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Cloning and Functional Expression of an MscL Ortholog from *Rhizobium etli*: Characterization of a Mechanosensitive Channel

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Abstract *Rhizobium etli* is equipped with several systems to handle both hyper- and hypo-osmotic stress. For adaptation to hypo-osmotic stress, *R. etli* possesses a single gene with clear homology to MscS, four MscS-like channels and one ortholog of MscL (ReMscL, identity $\approx$ 44% compared to *Escherichia coli* MscL). We subcloned and expressed the ReMscL channel ortholog from *R. etli* in *E. coli* to examine its activity by patch clamp in giant spheroplasts and characterized it at the single-channel level. We obtained evidence that ReMscL prevents the lysis of *E. coli* null mutant log-phase cells upon a rapid, osmotic downshock and identified a slight pH dependence for ReMscL activation. Here, we describe the facilitation of ReMscL activation by arachidonic acid (AA) and a reversible inhibitory effect of Gd^{3+}. The results obtained in these experiments suggest a stabilizing effect of micromolar AA and traces of Gd^{3+} ions in the partially expanded conformation of the protein. Finally, we discuss a possible correlation between the number of gene paralogs for MS channels and the habitats of several microorganisms. Taken together, our data show that ReMscL may play an important role in free-living rhizobacteria during hypo-osmotic shock in the rhizosphere.

Keywords Mechanosensitive channel · *Rhizobium etli* · Gating modulation

Daniel Balleza and Froylan Gómez-Lagunas contributed equally to this work.

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Introduction

Mechanosensitive (MS) channels are proteins with the ability to sense increases in tension in the lipid bilayer of the cytoplasmic membrane and to transduce that signal into the channel opening. They have been documented in bacteria, archaea and eukaryotic cells (Kloda and Martinac 2002; Pivetti et al. 2003). In *Escherichia coli*, four MS-channel activities have been characterized: large conductance (MscL), smaller conductance (MscS), miniconductance (MscM) and K^+ regulated (MscK) (Berrier et al. 1996). The first electrophysiological studies of MS channels were carried out by patches of *E. coli* giant spheroplasts subjected to negative pressure in a pipette (Martinac et al. 1987), and similar observations have been made in *Bacillus subtilis* (Szabó et al. 1992) and *Streptococcus faecalis* protoplasts (Szabó et al. 1993). To date, there have been several MscL orthologs cloned from bacteria and archaea (Moe et al. 1998; Moe et al. 2000; Kloda and

Martinac 2001; Folgering et al. 2005). MscL is a homopentameric protein and was the first to be cloned and found active when purified and reconstituted into bilayers of known lipid species (Sukharev et al. 1994), although it forms tetramers in *Staphylococcus aureus* (Liu et al. 2009). At present, MscL is the best-understood MS channel from any species (Sukharev et al. 2005; Blount et al. 2007). This protein is both necessary and sufficient to transduce changes in membrane tension into an ion flux (Häse et al. 1995; Blount et al. 1996a). Thus, as the first key to mechanosensory transduction, MscL presents a simple, accessible system to assess how tension in a lipid membrane affects the gating of an MS channel (Blount et al. 1996b; Häse et al. 1997; Sukharev et al. 1999).

Bacteria within the Rhizobiaceae family include gram-negative soil bacteria that display symbiotic interactions with specific legume hosts. To accomplish a successful symbiotic interaction, rhizobacteria are required to cope with the varying conditions that they encounter in the local soil environment surrounding the plant roots (the *rhizosphere*). Furthermore, to establish symbiosis, free-living soil rhizobia are endocytosed by the host and encapsulated within a modified plasma membrane called the "peribacteroid membrane" (PBM), forming the symbiosome, which consists of the PBM and its microsymbiont (Gibson et al. 2008). The majority of these bacteria are very sensitive to osmotic challenges in free life and symbiosis, which adversely affect their N_2-fixation capacity and, hence, the productivity of the entire legume plant (Miller and Wood 1996). Thus, rhizobacteria possess mechanisms designed for adaptation to environments of high and low osmolality. Under saline conditions, they respond by modulating their cytoplasmic contents and accumulate compatible solutes and osmoprotectants such as K^+, glutamate, proline, trehalose, ectoine and glycine- and proline-betaine. On the other hand, hypoosmotic adaptation by rhizobacteria involves the accumulation of periplasmic β-glucans and, under some circumstances, probably the activation of MS channels (Breedveld and Miller 1994; Miller and Wood 1996).

The transport systems of rhizobia have been partially investigated (Taté et al. 1998, 2004; Boncompagni et al. 1999; Yurgel and Kahn 2004; Guillén-Navarro et al. 2005; Krehenbrink and Downie 2008), including ion channels and porins (Chevalier and Delamarche 1992; Sutton et al. 1994; Rojas-Jiménez et al. 2005; Chiu et al. 2007). To our knowledge, however, potential MS channels in rhizobacteria have not been described. In order to study such channels in *Rhizobium etli*, the microsymbiont of the common bean (*Phaseolus vulgaris*), we began an intensive search for possible homologs of MS channels, taking advantage of the genome sequence project of the *R. etli* CE3 (Gonzalez et al. 2006). The aim of this study was to describe the putative ortholog protein ReMscL by cloning and using the patch-clamp technique to determine whether ReMscL, which is similar to *E. coli* EcoMscL, encodes a functional MscL channel. Here, we demonstrate that the *mscL* gene (gene ID 3892764) from *R. etli* CE3 indeed encodes a functional MscL channel. We further tested the effects of arachidonic acid (AA) and Gd^{3+} on channel gating and found a stabilizing effect on partially expanded conformations of the protein. Finally, a genomic comparison with other microbes is discussed in terms of their habitats.

Materials and Methods

Chemicals

AA (*cis*,*cis*,*cis*,*cis*-5,8,11,14-eicosatetraenoic acid), citrate (citric acid monohydrate, potassium salt), buffers and salts were of analytical grade (Sigma-Aldrich, St. Louis, MO).

Strains and Cell Growth

R. etli CE3 was grown at 30°C in PY medium (0.5% peptone, 0.3% yeast extract, 10 mM $CaCl_2$) containing 50 μg/ml nalidixic acid. Total DNA was purified according to Meade et al. (1982). *E. coli* strains MJF453 and MJF465 were used for all electrophysiological experiments (Table 1). *E. coli* cultures were routinely grown in Luria-Bertani (LB) medium containing ampicillin (100 mg/ml) or the appropriate antibiotics in a shaker incubator at 37°C and rotated at 200–250 cycles/min.

Subcloning the *mscL* Homolog from *R. etli*

The *mscL* gene homolog from *R. etli* was recovered by PCR using *Pfu* DNA polymerase and standard reaction conditions. Sequence data were utilized to design oligonucleotide primers that encompassed the open reading frame. We used *Nco*I (5′) and *Xho*I (3′) to facilitate directional cloning of the fragment into pB11d under the control of a *lac*-inducible promoter (Blount et al. 1996a; Batiza et al. 2002). The oligonucleotide primers used were as follows: 5′-CTT GCC ATG GAT GCT CAA TGA GTT CAA GGC CTT TAT CGC CCG CGG C-3′ and 5′-CCG CTC GAG TCA GAC GGC GGG GCG TTT GGC CAG CAG ATC-3′. Sequence analysis and comparison with the genomic portion of the recovered gene were performed to verify the PCR product and clones.

Osmotic Downshock and Analysis of Cell Viability

The survival of *E. coli* DH5, Frag1, MJF429 and MJF465 (carrying pB11d-*ReMscL* or empty plasmid controls) under

Table 1 Bacterial strains used in this study

Strain	Genotype	References
R. etli		
CE3	Wild-type *CFN42*, Sm^r	Noel et al. (1984)
E. coli		
DH5α	*supE44*, *ΔlacU169* (*ϕ80lacZΔM15*), *recA1*	Hanahan (1983)
Frag1	F^-, *rha*, *thi*, *gal*, *lacZ*	Epstein and Kim (1971)
MJF429	*Frag1*, *ΔyggB*, *ΔkefA::kan*	Levina et al. (1999)
MJF453	*Frag1*, *ΔkefA::kan*, *ΔmscL::Cm*	Levina et al. (1999)
MJF465	*Frag1*, *ΔyggB*, *ΔkefA::kan*, *ΔmscL::Cm*	Levina et al. (1999)

osmotic downshift conditions was analyzed as described previously (Levina et al. 1999; Batiza et al. 2002). Briefly, a single colony of each strain was used as the overnight (O.N.) inoculum in LB medium with the appropriate antibiotics. We then diluted, 100 ml of the cultures at 1:20 in fresh LB plus isopropyl-β-d-thiogalactopyranoside (IPTG), and cells were allowed to grow to an A_{600} of 0.8 and then diluted 1:20 in 20 ml of prewarmed (37°C) LB medium with (0.5 M Na, control) or without (0 M Na, downshock) NaCl in 125-ml flasks shaken at 37°C for 30 min. After 30 min, we diluted 5 ml of cells with sterile equiosmolar buffers (0.5 M NaCl), all of which were prewarmed. Samples were then plated on agar with antibiotics and incubated O.N. at 37°C before the number of colony-forming units (CFUs) was determined on triplicate plates the next morning.

Spheroplast Preparation

Giant spheroplasts were prepared essentially as described by Martinac et al. (1987) with some modifications (Sukharev et al. 1994; Blount et al. 1996a). Briefly, bacterial cultures were grown and ReMscL expression was induced O.N. using 1 mM IPTG. The culture was then diluted 1:100 in fresh medium and incubated for 2 h to an OD_{600} of 0.6. This culture was then diluted 1:10 in LB medium containing 60 μg/ml cephalexin (Sigma-Aldrich, St. Louis, MO), an antibiotic that blocks cell septation. The culture was incubated with shaking for 1.5 h at 37°C until the nonseptated filamentous cells ("snakes") reached 50–150 μm in length. They were gently collected and digested with 0.2 mg/ml lysozyme and 5 mM EDTA at room temperature to obtain giant spheroplasts that were stored at −80°C for 2–3 months without loss of channel activity.

Electrophysiological Recordings

Patch-clamping procedures were carried out as previously described (Hamill et al. 1981; Martinac et al. 1987). Currents were recorded using borosilicate microcapillary pipettes (Drummond Scientific, Broomall, PA) prepared with a micropipette puller (Flaming/Brown model P-97; Sutter Instrument, Novato, CA) and pulled to have an opening diameter of ~1 μm. Pipettes were of bubble number 4.8–5.0 in absolute ethanol, corresponding to a resistance of 4–5 MΩ in recording solutions (150 mM KCl, 20 mM $CaCl_2$, 10 mM HEPES adjusted to pH 7.8), and used without heat polishing. Sucrose (0.5 M) was added to increase the osmotic stability of the spheroplasts. A Leitz (Wetzlar, Germany) micromanipulator was used to move pipettes to suitable spheroplasts, which were observed with a phase-contrast microscope (CK-2; Olympus, Tokyo, Japan). Seals between the spheroplast membrane and patch-clamp pipettes were formed spontaneously or following brief application of negative pressure (<50 mmHg). Membrane patches were excised mechanically, and channels were activated by applying suction to the interior of the pipette via the same port used for seal formation. We used $Ag/AgCl_2$ electrodes and agar salt bridges (2% agarose in 2 M KCl) as the reference electrode. Single-channel currents were filtered at 2.5 kHz using an EPC-7 patch-clamp amplifier (Heka, Lambrecht, Germany) and digitized at 10 kHz with a Digidata 1440A data-acquisition system coupled to the pCLAMP 10 package (Axon Instruments, Foster City, CA). Negative pressure was applied to the patch pipette through the suction port of the pipette holder by mouth or syringe. It was then converted into a voltage signal using a pneumatic transducer tester (DPM-IB; Bio-Tek Instruments, Winooski, VT), measured with a digital pressure gauge (143PC05D; World Precision Instruments, New Haven, CT) and digitized together with the current signal. Channel recording was conducted on excised inside–out patches in symmetrical conditions. Recordings were performed at a holding potential between −40 and +40 mV for the determination of conductances. All recordings were performed at room temperature.

Results

ReMscL Cloning in a Knockout *E. coli* Strain and Its Role In Vivo

The genomic sequence of the *R. etli* CE3 strain was recently published (Gonzalez et al. 2006; http://www.cifn.unam.mx/retlidb/). We identified sequences for the following:

four putative porins, one aquaporin, two Cl^- channels, two K^+ channels, one Ca^{2+} channel, three ABC transporters, a homolog for MscL (ReMscL; locus RHE_CH00567, accession YP_468112) and five sequences with similarity to MscS (loci ypch00482, ypch00707, ypch00853, ypch00960 and ypch01233). Here, the putative ortholog of MscL was selected for study (ReMscL, 67.7% similarity and 43.7% identity with EcoMscL; Fig. 1a). We first attempted to obtain giant spheroplasts from *R. etli* directly; however, in contrast to previous studies performed using *E. coli* (Martinac et al. 1987), it was not possible to obtain giant *R. etli* spheroplasts appropriate for patch clamping. Initial experiments in which we attempted to obtain good "snakes" to digest with lysozyme failed, and no tight and stable seals could be generated on these structures. This could have been due to the exopolysaccharides, capsular polysaccharides, lipopolysaccharides, lipo-oligosaccharides and/or cyclic glucans on these bacteria (Breedveld and Miller 1994; Fraysse et al. 2003). Therefore, we subcloned and expressed the MscL channel ortholog from *R. etli* in *E. coli* MJF465 (see Table 1) to examine its activity using patch clamp in giant spheroplasts. To that end, we exploited an *E. coli* expression system designed previously (Blount et al. 1996b). This system allows for the expression of diverse channel proteins in a common membrane system, and the presence of endogenous MscS activity provides an internal calibrator to determine the pressure response of the exogenous channel activity.

After cloning, we investigated the possible physiological role of ReMscL in vivo using a hypo-osmotic downshock assay in *E. coli* (Levina et al. 1999; Batiza et al. 2002; Booth et al. 2007). This assay utilizes the triple MS channel-deficient mutant *E. coli* MJF465 ($MscK^-$, $MscS^-$, $MscL^-$), which is not able to survive a hypo-osmotic shock. Figure 1b shows the viability of the following three *E. coli* strains under hypo-osmotic downshock: Frag1 (WT), MJ429 ($MscS^-$, $MscL^+$) and MJ465 carrying empty plasmids (pB11d) or the ReMscL channel gene from *R. etli*. Our results confirm that the viability of the triple mutant MJF465 is reduced to ~10% after osmotic downshift and, most importantly, the complementing plasmid carrying the gene *mscL* from *R. etli* restores ~97% survival to *E. coli* MJF465. As a control, the survival of the MJ429 strain with an intact copy of EcoMscL was nearly identical. These data suggest that ReMscL is a functional MS channel in *R. etli* with a large conductance activated under hypo-osmotic conditions.

Electrophysiological Characterization of the ReMscL Channel

In comparison to *Rhizobium* cells, viable giant spheroplasts (d = 5–6 μm) result from hydrolysis of the cell wall and peptidoglycan layer of "snakes" from *E. coli* carrying the ReMscL gene. After forming a giga-Ω seal by suction through a patch-clamp electrode, membrane patches were excised from the spheroplasts and analyzed for the presence of channel activity. If channels were observed, the patch was clamped at a negative pressure between −50 and −250 mmHg. Figure 2a shows an example of a recording from ReMscL. The minimum suction required to activate the channel ranged between −100 and −150 mmHg (1 mmHg ≈ 133 Pa); on the other hand, the release of suction closed the channels. This result confirms that the *mscL* gene from *R. etli* encodes a functional MS channel when it is expressed in *E. coli* spheroplasts. No such activity was observed in spheroplasts carrying empty plasmids (not shown).

Figure 2b depicts the single-channel current–voltage relationship for the ReMscL channel in symmetric solutions (150 mM KCl). The conductance of the channel was calculated from the slope to be 3.3 nS. Each point represents the maximum amplitude observed at each voltage and was considered as the fully open state (O_f) of the channel. In comparison, EcoMscL had a conductance of 3.1 nS under the same conditions. Succinate is a large organic ion that has been probed in EcoMscL to calculate an open pore size of 30–40 Å (Cruickshank et al. 1997; Perozo et al. 2002a). We found that citrate was equally effective as a permeant ion for ReMscL, as judged by the reversal potential (data not shown). Using the MJF453 strain, we found that the pressure ratio of ReMscL to EcoMscS (P_L/P_S ratio) opening was 1.34 ± 0.6. Figure 3 illustrates the open probability (P_o) of ReMscL as a function of the negative pressure applied to the pipette. The P_o of the ReMscL channel was fitted to a Boltzmann distribution function. As for EcoMscL, we found that the P_o of ReMscL depends on cytoplasmic pH. At pH 7.8, the pressure required for ReMscL half-activation ($p_{1/2}$) was 166 mmHg, whereas at pH 5.4, $p_{1/2}$ = 192 mmHg. In comparison, for EcoMscL the $p_{1/2}$ at pH 7.0 is 76.3, while at pH 5.5, $p_{1/2}$ = 120.2 mm (Kloda et al. 2006). In addition, our results showed that an acidic pH of 5.4 did not affect the single-channel conductance of ReMscL (not shown). In short, we found that an acidic intracellular pH slightly changed the sensitivity of the channel to the applied pressure, suggesting that protons have a modulatory effect on the activity of the channel. This is shown in Fig. 3b, in which the P_o curves show a subtle rightward shift on the pressure axis with a difference of 2.4 pH units (from 7.8 to 5.4).

Closer examination of the current traces revealed that they exhibit multiple subconducting states. To study these intermediate states of the ReMscL channel, we analyzed their frequencies under the slow release of suction over several minutes at +10 mV. Under these conditions, the channel more regularly showed several subconductances.

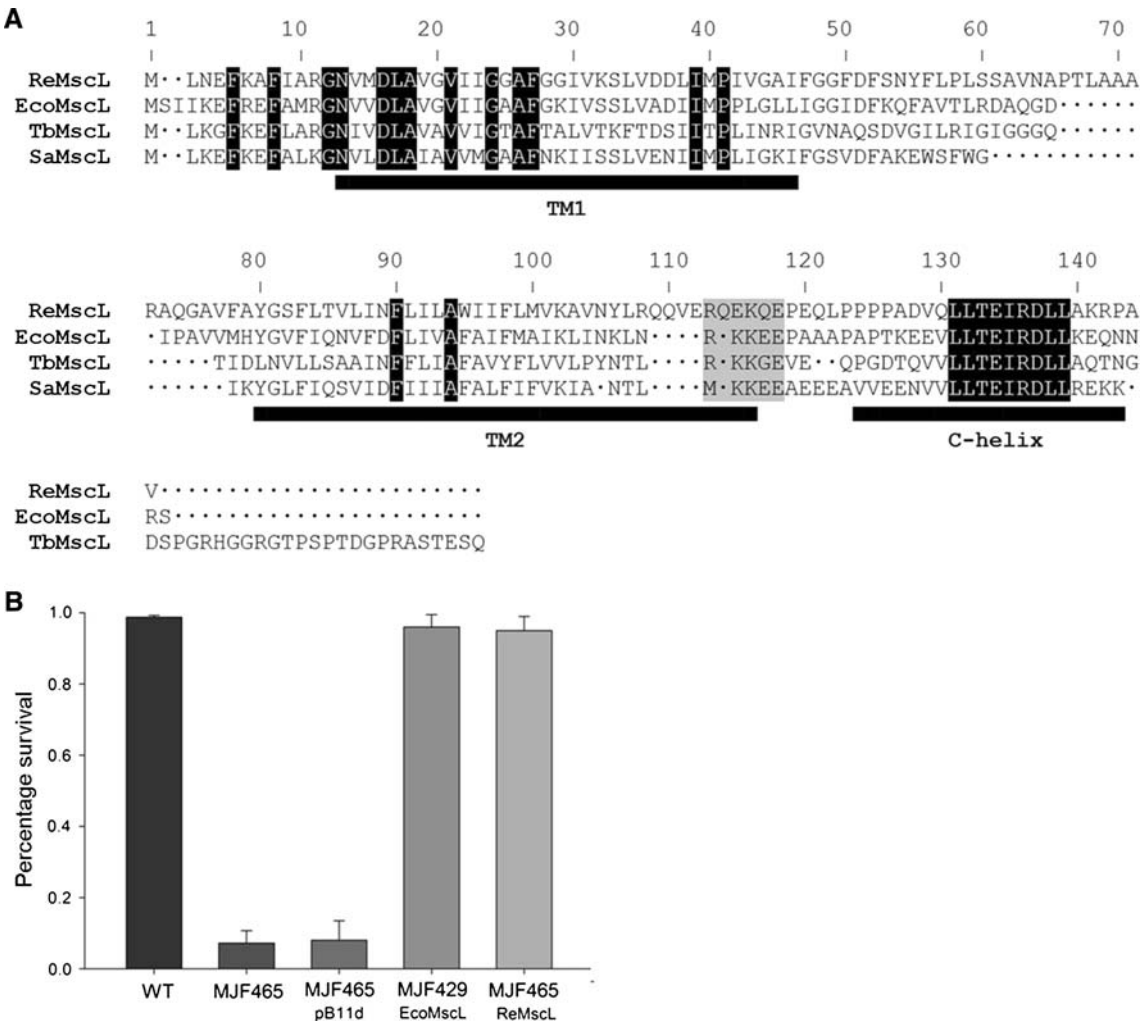

Fig. 1 **a** Alignment of MscL from *R. etli*, *E. coli*, *M. tuberculosis* and *S. aureus*. *Boxed amino acids in black* correspond to fully conserved residues. Transmembrane segments and cytoplasmic helix are shown as *black horizontal bars*. *Gray box* indicates the conservation of clusters of charged residues. **b** Survival of *E. coli* cells expressing the ReMscL channel upon osmotic downshock. Cells were grown to exponential phase in minimal medium (pH 7.0) in the presence of 0.5 M NaCl, then diluted 20-fold into minimal medium without NaCl (see "Materials and Methods"). Data show the survival of strains measured 30 min after downshock ($n = 3$). The presence of ReMscL increased survival of the *E. coli* null mutant (MJF465), which strongly suggests that ReMscL protects cells under conditions of severe osmotic downshift. Strains assayed: Frag1 (wild type), MJF429 (Δ*yggB*, Δ*kefA*) and MJF465 (Δ*yggB*, Δ*kefA*, Δ*mscL*)

After the fully open state (O_f), the channel usually entered a long-lived subconducting state ($L_{0.87}$). We observed at least five more subconductances: $S_{0.05}$, $S_{0.13}$, $S_{0.23}$, $S_{0.36}$ and $S_{0.65}$ (Fig. 4a). The frequency of each S state was variable from patch to patch, but the L state, $L_{0.87}$, was very frequent and previously interpreted as an alternative open state in EcoMscL (Chiang et al. 2004). Figure 4b shows a representative all-point amplitude histogram obtained from a 25-min continuous recording. Statistical analysis showed at least seven gaussian curves with amplitudes of 0.0 (closed), 0.05, 0.13, 0.65, 0.72 and 0.87 relative to the amplitude of the O_f . The $S_{0.23}$ and $S_{0.36}$ substates were infrequent (see lower inset in Fig. 4a, Table 2).

Micromolar Concentrations of AA and Traces of Gd^{3+} Ions Stabilize ReMscL in Partially Expanded Conformations

To study the pharmacology of ReMscL, we tested whether a free fatty acid, such as AA, could facilitate the activation of ReMscL. Inside–out patches were formed in symmetrical 150 mM KCl, and 10–40 μM AA was applied to the cytoplasmic side of the membrane. We observed that under sustained negative pressure (−110 mmHg) AA facilitated the opening of ReMscL channels within the first 60 s after treatment ($n = 5$). AA aided the opening of ReMscL channels, as shown in Fig. 5; this result was observed in ~80% of the patches tested. Once channel activation was

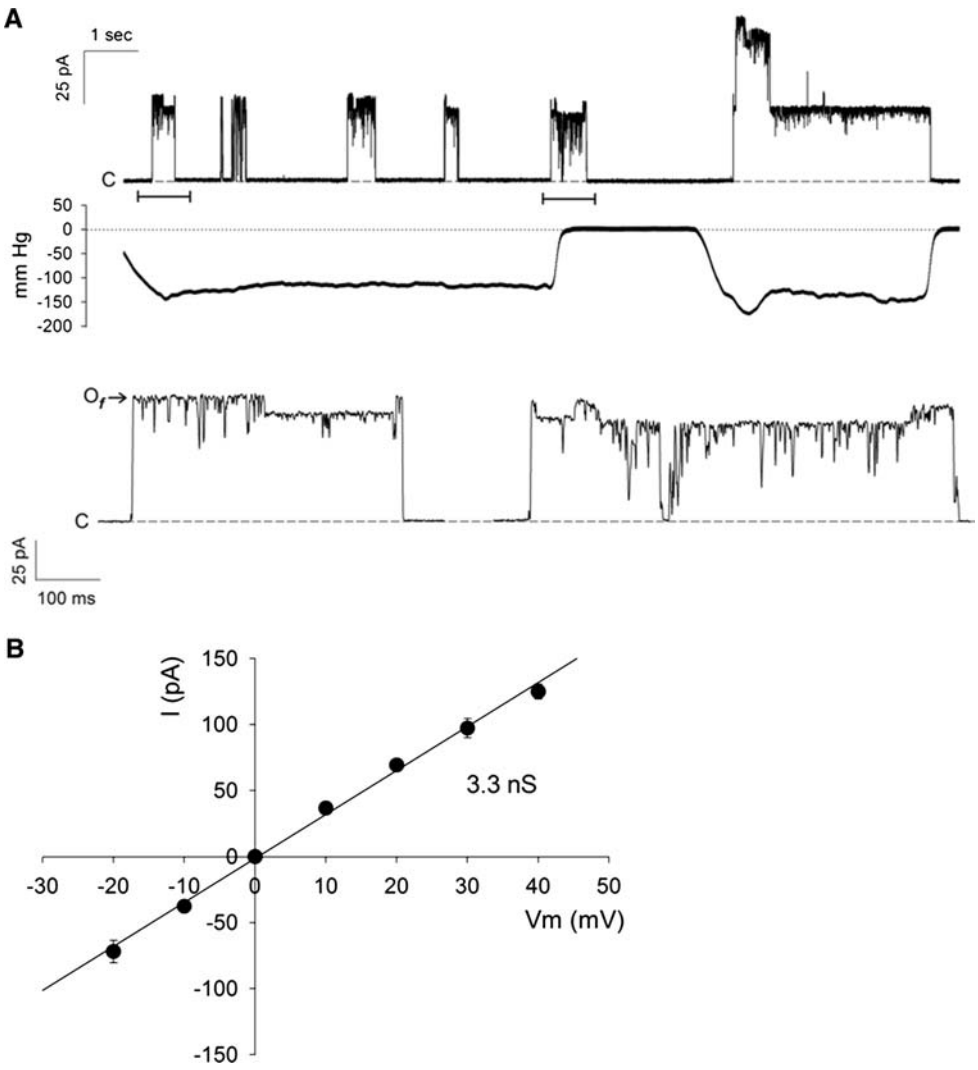

Fig. 2 **a** Representative single-channel traces recorded from inside–out patches of giant spheroplasts expressing ReMscL on *E. coli* MJF465. Pressure traces (measured in mmHg) are shown below the current trace. Holding potential was +10 mV, and low-pass filtering was set at 2.5 kHz. *Inset below* (*expanded underlines in upper trace*) shows the fully open state (O_f) of the channel (*arrow*), which was used to obtain the *I–V* relationships. *C*, closed state of the channel. **b** *I–V* plot of ReMscL under symmetrical conditions (150 mM KCl). The slope of the least squares line through the points gives a conductance of 3.3 ± 0.2 nS ($r^2 = 0.98$) ($n = 12$, mean ± SEM)

obtained under these conditions, each patch became very unstable, and eventually, the seal was permanently lost, presumably due to continued insertion of AA. In another series of experiments, we decided to induce the opening of ReMscL at pressures under which P_o is very low (−50 mmHg). Exogenous application of 10 μM AA led to channel activation in a time-dependent manner. Channel activity after ~1 min of AA application is shown in Fig. 5b and corresponds to several substates described previously (C, $S_{0.23}$ and $L_{0.87}$), typical for ReMscL. Under these conditions, the patches were lost after ~2 min ($n = 4$). Note that after the application of 10 μM AA, channel activity increased from $P_o \approx 0.01$ to $P_o \sim 0.45$, while control experiments in which channel activity was monitored in the absence of AA showed only spontaneous opening (Fig. 5b, c). In general, for independent experiments without the application of AA, there was no activation of the channel at −50 mmHg. However, in the presence of AA at micromolar levels, a small increase in channel activity was observed during the first 0.5–2 min. In one experiment we added 30 μM AA to patches without applying suction and observed some openings to substates $S_{0.13}$, $S_{0.23}$ and $S_{0.72}$ after treatment, with $P_o < 0.01$ (see supplementary Fig. S1). As a control, we applied the AA solvent dimethyl sulfoxide (DMSO) alone and found no change in the activity of ReMscL (not shown). Additionally, we evaluated the effects of AA on EcoMscL and found that, as in ReMscL, application of 20 μM AA facilitated opening of the EcoMscL channel by increasing the frequency of flickering, with fast openings from 2 to 50 ms ($n = 2$) (see supplementary Fig. S2).

In contrast, the ReMscL channel was reversibly inhibited by gadolinium. Complete blockage of channel activity by 10–40 μM Gd^{3+} was observed even at negative pressures of up to −200 mmHg or more ($n = 5$). This inhibition of channel activity could be reversed by intense washout and increasing the applied pressure to the patch to −250 mmHg or more. After Gd^{3+} treatment, we noted that

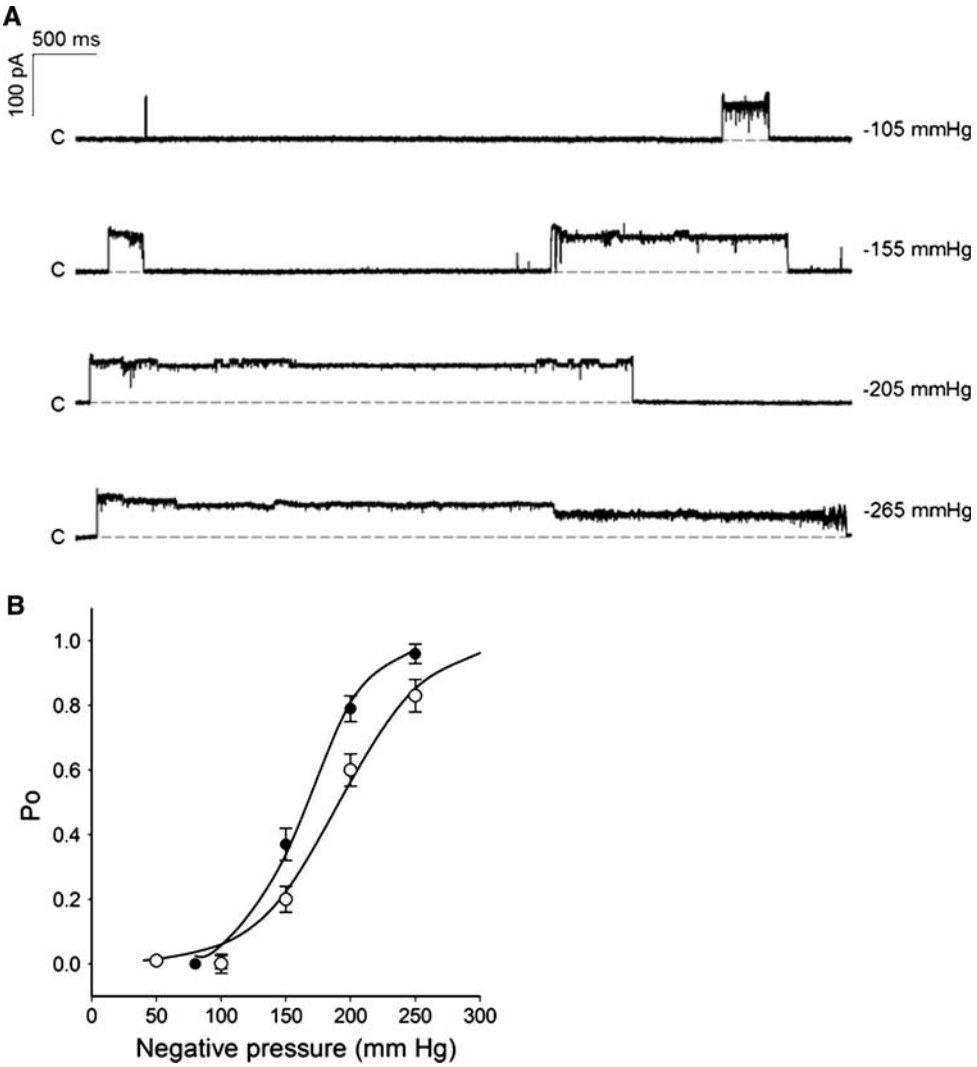

Fig. 3 Pressure dependence of the open probability for ReMscL expressed in *E. coli* spheroplasts. **a** Single-channel traces obtained in excised patches at +20 mV; activation pressure (mmHg) is shown at *right*. **b** Open probability (P_o) of ReMscL as a function of the negative pressure applied to the patch pipette fitted to a Boltzmann equation of the following form: $P_o = 1/\{1 + \exp[-\alpha(p - p_{1/2})]\}$. The rightward shift in the pressure axis corresponds to a change in cytoplasmic pH from 7.8 to 5.4 in the bath solution. Parameters, pH 7.8 ($n = 8$, *closed circles*): $p_{1/2} = 166$ mmHg, $\alpha = 0.04$; pH 5.4 ($n = 3$, *open circles*): $p_{1/2} = 192$ mmHg, $\alpha = 0.03$

the membrane patch was more stable. The open probability after washout dropped from 0.7 ± 0.1 to 0.2 ± 0.1, whereas the dwelling time for the closed state (τ_c) increased from 69 ± 25 to 231 ± 81 ms. Curiously, we observed that after washout only substates $S_{0.72}$ and $L_{0.87}$ were favored, although some evident openings at $S_{0.13}$ were also detectable (Fig. 6). We then attempted to recover fluidity of the membrane by application of AA after washout of Gd^{3+}. However, all of our attempts failed and new experiments are needed to clarify these observations.

A Possible Evolutionary Implication for MscL Orthologs

Because many bacterial and archaeal genome-sequencing projects have been completed (Comprehensive Microbial Resource, http://cmr.jcvi.org/), we can address the question of MS gene conservation. The genomes of rhizobia are large and complex, with the following sizes: 6.53 Mb (*R. etli*), 6.69 Mb (*Sinorhizobium meliloti*), 6.81 Mb (*S. medicae*), 7.59 Mb (*Mesorhizobium loti*), 7.75 Mb (*R. leguminosarum* bv. *viciae*) and 9.1 Mb (*Bradyrhizobium japonicum*). Many genes are devoted to transport, regulation and a wide range of catabolic systems (Boussau et al. 2004). With the goal of establishing a possible relationship between common habitats and the presence of MS channels in some species of rhizobia, we compared the genomes of *R. etli*, *S. meliloti*, *S. medicae*, *M. loti*, *R. leguminosarum* and *B. japonicum* with the genomes of *Azoarcus* (an N_2-fixing obligate endophyte), *Frankia* (a facultative symbiotic, N_2-fixing, gram-positive actinobacteria), select plant pathogens (*Agrobacterium tumefaciens*, *Pseudomonas syringae* and *Xanthomonas campestris*), a moderate (*Chromohalobacter*) and an extreme (*Halorhodospira*) halophile and select Archaebacteria (*Halobacterium*, *Methanosaeta* and *Methanosarcina*). The results of this search showed that both N_2-fixing bacteria and plant pathogens have at least one copy of MscL and several copies of MscS-like

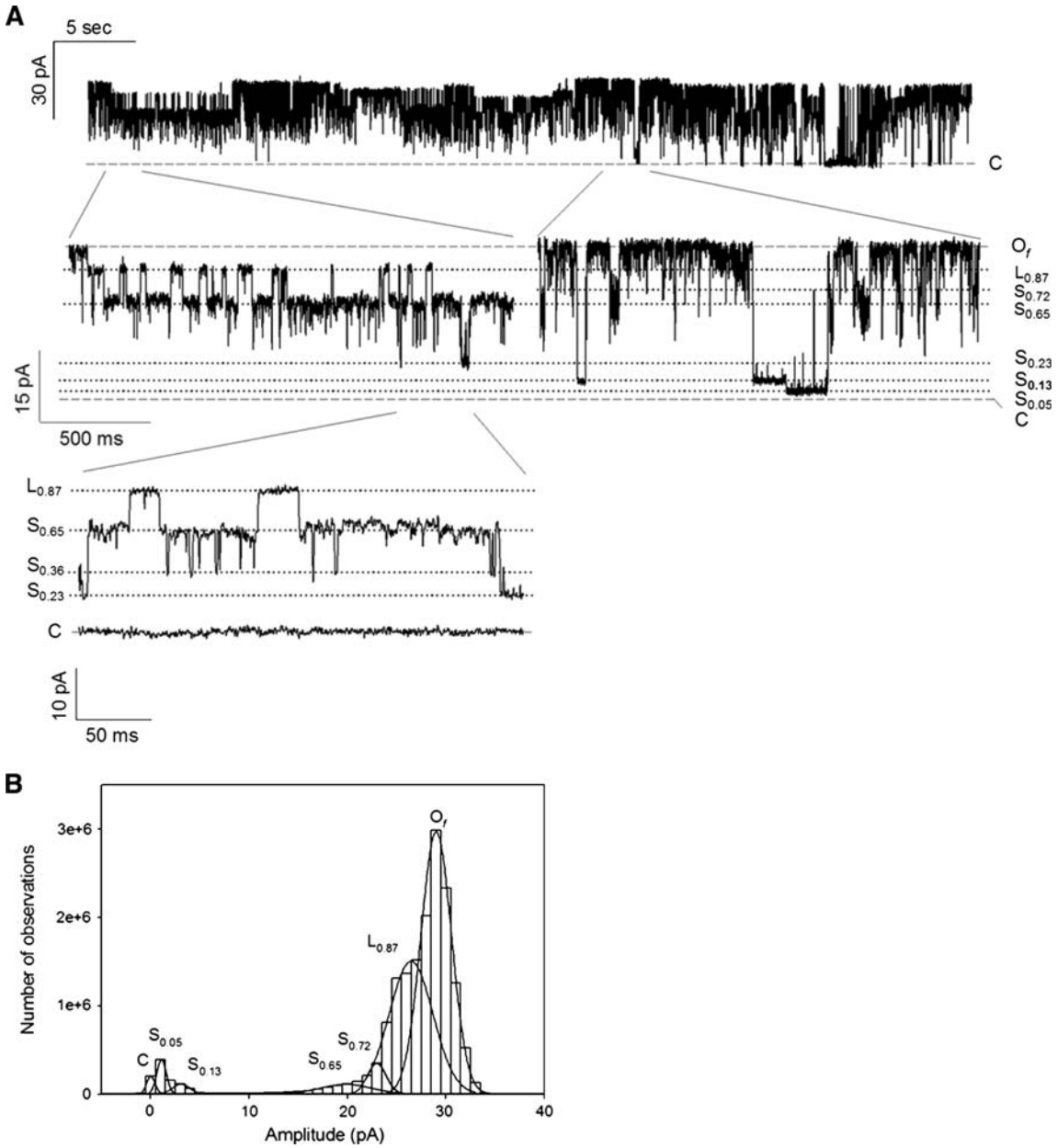

Fig. 4 Subconducting states of ReMscL. **a** Gating pattern of the ReMscL channel under prolonged suction recorded in an inside–out patch at +10 mV under constant suction (−150 mmHg). A 55-s trace of a 25-min recording is shown with openings as upward deflections and *dotted lines* representing the more frequent subconductances observed. **b** All-point amplitude histogram including the entire 25-min data set. It was fitted by gaussian distributions (peaks). Peak positions shown by numbers represent amplitudes of subconducting states relative to the amplitude of the fully open state (O_f)

sequences, depending on the species. One curious aspect, however, is that *M. loti* possesses three putative copies of MscL, whereas *Frankia alni* has two. On the other hand, bacterial halophiles and the archaeon *Halobacterium* (an extreme halophile) have no copies of MscL, while *Methanosaeta* and *Methanosarcina* (two anaerobic methanogens) each have one copy, along with several copies of MscS-like channels. These results are shown in Table 3 and a phylogenetic relationship of MscL orthologs based on (Kumar et al. 2008) is shown in supplementary Fig. S3.

Discussion

In this study, we cloned and characterized an *mscL* ortholog gene from *R. etli*, encoding an MS channel of large

Table 2 Conductances of the different substates of ReMscL

Substate	Conductance (nS)	Frequency	*n*
Open (O_f)	3.3 ± 0.2	0.5	12
$L_{0.87}$	2.9 ± 0.2	0.3	6
$S_{0.72}$	2.4 ± 0.2	<0.1	4
$S_{0.65}$	2.2 ± 0.2	<0.1	4
$S_{0.36}$	1.2 ± 0.2	<0.05	4
$S_{0.23}$	0.8 ± 0.1	<0.05	4
$S_{0.13}$	0.4 ± 0.2	<0.1	4
$S_{0.05}$	0.2 ± 0.1	<0.1	4
Closed (C)	0.0 ± 0.0	<0.1	12

conductance (MscL) with an important function in the physiology of bacteria under hypotonic conditions. This channel exhibited characteristics similar to EcoMscL in terms of both gating and mechanical properties, in addition to pH sensitivity. At the protein sequence level, ReMscL has 43.7% identity with EcoMscL and the two putative helical transmembrane segments are well conserved (see Fig. 1a; Balleza and Gómez-Lagunas 2009). The activation of ReMscL in spheroplast patches required −100 to −150 mmHg, although individual patch geometries affected its pressure dependence. As for EcoMscL, patches formed with large-diameter pipettes activated at lower pressures, suggesting that the parameter that drives ReMscL gating is tension, not pressure (Sukharev et al. 1999). Prolonged exposure of patches to high pressures often caused patch loss. We found that a change in pH from 7.8 to 5.4 did not affect the conductance of the channel but, instead, exerted a slight effect on channel sensitivity to negative pressure. Boltzmann distribution curves for ReMscL channels showed a rightward shift in the negative pressure slope factor, describing the sensitivity to negative pressure of the channels under acidic conditions on the cytoplasmic side. The putative pH sensor in EcoMscL is associated with the charged RKKEE cluster on the cytoplasmic face of the second transmembrane segment (Kloda et al. 2006). These residues have different ionization states affected by changes in pH that have effects on channel activity. This cluster is different from that found in the sequence of ReMscL: RQEKQE (see Fig. 1a). In comparison to EcoMscL, we noted that the activation of ReMscL was minor in response to a decrease in cytoplasmic pH (Fig. 3). At least in part, the presence of two intercalated glutamines must neutralize the overall charge of the ReMscL cluster. This suggests that the presence and order of non-ionizable residues within the charged cluster may also modulate the pH sensitivity, and their interactions with head groups in the lipid bilayer may underlie the mechanosensitivity of the channel. On the other hand, because the rest of the protein (~56%) is dissimilar to EcoMscL, the pH sensitivity could be influenced by some other region of the protein. The study by Kloda et al. (2006) indicates that protonation of Glu residues causes a rightward shift of the P_o curve, whereas protonation of Arg and Lys residues causes a leftward shift. If we compare the RQEKQE cluster from ReMscL with the RKKEE cluster at pH 5.5 or the RKKQQ mutant at pH 7.0 from EcoMscL, both show a rightward shift of the P_o curve. The change in pH, however, is different. For EcoMscL the effect was observed after a change of 1.5 pH units, whereas for ReMscL the difference was a change in 2.4 pH units, with a slight effect on pH sensitivity (Table 4). To clearly demonstrate the involvement of this cluster as a pH sensor, future experiments should include probing pH < 5.0 in the bath solution and mutational analysis of the charged cluster.

In addition to negative pressure, the activation of ReMscL was facilitated by polyunsaturated fatty acids (PUFAs). Trapping the EcoMscL channel in the fully open conformation via the effects of lysophosphatidylcholine (LPC) (Perozo et al. 2002a, b) or the light-induced *cis–trans* isomerization of the azobenzene moiety of di-(5-{[4-(4-butylphenyl)azo]phenoxy}pentyl)phosphate (4-Azo-5P) (Folgering et al. 2004) has been widely studied. To our knowledge, however, the effect of AA has not been explored in MscL channels, although its activating effect has been demonstrated in mechanogated 2P and S-type K^+ channels (for a recent review, see Dedman et al. 2009). We found that 20 μM AA stabilizes the opening of ReMscL and induces the opening of EcoMscL. To evaluate the effect of AA on ReMscL gating, we carried out experiments in patches with a very low P_o by applying mild suction. The increase in channel activity shown in Fig. 5 must be the result of the addition of AA as we did not observe any activation in the presence of DMSO alone. We do not know whether such activation is reversible, however, because the patches were frequently lost. Nevertheless, we observed that 10 μM AA induced fast openings from the C to $S_{0.23}$ and $L_{0.87}$ substates in a time-dependent manner, suggesting a stabilizing effect of the partially expanded conformation of the channel. Under control conditions, we observed at least eight subconducting states at −150 mmHg (see Fig. 4, Table 2). The frequencies of short-lived $S_{0.23}$ and long-lived $L_{0.87}$ substates in the presence of 10 μM AA suggest that the incorporation of AA into the lipid bilayer facilitates the energy-requiring transition for the gating of the channel from the closed state. It has been shown that in EcoMscL occupancy of the $S_{0.22}$ substate (analogous to $S_{0.23}$ in ReMscL) decreases with tension and is associated with the largest area increase in protein expansion, while occupancy of the $L_{0.78}$ substate (analogous to $L_{0.87}$ in ReMscL) increases with tension (Chiang et al. 2004). We suggest that AA leads to channel activation via changes in the biophysical properties of the bilayer or by affecting local

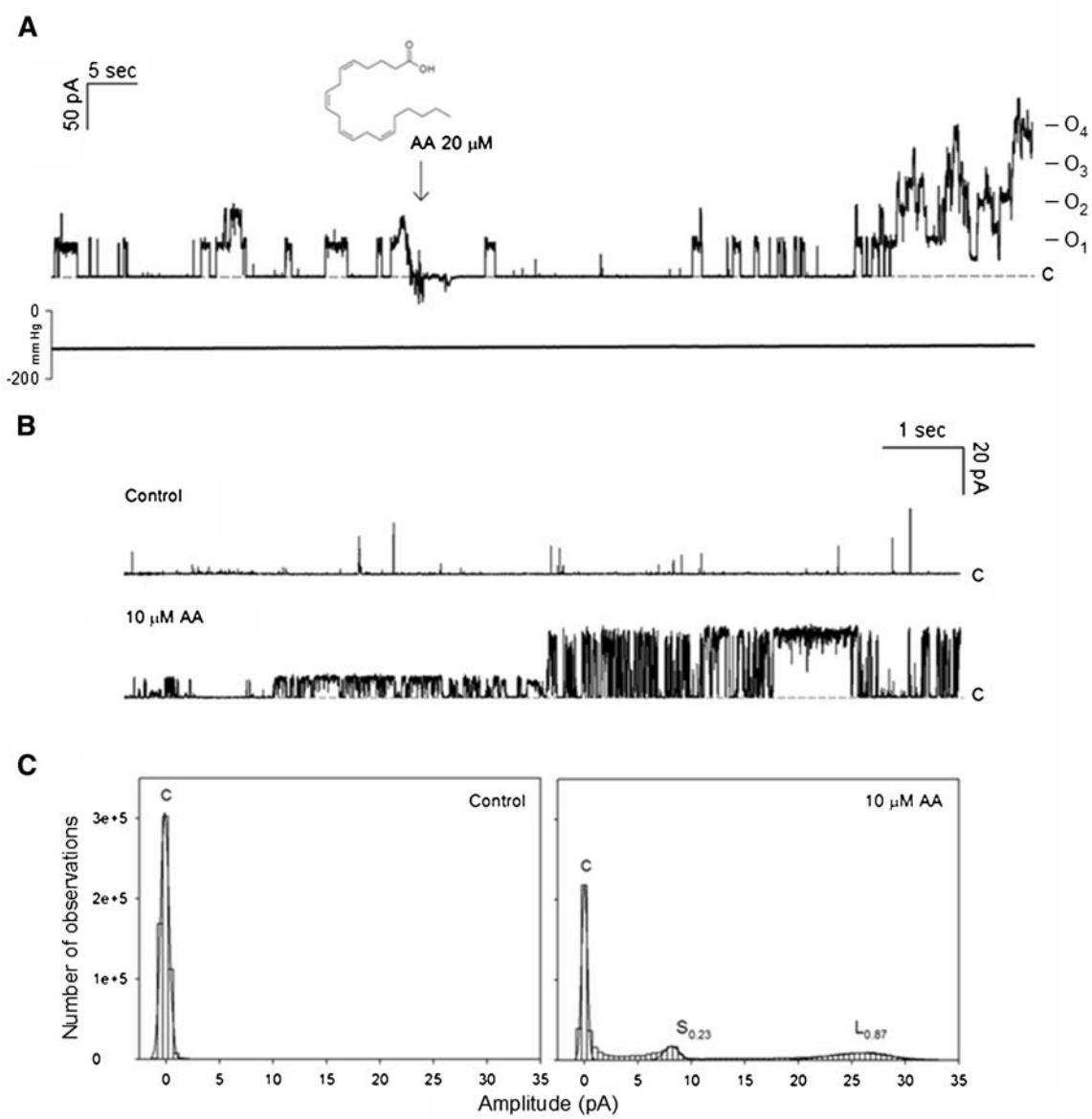

Fig. 5 Effect of AA on the activation of ReMscL. **a** Representative excised-patch (inside–out) recording of ReMscL in response to negative pipette pressure (−110 mmHg) during a +10 mV voltage step followed by the addition of 20 μM AA (*arrow*). The patch was lost within the first 60 s after the application of AA. **b** Effect of 10 μM AA at a constant negative pressure (−50 mmHg) and voltage (+10 mV). **c** Amplitude histograms before and after (~1 min) application of 10 μM AA to the bath solution. Substates $S_{0.23}$ and $L_{0.87}$ are evident. P_o increased from 0.01 (control) to 0.45 (10 μM AA)

curvature. This is in accordance with the bilayer couple hypothesis (Sheetz and Singer 1974), which proposes that compounds with a smaller polar head and a large hydrophobic tail (e.g., AA) are considered to be inverted cones. In fact, it has been demonstrated that the addition of cone-shaped phospholipids to one membrane leaflet creates surface tension, probably by bending the membrane and altering bilayer mechanical properties (Farge and Devaux 1992; Meves 1994, 2008; Maingret et al. 2000; Kloda et al. 2007). The partition of such molecules into the lipid bilayer depends on the asymmetry of the two leaflets, leading to local deformation (convex curvature) and facilitating the opening of MS channels (Martinac et al. 1990; Patel et al. 2001). AA (20:4) is a 20-carbon ω-6 PUFA described as a "slippery molecule," and its four *cis* double bonds are the source of its flexibility (Brash 2001). It may insert into the membrane as the asymmetrical insertion of lysolipids into membranes, which produces a torque on the channel proteins (Phillips et al. 2009). Thus, our results suggest that, due to its amphipathic character, AA may disturb the lipid–protein interface and thereby facilitate the activation of ReMscL, and modulating its gating. In EcoMscL, a two-gate

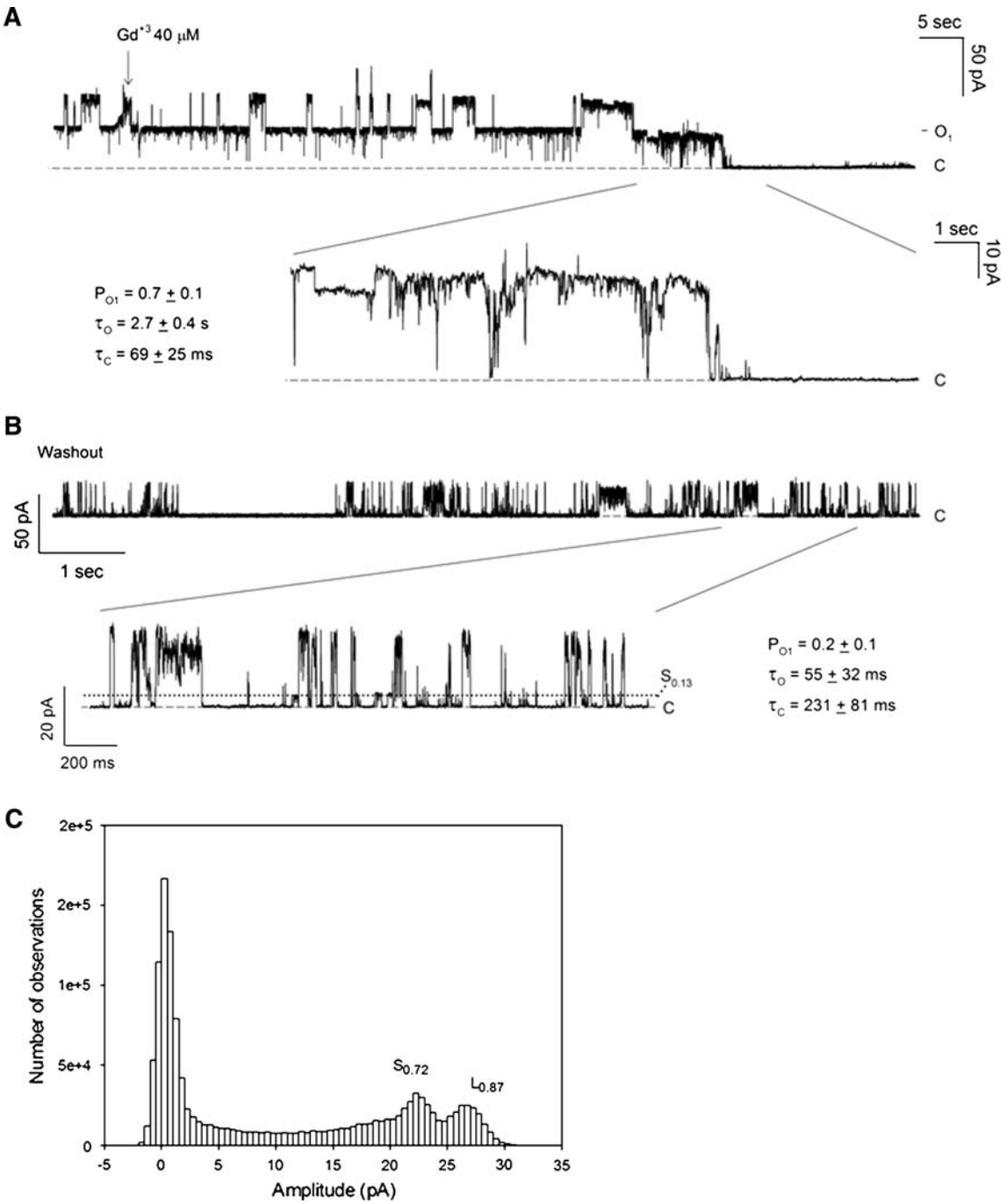

Fig. 6 Effect of gadolinium on ReMscL gating. **a** Channels were activated by −200 mmHg in an excised patch at +10 mV. *Arrow* indicates the point at which 40 μM Gd^{3+} was added to the bath solution. **b** Washout of inhibited channels and partial recovery of activity. Open probability and time constants for open (τ_o) and closed (τ_c) dwell times are shown for each condition. **c** Amplitude histogram after washout of Gd^{3+}; only substates $S_{0.72}$ and $L_{0.87}$ are clearly favored, although some openings to $S_{0.13}$ are visible

mechanism has been suggested in which the first subtransition (C → S) can be viewed as the opening of the central (TM1 ring) gate, resulting in an expanded water-filled "leaky" conformation (Anishkin et al. 2005). Although ω-3 PUFAs have been shown to change the internal force profile of the lipid bilayer in silico (Carrillo-Tripp and Feller 2005), elucidation of the mechanism leading to ReMscL opening by AA requires additional dose–response experiments and extensive molecular dynamics simulations.

Table 3 Completed genome projects for rhizobacterial and other microbial species with number of putative homolog MS channels

Species	Size (Mb)	MscL	% Id	% CR	MscS-like	GenBank accession
Rhizobia						
Bradyrhizobium japonicum	9.10	1	54.0	19.7	2	NC_004463
Bradyrhizobium sp. ORS2	7.45	1	54.0	18.9	2	NC_009445
Mesorhizobium loti	7.59	3	50.7	22.8	3	NC_002678/79, NC_002682
			51.1	22.6		
			50.0	25.7		
Mesorhizobium sp. BNC1	4.93	1	50.4	22.9	3	NC_008242–44, NC_008254
R. etli CE3	6.53	1	43.7	23.9	5	NC_004041, NC_007761–66
R. leguminosarum	7.75	1	44.6	25.9	5	NC_008378–84
Sinorhizobium medicae	6.81	1	44.7	25.5	7	NC_009620–22, NC_009636
S. meliloti	6.69	1	43.3	26.2	6	NC_003037, NC_003047, NC_003078
N_2-fixing facultative symbionts						
Azoarcus sp. BH72	4.37	1	55.3	21.3	1	NC_008702
Frankia alni ACN14a	7.49	2	42.0	22.5	2	NC_008278
			38.1	26.9		
F. sp. CcI3	5.43	1	42.9	24.1	1	NC_007777
Plant pathogens						
Agrobacterium tumefaciens	5.67	1	47.9	24.3	1	NC_003062–65
Pseudomonas syringae pv. tomato	6.53	1	67.2	17.9	4	NC_004578, NC_004632/33
P. syringae pv. phaseolicola	6.11	1	67.2	18.7	5	NC_005773, NC_007274/75
Xanthomonas campestris	5.15	1	57.0	23.7	1	NC_007086
Halophiles						
Chromohalobacter salexigens	3.69	0	–	–	4	NC_007963
Halorhodospira halophila SL1	2.67	0	–	–	1	NC_008789
Archaebacteria						
Halobacterium sp. NRC-1	2.57	0	–	–	2	NC_001869, NC_002607/08
Methanosaeta thermophila	1.88	1	46.6	27.6	2	NC_008553
Methanosarcina acetivorans	5.75	1	29.3	40.4	3	NC_003552

% Id, percentage identity; % CR, percentage of conservative replacements in comparison with EcoMscL

Table 4 Boltzmann parameters of the EcoMscL and ReMscL channels within the charged cluster and pH sensitivity

	E. coli (wt)[a] RKKEE		Mutant[a] RKKQQ	*R. etli* RQEKQE	
	pH 5.5	pH 7.0	pH 7.0	pH 5.4	pH 7.8
$p_{1/2}$ (mmHg)	120.2	76.3	130.3	192.2	166.0

[a] Kloda et al. (2006)

On the other hand, blockage with gadolinium was seen at micromolar concentrations. The Gd^{3+} ion is a well-known blocker of MS channels and has diverse, often nonspecific effects (Evans 1990; Häse et al. 1995; Hamill and McBride 1996; Caldwell et al. 1998; Ermakov et al. 2001; Petrov and Martinac 2007). Lanthanides possess high affinity for cellular membrane phospholipids, with K_d values in the micromolar range (Evans 1990); and we observed complete blockage after ~60 s of 40 μM Gd^{3+} application. In the majority of these experiments, the blockage was nearly instantaneous after treatment. In some experiments, however, we observed a gradual effect in the gating of ReMscL. The reversibility of the blocking effect was probed by an extensive washout of the bath solution as blocked channels are unable to reopen without washout. Nevertheless, this recovery was not complete because the channel gating was different, mainly favoring substates $S_{0.72}$, $L_{0.87}$ and several transitions to $S_{0.13}$ (Fig. 6). The frequency of such subconductances suggests that after Gd^{3+} treatment the fluidity of membrane changes and some

rigidity remains, as has been previously proposed (Ermakov et al. 2001). This prevents complete reopening of the channel under further suction, stabilizing again partially expanded conformations. Curiously, in EcoMscL the long-lived subconducting states $L_{0.78}$ and $L_{0.64}$ (probably analogous to $L_{0.87}$ and $S_{0.72}$, respectively, in ReMscL) are characterized by larger in-plane areas than the open state (Chiang et al. 2004). This observation raises the question of the kinetic effects of Gd^{3+} on the gating of MscL homologs. Taken together, the results obtained for subconductance activation with AA and reversible Gd^{3+} blockage suggest that the mechanical properties of the bilayer play an essential role in inducing conformational changes in the ReMscL channel, as has been previously proposed for MscL homologs and membrane proteins in general (Wiggins and Phillips 2004, 2005; Phillips et al. 2009). Thus, we believe that our results highlight important new questions for future research with regard to the effects of AA and Gd^{3+} on the responsiveness of the membrane to tension.

Regardless, the gating characteristics of ReMscL in its native environment could be different. Although we attempted to study ReMscL in *R. etli* spheroplasts, we were unable to do so. This is an important consideration in future studies because the lipid composition of *Rhizobium* is different compared to that of *E. coli* (López-Lara et al. 2003). As in *E. coli*, the major phospholipids in plant-associated bacteria are phosphatidylglycerol (PG), cardiolipin (CL) and phosphatidylethanolamine (PE) (Gill 1975; Shukla et al. 1980). Phosphatidylcholine (PC) is the major phospholipid in eukaryotic membranes, but only 10% of all bacteria are predicted to synthesize PC. In Rhizobiaceae, however, PC is essential for the establishment of a successful host–microbe interaction (López-Lara et al. 2003). Remarkably, an important adaptive response in *Rhizobium* during growth in hypo-osmotic environments involves substantial remodeling of the cell envelope. These changes include important alterations in periplasmic glucan levels (Breedveld and Miller 1994; Miller and Wood 1996), but changes in lipid composition have not been well studied.

Finally, taking advantage of genomic databases, we explored the genomes of several rhizobia and other microorganisms in search of genes encoding MS channels, to establish a relationship between this information and their typical habitats. Based on the results shown in Table 3, we propose that the presence of a single copy of MscL and several copies of MscS/MscK comprises an evolutionary strategy to handle a plethora of osmotic conditions in the environment. Thus, we propose that the basic genome structure for a prokaryote is to carry one copy of *mscL* to handle extreme turgor, with this gene being "common," while depending on the bacterial/archaeal species, the small conductances of MscS-like proteins may be required for the fine-tuning of different nonsevere osmotic conditions according to the species and its life cycle.

Conclusions and Perspectives

Taken together, our results strongly suggest that ReMscL is a key survival channel for free-living rhizobacteria. However, once *Rhizobium* gain intracellular access to their legume host, they differentiate into N_2-fixing bacteroids (Gibson et al. 2008). Little is known about the changes in the bacteroid envelope outer and inner membranes except that, in comparison to free-living rhizobia, the envelopes are modified in some way that causes bacteroids to exhibit osmotic fragility (Sutton et al. 1977; Müller et al. 2001). This is not likely to be a simple adaptation by bacteroids to the high osmolarity of the nodule cytoplasm (Boscari et al. 2006); and a more likely explanation would be a change in the structure of the protein composition of the plasma membrane during the conversion from the free-living forms to N_2-fixing forms. This presumably reflects a change in function and indicates that the new peribacteroidal membrane system plays an important role in the fixation process. On the other hand, when nodules disintegrate via senescence, viable bacteroids are released into the soil (Tsien et al. 1977; Müller et al. 2001; Mergaert et al. 2006), which is an obvious benefit for the enrichment of the rhizosphere microflora for further infections. Is the ReMscL channel functional in the differentiated and de-differentiated stages of *Rhizobium*? Is the expression of the protein somehow regulated through the developmental program of the symbiont? Our work presented here is a starting point to the answer these important questions.

Update

Recent work by Liu Z, Gandhi CS and Rees DC (Nature 2009;461:120–124) shows a structural study of an MscL homolog from *Staphylococcus aureus* (SaMscL, which displays 40% homology with *Mycobacterium tuberculosis*, MtMscL or TbMscL and 51% with EcoMscL) and demonstrates a tetrameric oligomeric structure for this channel. These two channels share similar conductance properties and differ only in open dwell times.

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