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ARTICLE

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Visualisation of the mechanosensitive channel of large conductance in bacteria using confocal microscopy

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Abstract The mechanosensitive channel of large conductance (MscL) plays an important role in the survival of bacterial cells to hypo-osmotic shock. This channel has been extensively studied and its sequence, structure and electrophysiological characteristics are well known. Here we present a method to visualise MscL in living bacteria using confocal microscopy. By creating a gene fusion between *mscl* and the gene encoding the green fluorescent protein (GFP) we were able to express the fusion protein MscL-GFP in bacteria. We show that MscL-GFP is present in the cytoplasmic membrane and forms functional channels. These channels have the same characteristics as wild-type MscL, except that they require more pressure to open. This method could prove an interesting, non-invasive, tool to study the localisation and the regulation of expression of MscL in bacteria.

Keywords Mechanosensitive channel of large conductance · Green fluorescent protein · Confocal microscopy · Patch-clamp

Abbreviations D/R: Dehydration/rehydration · GFP: Green fluorescent protein · GST: Glutathione S-transferase · HEPES: *N*-(2-Hydroxyethyl)piperazine-*N*′-ethanesulfonic acid · IPTG: Isopropyl-β-D-thiogalactoside · LB: Luria-Bertani · MscL: Mechanosensitive channel of large conductance · MscS: Mechanosensitive channel of small conductance · OD: Optical density · PCR: Polymerase chain reaction

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Introduction

The mechanosensitive channel of large conductance (MscL) serves as an osmosensor of hypo-osmotic shock in bacterial cells. MscL has been associated with a strong driving force for water influx upon hypo-osmotic shock into the cells, causing a transient increase in cellular turgor and cell swelling. By opening, these channels protect the integrity of the bacterial cell membrane (Levina et al. 1999). MscL has been extensively studied and its structural and functional characteristics are well established (Martinac 2004). Its 3D structure was determined by X-ray crystallography and shows that the channel is a homopentamer (Chang et al. 1998). Each subunit comprises two transmembrane segments TM1 and TM2 connected by a periplasmic loop. The channel pore is lined by five TM1 helices. MscL is located in the inner cytoplasmic membrane in bacteria and it has been estimated that up to 50 channels are expressed per bacterial cell (Häse et al. 1997). Here we present a method to visualise MscL in *Escherishia coli* cells, using confocal microscopy. We used the green fluorescent protein (GFP) from the bioluminescent jellyfish *Aequorea victoria* to label the protein in order to visualise the channel in vivo in bacteria. The GFP has been extensively used as a reporter gene or in a fusion with an endogenous gene in order to study its expression and localisation. It has also been used in bacteria to monitor the correct folding of overexpressed proteins and in particular of membrane proteins, where it was shown that GFP did not interfere with the correct folding and membrane localisation of the membrane proteins tested (Drew et al. 2001; Waldo et al. 1999). We used the GFP to label MscL and studied the expression of the fusion protein in bacteria with a knockout background for

MscL, its localisation, and the electrophysiological characteristics of the channels formed.

Here we show that MscL-GFP is expressed in bacteria and appears to be located in the cell membrane. Moreover, it forms functional channels with the same electrophysiological characteristics as the wild-type MscL channel (MscL-WT), except that MscL-GFP require more pressure to open.

We believe that this method can provide an interesting tool to study MscL regulation in response to different growth conditions or osmotic stress in bacteria.

Materials and methods

Bacterial strains

E. coli Xl1blue was used for plasmid propagation and manipulation. AW737KO (Sukharev et al. 1994), transformed with pREP4 (Qiagen, Crawley, UK), was used for expression and protein purification.

Growth conditions

All strains were grown in Luria-Bertani (LB) medium at 37°C with addition of appropriate antibiotics (ampicillin 100 μg/ml, kanamycin 25 μg/ml, chloramphenicol 25 μg/ml). Prior to manipulation, overnight cultures were diluted 1/20 in fresh medium and incubated at 37°C until the desired optical density (OD) at 600 nm was reached and the exponential growth curve was re-established. Protein expression was induced by the addition of isopropyl-*β*-D-thiogalactoside (IPTG) at a final concentration of 0.5 mM.

Plasmids

Four different constructs of MscL-GFP were generated in order to express the fusion protein MscL-GFP: pQE70msclgfp.1 contained the coding sequence for *mscl* followed by that of *gfp* then six histidines, all three sequences in phase and each separated by one arginine and one serine.

In order to improve the purification of MscL-GFP by affinity, two variants of this plasmid were constructed, where some amino acids or linkers were added between the sequence coding *gfp* and the six histidines: the plasmid pQE70msclgfp.2 is the same as pQE70msclgfp.1 except that two linkers, SRSGS and SSSGRS, were added between *mscl* and *gfp* and the six-histidine tag, respectively. For plasmid pQE70msclgfp.3 the linkers were SACRSTLEDPRVPVEK and KCIPTERRRS.

Finally, a fourth plasmid was constructed where the *mscl-gfp* gene fusion was added at the C-terminus of the glutathione S-transferase (GST) in order to try an alternative way of purification. All four plasmids were constructed as detailed in the following:

1. The coding sequence for GFP was amplified by polymerase chain reaction (PCR) from pGFPuv (BD biosciences, Clonetech, USA) using the primers CATAAGATCTATGAGTAAAGGAGAAGAACTTTTCACTG and GATGAGATCTTTGTAGAGCTCATCCATGCC. The PCR product was digested with *Bgl*II, ligated into pQE70mscl digested by *Bgl*II, and the resulting plasmid transformed into *E. coli* Xl1blue. A positive clone contained the GFP sequence in the right orientation; this plasmid was sequenced to verify that the insertion had occurred properly and that MscL, GFP and the six-histidine tag sequences were all in phase. The resulting plasmid was named pQE70msclgfp.1 and was transformed into AW737KOpREP4. This construct contains MscL, GFP and six histidines in phase, each separated by one arginine and one serine.
2. The plasmid pQE70msclgfp.2 was constructed as for pQE70msclgfp.1 but using the primers CATAAGATCT GGTA GCATGA GTAAAGGA GAAGAACTTTTCACTG and GATGAGATCTA CCAGA GGAGGATTTGTAGA GCTCATCCATGCC.
3. For plasmid pQE70msclgfp.3, the MscL coding sequence was amplified by PCR using the primers CATAAAGCTTGCATGCGCATTATTAAAGAATTTC and CGACCTGCAGGCAGAGCGGTTATTCTGCTCTTTCA. The PCR product was digested by *Hind*III and *Pst*I and introduced at the same sites into pGFPuv. The gene fusion *mscl-gfp* was then amplified by PCR using the same left primer and ATGAGATCTCCGGCGCTCAGTTGGAATGCATTTTTTGTAGAGC as a right primer, the resulting PCR product was purified, digested by *Sph*I and *Bgl*II and ligated into pQE70 digested by *Sph*I and *Bgl*II.
4. The plasmid pGexmsclgfp was constructed as for pQE70msclgfp.3 using CGCCAAGCTTGGAATTCGGATCCCTCGAGCATAGGCAGGGAGAATAC and CGACCTGCAGGCAGAGCGGTTATTCTGCTCTTTCA to amplify the *mscl* sequence from pGex1.1 (Häse et al. 1995). The PCR fragment was inserted in pGFPuv at sites *Hind*III and *Pst*I. The same left primer and CGTAGCGGCCGCTTATTTGTAGAGCTCATCCATGCC as the right primer were used to amplify *mscl-gfp* and the resulting product was ligated at sites *Eco*RI and *Nco*I of pGEX4T-1.

Spheroplasts preparation, protein purification and electrophysiology

Giant spheroplasts were prepared as described previously (Martinac et al. 1987) except that 1 mM IPTG was added for 2 h at 37°C after addition of cephalexin. Briefly, an overnight culture of AW737KOpREP4pmsclgfp.1 was diluted 1/20 in pre-

warmed LB medium containing the appropriate antibiotics and grown at 37°C until the OD at 600 nm reached 0.4–0.5. The cells were then diluted 1/10 in pre-warmed LB medium containing the appropriate antibiotics and cephalexin. The cells were grown for an additional 2–3 h at 37°C until strings of bacteria, called snakes, of the appropriate length formed as observed under a microscope. Then 1 mM IPTG was added to induce the expression of MscL-GFP and the cells were grown for an additional 2 h. Spheroplasts were then obtained as described previously (Martinac et al. 1987) and used for confocal microscopy and for patch-clamp recording. Patch-clamp recordings were performed in cell-attached or excised patches at room temperature (25°C) in symmetric solution containing 200 mM KCl, 40 mM $MgCl_2$, 5 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES), pH 7.2, adjusted with KOH. A gigaohm seal was formed by gentle suction applied to the pipette. Negative pressure was applied using a syringe and converted to voltage by a piezoelectric transducer (Micro Switch; Omega Engineering, Stamford, CT, USA). Pipettes were pulled using a P87 puller (Sutter Instruments Company, USA). For recordings we used an Axopatch 1D amplifier, and the pClamp 9 software package (all from Axon Instruments, Union City, CA, USA) for data collection and analysis.

For the purification of MscL-GFP, an overnight culture of AW737KOpREP4pmsclgfp.1 was diluted 20 times in 2 l of pre-warmed LB medium with the appropriate antibiotics added. The culture was grown until an OD at 600 nm of 0.6 was reached, then expression was induced by the addition of IPTG to a final concentration of 0.5 mM. The culture was grown for an additional 4 h, then cells were harvested. Protein purification was carried out as described previously (Sukharev et al. 1999). The different fractions from the affinity column (flow-through, wash and elution) were checked with 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and stained with Coomassie. The fusion protein was found in the flow-through fraction, which was used for reconstitution into liposomes. MscL-WT was prepared as described previously (Häse et al. 1995). Liposomes were prepared using a method similar to that described previously (Delcour et al. 1989; Sukharev et al. 1993). Briefly, after rinsing a glass tube with chloroform, phosphatidylcholine (20–30 mg, L-phosphatidylcholine type IV-S, Sigma, Australia) was dissolved in chloroform that was then evaporated by nitrogen for 15 min. The thin layer of lipid on the bottom and on the wall of the glass tube was then resuspended in dehydration/rehydration (D/R) buffer (200 mM KCl, 5 mM HEPES, pH 7.2 with KOH) to achieve a final concentration of 10 mg/ml. The lipid solution was then sonicated for 5 min. MscL or MscL-GFP were added to the lipid in a centrifuge tube and placed on a platform rocker for 1 h at room temperature (21, 22°C). Detergent (Triton X) was removed by incubating the protein–lipid solution with about 10 mg Bio-Beads (Bio-Rad, Australia) for another 3 h. Then, the Bio-Beads were separated by gravity and the suspension of protein–lipid was collected by spinning for 30 min at 90,000 rpm (TL-100, Beckman) at 4°C. The pellet was resuspended in 40 µl of D/R buffer, and 20 µl aliquots were then spotted onto a 100% ethanol-cleaned slide and dehydrated for 6 h in a vacuum dessicator at 4°C. The spots were then rehydrated with 10–20 µl D/R buffer and left overnight (around 12 h) for use. For patch-clamp recording, 3.5–4.0 µl of the liposomes was placed in the recording chamber (about 0.7 ml volume) containing the recording solution (200 mM KCl, 40 mM $MgCl_2$, 5 mM HEPES, pH 7.2 with KOH). Within 10–20 min, clear liposome blisters were observed and a tight seal between the blister and the glass pipette could then be formed. Single-channel currents were recorded using a borosilicate glass pipette, which had a resistance of 4 MΩ with recording solution in the pipette. A tight seal formed automatically between the pipette tip and the liposome or formed after a brief application of suction. After seal formation the pipette tip was briefly passed through the solution–air interface to rupture the outer face of the closed liposome vesicle. The patch current signal was amplified and filtered (1 kHz) with an Axopatch 1D amplifier (Axon Instruments) and digitised at 5 kHz with a Digidata 1200B using pClamp 8 acquisition software. The pressure was given by applying suction to the pipette, which was monitored with a piezoelectric pressure transducer (Omega Engineering).

Confocal microscopy

For confocal microscopy, bacteria were spun onto a microscope slide (Shandon Cytospin 4, 13,000 rpm, 5 min), covered with a drop of LB medium, then with a cover slip. The spheroplasts were put on a cover slip and observed unsealed under the confocal microscope. The samples were observed with a Bio-Rad MRC 1000/1024 UV laser scanning confocal microscope, using the 488 nm line from an argon ion laser and a 522/535 nm band pass emission filter. A Nikon ×60, 1.2 numerical aperture water immersion objective was used for all imaging.

Results

The fusion protein MscL-GFP is expressed in bacteria

In order to visualise MscL using confocal microscopy, a gene fusion between *mscl* and *gfp* was made as described in "Materials and methods" and carried on a bacterial expression plasmid. This plasmid was introduced in a bacterial strain with a knockout background for MscL called AW737KO. The expression of *mscl-gfp* is under the control of the phage T5 promoter, which is a strong promoter, regulated by two *lac* operator sequences, which increase *lac* repressor binding and ensure efficient

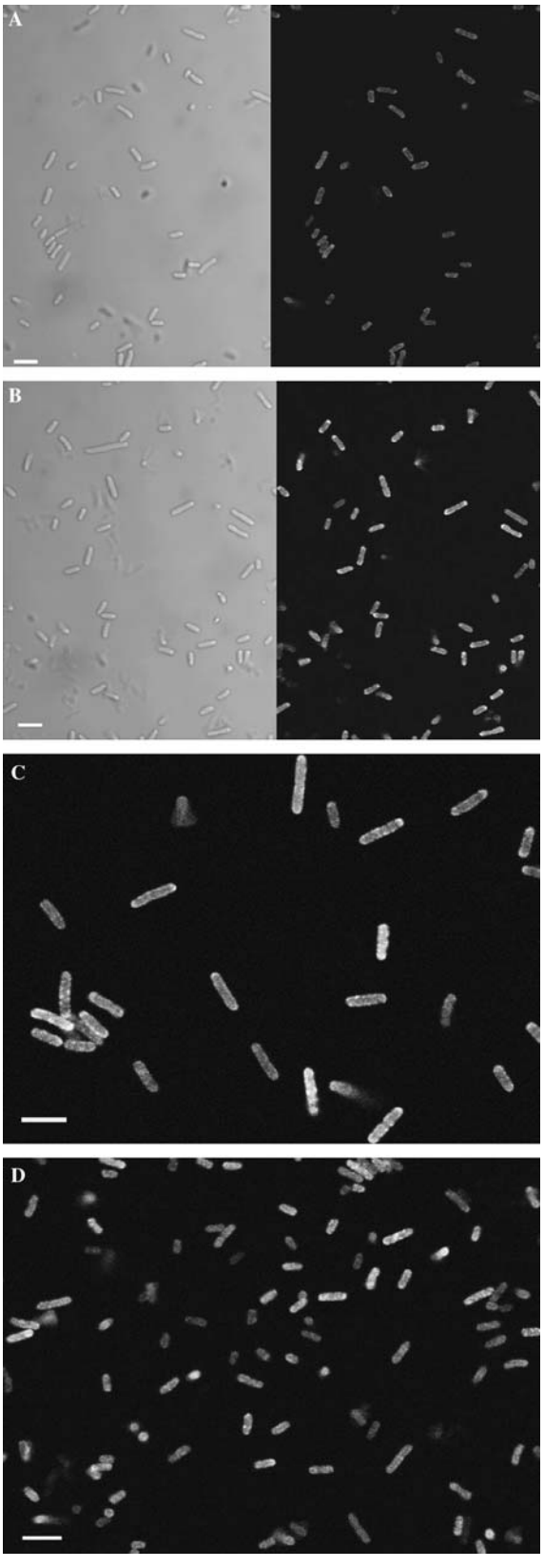

Fig. 1 Detection of fluorescence from MscL-GFP in bacteria. Transmission and confocal images of bacteria AW737KO-pREP4pmsclgfp. The fluorescence from the fusion protein MscL-GFP 1 h (**a**) or 2 h (**b**) after induction of expression of MscL-GFP by 0.5 mM isopropyl-*β*-D-thiogalactoside (*IPTG*). All the bacteria are fluorescent as observed by comparing the transmission images with the confocal images. The confocal images of the bacteria 2 h after induction of expression of MscL-GFP by 0.5 mM IPTG in culture in early exponential growth phase (optical density at 600 nm, 0.145) (**c**) and late exponential growth phase (optical density at 600 nm, 0.754) (**d**) show that there is no difference in the fluorescence intensity or distribution in bacteria where expression is induced in early or late exponential phase. *Scale bars* 5 μm

control of the powerful T5 promoter. The plasmid pREP4 (Qiagen), which allows the production of high levels of the *lac* repressor protein, was also introduced in these bacteria together with the plasmid carrying *mscl-gfp* to allow the tight control of the expression. Under these conditions, the expression of *mscl-gfp* is solely controlled by the addition of IPTG to the culture, and the availability of nutrients and energy to the cells.

The fluorescence from the MscL-GFP fusion protein can be detected in these bacteria after induction of the expression by IPTG. One hour after induction, the fluorescence is weak (Fig. 1a) but all the bacteria are fluorescent. Two hours after induction of expression, the fluorescence from MscL-GFP is much stronger and also present in all the cells (Fig. 1b). Under the same settings, no fluorescence is detected from the bacteria expressing MscL only (data not shown). If the expression of MscL-GFP is induced for 2 h in bacteria during the early log phase (Fig. 1c) and the late log phase (Fig. 1d) as measured by the culture's OD at 600 nm of 0.145 and 0.754, respectively, there is no noticeable difference in the fluorescence detected or the distribution of the fluorescence. In the early log phase (Fig. 1c), most of the bacteria are dividing, which is consistent with bacteria in active growth, and all of them are fluorescent, some brighter than others. In most bacteria, we observe a bright outline of the bacteria with a darker line inside, which could indicate that MscL-GFP is actually located in the bacterial membrane. In the late log phase (Fig. 1d), all the cells are fluorescent to a level similar as that of Fig. 1c, with some brighter than others, and the fluorescence also appears to be brighter in the membrane area. Only in bacteria where expression is induced in the stationary phase does the fluorescence appear weaker (data not shown), probably owing to the fact that nutrients are limited in the stationary phase and the energy levels are lower so the levels of expression of MscL-GFP might be reduced compared with those of bacteria in exponential growth.

The fusion protein MscL-GFP is localised in the membrane and forms functional channels

We made giant spheroplasts from the bacteria with a knockout background for MscL and expressing MscL-GFP in order to examine the localisation of the fluorescence and detect channel activity by patch-clamp. Fluorescence from these spheroplasts can be detected and seems to be mostly concentrated in the membrane

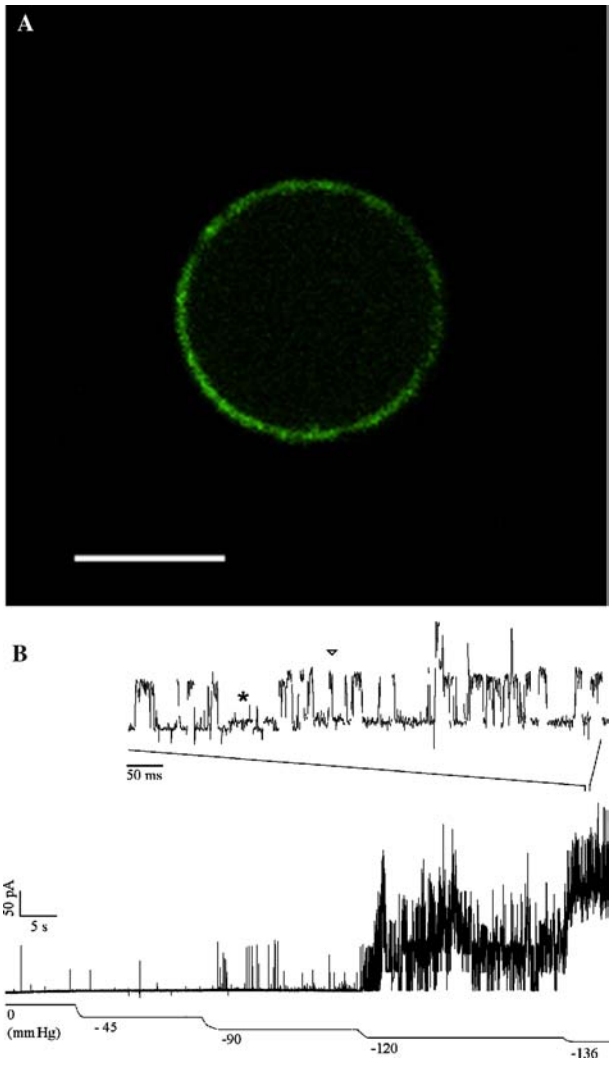

Fig. 2 Detection of fluorescence and channel activity from MscL-GFP in giant spheroplasts. **a** Confocal image of giant spheroplasts obtained from *Escherishia coli* strain AW737KOpREP4pmsclgfp.1 induced by 1 mM IPTG for 2 h showing that the fluorescence from MscL-GFP is mostly detected in the membrane area, suggesting that MscL-GFP is located in the cytoplasmic membrane. *Scale bar* 5 μm. **b** Patch-clamp recording of MscL-GFP and MscS channels activity in an excised patch of a giant spheroplast obtained from AW737KOpREP4pmsclgfp.1. MscL-GFP has a threshold of activation of approximately −90 mmHg and a conductance of 3.3 nS. About ten MscL-GFP were detected in this patch (not shown). The *inset* shows the MscL-GFP (*triangle*) and MscS (*star*) channel activity at a different time scale under a pressure of −136 mmHg. The pipette voltage is +30 mV. The pressure steps applied to the patch are shown at the *bottom*.

(Fig. 2a). The outline of the giant spheroplast, which corresponds to the cytoplasmic membrane area of the bacteria, is very bright, whereas the centre of the spheroplast is dark. This suggests that MscL-GFP is located mostly in the membrane. This is confirmed by the detection of MscL activity by patch-clamp in these giant spheroplasts. As shown in Fig. 2b, channels showing similar characteristics of activation by membrane tension and the same conductance as MscL are detected in membrane patches from these spheroplasts. Since the bacteria used have a knockout background for MscL, this activity is due solely to the MscL-GFP channels, thus showing that the MscL-GFP channels have the same electrophysiological characteristics as the MscL channel. It was shown earlier (Blount et al. 1996) that MscL-WT needs 1.4 times higher negative pressure than the mechanosensitive channel of small conductance (MscS) to open. Later observations (Martinac and Kloda 2003, Martinac unpublished data), showed that MscL and MscS in spheroplasts have an activation threshold near −80 and −40 mmHg, respectively (i.e. 2 times higher for MscL), thus suggesting that the activation threshold of MscL-GFP observed in our experiments (between −90 and −130 mmHg) is slightly higher than that of MscL-WT in spheroplasts. MscS activity is also observed (Fig. 2b) but the presence of these channels in the patches examined is much less than that of MscL-GFP because MscL-GFP is overexpressed in these bacteria.

The channels formed by MscL-GFP have the same characteristics as MscL but require more pressure to open

In order to study further the characteristics of the channels formed by MscL-GFP, we attempted to purify the fusion protein Mscl-GFP by affinity. The fusion protein contains a histidine tag in the C-terminus of GFP, which allows purification by affinity on a nickel-nitrilotriacetic acid column. However, the protein did not bind to the affinity column and was found in the flow-through (Fig. 3), and represented the majority of proteins present in this fraction. Two similar fusion proteins, MscL-GFP.2 and MscL-GFP.3 were constructed as described in "Materials and methods" and were passed through a column using the same method as for MscL-GFP, but neither of them could be purified by affinity (data not shown). A fourth fusion protein was created using the GST in the N-terminus of MscL-GFP, but this protein was present in inclusion bodies when expressed in the bacteria, thus forming a non-functional protein (data not shown). We thus used the initial fusion protein MscL-GFP and isolated the flow-through from the column, reconstituted it in liposomes and examined patches for MscL activity. Our patch-clamp results show that MscL-GFP reconstituted in liposomes forms functional channels with activity similar to MscL-WT (Fig. 4). However, the threshold pressure required to open the MscL-GFP channel is significantly higher compared with that of the MscL-WT channel. MscL-GFP requires 1.16 times more pressure than MscL-WT to open (with a probability $p < 0.01$, as calculated by the Student t test, Table 1). In addition, the free energy needed to open the MscL-GFP channel also significantly ($p < 0.001$) increases by about 1.6 times compared with that of the MscL-WT channel (Table 1).

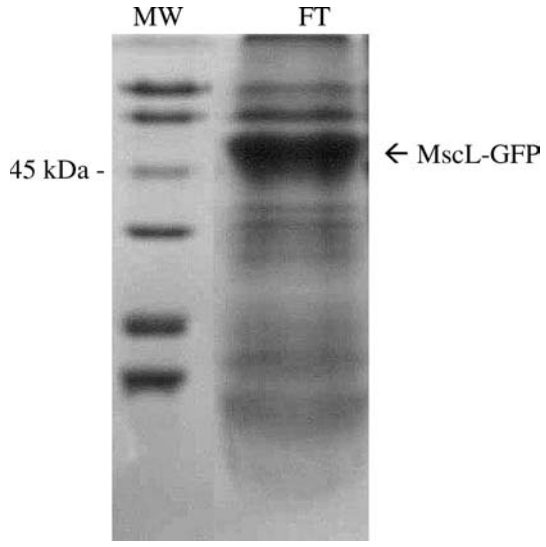

Fig. 3 MscL-GFP does not bind to the affinity column and is found in the flow-through. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis, stained by Coomassie showing the molecular weight marker (*MW*, low range, Bio-Rad, Australia) and the flow-through (*FT*) from the nickel affinity column after an extract of membrane proteins from bacteria overexpressing MscL-GFP from the plasmid pQE70msclgfp.1 had been added. The fusion protein MscL-GFP (approximately 45 kDa) containing the histidine tag does not bind to the nickel column and is found in this fraction, where it represents the majority of proteins.

Discussion

The results show that the addition of GFP at the C-terminus of MscL gives a fusion protein MscL-GFP, which is expressed in bacteria. This protein is present in the cytoplasmic membrane and forms functional ion channels with similar characteristics as MscL-WT except that they require slightly more pressure to open.

We cannot explain why the fusion protein could not be purified by affinity. A similar construct of a membrane protein with the GFP having a six-histidine tag at the C-terminus of the GFP was successfully purified by affinity using a similar method (Drew et al. 2001). We tried to modify the fusion protein by adding extra amino acids at the C-terminus of GFP before the histidine tag as described in "Materials and methods", in the belief that the six histidines were not exposed enough to bind to the column, but neither of the two constructs, MscL-GFP.2 and MscL-GFP.3, bound to the column. A fourth construct was attempted where the GST was added at the N-terminus of MscL-GFP, as described in the "Materials and methods", but the resulting fusion protein was found in the inclusion bodies. An initial construct where GFP was added at the N-terminus of MscL was also found in the inclusion bodies (data not shown). However, in our hands, these two constructs still emitted fluorescence even when present in the inclusion bodies. Drew et al. (2001) reported that when their GFP fusions were in the inclusion bodies they lost the ability to fluoresce (Drew et al. 2001). It might be due to the variant of the GFP that

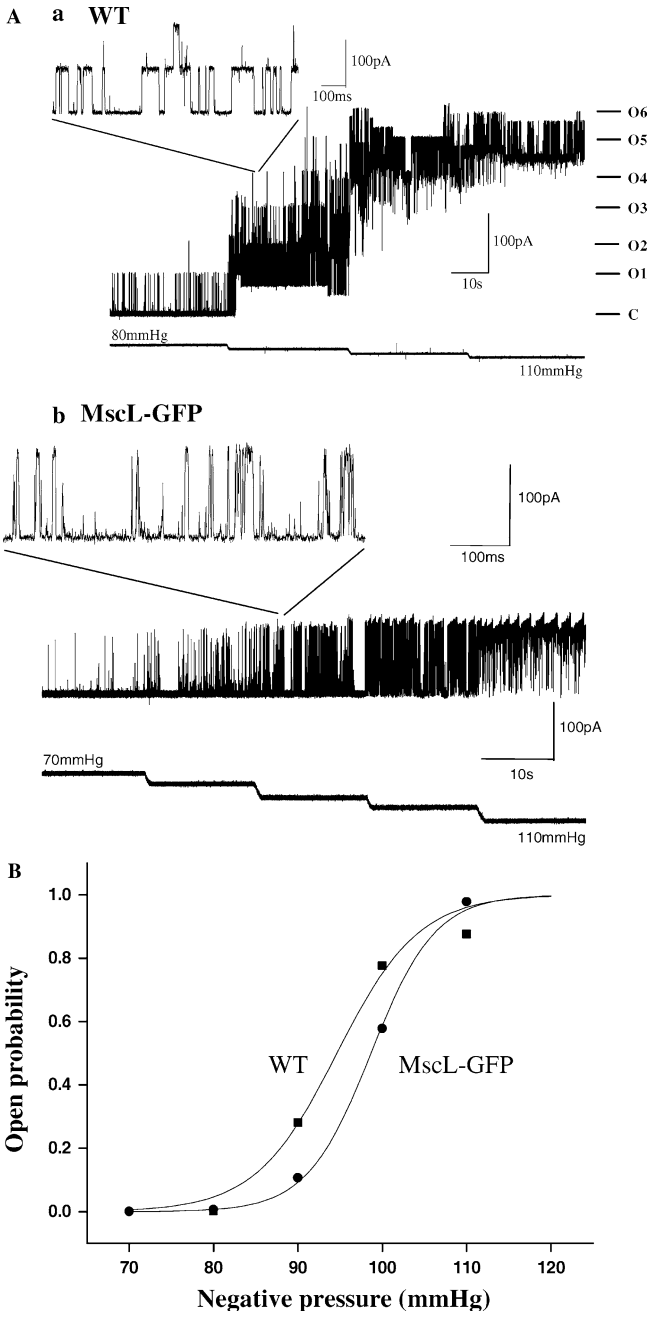

Fig. 4 Activation of wild-type (*WT*) MscL and MscL-GFP reconstituted into liposomes. **a** Current traces from liposome patches showing the channel activity of MscL-WT (*top*) and MscL-GFP (*bottom*) by negative pressure. The pipette holding potential is + 30 mV. *C* denotes the closed state of the channel, and O_n denotes the open state of *n* channels. The *lower trace* in each panel is the pressure trace with each pressure step being 10 mmHg. The *insets* show the single-channel activity on an expanded scale. **b** Boltzmann distribution curves of MscL-WT and MscL-GFP plotted for the channels shown in *a*. The Boltzmann distribution has the form $P_o/(1-P_o) = \exp[\alpha(p_{1/2})]$, where P_o is the channel open probability, p is the negative pressure, $p_{1/2}$ is the pressure at which the channel is open 50% of the time ($P_o = 0.5$) and α is the slope of the plot $\ln[P_o/(1-P_o)]$. ΔG_o is the difference in free energy between the closed and open conformations of the channel. In this sample, for MscL-WT, $p_{1/2}$ is 94.63 mmHg, $1/\alpha$ is 4.84 mmHg, and ΔG_o is19.55 kT, and for MscL-GFP, $p_{1/2}$ is 98.66 mmHg, $1/\alpha$ is 3.79 mmHg, and ΔG_o is 26.03 kT.

Table 1 Mechanosensitivity of the wild-type mechanosensitive channel of large conductance (MscL-WT) and the mechanosensitive channel of large conductance labelled with the green fluorescent protein (MscL-GFP) reconstituted in liposomes

Protein	Activation threshold (mmHg)	g (nS)	$p_{1/2}$ (mmHg)	$1/\alpha$ (mmHg)	ΔG_o (kT)
MscL-WT	73.24 ± 22.86 ($n=37$)	3.18 ± 0.31 ($n=37$)	-98.26 ± 7.75 ($n=9$)	5.14 ± 1.83 ($n=9$)	21.08 ± 6.78 ($n=9$)
MscL-GFP	85.45 ± 23.39* ($n=10$)	3.70 ± 0.17 ($n=10$)	-97.18 ± 17.64 ($n=3$)	2.94 ± 0.92** ($n=3$)	34.40 ± 7.39** ($n=3$)

The data are the mean $\pm$ the standard deviation. The activation threshold is the minimum negative pressure at which the channel opens, g is the conductance of a single MscL channel, $p_{1/2}$ is the negative pressure at which the channel is open 50% of the time, α is the slope of the plot $\ln [P_o/(1-P_o)]$, where P_o is the channel open probability, $1/\alpha$ is the sensitivity to negative pressure, and ΔG_o is the difference in free energy between the closed and open conformations of the channel.
*$p < 0.01$, **$p < 0.001$, compared with MscL-WT by the Student t test

we used, or to the greater sensitivity of the laser and confocal microscope we used compared with that of the a fluorescence spectrophotometer used by these authors.

The fact that MscL-GFP forms functional channels in spheroplasts, with similar characteristics as MscL-WT, is promising and indicates that the GFP does not interfere significantly with the function of the channel and that the fusion protein is properly inserted in the cytoplasmic membrane of the bacteria. MscL-GFP could be an interesting tool to study the regulation of expression of MscL in bacteria, during growth or in response to osmotic stress. Recently it has been shown that MscL expression, as well as that of the MscS, is up-regulated in bacteria upon entry into the stationary phase as well as in bacteria grown in high osmotic media (Stokes et al. 2003). This up-regulation could protect the bacteria against hypo-osmotic shock and is under the regulation of the stress sigma factor RpoS. In this study, *mscl-gfp* was carried on a plasmid DNA, and the expression of this fusion gene was controlled solely by the addition of IPTG. However, if the gene fusion *mscl-gfp* was introduced in the bacterial genome in place of the WT *mscl* gene then, we can expect that it would be regulated in the same way as *mscl* is in the bacteria during the growth of the cells or under different stress. The functional Mscl-GFP channel, replacing the endogenous MscL-WT channel could be used to follow the up-regulation of MscL upon entry in the stationary phase by confocal microscopy and perhaps even investigate if the channels are distributed homogenously around the cells or if there are differences in their ocalisation with different growth conditions of the bacteria or under different types of stress.

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