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Characterization of three novel mechanosensitive channel activities in *Escherichia coli*

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Abbreviations: aa, amino acids; MscK, potassium-dependent mechanosensitive channel; MscL, mechanosensitive channel of large conductance; MscM, mechanosensitive channel of mini conductance; MscS, mechanosensitive channel of small conductance; TM, transmembrane span; RBS, ribosome binding site

Mechanosensitive channels sense elevated membrane tension that arises from rapid water influx occurring when cells move from high to low osmolarity environments (hypoosmotic shock). These non-specific channels in the cytoplasmic membrane release osmotically-active solutes and ions. The two major mechanosensitive channels in *Escherichia coli* are MscL and MscS. Deletion of both proteins severely compromises survival of hypoosmotic shock. However, like many bacteria, *E. coli* cells possess other MscS-type genes (*kefA*, *ybdG*, *ybiO*, *yjeP* and *ynaI*). Two homologs, MscK (*kefA*) and YbdG, have been characterized as mechanosensitive channels that play minor roles in maintaining cell integrity. Additional channel openings are occasionally observed in patches derived from mutants lacking MscS, MscK and MscL. Due to their rare occurrence, little is known about these extra pressure-induced currents or their genetic origins. Here we complete the identification of the remaining *E. coli* mechanosensitive channels YnaI, YbiO and YjeP. The latter is the major component of the previously described MscM activity (~300 pS), while YnaI (~100 pS) and YbiO (~1000 pS) were previously unknown. Expression of native YbiO is NaCl-specific and RpoS-dependent. A Δ7 (MJF64I) strain was created with all seven *E. coli* mechanosensitive channel genes deleted. High level expression of YnaI, YbiO or YjeP proteins from a multicopy plasmid in the Δ7 strain leads to substantial protection against hypoosmotic shock. Purified homologs exhibit high molecular masses that are consistent with heptameric assemblies. This work reveals novel mechanosensitive channels and discusses the regulation of their expression in the context of possible additional functions.

Introduction

Maintaining intracellular homeostasis is imperative for all cells for their survival and growth. Bacteria thrive in diverse environments and, as such, have in place a multitude of systems to respond to changes in their surroundings.[1-5] They are able to adapt to fluctuations in pH, osmolarity, and in some cases temperature and also combat many toxic compounds.[6-8] Central to the maintenance of cytoplasmic ionic composition are the actions of specific ion channels and transporters. In bacteria, these enable the cell to maintain high intracellular levels of potassium (~300–600 mM), a physiological pH and an outward turgor pressure. However, during situations of extreme stress, bacteria will forfeit favorable homeostasis to implement rapidly-acting survival strategies.[9,10]

Bacterial mechanosensitive channels were discovered by the application of patch-clamp technology to giant spheroplasts.[11,12] They gate when cells experience a sudden transfer from a high osmolarity medium to one of low osmolarity (hypoosmotic shock) and an increase in membrane tension occurs as water rushes into the cell.[9,13] To avoid cell lysis, these proteins open large-diameter pores in the membrane[14-16] that indiscriminately release osmotically-active ions and solutes from the cytoplasm.[9] Channel openings are short-lived (< 150 ms for MscS)[17] and they close immediately when the increased-tension stress is alleviated.

Initial electrophysiological analysis of bacterial membrane proteins revealed multiple channel activities in response to pressure[12] and in 1994, Kung's group purified a major mechanosensitive protein from *E. coli*, the mechanosensitive channel of large conductance (MscL).[18] Our group has since identified three further pressure-activated channels: MscS, MscK and YbdG.[13,19,20] These belong to a second major mechanosensitive channel family named MscS. Searches of protein databases show members of the MscL and MscS families are present in many Archaea and some plants and fungi, as well as most bacteria.[21] *E. coli* possess only one MscL type protein but six MscS homologs: MscS (286

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Table 1. Frequency of different channel amplitudes in the presence and absence of 0.5M NaCl treatment

	MJF429		MJF611		MJF612	
	Std	+ NaCl	Std	+ NaCl	Std	+ NaCl
< 7 pA	4	5	4	2	2	4
7.5/9–13 pA	3	1	1	1	1	3
~20 pA	-	8	-	6	-	6
n patches	19	14	19	15	22	15

"-", no channel of this amplitude category measured. Electrophysiological recordings of three strains, MJF429, MJF611 and MJF612, harbouring different complements of known mechanosensitive channel proteins were used to prepare protoplasts in the presence and absence of 0.5M NaCl treatment prior to the plasting reaction. Appearance of the different channel amplitudes were then recorded in patches of these protoplasts.

aa), MscK (1120 aa) and YbdG (415 aa), as well as YnaI (343 aa), YbiO (741 aa) and YjeP (1107 aa).[13] Alignment of protein sequences and hydrophobicity plots highlights a core structural unit for each of the six homologs at the C-terminal end.[22] This unit consists of three transmembrane (TM) helices followed by an extra-membranous cytoplasmic domain and equates to the full length of the smallest homolog protein, MscS.

The MscS channel itself has been extensively studied. X-ray crystallography has produced structures of a closed state and an open state and combined with substantial electrophysiological, functional and biochemical data, MscS protein conformations and gating mechanisms have been proposed.[22-25] Our work on the MscK and YbdG proteins has determined that although structural similarities to MscS exist, the gating mechanisms of the three channels are quite different.[19,20] This has raised interest in the remaining genes for MscS homologs: do they encode functional channels and how do their characteristics compare with the other members of the family?

Previous studies reported the presence of multiple mechanosensitive channel activities when *E. coli* membranes were fused with liposomes, but genetic studies were not possible at that time to refine the relationship between activities and gene products.[26] In giant *E. coli* protoplasts derived from cephalexin-treated filamentous bacteria, three groups of mechanosensitive channel activities have been labeled to date: MscL, MscS and MscM.[26] MscL openings were shown to be the product of a single gene,[18] whereas the originally identified MscS activity was found to be the product of both the MscS and MscK channels.[13,18,19] Pinpointing the source of the more elusive MscM-labeled conductance was more difficult. We recently identified YbdG as possessing MscM-like activity, although deletions that removed the *ybdG* gene did not eliminate all MscM-sized openings.[20] Moreover the YbdG channel was not found to gate in patch clamp experiments unless a mutation had been introduced in the β domain.[20] In the current study we integrated the analysis of deletion mutants with variations in the conditions for preparation of protoplasts and with the expression of cloned genes, to identify novel mechanosensitive channel activities. We demonstrate that YbiO, YnaI and YjeP are present in membrane patches at a low frequency, that YbiO is specifically induced by NaCl and that all three genes encode for distinct mechanosensitive channels. We demonstrate that the failure of these homologs to provide protection against hypoosmotic shock is due to their low levels of either expression or integration into the membrane. The new data are discussed in terms of the global observation of mechanosensitive channel homologs in bacteria and the implications for cell physiology.

Results

New mechanosensitive channel activities. The mechanosensitive channels MscS, MscK and MscL have well-characterized channel openings in patch-clamp experiments. In wild-type *E. coli* cells their characteristic current amplitudes (at 20 mV holding potential in 200 mM KCl) make them easily recognizable: MscS, 25 pA; MscK, 17.5 pA; MscL, 90 pA. YbdG channels do not readily gate under standard electrophysiological conditions. Expression of a mutant YbdG protein (V229A[20]) increased the observation of 7.5 pA openings, thus allowing categorization as a potential component of the previously defined MscM activity. However, elimination of the *ybdG* gene did not prevent the observation of MscM-type openings.

Recordings from cells deleted for *mscS* and *mscK* (strain MJF429; **Table S1**) showed infrequent mechanosensitive channel openings of various sizes, along with the expected MscL activity (**Table 1**; **Fig. 1A**). These activities could be placed, subjectively, into different categories based on amplitude and gating properties: (1) small openings of less than 7 pA that were either short-lived, multiple opening/closings or that were open throughout the applied pressure stimulus; (2) sustained openings of ~7.5 pA (comparable to the previously reported MscM activity[26]) or slightly larger sustained openings (up to ~13 pA). Strains lacking YbdG (MJF611 and MJF612) and/or MscL (MJF612; **Table S1**) still occasionally exhibited these unidentified openings (**Table 1**) suggesting that they were novel activities and not sub-states of MscL.

High-salt treatment increased the incidence of a 20 pA activity. In an attempt to increase observations of the rare, unidentified mechanosensitive activities, a NaCl-treatment step was added during protoplast preparation. The standard protocol includes incubating growing cells with cephalexin for 2.5 h to form filamentous 'snakes', due to inhibition of septum formation and cell division, followed by a lysozyme treatment (plasting; see Methods for full details). This contrasts with the growth protocol for hypoosmotic shock experiments that always include a step in which cells are grown at high osmolarity. We, therefore, extended the cephalexin incubation by a further 1 h in the presence of 0.5 M NaCl prior to carrying out the plasting reaction. Recordings were made for three strains that lacked both MscS and MscK. After incubation with NaCl in the plasting medium, step-wise increments in current of ~20 pA were observed in about half of all patches tested (n = 20/44 patches, total for three strains; **Table 1**). This newly revealed channel activity amplitude ranged from 16–24 pA and the channels remained open for the duration of pressure applications (**Fig. 1B**). This specific type of channel opening was never observed in the absence of the NaCl treatment (n = 0/60 patches for three strains; **Table 1**) and it

appeared that NaCl induced multiple units of this protein, as simultaneous openings of this type were often observed in excised patches (up to 20 channels). The smaller amplitude channels (**Table 1**) were also noted in the NaCl-treated protoplast patches but there was no consistent increase in the frequency of observations of these activities, (**Table 1**). This modified protocol was extended to other mutant strains either containing *ynaI* on the chromosome but null for *ybiO* and *yjeP* or expressing *ybiO* and *yjeP* but with *ynaI* deleted. The new NaCl-instigated channel only appeared in membranes from cells expressing YbiO and YjeP, suggesting one, or both, of these proteins as the source of this new channel activity (**Fig. 1 and Table 1**).

The role of NaCl in increasing the frequency of observation of the new 20 pA mechanosensitive channel activity was investigated by isoosmotic substitution for the NaCl during the plasting reaction. Strains MJF611 and MJF612 were assessed and all channel openings measured. While 6/15 patches from each of these strains exhibited the new channel after NaCl treatment, 0.5 M KCl did not produce this increase in occurrence (12 patches tested for each strain). Further tests showed that 0.5 M $NaNO_3$ (5 patches) or 0.9 M sorbitol (12 patches), inositol (12 patches) or glucose (6 patches) also all failed to induce extra channel activities (all sugars were applied at 0.9 M to replicate the osmolarity increase caused by addition of 0.5 M salt). Thus, the induction appears NaCl-specific (**Table S2**).

Previous work has shown that expression of MscL, MscS and YbdG mechanosensitive channels is affected by the stress response/stationary phase factor RpoS, which is induced by NaCl.[27-29] Both MscL and MscS are partially under the control of this stress-related sigma factor, while YbdG expression is impaired by the activity of RpoS.[20] Strain MJF429Δ*rpoS* was created by transduction and analyzed by patch-clamp after growth of 'snakes' in the presence of 0.5 M NaCl. Only MscL activity and openings ≤ 7 pA were detected; no channel openings of *c.* 20 pA were recorded (n = 10 patches; **Table S2**), suggesting that the action of NaCl to raise this activity requires RpoS function.

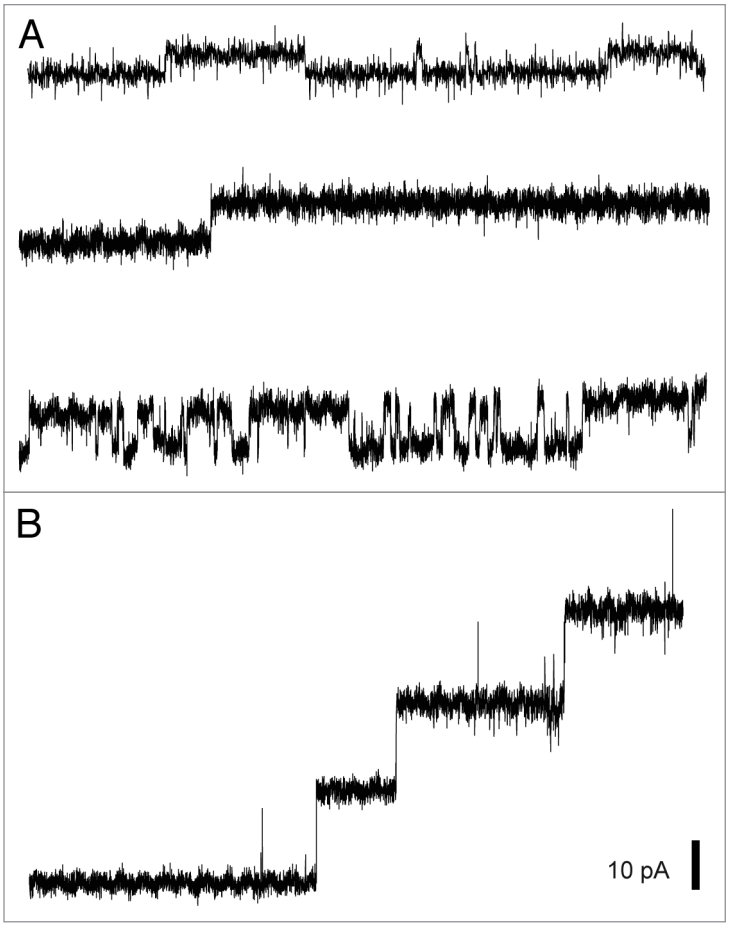

Figure 1. Channel activities in strains deleted for the identified mechanosensitive channels. (**A**) Electrophysiological recordings of pressure induced activities seen in membrane patches of protoplasts from a strain lacking MscL, MscS, MscK and YbdG. (**B**) Treatment of protoplasts with a 1 h exposure to 0.5M NaCl following the cephalexin treatment resulted in the appearance of a new channel activity.

YbiO, YjeP and YnaI are the proteins underlying the novel activities. The MscS family of channels is larger and much more variable in size and sequence than the MscL family.[21] Bioinformatic analyses identified, in addition to MscS, MscK and YbdG, three homologs in the MscS family of mechanosensitive channels: YbiO, YjeP and YnaI.[13,21] YnaI, like MscS and YbdG belongs to the smaller topological class of channels and is projected to have 5 TM spans.[22] YbiO and YjeP belong to the larger class of channels with 11 putative TM spans and extensive periplasmic domains.[21] A strain was created that lacked the *mscL* gene and all six members of the *mscS* family, designated MJF641 (Δ7 Black S, Rasmussen A, Edwards MD, Galbiati H, Miller S and Booth IR, unpublished data) (**Table S1**). Membrane patches from protoplasts of this strain, prepared using the standard protocol, were subjected to pressure gradients up to -400 mmHg (a level greater than that required to activate all mechanosensitive channel openings in *E. coli* patches). No channel-opening deviations in the current baseline were observed (n = 7; **Fig. 2A**). Furthermore, no activities were detected in patches derived from protoplasts of MJF641 prepared with the additional high-salt treatment (pressures up to -400 mmHg; n = 12). Thus, all the additional pressure-stimulated channel openings observed electrophysiologically in membrane patches, including the newly detected channel activity upregulated by high NaCl, are encoded by the known genes for mechanosensitive channel homologs.

Characterization of YbiO, YjeP and YnaI activities. Creation of an electrophysiologically-silent phenotype (MJF641) allowed assessment of the three remaining uncharacterised MscS family proteins. Clones of YbiO, YjeP and YnaI (in an IPTG-inducible plasmid; **Table S1**) were expressed in MJF641 with 60–120 min induction. Expression of pTrcYbiO gave rise to stable channel openings of ~17 pA (range: 16–19 pA; n = 12) that remained open until the pressure stimulus was released (**Fig. 2B**). Thus, YbiO protein appears to be the source of the NaCl-induced channel activity. Although, it was noted that

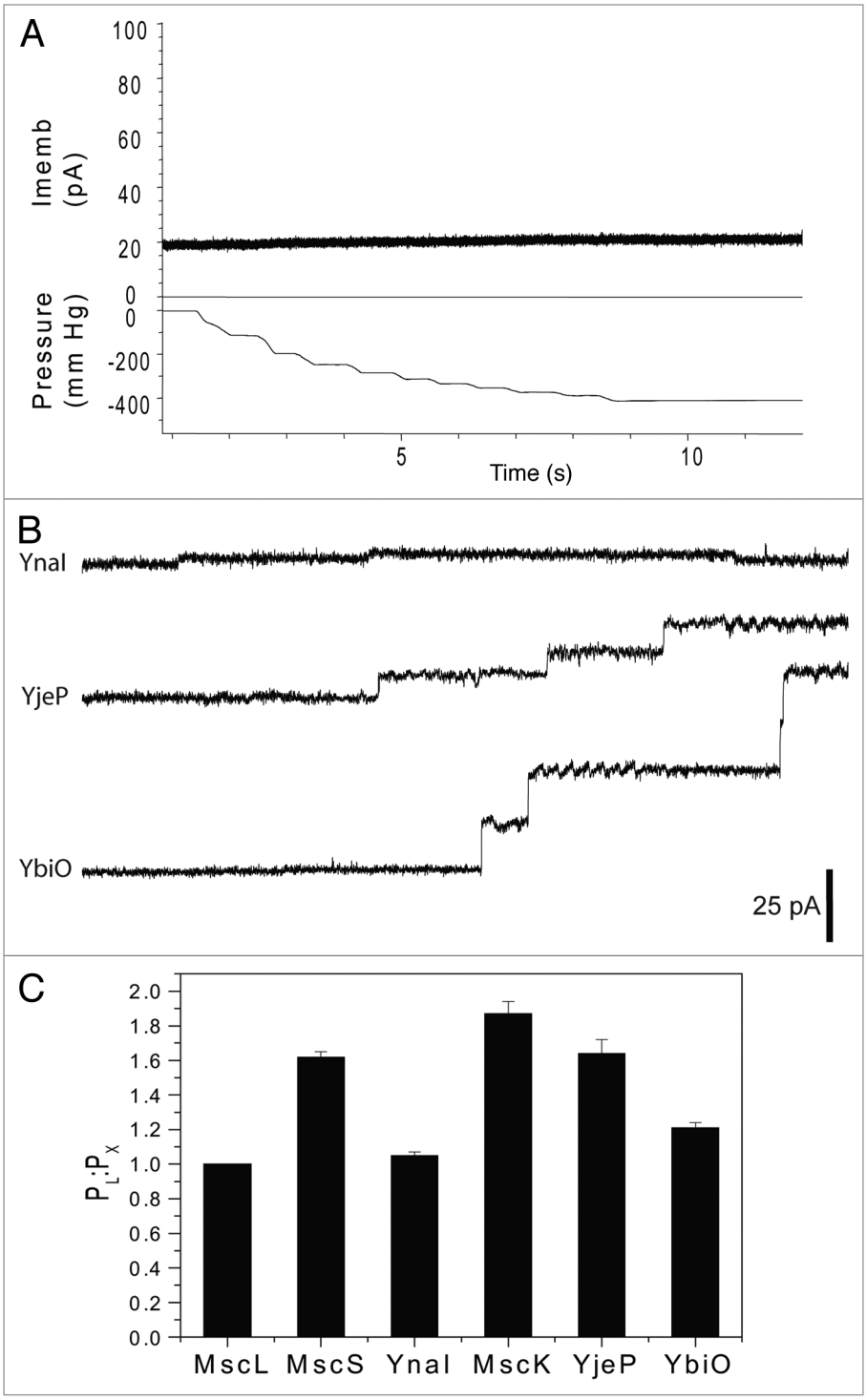

Figure 2. Electrophysiological characterization of YnaI, YbiO and YjeP. (**A**) Electrophysiological recordings were made of protoplast of MJF641 ± 0.5M NaCl. No channel activity was detected in these recordings up to -400 mmHg applied pressure, typically sufficient to activate all known channels. (**B**) Electrophysiological recordings from protoplasts of YnaI, YbiO and YjeP clones in MJF641 cells. (**C**) Expressing the clones in MJF611 allowed the pressure sensitivity of these channels to be measured relative to MscL (P_L:P_X).

there was some variability in the conductance of both the NaCl-instigated channel and the plasmid-produced YbiO channel in MJF641.

YjeP and YnaI overexpression produced lower conductance channels: YjeP was associated with multiple-sized, prolonged steps of between 5 and 8 pA (n = 9; representative trace in **Fig. 2B**), while YnaI channel openings were mostly of about 2 pA magnitude (n = 12; **Fig. 2B**). Due to the very small currents passing through YnaI, recordings were also made at a holding potential of +40 mV in some patches (as well as the standard +20 mV), to confirm that the tiny pressure-activated deviations in current baseline displayed ion channel properties (data not shown). Thus, each of the three newly characterized mechanosensitive channels displays unique single-channel conductance properties.

In membrane patches, MscL channels gate at pressures close to levels that could burst the bilayer. Lower pressures are required to gate MscS and MscK channels, which are conventionally reported as a ratio between the pressure to open each channel and the pressure to open MscL, in each test patch.[30] To compare the gating pressures for YbiO, YjeP and YnaI to that of MscS, the new channels were individually overexpressed from a plasmid in strain MJF611 (MscL$^+$). This strain does contain functional chromosomal copies of YbiO, YjeP and YnaI, but the analysis of the cloned genes was simplified by the very low frequency of observation of YbiO, YjeP and YnaI in the absence of the cloned genes. Further, the conductance of the channels had been characterized in MJF641 (Δ7) channel-less mutant strain and thus could easily be identified. YnaI and YbiO channels open at pressures near to that required to gate MscL, with pressure ratios (P_L:P_X, where x is the identity of the new homolog channel) of 1.05 ± 0.02 (n = 6) and 1.21 ± 0.03 (n = 6), respectively. YjeP demonstrated similar pressure sensitivity to MscS with a pressure ratio of 1.64 ± 0.07 (n = 5) (**Fig. 2C**).

Abundance of channel in the membrane varies among the three proteins. Microarray data displayed at the Oklahoma database (http://genexpdb.ou.edu/main/)[31] for *E. coli* transcription indicates that all of the homologs are expressed and show induction and/or repression in response to a wide range of specific growth conditions (**Table S1**). To quantify expression under the conditions used for generating spheroplasts we measured mRNA levels by qRT-PCR in cells grown in

LB, LB plus 0.5 M NaCl and in the presence or absence of functional RpoS. The reference genes chosen as standards (*rpoB* and *polA*) were found to vary in their expression under the different conditions and could not be used as controls. Thus, since we have previously studied the regulation of *mscS*[28] we used this as an internal standard, setting the qRT-PCR value to 1. Positive signals were obtained for all three homolog mRNAs with *ynaI* and *ybiO* proving to be more abundant than *mscS* and *yjeP* (**Fig. 3**) under all conditions. We have previously shown that *mscS* transcription is increased at high salt and the expression ratios for the other homologs were identical in the presence and absence of salt, indicating their induction under high NaCl. Deletion of RpoS led to a significant lowering of the *ybiO* signal compared with the other two homologs and this was counteracted by high NaCl. Thus, the data are consistent with slight induction of all the MscS homologs during growth at high osmolarity. However, only *mscS* and *ybiO* showed strong requirement for RpoS, with the change in expression being greatest for YbiO.

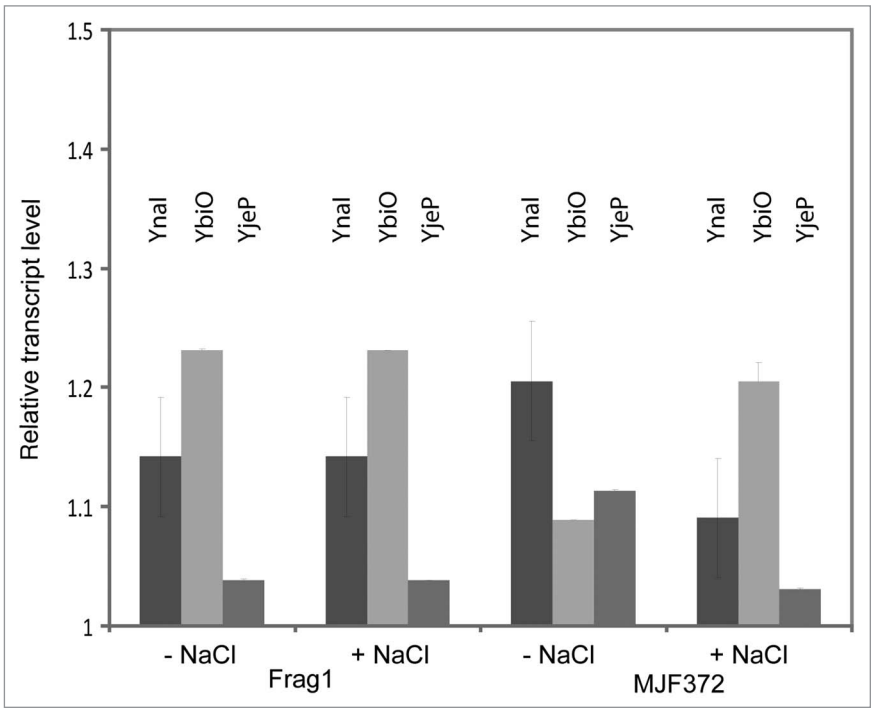

Figure 3. qRT-PCR expression of *ynaI*, *ybiO* and *yjeP*. Regulation of *ynaI*, *ybiO* and *yjeP* transcription as determined by qRT-PCR. Frag1 or MJF372 (Frag1 *rpoS::Tn10)* cells were grown in LB with and without an additional 0.5M NaCl. Total RNA fraction were isolated and transcribed into cDNA. Transcript levels were normalized to *mscS* in the corresponding growth condition.

Attempts to measure the abundance of the YnaI, YjeP and YbiO proteins expressed from their chromosomal loci, using specific antibodies,[32] proved unsuccessful – the expression level was below the level of detection. Thus, their assembly and integration into the membrane was compared with MscS using His-tagged proteins from the synthetic clones. Using our standard protocol (30 min with 0.3 mM IPTG prior to cell harvest and membrane preparation) we observed that YnaI accumulates in the membrane to levels comparable to that of MscS expressed from the same promoter/RBS combination. Both larger homologs, YbiO and YjeP, accumulate to a lesser extent than MscS and YnaI. YjeP is moderately reduced in abundance while YbiO accumulates to significantly lower levels in the membrane (**Fig. 4A**). 5-fold longer induction times were required to produce levels of expression of YbiO equivalent to that of MscS or YnaI as measured by western blot analysis (not shown) and physiological assays (see below).

The levels of protein found in the membrane were echoed in the functional assay results. When subjected to a 0.3 M hypoosmotic shock, basal levels of expression of all three homologs were insufficient to afford protection to the channel-less mutant strain, MJF641. Analysis of cell survival following a short 20 min induction of channel expression revealed that YnaI is able to provide almost complete protection against the osmotic shock with this level of increased expression. In contrast, YjeP and YbiO channels required a significantly longer induction period (2.5 h) in order to achieve full protection (**Fig. 4B**). Thus, by the physiological assay, all six of the MscS homologs have the capacity (when abundance is sufficient) to protect cells against hypoosmotic shock, but the level of their abundance in the membrane is a primary determinant of their effect on cell survival.

Purification and oligomeric organization. We purified the three new channel proteins to investigate their stability and to characterize their oligomeric state. Conditions found to yield stable MscS, MscK and YbdG were applied to membranes containing the His-tagged variants of YbiO, YnaI and YjeP. All six homologs were found to be stable in DDM (0.04%) and could be purified to homogeneity in a two-step purification protocol (**Fig. 5**). The fractions from the gel filtration chromatography were re-analyzed on the Superose-6 column (see Materials and Methods) for light scattering analysis (see below). After the gel filtration step we observed a single symmetrical peak for YnaI, but both YbiO and YjeP proteins gave multiple peaks with some evidence of higher order aggregates. Consistent yields were obtained for each of the proteins but the specific yield corresponded with the observed abundance in membranes described above (**Fig. S3**). Thus, yields for YnaI and YbdG were similar to MscS, but the yields of YjeP and MscK were reduced by ~70% and that for YbiO by ~95%. Each homolog elutes from the size-exclusion column in a major peak corresponding to a high molecular weight complex. For further characterization of the oligomeric state of these complexes we used multi-angle light scattering in line with a UV and reflective index detector to determine the protein mass, independent of the associated lipids and detergent.[33] Masses for YnaI and YbiO were consistent with a heptameric state of the complexes (**Fig. 5**). The protein mass for YjeP is higher than expected for the heptamer, which is probably due to overlap of the principal peak with light scattering from higher aggregates. These aggregates were not well separated on the Superose 6

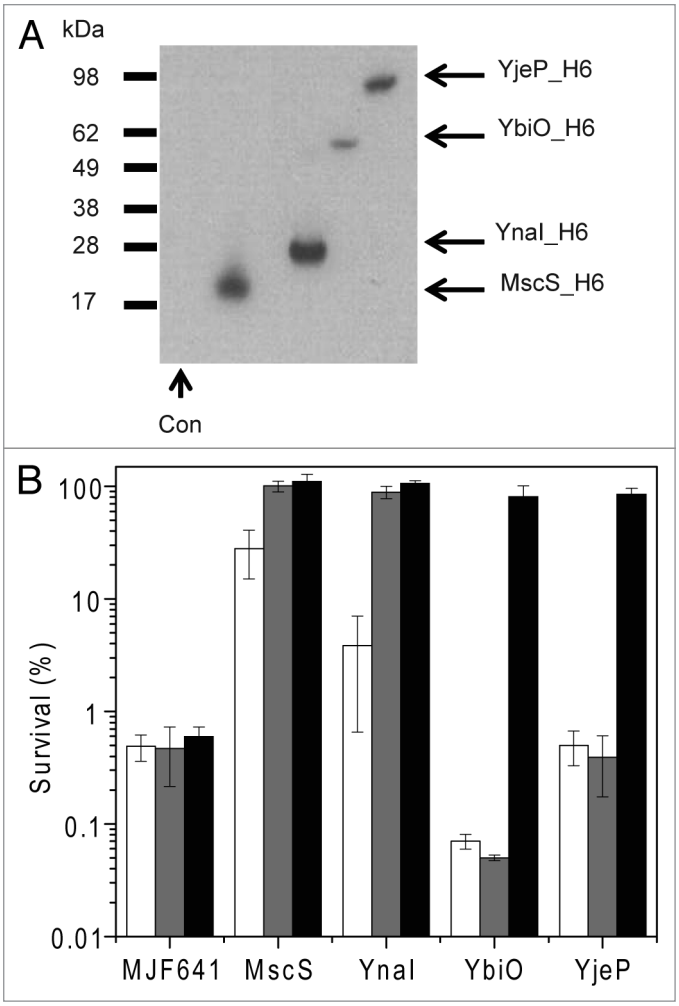

Figure 4. Expression and functional analysis of YnaI, YbiO and YjeP. (**A**) 15 μg total membrane proteins from membrane preparations isolated from MJF641 cells expressing plasmids bearing each of the three genes was separated by SDS-PAGE. Western blots were probed with an anti-His HRP conjugated antibody (Qiagen), and developed by the standard ECL method. (**B**) Overexpression of YnaI, YbiO and YjeP confers protection against a 0.3M NaCl hypoosmotic shock. MJF641 cells expressing MscS, YnaI, YbiO and YjeP were adapted in LB medium containing an additional 0.3M NaCl then diluted 20-fold into LB medium. Plasmid expression was either basal (open bars), or induced with 0.3 mM IPTG for 30 min (shaded bars) or 2.5h (closed bars).

column due to the high mass of the main species (the mass of a heptamer of our YjeP construct would be 861 kDa, not considering the associated lipids and detergent). In summary, the results suggest that all three new homologs are likely to form heptamers similar to MscS,[25] YbdG[20] and MscK (Rasmussen T, Li C, Booth IR, unpublished).

Discussion

The data presented here clearly define YjeP as the major component of the MscM channel and identify two new channels in *E. coli*. YjeP gates spontaneously in response to increased membrane tension and opens at pressures similar to those for MscS and MscK, which is consistent with previous reports that MscM opens before or around the tensions at which these larger channels are observed. The conductance is similar to that expected for MscM (~300 pS)[26] and the channel is increased in abundance in the Δ7 genetic background (MJF641) when the *yjeP* gene is expressed under IPTG-inducible *lac* promoter. Physiological studies establish that overexpression of YjeP protects cells against hypoosmotic shock. Analysis of the purified channel protein in DDM yields data consistent with a heptameric complex. Previously, we had proposed that MscM might be composed of the signals from several channels, including YbdG,[20] in a manner similar to the original descriptions of MscS. YbdG alone could not account for the MscM activity since null mutations did not eliminate the MscM channel activity from patches.[20] Indeed, a further confounding factor was that we could not cause native YbdG channels to gate spontaneously despite exploring a wide range of experimental regimes. The measured conductance of YbdG at ~300 pS, was dependent on introducing a gating mutation in the β domain (V229A).[20] In contrast, as indicated above, YjeP gates spontaneously on application of pressure and we believe that this is the most frequently encountered channel of the MscM size. For this reason we propose that YjeP is MscM and suggest that it be named accordingly.

The analysis of the homologs revealed two new channel activities, either of which is capable, when highly expressed, of protecting cells against hypoosmotic stress. YnaI is the first of the five TMS MscS proteins to have been measured by electrophysiology. Archaeal channels (MscMJ and MscMJLR)[34] that are close relatives of YnaI ($2e^{-29}$ and $4e^{-24}$, BLAST) have previously been characterized. Intriguingly, the two channels display very different conductance to YnaI, with values of ~300 nS and 2000 nS, respectively for MscMJ and MscMJLR.[34] The very low conductance of YnaI and its higher probability of opening at pressures similar to those needed to activate MscL, are the main reasons for its previous failure to be detected. It is notable that the general rule that the tension required to gate a channel correlates with its conductance breaks down for both YnaI and YbiO. Both are activated at tensions close to that for MscL, but their conductances are either very small (YnaI) or moderately large (YbiO). YbiO exhibits a conductance similar to MscS and MscK, gates at tensions between MscS and MscL, but appears to have a much lower intrinsic abundance in the membrane. In part this is due to the extremely low frequency with which the transcribed gene yields mature protein in the membrane. This can be inferred from the observation of very low abundance of the protein despite high levels of induction in response to NaCl revealed by qRT-PCR. All of the larger homologs have an N-terminal signal sequence that most certainly directs them into a different membrane-insertion pathway than is the case for MscS, YnaI and YbdG.[35,36] However, it is clear that once the proteins have been inserted in the membrane they can readily be recovered by detergent extraction and appear to retain a heptameric assembly. The two more abundant proteins (determined by western blots), MscK and YjeP, have large signal sequences (n > 33 amino acids) whereas YbiO has a much shorter signal sequence. This may be a factor determining their efficiency of assembly into the membrane. However, it is

clear that were it not for the expression problem, YbiO would be a major MS channel in *E. coli*.

The three new homologs display significantly different conductances from each other while retaining overall similarity in the pore sequences (**Fig. 2B**). An obvious question is whether any of the homologs can form partnerships with the others to create a greater diversity of channels. At this point there is no strong evidence for the formation of heteroligomers. However, we observed that overexpression of the single homologs in the Δ7 strain led to more regular channel openings in terms of conductance (**Fig. 2B**) compared with the channels in patches from mutants expressing the channels from the chromosome. This may indicate that some heterogeneity may arise from mixed channels, but there is currently no biochemical proof for such arrangements. In characterizing the individual homologs it was clear that there is no correlation between conductance and size of the protein complex, with each class large (> 700 amino acids) and small (< 500 amino acids) having members with low and high conductance. This observation mirrors that in *Methanocaldarius jannaschii*.[34] YnaI poses interesting challenges given the very low conductance of the channel. One possibility is that the upper part of the channel is narrowed compared with MscS, since there are a number of substitutions that introduce residues with larger molecular volume than the MscS equivalent residue. Other molecular dynamics studies of the lateral portals has concluded that may play a role in limiting conductance,[37] which is consistent with earlier data.[38] However, without detailed molecular dissection such an analysis remains speculative. The protective ability of YnaI suggests that small solutes can pass through the channel, which suggests that the minimum diameter of the open state is probably greater than the diameter of the hydrated potassium ion (> 6–8 Å diameter).[34]

Finally, it is clear from this analysis that the detection of mechanosensitive channel activities is dependent on the abundance of the proteins in the membrane. MscS and MscL were previously shown to be transcribed by RNA polymerase with either the vegetative sigma factor σ^{70} or the RpoS gene product (σ^{S}).[28] YbiO has previously also been identified as a gene predominantly expressed when σ^{S} is abundant.[39-41] In addition, RegulonDB indicates that *ybiO* has a σ^{54} promoter upstream of that for σ^{S} and it may be more than coincidence that the channel gene lies immediately downstream of the *glnHPQ* genes that are subjected to strong nitrogen regulation, which is the role of the σ^{54}.[42] More complex patterns of regulation for these and the other MscS homologs are revealed by the many microarray experiments logged at the Oklahoma database (**File S1**).[31] YnaI has been detected as a gene under the control of the acid-stress regulator EvgA.[43,44] Additional control mechanisms including DNA topology-mediated control and potential attenuation-based regulation are also evident from the gene structure and microarray experiments (see **Table S5**).

The MscM gene, *yjeP*, is part of an operon with phosphatidylserine decarboxylase (PSD), which generates the major phospholipid in *E. coli,* phosphatidylethanolamine. This is an intriguing observation. Expression of the *psd-yjeP* operon is under the control of σ^{E} and the two component regulator CpxAR, both of which respond to extracytoplasmic stress.[45,46] It is possible that

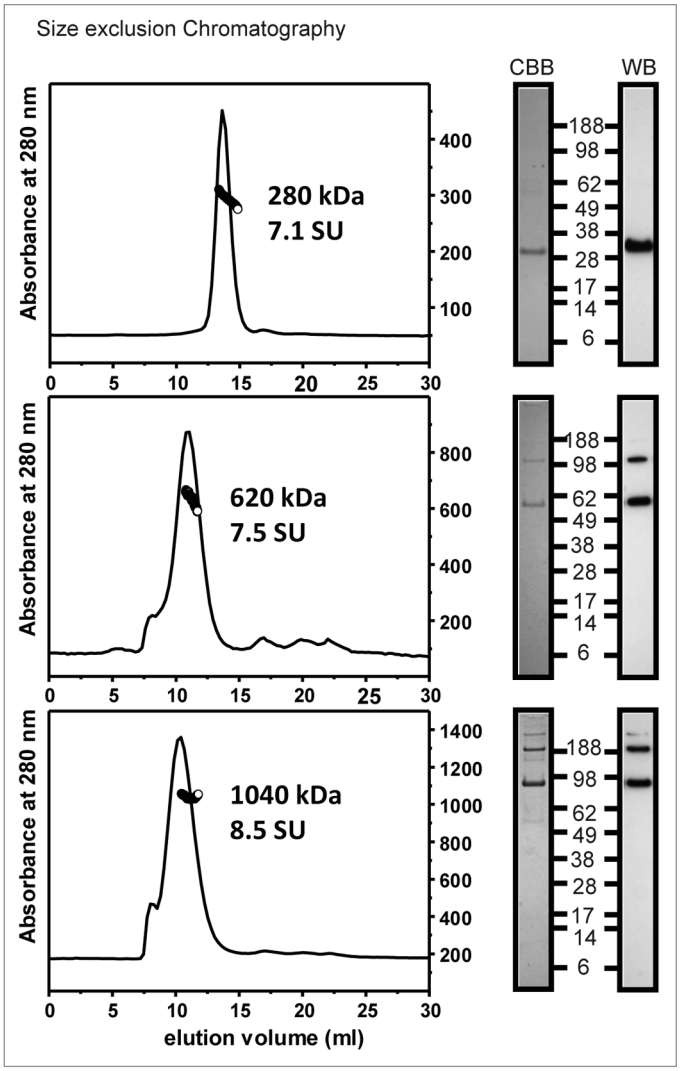

Figure 5. Purification and multi angle light scattering of MscS homologs. Profiles of size exclusion chromatography are shown with the absorbance at 280 nm (lines) and the calculated protein masses in the peak region (circles). The protein masses and the corresponding number of subunits (SU) are indicated. Coomassie stained SDS PAGE and western blots, detecting the C-terminal His-tag, of the purified homologs are shown on the right. Data are shown for YnaI (top), YbiO (middle) and YjeP (bottom).

while PSD is required for correct integrity of both the inner and outer membranes, the MscM channel may be induced to modulate turgor under extreme conditions. More complex patterns of regulation indicate that each of the homologs is regulated independently and thus, despite the commonality of their gating mechanisms and their potential contributions to ion balancing, there is no evidence of a common transcriptional control.

Initial genome analyses of *mscS* distribution among bacterial species revealed large numbers of homologs present in different species, including *E. coli*.[13,21] Paradoxically, electrophysiological characterization of *E. coli* membrane patches only identified two classes of mechanosensitive channel activity.[47] The limitations of this type of analysis were revealed both by initial membrane fusion experiments that suggested the existence of multiple

(n > 3) channel activities in *E. coli*.[26] The limitation of the electrophysiological analysis was further demonstrated when the MscS activity was shown to be two separate channels that can be differentiated both by their genetic origins and by the intrinsic properties.[13] Thus, it seemed likely that the uncharacterised MscM activity would arise from one of the homologs that had not been investigated at the molecular genetic level. Here we have characterized these activities and show that, while indeed *yjeP* does encode the principal component of the MscM channel activity, two unique channels are encoded by *ybiO* and *ynaI*. Previous failures to identify these channels can be explained by their low levels of expression from their chromosomal loci. Overexpression revealed their ability to protect cells in a manner akin to MscS or MscL, but, despite their complex regulation (**Fig. S1**), they never accumulate to sufficiently high levels to afford protection in mutants lacking MscS and MscL. This observation may reveal a deeper truth, that there is no sensing mechanism for MS channel abundance that provides a feedback signal to integrate the expression of the 'lower abundance' homologs, since if this were the case the phenotype of the MscS/MscL double mutant would not have been observed. A wider implication is that the presence of MscS homologs in the genome cannot be taken as proof that the channels are physiologically functional (i.e., capable of protection against hypoosmotic shock). This property resides not just in their sensitivity to increased tension, but also in their sufficiently high expression levels to afford rapid release of solutes. We have recently explored this rationale as the basis for the observed protection of *Vibrio alginolyticus* against hypoosmotic shock by expression of *E. coli* MscL.[48] The Vibrios have multiple MscS homologs but it can be inferred that they are insufficiently expressed to afford protection. Interestingly for the relationship between channels and physiology, the pathogenic Vibrios (e.g., *V. cholerae*) are unique in this genus in their possession of MscL naturally. Thus, channel diversity may have a role in defining the ecological niche of the organism.

Materials and Methods

Strains. A complete list of strains is shown in **Table S3**. Strain MJF641 was created by transduction of MJF517 to Apr^R using strain MJF611 (*ybdG::apr*) as a donor, the subsequent strain (MJF640) was transduced to Tet^R using strain MJF484 as a donor and transductants were screened by PCR for confirmation of co-transduction of Δ*f343*. All MscS family null mutants were verified by DNA sequencing of PCR products generated by gene flanking primers listed in **Table S3** and compared with genomic sequences obtained from Ecocyc using DNAstar Lasergene software. Strains were grown at 37°C in LB medium (10 g tryptone, 5 g yeast extract, 5 g NaCl/L) and stored at 4°C on agar plates (14 g/L) supplemented with antibiotic where appropriate.

Creation of null mutants and gene clones. The strategy for the creation of the *yjeP* null mutant by allele replacement mutagenesis has been previously described[13] and equivalent procedures were used to construct null mutants in *ynaI* and *ybiO* using primers listed in **Table S3**. The null mutants were confirmed by PCR. His tagged clones for *yjeP*, *ybiO* and *ynaI* were created synthetically by DNA2.0 in the pTrc99a[32] backbone. Creation of the non-His tagged constructs of *ybiO* (pF786) and *ynaI* (pF343) was achieved by amplification of the genes using primers listed in **Table S3**. pF786 contains a 135 bp upstream region due to uncertainties regarding the actual start site of the gene in the original genome literature. This places the structural gene ~145 bp downstream of the *pTrc* promoter and ribosome binding site, which may cause the lowered activity seen with this construct in whole cell assays (**Fig. S2**). This cloned region contains the $\sigma^{70/S}$ promoters and potentially a σ^{54} promoter (RegulonDB). To counter this, the synthetic gene *ybiO* was created using the K12 MG1655 corrected open reading frame from the newly recognized translational start site, yielding a protein of 741 amino acids with the addition of the His-tag (amino acid sequence LEHHHHHH). Plasmid pF343 is distinguished from pYnaI only in that the latter has the same His-tag sequence as the synthetic pYbiO clone. pYnaI, pF343, pF786 and pYbiO use pTrc99a[32] plasmid as their core. pYjeP was also made using this vector. The accuracy of the PCR products was verified by DNA sequencing on both strands. Synthetic DNA clones were made by DNA2.0 to our design.

Electrophysiology. Giant *E. coli* protoplasts of YnaI, YbiO and YjeP transformants were prepared as previously described[1] and expression was induced by 1h incubation with 0.3 mM IPTG during the final hour of cephalexin treatment. For NaCl treated protoplasts, the culture medium was supplemented with NaCl to a final concentration of 0.5M NaCl for an additional 2 h after the cephalexin treatment before the remainder of the protoplast preparation was followed as described. Excised membrane patches were analyzed at room temperature in symmetrical solutions (200 mM KCl, 90 mM $MgCl_2$, 10 mM $CaCl_2$ and 5 mM Hepes buffer pH 7). Data were acquired at a holding potential of 20 mV and a sampling rate of 50 kHz with 5 kHz filtration using an Axopatch 200B amplifier and PClamp9 software (Axon).

Hypoosmotic shock assay. To test channel function, hypoosmotic shock assays were performed, as previously described, using 0.3M NaCl downshock.[13,32] In addition, 0.3mM IPTG was incorporated for the duration of the incubation time in LB + 0.3M NaCl to evaluate the function of the channels under conditions of maximal protein accumulation possible within the constraints of the hyposmotic assay protocol.

Growth conditions and western blot. Protein expression profiles of the His-tagged clones were analyzed after growth in LB ± 0.5M NaCl and induction with 0.3 mM IPTG for 0, 0.5 or 2.5 h. All membrane samples were obtained 15 μg total membrane protein was separated on a 4–12% Bis-Tris SDS/PAGE gel (Invitrogen) and analyzed by western blot detection (Penta-HisHRP conjugated antibody, Qiagen) as previously described. Peptide-specific antibodies were generated in rabbits against specific regions of YjeP, YbiO and YnaI. The antisera were purified and used as described previously for antisera against MscS.[32]

qRT-PCR. Quantitative real time PCR analysis of the expression of the three homologs was conducted as described previously for the *ybdG* gene[20] using the primers described in **Table S4** Initially, *rpoB* and *polA* were used as internal controls. However, their mRNA abundance was found, unexpectedly, to vary with the growth conditions. Thus the *mscS* mRNA was used as the

reference point since we had previously characterized the expression of this gene in detail.[28]

Protein purification and characterization. The purification followed a similar protocol than described earlier for MscS[49,50] with some modifications. The pTrc constructs with the C-terminal His-tag were transformed into the Δ7 strain and grown in a 0.5L LB culture at 37°C to an OD_{650nm} of about 0.8. After lowering the temperature to 30°C, expression was induced with 0.8 mM IPTG for 4 h. The cell pellet was suspended in 20 ml PBS buffer (phosphate-buffered saline buffer, pH 7.5: containing 8 g of NaCl, 0.2 g of KCl, 1.15 g of $Na_2HPO_4 \cdot 7\ H_2O$ and 0.2 g of KH_2PO_4 per liter) and stored at -80°C until further used. Cells were ruptured in a French Press (Thermo Scientific) and bacterial membranes were separated by ultracentrifugation (100,000 g for 1h at 4 C). The membranes were suspended in a buffer containing 1.1% dodecylmaltoside (DDM, Glucon), 50 mM sodium phosphate pH 7.5, 300 mM NaCl, 10% glycerol, 25 mM imidazole, 0.2 mM phenylmethylsulfonylfluoride (PMSF, Sigma) using a Teflon-in-glass homogenizer and incubated for 1h at 4°C. In some experiments the DDM concentration was increased to 1.4 or 1.7% as indicated in the result section. After centrifugation at 3,000 g for 10 min and filtration through 0.2 μm syringe filter, the solution was loaded to a pre-packed 0.5 mL nickel-nitrilotriacetic agarose column (Sigma). The column was then washed with 15 ml of a buffer containing 0.05% DDM, 50 mM sodium phosphate pH 7.5, 300 mM NaCl, 10% glycerol and 25 mM imidazole and stored over night at 4°C. The protein was eluted in 1 ml fractions with a buffer similar to the washing buffer but containing 300 mM imidazole. The peak fraction was then further purified using a Superose 6 10/300 column (GE Healthcare) at 0.5 ml/min with a buffer containing 0.04% DDM, 50 mM sodium phosphate pH 7.5, 150 mM NaCl and 0.005% azolectin (Sigma). Yields were determined by peak integration using the software ASTRA (Wyatt). The identities of the purified homologs were confirmed by tryptic digest and mass spectroscopy (Aberdeen proteomics facility).

For the determination of the protein masses a miniDAWN light-scattering instrument and Optilab T-rEX refractometer (Wyatt, CA) coupled to AKTA purifier with UV detector (GE Healthcare) was used.[51,52] For this experiment, the peak fractions of the size exclusion step in the purification were re-injected onto the Superose 6 column and the conjugate module of the ASTRA software (Wyatt) was used for the analysis. Extinction coefficients were calculated using the ExPASY online suite and the refractive index increments were calculated on the basis of the amino acid sequence using the software Sedfit.[51] For DDM a refractive index increment of 0.1435 was determined. Rabbit aldolase (Sigma) was used in a control experiment. If the purification conditions were not optimal (for example, if the DDM concentrations during solubilisation were too high), an additional peak corresponding to a dimer of heptamers is seen on size exclusion chromatography. These were often accompanied by higher aggregates and therefore are not likely to be biological relevant complexes. **Figure S2** shows as example the size exclusion profiles for YnaI solubilised at different detergent concentrations. The dimerization of the heptamers may be triggered by de-lipidation as a higher amount is seen at higher detergent concentrations. This dimerization has also been observed in blue native gels for MscS mutants.[50]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental materials may be found here:
www.landesbioscience.com/journals/channels/article/20998

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