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ARTICLE

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Mechanosensitive channels of bacteria and archaea share a common ancestral origin

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Abstract The ubiquity of mechanosensitive (MS) ion channels set off a search for their functional homologues in archaea, the third domain of life. A new MS channel was identified in the archaeon *Methanococcus jannaschii* by using the TM1 transmembrane domain of the bacterial MS channel of large conductance, MscL, as a genetic probe to search the archaeal genomic database for MS channel homologues. The hypothetical protein MJ0170 (MscMJ) was found to harbor two MscL-like TM1 structural motifs and showed a high degree of sequence and secondary structure conservation with MscS (YggB) homologues. The alignment of sequences of MscL, MscS and MscMJ homologues further revealed that bacterial and archaeal channels form a phylogenetic tree composed of three main branches and share a common ancestral origin. This suggests the evolution of prokaryotic MS channels via gene duplication of a MscL-like progenitor gene followed by divergence, further indicating that the common ancestor of the prokaryotic MS channels most likely resembled MscL. When expressed in *E. coli* and functionally examined by the patch clamp, the MscMJ protein behaved as a MS channel with a conductance of 270 pS in 200 mM KCl and a cation selectivity (P_K/P_{Cl}) of approximately 6. The structural and functional homologue of MscMJ, MscMJLR, was identified as a second type of MS channel in *M. jannaschii*. The channel has a conductance of approximately 2 nS, rectifies with voltage and shares cation selectivity with MscMJ. The stoichiometry of both types of MS channels revealed that the free energy of activation, $\Delta G_0 \approx 7kT$, obtained for MscMJ matches the one calculated for MscS, $\Delta G_0 \approx 5kT$, whereas the free energy of activation of $\Delta G_0 \approx 18kT$ of MscMJLR resembles more the $\Delta G_0 = 14$–$19kT$ reported for MscL. The presence of two types of MS channels discovered in the cell envelope of *M. jannaschii* indicates that multiplicity of MS channels in prokaryotes is a necessary element for their survival in the habitats frequently challenged by sudden changes in osmolarity. Further functional and phylogenetic study of MS channels from all three domains of the universal phylogenetic tree may help to understand the evolution and common biophysical principles that govern mechanosensory transduction.

Keywords Ion channels · Mechanosensitivity · Prokaryotes · Phylogeny · Patch clamp

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Introduction

Mechanosensation is a physiological process by which a distortion of the cell membrane is converted into an electrical and/or biochemical signal. Since all aspects of cellular dynamics, ranging from cell division and cell growth to cell differentiation, involve a change in volume or shape, mechanosensation is believed to have originated very early during the evolution of life. Mechanosensation underlies many physiological processes in higher organisms. Touch and pain sensation (Burnstock and Wood 1996), hearing (Howard et al. 1988; Hackney and Furness 1995) and blood pressure regulation (Chapleu 1992), as well as pathophysiology of cardiac arrhythmia (Dean and Lab 1989; Franz et al. 1992), neuronal degeneration (Driscoll and Chalfie 1991; Tavernarakis and Driscoll 1997) and Duchenne muscular dystrophy (Franco and Lansman 1990), were all found to be linked to mechanosensation. Since mechanosensitive (MS) ion channels function as mechano-electrical transducers by converting mechanical stimuli into electrical or chemical signals in living cells, they most likely orchestrate numerous cellular processes associated with mechanosensory transduction. Furthermore, since MS channels have been found to play a role

in osmoregulation, it is possible they evolved very early in the history of life on Earth to detect and compromise for environmental challenges (Martinac 1999). In this way, their presence in cellular envelopes would ensure not only the survival but also the adaptation of the first organisms to a variety of habitats they colonized. Indeed, MS channels have been discovered in organisms of different phylogenetic origin (Fig. 1) such as bacteria, fungi, plants and mammalian cells (Morris 1990; Martinac 1993; Sachs and Morris 1998), as well as recently in archaea (Le Dain et al. 1998; Kloda and Martinac 2001a, 2001b, 2001d), which further indicates their early evolutionary origin.

Archaea, a third domain of life

In 1977, a comparison of 16S ribosomal RNA sequences lead to a proposal that life on Earth comprises three domains, bacteria, archaea and eukarya, instead of the classically accepted two lineages of prokaryotes and eukaryotes (Woese and Fox 1977; Woese et al. 1990; Woese 1994). Phylogenetically, archaea are neither bacteria nor eukarya but share characteristics of both lines of descent and often are described as an intermediary domain of life (Gray 1996) (Fig. 1, Table 1). Although, at the molecular level, archaea seem to resemble eukarya (Keeling and Doolittle 1995), studies based on the comparison of the pattern of protein families and their distribution suggest that archaea may share a common heritage with the last prokaryotic universal ancestor of all present forms of life (Ouzonis and Kyrpides 1996; Stein and Simon 1996).

The hypothesis that life on Earth comprises three kingdoms was reinforced in 1996 by the first complete genome sequence of a hyperthermophilic archaeon, *Methanococcus jannaschii* (Bult et al. 1996). Comparative sequence analysis with members of the two other

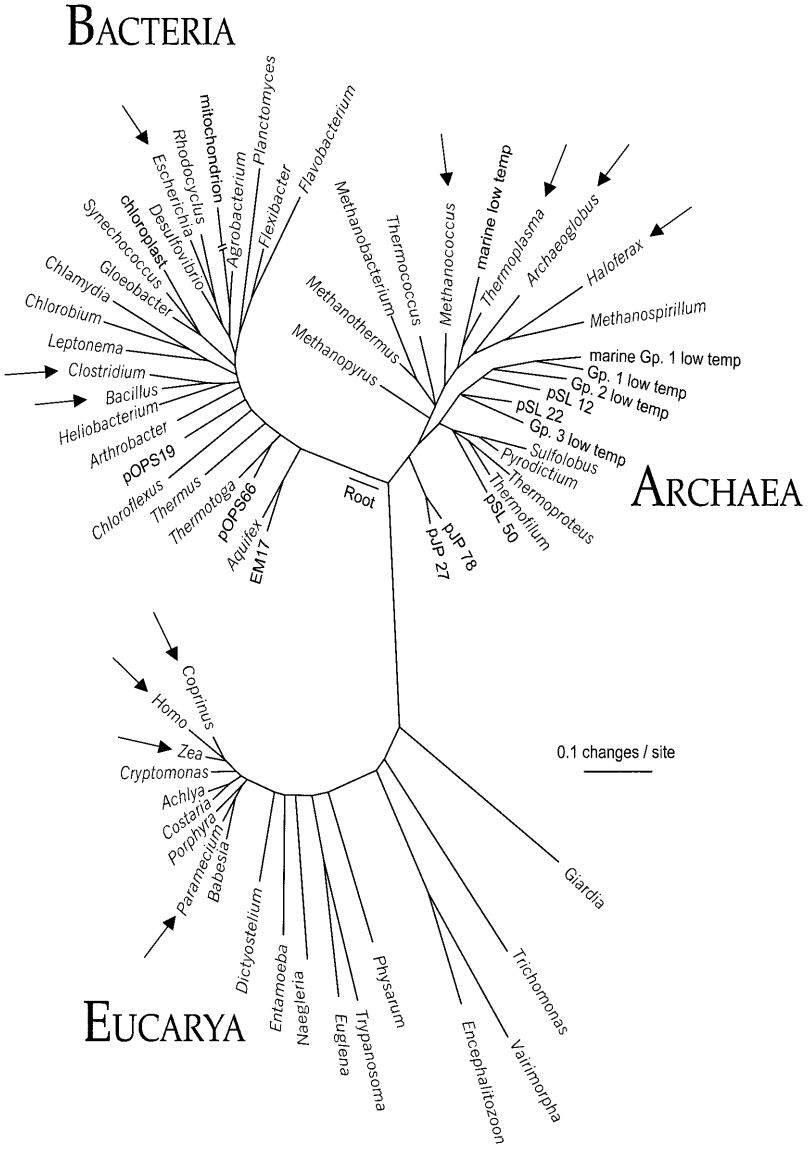


Fig. 1 Universal phylogenetic tree. The tree is based on the comparative sequencing of 16S ribosomal RNA and shows three lines of descent: archaea, bacteria and eukarya (modified from Pace 1997). *Arrows* point to prokaryotic and eukaryotic phyla identified with MS channels

Table 1 Summary of major differentiating and common molecular traits among three phylogenetic domains: bacteria, archaea, and eukarya (Woese 1981; Kates 1993; Brock et al. 1994)

Trait	Bacteria	Archaea	Eukarya
Cell size	About 1 μM	About 1 μM	About 10 μM
Membrane-bound nucleus	Absent	Absent	Present
Cell wall	Muramic acid present	Muramic acid absent	Muramic acid absent
Membrane lipids	Ester linked, straight-chain fatty acids	Ether linked, branched hydrocarbons	Ester linked, straight-chain fatty acids
Cytoskeleton	Absent	Absent	Present
Motility: rotary motor and rigid flagella	Present	Present	Absent
Porins	Present	Present	Present
MS channels	Present	Present	Present
Ribosome sedimentation	70S	70S	80S (70S organelles)
Circular chromosome	Present	Present	Absent
Plasmids	Present	Present	Rare
RNA polymerases	One (4 subunits)	Several (8–12 subunits)	Three (12–14 subunits)
mRNA binding site AUCACCUCC	Present	Present	Absent
TATA box and binding protein	Absent	Present	Present
In vitro transcription	Yes	No	No
Operons	Present	Present	Absent
Introns in tRNA	Rare	Present	Present
PolyA tail of mRNA	Absent	Absent	Present
Transfer RNA thymine unit	Present	Absent	Present
Initiator tRNA	Formylmethionine	Methionine	Methionine
Sensitivity to diphtheria toxin	No	Yes	Yes
Sensitivity to chloramphenicol, streptomycin, and kanamycin	Sensitive	Insensitive	Insensitive
Methanogenesis	No	Yes	No
Reduction of S to H_2S	Yes	Yes	No
Nitrogen fixation	Yes	Yes	No
Photosynthesis	Yes	No	Yes

domains revealed that although some genes (especially those related to energy production, cell division and metabolism) are similar to those found in bacteria, other genes (especially those involved in replication, transcription and translation) resemble more their eukaryotic counterparts. Surprisingly, 56% of protein coding genes of this archaeon have no matches in the existing databases (Morell 1996). Since MS channels have been implicated to play a role in the regulation of turgor pressure, which is essential for division and growth of bacterial cells (Csonka and Epstein 1996), it would be expected for archaeal MS channels to be closer relatives of bacterial rather than eukaryotic MS channels. Indeed, archaeal genes involved in the transport of inorganic ions such as potassium and sodium across the cell membrane were found to be very bacteria-like, indicating that both lines of descent derived the ion transport pathway from a common ancestor (Morell 1996). Since it has been postulated that all three primary lineages share a common evolutionary ancestor (Pace 1997; Woese 1998) (Fig. 1), it is possible that a single ancestral prototype evolved into a variety of MS ion channel genes.

Archaea are truly life's extremists, occupying some of the most harsh environments on the planet including hot springs, extremely alkaline or acid waters, extremely saline waters and deep-sea volcanic vents at temperatures well over 100 °C (Woese 1981; Barinaga 1994; Brock et al. 1994; DeLong 1998). Generally, on the basis of the environmental niches they occupy, archaea fall into three main groups: extreme halophiles, methanogens and thermoacidophiles. Extreme halophiles are salt-loving microorganisms and require concentrations of salts close to saturation to maintain cellular integrity. Methanogens are methane-producing, obligate anaerobes, while thermoacidophiles live at high temperatures and low pH.

Despite great differences in the habitats in which they live, archaea are linked by common properties, which distinguishes them from other domains (Table 1). In addition to differences in transfer RNA and ribosomal RNA sequences and modification patterns, as well as enzyme subunit structure, archaea also differ in cell wall and lipid bilayer structure from the other two domains. However, unlike eukarya, both bacteria and archaea do not have a cytoskeleton. As discussed further, it is the lipid bilayer which acts as the tension-bearing element, transmitting the mechanical force to the MS channels in prokaryotic cell membranes. The presence of MS channels (gated by the force transmitted via the lipid bilayer alone) in cells belonging to all three domains of the phylogenetic tree indicates the universal biophysical principle underlying the activity of these channels.

A detailed description of the structural diversity of prokaryotic cell wall and envelope is beyond the scope of this paper and the reader is referred to a recent review by Kloda and Martinac (2001c). Briefly, the prokaryotic

cell wall is a firm structure, which provides mechanical support and protects the fragile cytoplasmic membrane of prokaryotic cells. The major constituent of the bacterial cell wall is peptidoglycan, a rigid molecule composed of *N*-acetylglucosamine and *N*-acetylmuramic acid. On the basis of the Gram stain technique, bacterial cells can be divided into Gram-positive or Gram-negative, depending on the content of peptidoglycan in their cell wall. Unlike Gram-positive bacteria, the cytoplasm of Gram-negative bacteria possesses an outer membrane in addition to an inner (cytoplasmic) membrane. The outer membrane is in contact with the cell wall and consists of two monolayers. The inner monolayer consists of phospholipids, whereas the outer monolayer is composed of lipopolysaccharides (LPS) (Nikaido 1996). Although archaeal cell walls stain Gram-positive or Gram-negative, their structure and chemistry is profoundly different from the bacterial cell wall (Kandler and Konig 1993; Brock et al. 1994; Doolittle 1999). In contrast to the bacterial cell wall, the archaeal cell wall lacks peptidoglycan. Furthermore, Gram-negative archaea have their plasma membranes surrounded by an electron-dense, proteinaceous S-layer.

The cell envelopes of archaea differ largely between species in their structure and composition, which most likely reflects adaptation to different extreme environments. Unlike bacterial cell membranes which are composed of phospholipids, the major constituent of archaeal membranes is diphytanylglycerol diether or tetraether or both (Doolittle 1999). A variety of lipids can combine in different ratios to form membranes of various thickness and rigidity in different archaeal species. For example, the membrane of halophiles has only the glycerol diether lipid bilayer while thermophilic archaea tend to have a diglycerol tetraether lipid monolayer membrane structure (DeRosa et al. 1986; Kates 1993). *M. jannaschii* contains a high proportion of macrocyclic diether glycerolipids, a unique type of lipid which might be unique to methanogens living in deep-sea hydrothermal vents (Comita and Gagosian 1983; Ferrante et al. 1990). Interestingly, in the cell-wall-less *Thermoplasma*, the length of the hydrocarbon chains can be controlled by the introduction of pentacyclic rings, which provide rigidity and render the membrane stable in hot, acidic conditions (Langworthy 1982; Langworthy et al. 1982).

MS channels in prokaryotes

MS ion channels were first identified at the molecular single-channel level in 1984 in eukaryotic cell preparations of chick skeletal muscle and frog muscle (Brehm et al. 1984; Guharay and Sachs 1984). The advent of the patch clamp technique and improved methods of preparation opened access to study MS channels in microbial cells. In 1987, MS channels were discovered in giant spheroplasts of the Gram-negative bacterium *Escherichia coli* (Martinac et al. 1987) and in reconstituted bacterial membrane fractions (Berrier et al. 1989; Delcour et al. 1989). The methods of study of prokaryotic MS channels are summarized in Fig. 2. Briefly, cells treated with cephalexin grow into snake-like filaments of 50–150 μm in length as a result of arrested septation. With further treatment with EDTA and lysozyme, which weakens the cell wall, these structures form giant spheroplasts approximately 5–10 μm in diameter (Ruthe and Adler 1985), which are large enough to be used for the patch clamp recording. Alternatively, the membrane fraction is prepared by passing the cells through a French press and the outer and inner membranes can be further separated by sucrose gradient centrifugation. The membrane fractions are fused with azolectin liposomes followed by a dehydration-rehydration cycle.

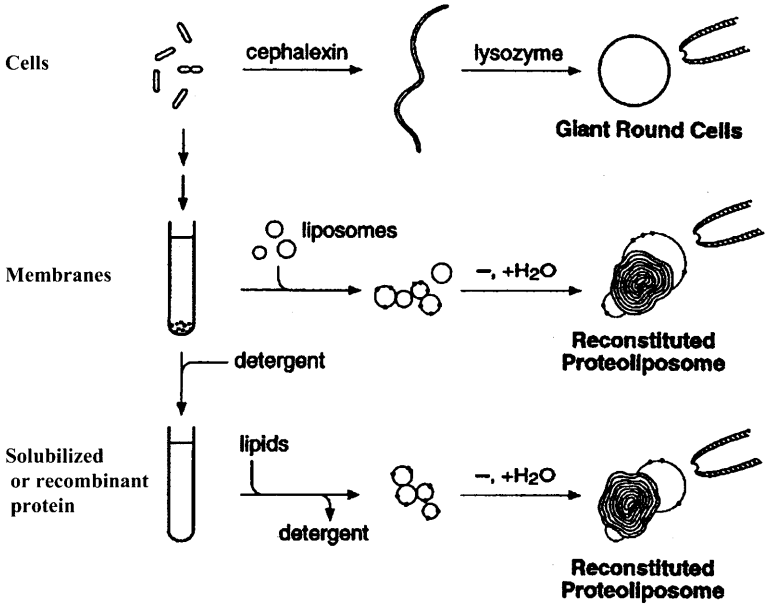


Fig. 2 Methods of study of prokaryotic MS channels (adapted from Sukharev et al. 1997). *Top row*: giant spheroplasts; *middle row*: reconstitution of membrane fraction into azolectin liposomes; *bottom row*: reconstitution of either solubilized MS channel-rich cellular membranes or purified recombinant proteins

When placed in a recording solution containing $MgCl_2$ the liposomes collapse and form unilamellar blisters, which yield high gigaohm seals upon contact with the patch pipette. The MS channels of *E. coli* were found to be functional upon solubilization of the membrane fraction with mild detergent such as octylglucoside, followed by reconstitution upon removal of the detergent (Sukharev et al. 1994b). This ability allows the study of recombinant MS channels expressed in *E. coli* as either a GST-fusion protein or a 6×His-tagged protein followed by single-step purification by affinity chromatography (Häse et al. 1995; Blount et al. 1996a).

Three types of MS channels were identified in *E. coli*, which based on their conductance were named as MscM (M for mini), MscS (S for small) and MscL (L for large) (Berrier et al. 1996). The channel conductance is paralleled by the amount of negative pressure required for activation. In contrast to MscL, which is non-selective, MscS was found to be more selective for anions over cations ($P_{Cl}/P_K \approx 3$) (Martinac et al. 1987; Sukharev et al. 1993), while MscM was reported to exhibit a slight preference for cations over anions (Berrier et al. 1989, 1996). The activity of MscL and MscM is not dependent on voltage; however, MscS displays voltage dependence characterized by 15 mV per e-fold change in the channel open probability and increased activity with membrane depolarization (Martinac et al. 1987). Furthermore, MscS exhibits rectification with a conductance of approximately 0.97 nS at positive voltages and 0.65 nS at negative voltages (Martinac et al. 1987). To date, bacterial MscL is the most commonly studied MS channel. The tertiary structure of MscL from *Mycobacterium tuberculosis* (Tb-MscL) was solved by X-ray crystallography to 3.5 Å resolution and revealed that the functional channel is a homopentamer (Chang et al. 1998).

Recently, two genes of *E. coli*, *yggB* and *kefA*, were cloned and were found to encode membrane proteins associated with the MS channel with properties similar to MscS (Levina et al. 1999). While YggB is a smaller 286 amino acid residue protein with four or five hydrophobic domains, the KefA is approximately four times larger (1120 amino acids) with at least eight hydrophobic segments. In addition, YggB has an important physiological phenotype. Mutants lacking both MscL and YggB are severely compromised in their survival if challenged by an osmotic downshock (Levina et al. 1999). Many organisms possess multiple homologues of YggB or KefA that most likely reflect a need for a variety of MS ion channels with different responses upon challenge with different osmotic cues.

Until recently, nothing was known about MS channels in archaea, the third domain of the phylogenetic tree. Using patch clamp techniques, MS channels were identified for the first time in three archaeal species occupying three different environmental niches: *Haloferax volcanii*, *Thermoplasma acidophilum* and *M. jannaschii* (Le Dain et al. 1998; Kloda and Martinac 1999, 2001a, 2001b, 2001d).

Membranes of *H. volcanii* were found to harbor two types of MS channels named MscA1 and MscA2 (Le Dain et al. 1998). MS channels of *H. volcanii* share several features with bacterial MS channels, such as activation by the bilayer mechanism (Martinac et al. 1990; Hamill and McBride 1997; Hamill and Martinac 2001), voltage dependence, sensitivity to pressure per e-fold change in open probability and blockage by gadolinium. Similar to MscS, both MS channels of *H. volcanii* exhibit rectification with higher conductance of 0.85 nS at positive voltage and a lower conductance of 0.49 nS at negative voltage. Interestingly, MscA1 exhibits inverse rectification with voltage polarity compared to MscA2 by having a lower conductance of 0.38 nS at positive voltages and a higher conductance of 0.68 nS at negative voltages (Le Dain et al. 1998).

Recently, two novel archaeal MS channels were identified and cloned using the TM1 domain of *E. coli* MscL as a genetic probe against the genomic database of the archaeon *M. jannaschii* (Kloda and Martinac 2001a, 2001d). When examined by the patch clamp technique, both channels displayed MS properties and, similar to bacterial MS channels, they differed in conductance. Both channels were found to be selective for cations with a similar permeability ratio for potassium versus chloride (P_K/P_{Cl}) of between 5 and 6. In this respect they resemble the eukaryotic stretch-activated cation channels (SA-CAT) (Hamill and McBride 1996). Furthermore, the channels of *M. jannaschii* were found to differ in the rectification pattern. As a result, the non-rectifying channel with a smaller conductance of approximately 270 pS was named as MscMJ (for mechanosensitive channel of *M. jannaschii*), while the rectifying one characterized by larger conductance of approximately 2 nS was given the name MscMJLR (for mechanosensitive channel of *M. jannaschii*, large, rectifying). Interestingly, MscMJ rectifies strongly at high ionic strength and, similar to MscA1 and MscA2, the MS channels of *H. volcanii*, it exhibits inverse rectification with voltage polarity compared to MscMJLR. Thus, the presence of two MS proteins with a "mirror image" rectifying conductive property in two archaeal species, occupying different environments, suggests that these two heterogeneous MS channels may operate at different levels of cellular turgor, similar to the two bacterial channels of *E. coli*: the rectifying MscS and non-rectifying MscL.

A distant homologue or a functional analogue of *E. coli* MscL was identified in the cell-wall-less archaeon *Thermoplasma volcanium* using a functional approach similar to the one used for molecular identification of MscL (Sukharev et al. 1994a, 1994b). In a standard recording solution of 200 mM KCl and 40 mM $MgCl_2$ the channel exhibits a conductance of approximately 1.5 nS. Twenty N-terminal amino acid residues of the 15 kDa putative protein matched with 75% identity the start of the unknown open reading frame in the genome of the related species *T. acidophilum* (Kloda and Martinac 2001b). The secondary structure analysis

revealed two putative α-helical membrane spanning domains, suggesting a possible structural homology with MscL. Interestingly, both helices can be modeled by helical wheel alignment as amphipathic. Thus unlike MscL, in which the TM1 domain creates the bulk of the pore of the pentameric channel (Chang et al. 1998), both helices could line the pore of the *T. volcanii* MS channel.

Besides gating patterns summarized in Fig. 3, archaeal MS channels share several other features with bacterial MS channels. Similar to their bacterial counterparts, archaeal MS channels can be blocked by submillimolar concentrations of gadolinium. Furthermore, similar to *E. coli* MscS (Martinac et al. 1990), MscMJ of *M. jannaschii* can be activated by the amphipathic compounds trinitrophenol (TNP) and chlopromazine (CPZ) (Kloda and Martinac 2001a). TNP was also found to affect the activity of the MS channel of *Thermoplasma*, whereas it has no significant effect on MscMJLR, the large, rectifying channel of *M. jannaschii* (Kloda and Martinac 2001b, 2001d). Interestingly, TNP was shown to activate the eukaryotic MS channel TREK-1, while CPZ affected the channel by closing it (Patel et al. 1998). The effect of gadolinium and amphipaths indicates the activation by mechanical force transmitted via the lipid bilayer alone, owing to the preferential insertion into inner or outer leaflets of the bilayer (in the case of amphipaths) or modifying the chemical properties of the lipid bilayer (in the case of gadolinium) (Martinac et al. 1990; Ermakov et al. 1998). Recent evidence confirms that the bilayer model may apply not only to prokaryotic but also to eukaryotic MS ion channels (Zhang et al. 2000), indicating a universal mechanism of activation of MS channels in organisms from all three domains of life (Hamill and Martinac 2001).

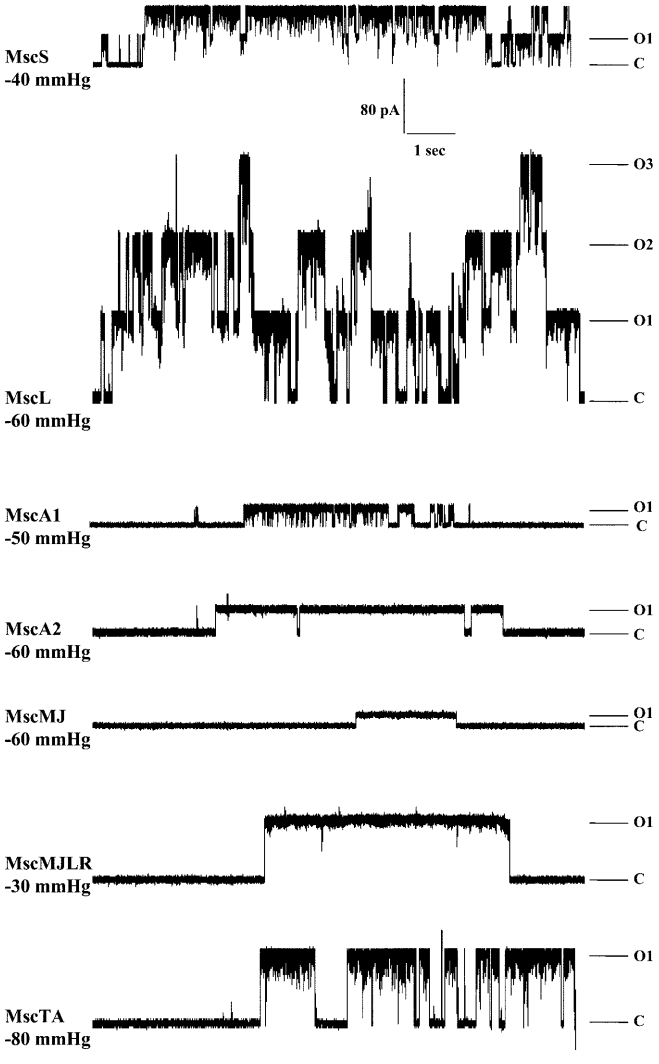


Fig. 3 Multiplicity of MS channels in prokaryotes. Current traces of *E. coli* MscL and MscS are followed by traces of MscA1 and MscA2 of *H. volcanii* and MscMJ and MscMJLR of *M. jannaschii*. The last trace represents the activity of MscTA, the channel of *T. acidophilum*. Current traces were recorded at +40 mV

Sequence and structural similarities indicate a common ancestral origin

Identification and cloning of MscMJ has provided the first opportunity to compare and contrast the amino acid sequences and putative secondary structure of archaeal and bacterial MS channels. The local alignment of the sequence of MscMJ and MscL revealed 38.5% identity with the TM1 of MscL (Fig. 4). It was this high level of sequence preservation which allowed identification of the MJ0170 putative protein as a candidate for an MS channel (Kloda and Martinac 2001a, 2001c). Furthermore, 31.8% identity was found within a stretch of 22 amino acids corresponding to the TM2 of MscL. Interestingly, the third highly preserved segment of 20 amino acids showed 40% identity to the TM1 periplasmic loop of MscL, indicating that not one but two TM1-like structural motifs were preserved within the sequence of MscMJ. MscMJ was also found to share a high degree of homology with YggB, a protein underlying the activity of MscS (Levina et al. 1999), and all three proteins, MscL, MscS and MscMJ, share a C-terminal cluster of charged residues. A similar charged cluster can also be identified within the C-terminus of MscMJLR, the large, rectifying homologue of MscMJ, the MS channels of *T. acidophilum*, a cell-wall-less archaeon, as well as the eukaryotic MS channel TREK-1 (Patel et al. 1998). Such preservation of a structural motif in the MS channels of organisms belonging to all three domains of life indicates their evolutionary relationship and the yet unknown functional importance of charged residues to the channel function. Indeed, the MscL mutant harboring the deletion within this region is not functional (Blount et al. 1996b; Häse et al. 1997). Furthermore, the C-terminal cluster of charged residues has been proposed to stabilize the closed configuration for MscL (Hamill and

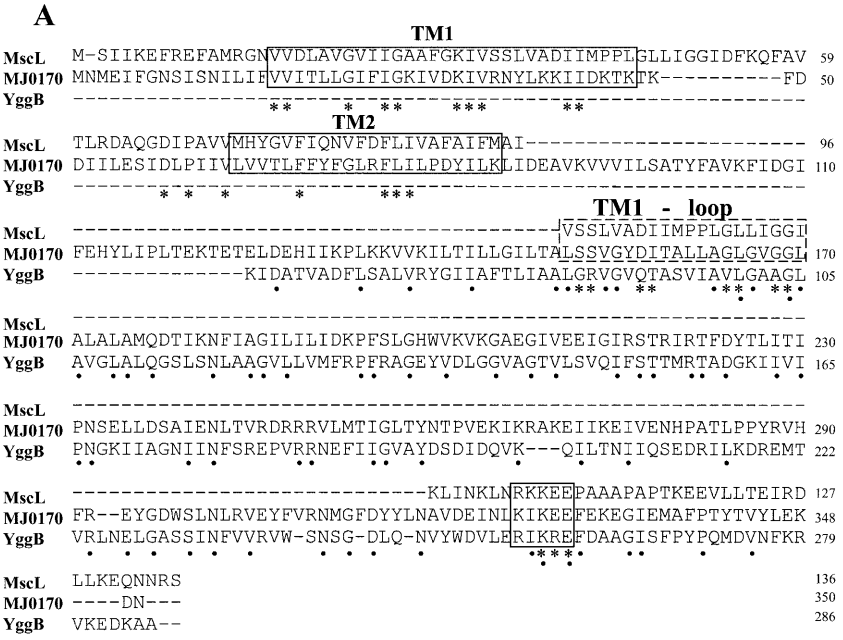


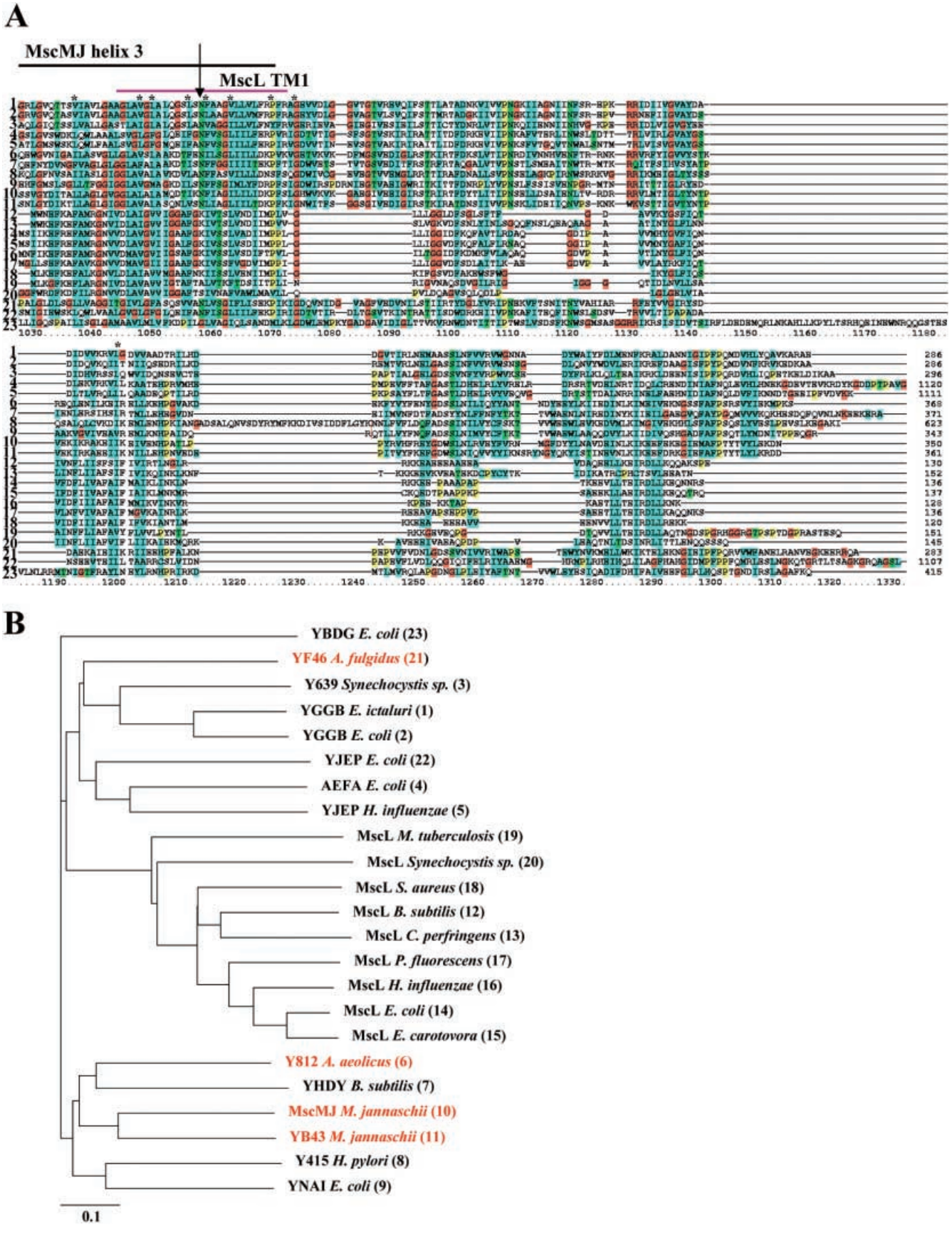
Fig. 4 Regions of homology between MscL, MscMJ (MJ0170) and MscS (YggB) (adapted from Kloda and Martinac 2001a). The identical residues found in both MscL and MscMJ amino acid sequences are marked with *asterisks*, whereas those common to the MscS and MscMJ sequences are marked with *dots*. The MscL TM1 and TM2 transmembrane domains and a cluster of charged residues that match the MscMJ sequence are *boxed*

Martinac 2001), indicating its vital role in the MS channel gating.

The multiple sequence alignment of homologues of prokaryotic MS channels with large and small conductance revealed that bacterial and archaeal channels form a phylogenetic tree composed of three main branches (Fig. 5), suggesting the common ancestry of prokaryotic MS channels. Sequence homology of archaeal and bacterial MS channels is further paralleled by similarities in the secondary structure. The hydropathy plot analysis showed that the profile of *E. coli* MscS (YggB) is similar to the profile of both *M. jannaschii* MS channels, MscMJ and MscMJLR. Furthermore, hydrophobicity plots of all three proteins show similarity to the C-terminal portion of KefA protein, which was also found to underlie the activity of MscS (Levina et al. 1999). Interestingly, their hydrophobic regions also show a high probability of adopting helical conformation and thus, similar to MscL, the channel pores formed by these proteins might be enclosed by α-helices.

The absence of the MscL gene from the presently known archaeal and eukaryotic genomes, and the preservation of the MscL-like structural motifs within sequences of many homologues which underlie the activity of the second type of MS channels in prokaryotes with smaller conductance, suggests the evolution of MS channels via gene duplication of the MscL-like progenitor gene which occurred before separation of bacteria and archaea (Kloda and Martinac 2001a, 2001c). The gene duplication was most likely followed by subsequent gene divergence from the universal common ancestor followed by diversification and spread across different phyla (Fig. 1). Moreover, archaeal MS channels may represent evolutionary intermediates between bacterial and eukaryotic MS channels and thus may be used as a stepping stone to trace the MscL-like ancestry to some of the eukaryotic MS ion channels as well.

Fig. 5A, B Phylogeny of prokaryotic MS channels (modified from Kloda and Martinac 2001a). **A** Multiple sequence alignment of MscMJ and MscL homologues (Clustal X 1.8). The alignment includes C-terminal portion of MscMJ and its homologues comprising most of the helix 3 (marked with a *horizontal black line*) and the complete sequences of MscL homologues. The colored residues share similarity with the consensus, which is defined by the maximized alignment of all sequences. The *yellow* color represents proline residues, *brown* represents glycine residues or its divergent substitution with basic residues, the *green* color is assigned to polar residues, while *blue* represents hydrophobic residues or their divergent substitutions with mostly acidic residues (see Clustal X 4 color index for more information on the color scheme chosen for this figure). Highly conserved residues are marked with an *asterisk*. The most extensive consensus sequence between all aligned proteins is marked by the helix 3 of MscMJ (*black horizontal line*) and the TM-1 domain of MscL (*purple horizontal line*). The *arrow* points to a highly conserved asparigine residue (N182) within the helix 3 of MscMJ and its homologues, which is replaced with lysine in *E. coli* MscL (K31) and most of its homologues. The sequences are numbered and their names given in **B**. **B** Phylogenetic tree of complete aligned sequences of MscMJ homologues and MscL homologues, showing the common ancestry of prokaryotic MS channels. The archaeal homologues of MscMJ are *red*. Numbers in parentheses correspond to the sequence number in **A**. Method: *E. coli* MscL and MscS (YggB) as well as MscMJ protein sequences were chosen to search the existing protein databases (GenBank, Protein DataBank and SwissProt) using BLAST (Altschul et al. 1997) for their homologues. Multiple sequence alignment was performed on retrieved homologues using the Clustal X program (Thompson et al. 1997). This alignment was used to construct a phylogenetic tree (Bootstrap Neighbor-Joining Tree) with 1000 bootstrap trials. Bootstrapping draws a number of trees with random samples from the multiple alignment and analyzes in terms of how many times each grouping from the original tree occurs in the sample tree. The tree was viewed with TreeView program, which outputs evolutionary distances as number of nucleotide substitutions per site


A
MscMJ helix 3
MscL TM1
B
YBDG E. coli (23)
YF46 A. fulgidus (21)
Y639 Synechocystis sp. (3)
YGGB E. ictaluri (1)
YGGB E. coli (2)
YJEP E. coli (22)
AEFA E. coli (4)
YJEP H. influenzae (5)
MscL M. tuberculosis (19)
MscL Synechocystis sp. (20)
MscL S. aureus (18)
MscL B. subtilis (12)
MscL C. perfringens (13)
MscL P. fluorescens (17)
MscL H. influenzae (16)
MscL E. coli (14)
MscL E. carotovora (15)
Y812 A. aeolicus (6)
YHDY B. subtilis (7)
MscMJ M. jannaschii (10)
YB43 M. jannaschii (11)
Y415 H. pylori (8)
YNAI E. coli (9)
0.1

Mechanosensitivity of prokaryotic MS channels

Prokaryotic MS channels are gated by membrane tension that develops within the lipid bilayer as a result of external mechanical force. The effect of tension on the channel open probability follows the Boltzmann distribution function. For MS channels the Boltzmann function is expressed in terms of $p_{1/2}$ and α and has the following form:

$$P_o = [1 + \exp \alpha(p_{1/2} - p)]^{-1} \quad (1)$$

where P_o is the open probability, p is the negative pressure at which the channel open probability is 50% and α is the channel sensitivity to pressure. According to a two-state Boltzmann model with a change of area $t\Delta A$ being the dominant energy term (Sukharev et al. 1999), it follows according to the model by Howard et al. (1988) that the free energy ΔG is a linear function of membrane tension t:

$$\Delta G = t\Delta A - \Delta G_0 \quad (2)$$

where ΔA is the difference in membrane area occupied by the open and closed channel at a given membrane tension t, and ΔG_0 is the difference in the free energy between the closed and open channel conformations in the absence of t, whereas $t\Delta A$ is the work required to keep an MS channel open by external mechanical force at the open probability $0 < P_o < 1$. The Boltzmann equation can be re-written in energy terms as follows:

$$P_o = [1 + \exp(\Delta G_0 - t\Delta A)]^{-1} \quad (3)$$

Since the membrane tension t is nearly proportional to the pressure within the range of pressures required for MscL activation, it is well approximated by Laplace's law ($t = pR/2$), where R is the radius of the curvature such that $t - t_{1/2} = (p - p_{1/2})(r/2)$. At the open probability of $P_o = 0.5$, $p = p_{1/2}$ and $t = t_{1/2}$, the free energy difference $\Delta G = 0$. Consequently, $t_{1/2} = \Delta G_0/\Delta A$ and $p_{1/2} = 2\Delta G_0/r\Delta A$, whereas $\alpha = r\Delta A/2kT$. Thus, multiplying $p_{1/2}$ and α gives a convenient expression for an estimate of the free energy of activation ΔG_0:

$$\Gamma_{MSC} = \alpha p_{1/2} = \Delta G_0/kT \quad (4)$$

Structural and functional similarities and differences between the prokaryotic MS channels

In addition to sequence and secondary structure homology, archaeal MS channels share MS properties with bacterial MS channels (Fig. 6 and Table 2). Furthermore, similar to bacteria, archaea also harbor at least two types of MS channels in their cell envelopes, which differ in not only conductance but also free energy of activation (Table 2). MscL and MscS, two *E. coli* MS

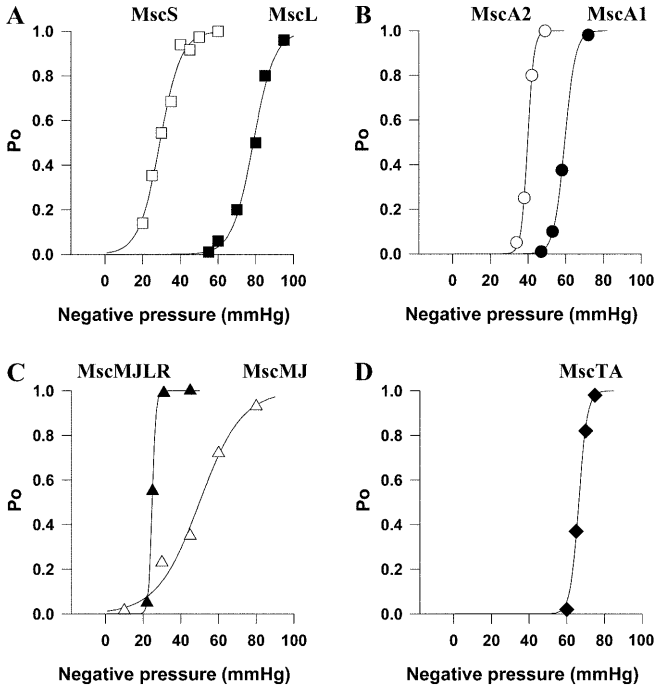


Fig. 6A–D Boltzmann distribution curves for MS channels in prokaryotes. **A** MscS and MscL of *E. coli*, based on Martinac (unpublished) and Kloda and Martinac (2001b, 2001c). **B** MscA1 and MscA2 of *H. volcanii*, adapted from Le Dain et al. (1998). **C** MscMJ and MscMJLR of *M. jannaschii*, adapted from Kloda and Martinac (2001a; 2001d). **D** MscTA of *T. acidophilum*, based on Kloda and Martinac (2001b). The Boltzmann function for MS channels relates applied negative pressure and open probability (P_o) fitted by non-linear regression and has the form: $P_o/(1-P_o) = \exp[\alpha(p-p_{1/2})]$, where $p_{1/2}$ is the suction (mmHg) at which the channel is open half the time and α is the slope of the plot of $\ln[(P_o/(1-P_o)]$ versus negative pressure and describes the sensitivity to pressure of the channels in the particular patch. Values for Boltzmann parameters are given in Table 2

channels with large and small conductance, require free energy of activation of $\Delta G_0 = 14$–$19kT$ and $\Delta G_0 \approx 7kT$, respectively. In analogy, two archaeal species, *H. volcanii* and *M. jannaschii*, have been identified with two types of MS channels whose conductance is also paralleled by the amount of free energy of activation required to open the channel. In *H. volcanii* the channel of larger conductance, MscA2, requires a much higher energy of activation of $\Delta G_0 \approx 29kT$ compared to MscA1, the channel with lower conductance and free energy of activation $\Delta G_0 \approx 15kT$. In *M. jannaschii*, the channel of smaller conductance named MscMJ requires only $\Delta G_0 \approx 5kT$ compared to $\Delta G_0 \approx 18kT$ required for the opening of MscMJLR, the channel of larger conductance. Interestingly, the free energy of activation of MscMJ resembles the one calculated for MscS, whereas the free energy of activation of MscMJLR is very similar to the energy of activation obtained for MscL. Furthermore, MscL and MscMJLR both resemble MscA1 in their free energy of activation. However, MscA2 resembles more MscTA of *T. acidophilum*, which is characterized by $\Delta G_0 \approx 35kT$.

Table 2 Summary of the Boltzmann characteristics and conductance for the known prokaryotic MS channels. The values of the shown parameters were compiled from original research papers

MS channel	$p_{1/2}$ (mmHg)	$1/\alpha$ (mmHg)	ΔG_0 (kT)	Conductance (nS)	Ref
MscL	75	4.4–5	14–19	3.3–3.8	Häse et al. (1995); Sukharev et al. (1999); Kloda and Martinac (2001b)
MscS	36	5	7	0.97 (+ve), 0.65 (–ve)	Martinac et al. (1987); Martinac (unpublished); Kloda and Martinac (2001c)
MscA1	34	2.3	15	0.38 (+ve), 0.68 (–ve)	Le Dain et al. (1998)
MscA2	43	1.5	29	0.85 (+ve), 0.49 (–ve)	Le Dain et al. (1998)
MscMJ	57	11	5	0.27	Kloda and Martinac (2001a)
MscMJLR	29	1.7	18	2.2 (+ve), 1.7 (–ve)	Kloda and Martinac (2001d)
MscTA	78	2.4	35	2.8	Kloda and Martinac (2001b)

The sequence comparison showed that, in terms of primary structure, MscMJ can be best described as a hybrid between MscL and MscS; however, in terms of conductance and selectivity it resembles more MscM, the bacterial MS channel of small conductance. In contrast, MscMJLR shares sequence homology to MscS, has selectivity similar to MscM and free energy of activation very close to MscL. Thus, similarity at the level of primary sequence between archaeal and bacterial MS channels correlates with similarities in their MS properties. In general, the larger the conductance, thus the pore size, the more free energy of activation is required to open MS channels in bacteria as well as in archaea. Furthermore, similar to bacteria, a multiplicity of MS channels may be required for survival of archaeal cells. This may reflect the need for channels operating at different levels of cellular turgor, which is most likely dictated by the different environmental cues of the living habitats of prokaryotic cells. Such structural and functional relatedness of prokaryotic MS channels may reflect their common ancestry and divergence over time. Furthermore, preservation of structural motifs common to both bacterial and archaeal MS channels may point to a common element necessary for mechanosensation. In addition to α-helical membrane-spanning domains, all functional prokaryotic MS channels share another structural motif, the C-terminal cluster of charged residues whose exact function is yet to be determined.

It was mentioned above that ΔG_0 is a product of both sensitivity to pressure (α) and the pressure necessary for 50% channel activation ($p_{1/2}$). Thus both parameters seem to be important for reaching the necessary level of free energy to activate the channel. This reflects in a heterogeneity of $p_{1/2}$ and $1/\alpha$ of prokaryotic MS channels (Table 2). Although the general trend is that the larger the conductance the more pressure is required to activate the channel, the MS channels of *M. jannaschii* seem to be an exception. The MscMJLR, the channel of larger conductance, requires less activation pressure. In contrast, MscMJ has a smaller conductance but requires more pressure to open the channel. However, these two channels also differ significantly in the sensitivity to pressure per e-fold change in the channel open probability. The small conductance MscMJ with $1/\alpha =$ 11 mmHg is significantly less sensitive to applied pressure compared to the channel of larger conductance, MscMJLR, characterized by $1/\alpha$ of 1.7 mmHg per e-fold change in open probability. Thus in archaea the emerging trend is that the MS channel conductance parallels not only the free energy of activation but also the channel sensitivity to pressure per e-fold change in the channel open probability. Indeed, the two channels of *H. volcanii* also differ (although to a lesser extent) in the $1/\alpha$ value, with a larger MscA2 being more sensitive to pressure ($1/\alpha = 1.5$ mmHg) compared to MscA1 ($1/\alpha = 2.3$ mmHg), the channel of smaller conductance. In contrast, MscS and MscL of *E. coli* are both characterized by a similar sensitivity to pressure per e-fold change in channel open probability of ~5. Thus the MS channels of archaea may differ from the MS channels of bacteria in the mechanism of adjusting the channel activity to a cellular turgor specific to the cell and its environment. Such difference may arise from not only differences in the habitats but also differences in prokaryotic cell envelopes and different chemical properties of their lipid bilayer. Differences in the composition of the lipid bilayer may account for the very high energy of activation calculated for MscA2, the MS channel of *H. volcanii*, and MscTA, the MS channel of *T. volcanium*. Such high energy of activation may result from the increased sensitivity of these channels to hydrophobic mismatch. Indeed, lipids of shorter length were found to facilitate the activation of MscTA and significantly lowered the free energy of ΔG_0 in MscL (Kloda and Martinac 2001b).

Concluding remarks

The Earth was formed 4.6 billion years ago and, for most of the time since formation, life on Earth has

consisted solely of microorganisms (Woese 1981). The primitive Earth atmosphere was characterized by anoxic conditions and high temperatures, which set the stage for the emergence of the first primitive microorganism and hence biological evolution. Besides the ability to replicate and thus pass genetic information to the next generations and the ability to transform nutrients and energy into building blocks, the first living form must have evolved a way of adaptation to constant environmental challenges. To make a survival in varying environments possible, the earliest organisms would also require the ''emergency valves'' for release of osmotic stress. In the light of evidence that MS channels play a role in regulation of turgor pressure which is essential for the division and growth of bacterial cells (Csonka and Epstein 1996), it is likely that these molecules evolved very early during evolution to fulfill functions which are crucial for survival.

The phylogenetic tree is based on three main lines of descent: bacteria, archaea and eukarya (Fig. 1). Although the root of the tree points deeply into the bacterial lineage, the archaeal branch of the tree is at the point closest to the root. This suggests that archaea might be more closely related to the last universal ancestor than any other present forms of life. The discovery of MS channels in the third domain of the phylogenetic tree has already given a new picture of evolution of these important molecules. However, the finding that they share sequence similarity, possible structural motifs, and functional properties with bacterial MS channels poses more questions to answer. The most obvious ones are: what was the first MS channel like and did it resemble any contemporary prokaryotic MS channels? Most likely, eons of evolution would result in extensive modification of the primordial MS channel template preserving only structural motifs of functional importance. Members of archaea inhabit environmental extremes, reflecting conditions under which first life originated. Can we thus consider MS channels of archaea as evolutionary relics, which may resemble MS channels of the last universal ancestor? This review suggested that it was the MscL-like progenitor molecule that gave the origin to a variety of MS channels in prokaryotes. However, in years to come, more precise answers to a variety of questions concerning the evolution of MS channels should be forthcoming with molecular identification and comparative methods of study of their representatives from all three domains of life. Such a phylogenetic approach should provide new insights into the possible structure-function relationship as well as the origin of MS channels.

The above discussion provides only a brief overview of prokaryotic MS channels and the evolutionary implications of their structural and functional aspects. We hope that with this treatment, which completes our review, we have stimulated the reader with curiosity and a desire to learn more about the evolutionary origins and function of these important biological molecules.

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