives, these data hint at the gene rearrangements and/or loss during speciation.

3.2. Gene synteny: a case study of mscL in ammonia-oxidizing archaea

Analyses of gene synteny can allow more accurate predictions of whether genes have been acquired by horizontal gene transfer or whether ancestral genes have been conserved (essentially vertical inheritance). In ammonia-oxidizing archaea (Thaumarchaeota), MscL homologues are found in syntenic regions within a genus, but this synteny is not conserved between thaumarchaeal genera (Fig. 5). Genes occurring at syntenic breakpoints may be more likely to have an adaptive function [89], which may explain the localisation of MscL homologues in such non-syntenic positions. One striking example is the insertion of *mscL* gene within a synton in the fresh-water archaeon *N. uzonensis* (Fig. 5). There is a strong correlation between possession of MscL and the ability to colonise osmotically-variable environments [3,69,90] (Fig. 6). The MscL homologues found in thaumarchaea share the greatest sequence identity with each other, indicating that they were likely shared by vertical inheritance after their initial acquisition rather than multiple horizontal gene transfer events. On the other hand, thaumarchaeal MscL genes have a very low sequence identity with their homologues found in euryarchaea but share a similar level of identity with Lactobacilli genes, which would be consistent with acquisition of the thaumarchaeal MscL sequences by horizontal gene transfer(s) from archaea to bacteria or vice versa in the past. The physiological and ecological roles of mechanosensitive channels in thaumarchaea remain entirely uncharacterised, but it is fascinating that genomes obtained from marine organisms frequently lack MscL homologues (Fig. 6). In contrast, a MscL homologue is found in all the genomes originating from a range of ecosystems, including terrestrial, fresh-water and estuarine habitats. The presence of MscL in thaumarchaea cultivated from estuarine sediments was previously proposed to be an adaptation to lower salinity [91]. Phylogenetic analysis of 40 concatenated ribosomal proteins suggests that freshwater thaumarchaeal lineages e.g. *Nitrosocaldus* and *Nitrososphaera* are deeper-branching than the genus *Nitrosopumilus* [92]. The latter contains the only cultivated marine representatives of ammonia-oxidizing archaea. It is therefore plausible that MscL was lost as an adaptation to the highly saline marine habitat [90].

A bioinformatics analysis of actinobacteria related to *Micromonospora*, which are predominantly soil organisms [90], has shown that the marine species, such as *Salinispora*, have arisen multiple times at different points in the lineages of the genus and are closely related to non-marine species [90]. Gene acquisitions, such as compatible solute transport, can be identified that appear to have favoured the specialisation of the marine organisms to this niche and a similar observation has been made for *Nitrosopumilus* [91]. Note, however, that the accumulation of compatible solutes in response to osmotic stress is almost universal and is not a specific feature found only in marine-adapted organisms [93,94].

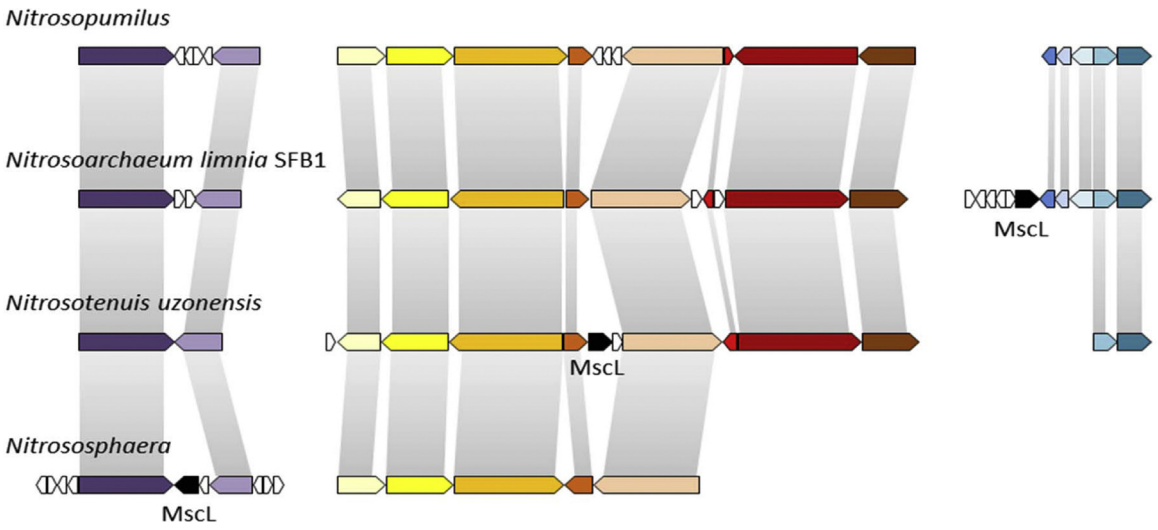

Fig. 5. Synteny analysis of MscL in thaumarchaeal genera. Syntenic genes are indicated by common coloured blocks joined by vertical (genes in the same orientation on the chromosome) and slanted (genes with reversed orientation) lines. MscL is absent from the all sequenced marine organisms belonging to genus *Nitrosopumilus*, but is found in other species that are found in variable environments. A black arrow indicating the orientation and position within the genome shows the position of the *mscL* gene in these organisms. Synteny analysis was undertaken using the MicroScope programme [114].

In *Salinispora*, loss of MscL is strongly correlated with the inability to exist outside of this environment. Similarly we have previously noted that possession of MscL is rare among *Vibrio* species and its presence correlates loosely with species (e.g. *V. cholera*) that must survive osmotic transitions during their transmission as pathogens [3]. Re-introduction of the *E. coli* MscL channel into *V. alginolyticus* was shown to facilitate specific transitions from high to low osmolarity [95]. A similar observation has been made for *Salinispora* [96]. The inference, therefore, is that over evolutionary time these organisms either lost *mscL* by reductive gene destruction or did not acquire the *mscL* gene by horizontal gene transfer.

For *Salinispora* it was suggested that gene loss was more likely than gene acquisition [90]. Moreover, the study by Penn and Jensen [90] indicates that there are several species that have no *mscL* gene (see Fig. 1 of [90]) and our own studies indicate that the correlation between occupation of a marine environment and lack of MscL may frequently break down (unpublished data). Thus, one slightly surprising observation is that *Verrucosispora maris*, which was isolated from a deep marine environment [97], has MscL and it is located in the genome as part of the same syntenic block found also in *Micromonospora*. It is clear that the absence of MscL does provide a niche limitation, as we have previously speculated [3], and as is

16S rRNA phylogeny	Organism	Cluster	Environment	MscS	MscL	Reference
	Nitrosopumilus maritimus SCM1	1.1a	Marine	3	0	91
	Nitrosopumilus sp. SJ		Marine sediment	2	0	115
	Nitrosopumilus koreensis AR1		Marine sediment	3	0	116
	Nitrosopumilus sediminis AR2		Marine	2	0	117
	Nitrosopumilus salaria BD31		Marine	1	0	118
	Nitrosoarchaeum koreensis MY1		Soil	1	1	119
	Nitrosoarchaeum limnia SFB1		Estuary	1[a]	1	120
	Nitrosoarchaeum limnia BG20		Estuary	1	1	121
	Nitrosotenuis uzonensis N4		Thermal spring	1	1	122
	Nitrososphaera gargensis	1.1b	Thermal spring	2[a]	1	123
	Nitrosocaldus sp.	ThAOA	Thermal spring	1[b]		124

Fig. 6. MscL is commonly absent from marine organisms. The figure illustrates the phylogenetic relationship between a small group of ammonia oxidizing archaea, the niche from which they were isolated and their possession of either MscL or only MscS or both [91,115–124]. [a]Although the genome annotations of *N. limnia* SFB1 and *N. gargensis* suggests the presence of more MscS homologues than indicated on the table, several of these coding sequences are most likely not mechanosensitive channels. [b]No *Nitrosocaldus* genomes are publically available, but MscS homologue from genus *Nitrosocaldus* has been deposited in NCBI under accession number BAE95206.

supported by experimental studies [90,95]. However, the picture looks much more complex than this simple argument.

3.3. Modular evolution

MscL is a structurally conservative, moderately highly conserved protein. Its evolution has been considered elsewhere [68,82]. The MscS family of proteins displays considerable plasticity in their current forms and the variants may have arisen by a variety of routes, including duplication, fusion, fission and evolved expression controls (Fig. 2). The archetypal MscS from *E. coli*, for which multiple crystal structures have been solved [27,73,98], is a homoheptamer; each monomer has three membrane-located spans, the first two of which (TM1-2) form the sensor of membrane tension. The third membrane helix (TM3) is in two parts – a hydrophobic section (TM3a) that lines the pore and an amphipathic section (TM3b) that lies at the membrane interface and integrates with the extensive cytoplasmic domains. At the sequence level the most highly conserved amino acids lie in the TM3a and TM3b sections [17,64,66,67], although there are other conserved residues in the cytoplasmic domain and in TM2. These conserved elements are the basis for annotation of proteins as members of the MscS family. However, there is extensive diversity outside of this core.

Each subunit contributes to the cytoplasmic domain, with portals created both at the interfaces between the subunits and an axial portal at the base of the channel formed by the carboxy-termini of the subunits coming together to form a beta barrel. One very specific variation that has been studied in some detail is the closure of the lateral portals and the expansion of the axial portal in the MscS from *Thermoanaerobacter tengcongensis* [99].

Additional transmembrane domains are one of the most frequently encountered variations [16,17,100]. Thus, YnaI and YbdG in *E. coli* have two additional helices that have the capacity to span the membrane and this appears to be a frequently recurring model for MS channels in bacteria, archaea and plant organelles (Fig. 2; [65]). This domain can be expanded to eight transmembrane helices, as in YbiO, YjeP and MscK. Little is known of the organisation of these extra helices. Other homologues have diverse arrangements of the transmembrane helices [81].

Periplasmic domains are another major variation. These are evident in YbiO, Yjep and MscK of *E. coli*. YjeP and MscK are very similar proteins with extensive sequence similarity across ~90% of their whole length. YbiO is completely unrelated to these two proteins, with only limited sequence similarity. The proteins differ also in the size of their periplasmic domains (~450 and ~110 amino acids, respectively, for YjeP and YbiO). *Pseudomonas aeruginosa* PA14 has three MscK homologues that are non-syntenic with their *E. coli* counterparts, display considerable similarity in their membrane domains, but their periplasmic domains are quite different. In *Rhodopseudomonas palustris* there is a single MscS-type channel that resembles *E. coli* YnaI most closely, but has an additional unique periplasmic domain. These periplasmic domains appear to have little precedent in the genome sequence database, although some do show potential structural counterparts when analyzed relative to the crystallographic database using programmes such as Phyre2 [101]. In contrast to these hypothetical structures there is clear evidence for gene fusion that has given rise to the PBP1-MscS [81] variants in bacteria and the EF-hand variants in yeast [20] (Fig. 2). Another channel protein with a unique modification is the MscS in *Corynebacterium glutamicum*, which has a unique sequence extension at the carboxy-terminus and is involved in glutamate excretion [102]. Thus, the picture emerging for MscS proteins is that they can be decorated at both the amino and carboxy-terminus with domains for which functions have yet to be described but which clearly are conserved intragenus, if not across more diverse organisms.

3.4. Transcriptional, translational and post-translational regulation

One of the most important aspects of evolution for any gene, is the establishment of controls over transcription and translation since these determine both protein abundance and the timing of expression. Paradoxically, we know much less about this aspect of mechanosensitive channels since bioinformatics can only predict potential transcriptional control mechanisms and is actually very poor in predicting translation. Fortunately, high throughput methods are beginning to establish parameters of relative translation and relatively simple assays of mRNA abundance can inform on timing [77]. Early studies on *mscL* and *mscS* expression established that, in *E. coli*, transcription of these genes can be achieved by RNA polymerase (RNAP) under the control of either the vegetative sigma factor or the stress factor, RpoS [79,103]. Other factors clearly also controlled expression, particularly changes that occur during growth at high osmolarity. As the study extended to other MS channels in *E. coli* it became clear that there was no simple and consistent pattern that linked all the channels through their expression [74]. Recent work on *E. coli* channels has concentrated on protein abundance and its relationship to the physiological role of the channels. Thus, the Phillips' group found that the abundance of MscL subunits was much higher than previously predicted and this has been supported by other studies [77,79]. Indeed the reported abundances for all of the *E. coli* channel subunits fits quite well with their reported contributions to survival and with the idea that low expression levels are a major contributor to phenotypes observed with mutants [74].

3.5. Timing is everything!

Recently, we have made the case that the critical period for cells experiencing the severe hypoosmotic shock is the first 50–100 ms [69]. This is the time period in which solutes must be lost in bulk from the cell to avoid wall and membrane damage caused by increased turgor pressure arising from water rushing into the cell. Death may follow much more slowly (greater than 20 min post-shock) and may be growth-associated as cells start to accumulate solutes to retore turgor. Only channels that are both abundant in the membrane and have a large enough pore will bring protection. Thus, for the smaller *E. coli* channels, such as YnaI, partial protection only is achieved through increasing the expression well above the levels normally seen in cells [74]. It is a complex algorithm, but clearly one where evolution can have exerted selection for the most optimal balance of channel conductance and gene expression. In *E. coli* the dominance of the MscL and MscS channels has allowed the diversification of signals controlling expression and abundance of the other channels with the potential that their expression under different ecological pressures provides physiological benefits.

4. Relationship of mechanosensitive channels to calcium signalling

Most bacterial cells maintain Ca^{2+} ion concentrations low in the cytoplasm via the activity of antiports that extrude the cation [42,45]. Thus, the potential exists for signalling functions that rely on transient opening of channels to create a Ca^{2+} spike that can be utilised by the cell as a signal. Recent work points to Ca^{2+}-activated K^+ channels in cyanobacteria that may set the membrane potential. Mutants lacking such channels exhibit a lowered membrane potential and acquire resistance to heavy metals [104], pointing to a potential physiological role for Ca^{2+}-mediated gating. A number of

Ca^{2+}-binding protein homologues have been detected in bacterial and archaeal genomes; their functions and roles in Ca^{2+} signalling are beginning to be systematically investigated [46,105].

An MS-like channel activity has been implicated in Ca^{2+} movements during spore germination in *Bacillus subtilis* [21]. Previously, strains deficient in MscS and MscL had been studied and found not to have defects in sporulation [106–108]. However, it was known that some bilayer-active compounds could initiate the release of calcium dipicolinate (CaDPA) from spores and thus initiate germination [21]. The SpoVA proteins were identified as intimately linked with control over CaDPA content and a detailed analysis of the SpoVAC gene product indicated a potential role in CaDPA release from spores. SpoVAC was found to exhibit mechanosensitivity – both assayed by applied pressure in electrophysiological assays and in response to membrane-active amphiphiles that activate classical bacterial MS channels [21].

In yeasts, specific MscS variants, the EF-MscS [19,20,81], play a role in survival of hypoosmotic shock. The fission yeast, *Schizosaccharomyces pombe* has two EF-MscS channel genes (Fig. 2), *msy-1* and *msy-2* (Msy1 and Msy2 channels, respectively). Homologues of these channels were found in a range of fungi, but not in the common food yeast, *Saccharomyces cerevisciae*. These proteins have six transmembrane helices (rather than the five commonly associated with some bacterial homologues and those in *Arabidopsis* [109]) and a short amino-terminal domain predicted to lie in the cytoplasm. An EF domain implicated in Ca^{2+} signalling is cytoplasmically-located and proximal to the pore helices. Immunocytochemistry of tagged proteins located them to different areas of the endoplasmic reticulum (ER), Msy1 in the perinuclear ER and Msy2 in the cortical ER [19]. Mutants lacking the channels were found to be very sensitive to hypoosmotic shock, were observed to swell to a much greater degree than the parent and to die after shock. The yeast cells exhibited modified behaviour with respect to Ca^{2+} transients during hypoosmotic shock and also exhibited a greater loss of viability when hypoosmotic shock was imposed in the presence of Ca^{2+}. The presence of the EF hand-sequences implicated this domain in Ca^{2+} signalling and it was observed that mutations in the EF-motif of Msy1 blocked the ability of the cloned channel to complement the *msy-1 msy-2* double mutant [20]. Mechanosensitive channel activity was associated with Msy1 in patch clamp experiments with *E. coli* but Msy2 currents could not be detected. Neither channel could protect *E. coli* mutants lacking MscS, MscK and MscL against hypoosmotic shock. Thus, overall, these unique variants appear to be functional mechanosensitive channels that have a unique sensitivity to Ca^{2+} consistent with their structural modifications [20].

5. Conclusion

Evolutionary trees constructed for mechanosensitive channels have deep roots, indicating their origin early in the divergence of bacterial species from their common ancestors. Multiple lineages are evident from comparisons of the pore-lining sequences [64]. Synteny analyses indicate that some channel genes are inherited by horizontal evolution, others by vertical descent and, in some organisms, they may be lost by genome reduction. Their role in protecting cells against hypoosmotic shock is well-established [17,69,110,111]. Proteins that behave like mechanosensitive proteins have been implicated in the release of CaDPA during germination of spores and in osmotic regulation in fission yeast [20]. Still other functions will be elucidated that might explain some of the complex domain structures that have been evolved in the MscS family. This important class of proteins exhibit all the hallmarks of an ancient family – divergent structures, widespread occurrence across kingdoms, modified regulatory patterns and finally selective gain and loss of structural genes.

Conflict of interest

There are no known conflicts of interest.

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