The EMBO Journal Vol.18 No.7 pp.1730–1737, 1999

Protection of *Escherichia coli* cells against extreme turgor by activation of MscS and MscL mechanosensitive channels: identification of genes required for MscS activity

Natalia Levina[1], Sabine Totemeyer[2], Neil R.Stokes, Petra Louis, Michael A.Jones[3] and Ian R.Booth[4]

Department of Molecular and Cell Biology, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen AB25 2ZD, UK

[1]Present address: Biology Department, York University, 4700 Keele Street, North York, Ontario, MB3J 1P3, Canada
[2]Present address: Microbial Pathogenesis Unit, International Institute of Cellular and Molecular Pathology, University of Luvain, Medical Faculty, Avenue Hippocrate 76, PO box 74.49, 1200 Brussels, Belgium
[3]Present address: Institute for Animal Health, Compton, Newbury, RG20 7NN, UK

[4]Corresponding author
e-mail: gen118@abdn.ac.uk

N.Levina, S.Totemeyer and N.R.Stokes contributed equally to this work

Mechanosensitive channels are ubiquitous amongst bacterial cells and have been proposed to have major roles in the adaptation to osmotic stress, in particular in the management of transitions from high to low osmolarity environments. Electrophysiological measurements have identified multiple channels in *Escherichia coli* cells. One gene, *mscL*, encoding a large conductance channel has previously been described, but null mutants were without well-defined phenotypes. Here, we report the characterization of a new gene family required for MscS function, YggB and KefA, which has enabled a rigorous test of the role of the channels. The channel determined by KefA does not appear to have a major role in managing the transition from high to low osmolarity. In contrast, analysis of mutants of *E.coli* lacking YggB and MscL shows that mechanosensitive channels are designed to open at a pressure change just below that which would cause cell disruption leading to death.

Keywords: mechanosensitive channels/MscL/MscS/osmoregulation/turgor maintenance

Introduction

The survival of bacteria in the natural environment is conditional upon mechanisms by which the cells can adjust to extremes of temperature, pH and osmotic pressure. The response to osmotic stress in bacteria has been best characterized in *Escherichia coli* (Booth *et al.*, 1994; Csonka and Epstein, 1996; Poolman and Glaasker, 1998). Cell turgor, which is important for cell wall extension (Koch, 1983; Holtje, 1995), is maintained relatively constant despite variations in external osmolarity through the controlled accumulation and release of K^+ and organic solutes (e.g. glutamate, betaine, proline and trehalose) via regulated transport systems (Booth *et al.*, 1994). Water movements are facilitated by an aquaporin (Calamita *et al.*, 1996). When the external osmolarity increases (upshock), the core physiological responses of this organism are dependent upon the activity of these proteins (Booth *et al.*, 1994; Poolman and Glaasker, 1998). Initial loss of water is countered by the controlled accumulation of solutes in the cytoplasm, and the consequent inflow of water restores the outwardly directed turgor pressure. When transferred to medium of low osmolarity (downshock), water inflow gives rise to a substantial increase in the turgor pressure, which could burst the cell envelope. Mechanosensitive channels are major routes for the release of cytoplasmic solutes to achieve a rapid reduction of the turgor pressure during the transition from media of high osmolarity to low (Berrier *et al.*, 1992).

The presence of different mechanosensitive channels in a diversity of bacteria has been detected by the application of patch–clamping (Zoratti and Petronilli, 1988; Berrier *et al.*, 1992; Szabo *et al.*, 1993; Sukharev *et al.*, 1994; Le Dain *et al.*, 1998). In *E.coli* cells, there are between three and five stretch-activated channels (Berrier *et al.*, 1996; Sukharev *et al.*, 1997). However, with the exception of the MscL channel, genetic studies have so far failed to identify the structural genes for these channels. The *mscL* gene was identified by patch–clamp analysis of purified and reconstituted membrane proteins from *E.coli* accompanied by N-terminal sequencing and database analysis. The identification of the structural gene facilitated the creation of a deletion mutant lacking MscL, and this was found only to lack the largest of the mechanosensitive channels. No significant growth or survival phenotype was identified in the mutant, suggesting a redundancy in the function (Sukharev *et al.*, 1994). More recently, this mutant has been shown to be deficient in the extrusion of small proteins such as thioredoxin during osmotic downshock, but competent for the release of small osmotically active molecules (Ajouz *et al.*, 1998). Biochemical and genetic studies on the mechanism of gating of the *E.coli* channel (Blount *et al.*, 1996a,b,c; Ou *et al.*, 1998; Saint *et al.*, 1998) have been complemented by the crystal structure of the MscL protein from *Mycobacterium tuberculosis* (Chang *et al.*, 1998).

Recently, we characterized an *E.coli* gene product, KefA (Y07802; also called AefA), which has a role in the normal regulation of ion balance and turgor pressure during growth at high osmolarity (G.Gouesbet, M.A.Jones, N.R.Stokes, D.McLaggan, W.Epstein and I.R.Booth, unpublished data). Missense mutations in this gene have been shown to alter the pressure response of the mechanosensitive channels (Cui *et al.*, 1995; Cui and Adler, 1996). In this study, we show KefA to be a member of a large bacterial protein family which contains proteins of two principal size classes: large proteins (>700 residues),

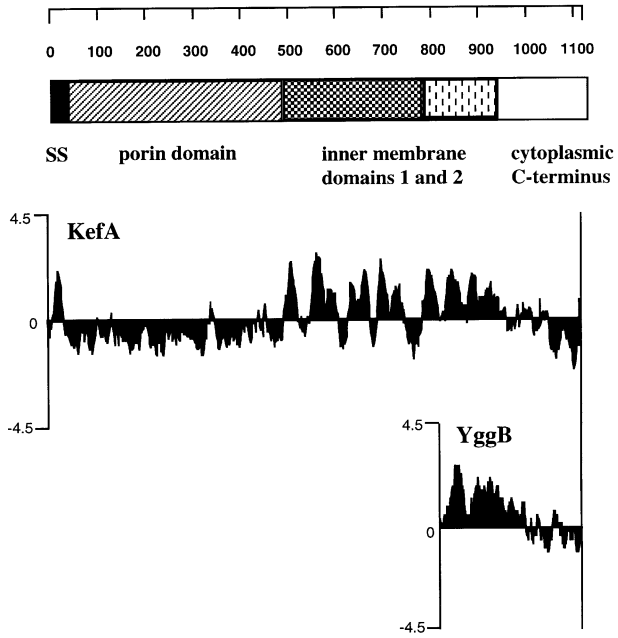


Fig. 1. Hydrophobicity plot for KefA and YggB. The hydrophobicity plots were constructed by standard methods (Kyte and Doolittle, 1982) using a window of 19 residues in the DNASTAR suite of programmes. On the basis of sequence homologies and alkaline phosphatase fusion data, the KefA protein is predicted to consist of up to five domains (G.Gouesbet, M.A.Jones, N.R.Stokes, D.McLaggan, W.Epstein and I.R.Booth, manuscript in preparation). An N-terminal signal sequence is followed by a hydrophilic domain that is either periplasmic or inserted in the outer membrane, two inner membrane domains and a C-terminal cytoplasmic domain. The YggB protein shows greatest homology to the second transmembrane domain and the C-terminal hydrophilic domain.

which have only been found in Gram-negative bacteria, and smaller proteins (~300–500 residues), which are almost ubiquitous in bacterial genomes. For *E.coli*, two open reading frames (ORFs), YggB (P11666) and F343 (D90771), belong to this group. Mutants lacking both KefA and YggB show no MscS activity but retain MscL and a smaller channel, MscM. Mutants lacking YggB and MscL are severely compromised in their survival of osmotic downshock. This is the first demonstration of a physiologically important phenotype associated with loss of mechanosensitive channel activity, and demonstrates that rapid release of solutes is required for survival of extreme turgor generation.

Results

Inactivation of YggB leads to loss of MscS activity in E.coli protoplasts

KefA is a large multi-domain protein (1120 amino acids) which has been implicated in the control of ionic homeostasis (D.McLaggan, M.A.Jones, W.Epstein and I.R.Booth, unpublished data). A BLASTP search (Altschul *et al.*, 1997) revealed that proteins related to KefA fell into two size categories (Figure 1): large proteins (>700 residues) and smaller proteins (~300–500 residues). The conserved region that establishes the relationship between the two classes of proteins corresponds to the second membrane domain and the C-terminal domain of KefA (Figure 1). To date, large KefA homologues have only been detected

Table I. Distribution of putative homologues of YggB

Organism	Database entry	Similarity		
		E value	I/S[a]	Length[b]
Edwardsiella ictaluri	AF037440	$7e^{-80}$	52/66	286 (285)
Synechocystis	D64002	$7e^{-36}$	33/51	296 (231)
Helicobacter pylori	AE000607	$2e^{-31}$	30/53	274 (231)
Archaeoglobus fulgidus	AE000996	$3e^{-31}$	31/50	283 (232)
Pyrococcus horikoshii	AB009476	$4e^{-26}$	33/59	335 (156)
Treponema pallidum	AE001253	$5e^{-21}$	24/45	301 (228)
Aquifex aeolicus	AE000718	$9e^{-19}$	24/46	436 (237)
A.aeolicus	AE000708	$1e^{-17}$	23/45	368 (222)
Methanococcus jannaschii	MJ1143	$2e^{-16}$	27/43	361 (251)
Haemophilus influenzae	HI0195.1	$4e^{-15}$	25/43	1111 (236)
E.coli	Y07802 (AefA)[c]	$3e^{-14}$	24/46	1120 (224)
M.jannaschii	MJ0170	$3e^{-13}$	24/43	350 (226)
Borrelia burgdorfori	AE001150	$3e^{-12}$	25/47	280 (164)
Methanobacterium thermoautotrophicum	AE000936	$6e^{-12}$	20/42	248 (195)
Bacillus subtilis	Y14082	$7e^{-12}$	22/42	371 (227)
Synechocystis	D64004	$1e^{-10}$	21/40	505 (223)
Synechocystis	D90916	$1e^{-09}$	22/40	479 (235)
H.pylori	AE000557	$2e^{-08}$	21/39	623 (254)
Synechocystis	D90900	$4e^{-08}$	23/50	765 (161)
Salmonella typhimurium	U94729	$3e^{-07}$	23/41	377 (197)
E.coli	P39285 (YjeP)	$3e^{-07}$	23/46	1107 (164)
Synechocystis	D64005	$4e^{-06}$	23/41	617 (201)
E.coli	D90771 (F343)	$5e^{-06}$	19/41	343 (236)
Synechocystis	D90904	$6e^{-05}$	24/45	704 (170)
Mycobacterium tuberculosis	Z95150	0.002	22/44	308 (153)
B.subtilis	Z99111	0.008	25/45	267 (155)
B.subtilis	Z99108	0.014	27/51	280 (103)
E.coli	AE000183 (F786)[d]	0.2	21/35	786 (241)

The putative homologues were identified using the BLASTP programme (Altschul *et al.*, 1997) against the non-redundant database.
[a]I/S indicates percentage of identical and similar residues, respectively.
[b]The predicted length of the protein is shown; the figure in brackets indicates the number of residues over which the sequence similarity is significant.
[c]The *kefA* gene was identified through lesions that affected the potassium-sensitivity of *E.coli* cells (D.McLaggan, M.A.Jones, W.Epstein and I.R.Booth, unpublished data). Analysis of the physiology of the mutants did not allow the conclusion that the gene product was specifically involved in K^+ pool regulation and the gene sequence was lodged in the database as AefA.
[d]The F786 protein is included even though the score is close to a random fit. The organization of the protein closely follows that of KefA and YjeP, and the protein shows significant similarity along its length to both of these proteins ($1e^{-05}$ and 0.011 for KefA and YjeP, respectively) (our unpublished data).

in Gram-negative bacteria, but the smaller proteins are present in the majority of bacteria (Table I). Many organisms have more than one ORF that belongs to the smaller KefA family. *Escherichia coli* possesses three members of the large KefA family (KefA, Yjep and F786) and two of the smaller proteins (YggB and F343). The conservation of these ORFs coupled with the proposed role of KefA in ionic homeostasis led us to investigate them as candidate genes for mechanosensitive channels.

The polymerase chain reaction (PCR) was used to amplify the *yggB* ORF and its flanking regions, and the gene was cloned and a chromosomal deletion was created

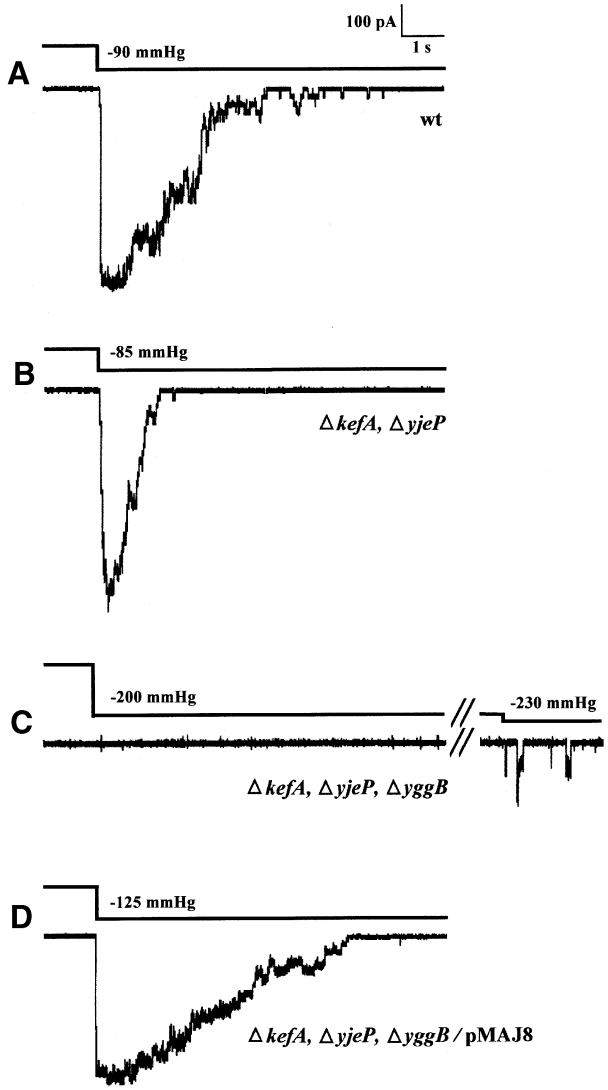


Fig. 2. YggB encodes the major MscS activity. Patch–clamp recordings were made on (**A**) Frag1, (**B**) strain MJF421 (Frag1, Δ*kefA*, Δ*yjeP*), (**C**) strain MJF431 (Frag1, Δ*kefA*, Δ*yjeP*, Δ*yggB*) and (**D**) MJF431/pMAJ8 as described in Materials and methods. In (C), the pressure was increased to confirm the presence of the MscL activity. The pressure on the patch is shown as a solid line immediately above the recording. The pressure required to activate the channels varies with patch geometry (Blount *et al.*, 1996b) and there is no significance in the differences in pressure required to see MscS channel activity in individual experiments.

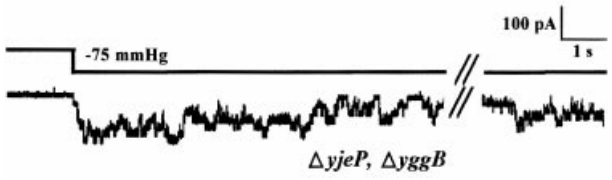


Fig. 3. KefA channel activity. Channel activity associated with KefA was analysed using strain MJF461 (Frag1 Δ*yjeP*, Δ*yggB*) as described for Figure 2. The total length of this recording was ~3 min.

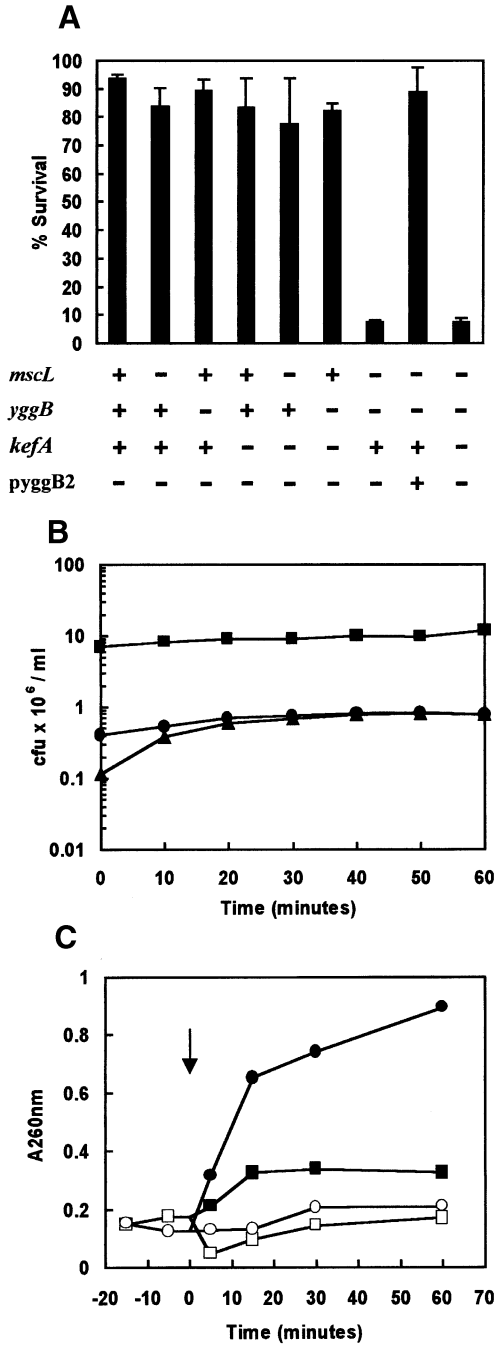
by allelic replacement (see Materials and methods). A full length homologue of KefA, YjeP (P39285), is present in *E.coli*, and a strain carrying a deletion of this gene was constructed for the studies reported here. Strains were created that possessed different combinations of MscL, KefA, YjeP and YggB. The growth of the mutants was found to be similar to wild type at both low and high osmolarity in the presence and absence of compatible solutes (data not shown). Giant protoplasts were made by standard methods and the stretch-activated channels analysed by cell-attached recording (see Materials and methods).

Patch–clamp analysis of giant protoplasts derived from the parental strain, Frag1, exhibited three distinct stretch-activated channels as previously described by other workers (Sukharev *et al.*, 1997): MscM, approximate conductance 0.1–0.3 nS; MscS, 0.8–1 nS; and MscL, 2.5–3 nS. The pressure thresholds for these channels were similar to the previously reported values in all of the strains studied (data not shown). Furthermore, the MscS activity of Frag1 ($n = 10$) was observed to undergo desensitization in the manner reported previously (Figure 2A) (Sukharev *et al.*, 1997). Inactivation of KefA and YjeP did not alter the apparent characteristics of the MscS channel ($n = 11$), since similar MscS activity was observed in Frag1 and MJF421 (Δ*kefA*, Δ*yjeP*), and normal desensitization occurred (Figure 2B). In contrast, inactivation of the *yggB* gene in strain MJF431 (Δ*kefA*, Δ*yjeP*, Δ*yggB*) led to complete loss of the MscS mechanosensitive channel activity ($n = 18$) (Figure 2C). When increased pressure was applied to protoplasts of MJF431, MscL activity was observed as expected (channel activities with unitary conductance 3.2 ± 0.5 nS were observed when the pressure was increased; $n = 12$) (Figure 2C). Introduction of YggB, on a moderate copy number plasmid, restored MscS activity (Figure 2D; $n = 3$).

The KefA channel has MscS-like properties

The identification of MscS channel activity with YggB, which is a homologue of KefA, suggested that the latter might exhibit channel activity that is masked in wild-type cells by the YggB-encoded activity. MscS activity is characterized by short bursts of activity that last for a few seconds (Figure 2A). During the analysis of mutants lacking YggB but possessing KefA, we noted that some patches exhibited a small number of channels that did not desensitize upon extended pressure application (Figure 3). In contrast to MscS, the new channel remained active for >30 s, and in some protoplasts the channel was active for >3 min (Figure 3) before recordings were terminated by loss of the seal. The new channel activity was not detected in all patches; for strains MJF461 (Δ*yggB*, Δ*yjeP*) and MJF451 (Δ*yggB*), ~70% of patches (6/8 and 12/19, respectively) were found to have this channel. In individual patches this channel appears to be 5- to 10-fold less abundant than MscS. The unitary conductance of the channel was similar to that of MscS (1 ± 0.09 nS; $n = 3$). Strains lacking YggB, KefA and YjeP did not show this channel activity (strain MJF431, 18 patches; Figure 2C). Similarly, strain MJF429 (Δ*kefA*, Δ*yggB*), which possesses YjeP, did not display this activity ($n = 6$), and this suggests that the channel activity is encoded by the KefA gene product. The sequence organization of KefA and YjeP, coupled with alkaline phosphatase fusion data and preliminary electron microscope images, suggests that these proteins may span both the inner and the outer membrane (G.Gouesbet, M.A.Jones, H.de Cock,

N.R.Stokes, D.McLaggan, W.Epstein and I.R.Booth, unpublished results). Such organization may compromise measurement of KefA and YjeP activity by patch–clamp analysis since the patch pipette would need to circumscribe an area containing both membranes. Attempts to restore channel activity were made using the cloned *kefA* gene; however, these experiments were unsuccessful due to premature arrest of protoplast formation when the *kefA* gene was being expressed even using a low copy number plasmid (pHSG575; Takeshita *et al.*, 1987) (data not shown). All of the data are consistent with the *kefA* gene encoding a mechanosensitive channel with similar conductance to MscS that does not desensitize upon extended pressure.



Physiological role of mechanosensitive channels

The creation of mutants lacking the YggB, KefA and MscL channels allowed a direct test of the role of these channels in protection of cells during sudden reductions of the external osmolarity (downshock). Organisms were grown in minimal medium in the presence of 0.5 M NaCl and the viability determined upon dilution into medium lacking NaCl (Figure 4A). Survival of cells was unaffected when the parent (Frag1) or single mutants, MJF367 (Δ*mscL*), MJF451 (Δ*yggB*) and MJF379 (Δ*kefA*) were diluted into low osmolarity media (Figure 4A). Similarly, mutants lacking KefA and MscL (strain MJF453), and KefA and YggB (MJF429) also survived the transition to low osmolarity medium (Figure 4A). However, a >10-fold loss of viability occurred when cultures of the double mutant lacking both YggB and MscL (MJF455) were rapidly transferred to medium of low osmolarity (Figure 4A). Restoration of the MscS channel by complementation with a *yggB* clone prevented loss of viability on downshock (Figure 4A). In strain MJF455, loss of viability occurred in the first few seconds after dilution into low osmolarity medium (Figure 4B). Light scattering at 650 nm decreased immediately upon downshock; the change was greatest in the double mutant MJF455 and, in contrast to Frag1, there was no recovery (data not shown). This change in OD_{650} was accompanied by a decrease in the total cell numbers (data not shown). Cell lysis, as revealed by an increase in A_{260} absorbing material in the medium, occurred after downshock of the mutant but was not significant in the parent, Frag1 (Figure 4C). A parallel increase in A_{280} absorbing material was also observed (data not shown). These data suggest that loss of viability preceded cell lysis.

The viability of the triple mutant MJF465 (Δ*mscL*, Δ*yggB*, Δ*kefA*) was only distinguishable from the double mutant MJF455 (Δ*mscL*, Δ*yggB*) at early sampling times when there was an apparent transient loss of viability with recovery occurring over a few minutes (Figure 4B). These data suggest that the KefA system is not essential for cell survival but its absence leads to cell damage from which the organism can recover.

Cell death in mutants lacking the mechanosensitive channels occurred upon downshock >0.2 M NaCl (Figure 5A). To relate this to the osmotic change required to

Fig. 4. Survival of mechanosensitive channel mutants upon downshock. (**A**) Cells were grown to exponential phase in minimal medium (pH 7) in the presence of 0.5 M NaCl and diluted 20-fold into minimal medium without NaCl (pH 7). The data show the survival of the strains measured 30 min after a 0.5 M NaCl downshock. Survival after iso-osmotic dilution is set to 100% and no significant differences were observed under these conditions for any of the strains. Strains assayed: Frag1 (wild type); MJF367 (Δ*mscL*); MJF451 (Δ*yggB*); MJF379 (Δ*kefA*); MJF453 (Δ*kefA* Δ*mscL*); MJF429 (Δ*kefA*, Δ*yggB*); MJF455 (Δ*mscL* Δ *yggB*); MJF455 (Δ*mscL*, Δ*yggB*)/ pyggB2; MJF 465 (Δ*kefA* Δ*yggB* Δ*mscL*). (**B**) Cells of strains Frag1 (■), MJF455 (Δ*yggB*, Δ*mscL*) (●) and MJF465 (Δ*yggB*, Δ*mscL*, Δ*kefA*) (▲) were treated as in (A) and samples taken at timed intervals, and the apparent viability determined as described above. Data for iso-osmotic dilution are omitted for clarity and were found to be identical to the data for Frag1. (**C**) Cells were grown as described in (A), filtered and re-suspended in the presence (open symbols) or absence (closed symbols) of 0.5 M NaCl. At intervals, 3 ml samples were centrifuged and the OD_{260} of the supernatant recorded. Symbols: (■,□) Frag1; (●,○) MJF455 (Δ*mscL*, Δ*yggB*). All of the data are representative of triplicate experiments and error bars display standard deviation from the mean for one experiment.

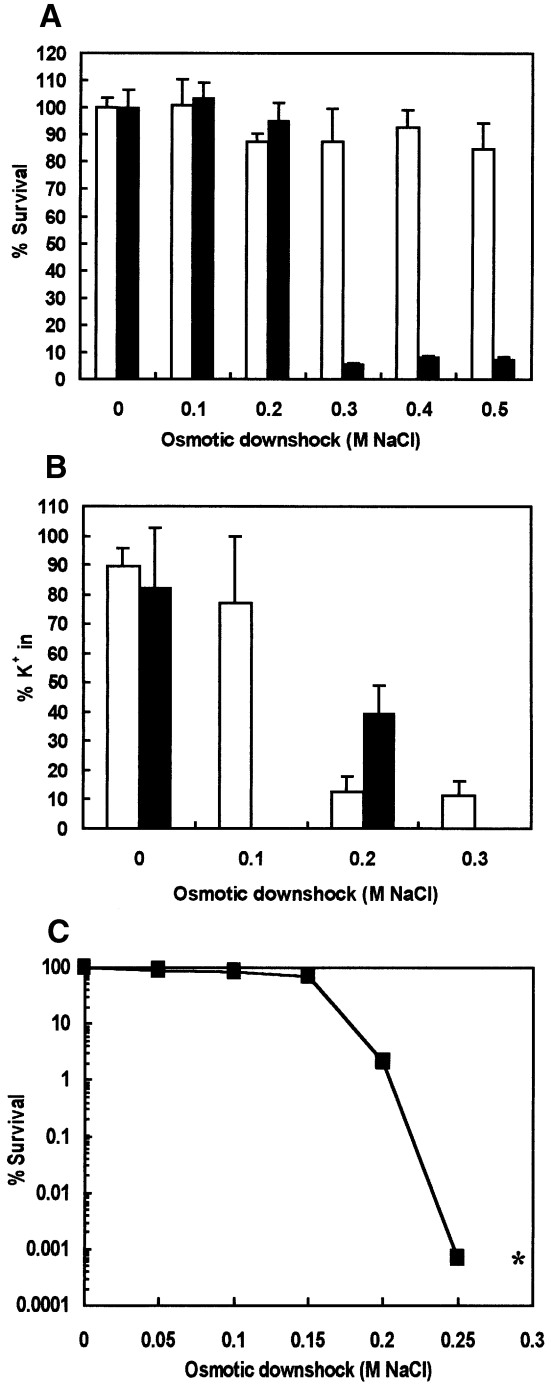


Fig. 5. Activation threshold of mechanosensitive channels in *E.coli*. (**A**) Cells of Frag1 (open bars) and MJF455 (Δ*mscL* Δ*yggB*) (closed bars) were grown and treated as in Figure 4 and diluted 20-fold into the media containing different NaCl concentrations to create the downshock indicated. The viability was determined after 30 min incubation. (**B**) Cells of Frag1 (open bars) and MJF455 (closed bars) were grown to mid-exponential phase and then filtered and re-suspended into media of different osmolarities. Samples were taken immediately and the K^+ content of the cells measured as described in Materials and methods. (**C**) Cells of Frag1 were grown to exponential phase in minimal medium (pH 7) supplemented with 0.3 M NaCl and then diluted 20-fold into minimal medium (pH 3.6) supplemented with a range of NaCl concentrations. An asterisk indicates that no viable cells could be recovered after downshock at pH 3.6. All of the data are representative of triplicate experiments and error bars display standard deviation from the mean for one experiment.

activate the mechanosensitive channels, we assayed K^+ efflux upon downshock. Strain Frag1 lost ~85% of cellular K^+ upon a 0.2 M NaCl downshock, but was barely affected by smaller changes in the external osmolarity (Figure 5B). Increasing the downshock did not lead to greater K^+ loss. The double mutant MJF455 (Δ*mscL*, Δ*yggB*) retained a greater fraction of its K^+ pool on a 0.2 M NaCl downshock (Figure 5B), suggesting that MscS and MscL activity is significant under these conditions. Bacterial mechanosensitive channels are relatively non-specific and consequently upon opening will facilitate major changes in the ion pools, leading to cytoplasmic acidification when the external pH is low (Berrier *et al.*, 1992; Schleyer *et al.*, 1993). The retention of an alkaline cytoplasmic pH is essential to the viability of *E.coli* cells. A combined pH shock (pH 7 to pH 3) with an osmotic downshock (medium plus 0.3 M NaCl to medium alone) reduced the viability of *E.coli* cells 10^6-fold faster than cells shifted to pH 3 in iso-osmotic medium (J.Glover, L.J.Malcolm, N.R.Stokes, M.A.Jones and I.R.Booth, unpublished data). We developed this assay to analyse further the threshold osmotic downshock at which the channels were substantially activated. Cells were grown at pH 7 in the presence of 0.3 M NaCl and shifted to media of different osmolarities at pH 3.6 and the viability determined after 30 min (Figure 5C). Cells shifted to pH 3.6 in iso-osmotic medium retained >95% viability, whereas those shifted to medium lacking NaCl died immediately upon transfer (Figure 5C). A significant loss of viability only occurred when the osmotic downshock exceeded 0.15 M NaCl (Figure 5C). Thus, the threshold osmotic downshock at which the mechanosensitive channels become significantly activated is set below that at which cell damage would occur in the absence of the channels (Figure 5A). Clearly, therefore, the channels have a major role in defence of the cell against sudden shifts in osmolarity.

Discussion

We have characterized two *E.coli* genes that are required for MscS mechanosensitive channel activity. Deletion of *yggB* eliminates the major MscS activity and reveals a similar channel activity that is less abundant and that does not de-sensitize on extended application of pressure. The latter activity is removed by *kefA* deletions. Formally, we cannot conclude that *yggB* and *kefA* are the channel genes since they could act as regulators of the MscS activity. However, both proteins are strongly predicted to be membrane-located, and missense mutations in the *kefA* gene lead to changes in cell physiology that are consistent with altered ion conductance (D.McLaggen, M.A.Jones, W.Epstein and I.R.Booth, unpublished data). Furthermore, the strongest regions of conservation between KefA and YggB are in the transmembrane strands and in the C-terminal cytoplasmic domain, which is also consistent with the similar ion conductance of the channels. Finally, although the YggB protein differs significantly in size and organization from the MscL protein [286 (31 kDa) and 136 (14 kDa) amino acids for YggB and MscL, respectively], there are similarities in the transmembrane strands that suggest conservation of function. A conserved pattern of periodic glycine residues has been shown to be critical

for gating of the *E.coli* MscL protein (Moe *et al.*, 1998; Ou *et al.*, 1998). A similar pattern of glycine residues can be found in YggB (residues 95–133), and this region is highly conserved in the YggB (and KefA) family of proteins (our unpublished data) despite considerable variation in the length of these proteins (Table I). These data are consistent with YggB and KefA being the structural genes for the channels.

The F343 open reading frame (ORF) of *E.coli* is similar to YggB in sequence and organization, but was not immediately evident as an equivalent activity in patch–clamp analysis. It may be that this protein is not expressed under the conditions of growth used here; e.g. one potential homologue of F343, KefA and F786 in *Lactococcus lactis* (AF005098) is induced by chloride ions (Sanders *et al.*, 1998). In addition, *E.coli* has a further homologue of the KefA/YjeP group of proteins, F786 (AE000183), the function of which remains unknown. Similarly, the function of YjeP is unknown because the *E.coli* deletion mutant has no phenotype. However, a null mutant in this gene in *Erwinia crysanthemi* causes a K^+-specific growth defect at high osmolarity and leads to abnormal regulation of ionic homeostasis during compatible solute accumulation (T.Touze, G.Gouesbet, A.J.Roe, I.R.Booth, A.Benassi and C.Blanco, unpublished data). Homologues of YggB are present in all eubacterial and archaeal genomes that have been sequenced with the exception of *Mycoplasma* and *Chlamydia*, and both of these organisms also lack MscL homologues. Since these organisms essentially prefer iso-osmotic environments, the potential lack of the major classes of mechanosensitive channels is understandable. In contrast, another intracellular parasite, *Rickettsia prowazekii*, has a small homologue of KefA (AJ235270; $E = 1e^{-04}$; 388 residues with 47% similarity over 121 residues) that is not identified by a BLASTP search with YggB (our unpublished data). Clearly, these gene products are sufficiently important to have been conserved by virtually all bacteria and possibly in some eukaryotes. A potential homologue of KefA is found in *Arabidopsis* (AF013293; $E = 0.014$, 353 residues, 46% similarity over 126 amino acids of the region in KefA that is equivalent to YggB). When the *Arabidopsis* sequence is used as the probe in a BLAST search, several bacterial members of the YggB/KefA family are suggested to have significant similarity (our unpublished data).

Many organisms possess multiple homologues of KefA/YggB (Table I). This may reflect a requirement for a number of mechanosensitive channels each with a different pore size, possibly induced by different environmental cues, and responding to different degrees of mechanical stress. This would offer the cell a graded response to changes in the osmolarity of the environment. The mechanosensitive channels open in response to high turgor (Berrier *et al.*, 1992). In our analysis, the pressure threshold for strong activation is an osmotic downshock equivalent to 0.2 M NaCl (Figure 5). Mutants lacking the MscS and MscL channel activities retain higher levels of K^+ during a downshock of 0.2 M NaCl and the limited solute release that does occur may reflect the contribution of KefA and MscM to osmotic adjustment. The increased retention of solutes in MJF455 (Δ*yggB*, Δ*mscL*) clearly has severe consequences for cell viability since an osmotic downshock of 0.5 M NaCl leads to extensive cell lysis (Figure 4C). This suggests that the channels are primed to open at pressures just below those that would compromise cell integrity. This is the first demonstration that cells lacking these channels are severely compromised in their ability to withstand sudden changes in the osmolarity of the environment.

Table II. Strains

Strain	Genotype	Reference
Frag1	F^-, *rha, thi, gal, lacZ*	Epstein and Kim (1971)
S17-1λpir	RPA-2, Tc::Mu-Km::Tn7 (λpir)	Simon *et al*. (1983)
MJF367	Frag1, Δ*mscL*::*Cm*	this study
MJF379	Frag1, Δ*kefA*::*kan*	(G.Gouesbet, M.A.Jones, N.R.Stokes, D.McLaggan, W.Epstein and I.R.Booth, unpublished data)
MJF421	Frag1, Δ*kefA*::*kan*, Δ*yjeP*	this study
MJF425	Frag1, Δ*yjeP*,	this study
MJF429	Frag1, Δ*yggB*, Δ*kefA*::*kan*	this study
MJF431	Frag1, Δ*kefA*::*kan*, Δ*yggB*, Δ*yjeP*	this study
MJF451	Frag1, Δ*yggB*	this study
MJF453	Frag1, Δ*kefA*::*kan*, Δ*mscL*::*Cm*	this study
MJF455	Frag1, Δ*mscL*::*Cm*, Δ*yggB*	this study
MJF461	Frag1, Δ*yggB*, Δ*yjeP*	this study
MJF465	Frag1, Δ*mscL*::*Cm*, Δ*yggB*, Δ*kefA*::*kan*	this study

Materials and methods

Strains

A complete list of strains is shown in Table II. Frag1 was obtained from Wolf Epstein (University of Chicago, IL) and strain MJF367 was created by transduction of Frag1 to Cm^R using strain AW405 (*mscL::cm*) (Sukharev *et al.*, 1994) as donor. Strain AW405 *mscL*::*Cm* was provided by Ching Kung (University of Wisconsin, WI). Strains carrying the *mscL::cm* and *kefA::kan* replacement alleles were created by transduction to Cm^R and Kan^R, respectively, using strains MJF367 and MJF379 (Δ*kefA::kan*) (G.Gouesbet, M.A.Jones, N.R.Stokes, D.McLaggan, W.Epstein and I.R.Booth, unpublished data) as donors. All other strain constructions were performed by direct allele replacement (see below).

Gene cloning and mutagenesis

Wild-type sequences for *yggB* and *yjeP*, and flanking regions required for allele replacement mutagenesis were obtained by PCR using *Taq* polymerase and primers based upon the published *E.coli* genome sequence. A list of primers used in this study is given (Table III). The construction of null mutants affecting *yggB* and *yjeP* was by overlap PCR (Ho *et al.*, 1989) and allele replacement using a sucrose-selectable suicide vector, pDM4 (Kaniga *et al.*, 1991; Milton *et al.*, 1996). The strategy for construction of null mutants by allele replacement is described in detail for *yggB* and equivalent procedures were used to construct null mutants in *yjeP*. Four oligonucleotide primers Y1F, Y2R, Y3F and Y4R (Table III) were designed to generate a fusion DNA fragment containing a 16 bp overlap, covering the regions 5′ and 3′ to *yggB* and creating a deletion of *yggB*. Two DNA fragments were amplified separately using the primer pairs Y1F/Y2R and Y3F/Y4R by PCR. These DNA fragments were purified, mixed in a 1:1 ratio and used as a template in PCRs with the primers Y1F and Y4R, which contain the restriction sites *Xba*I and *Sph*I, respectively. The resulting DNA fragment was then digested with these restriction enzymes and ligated into the suicide plasmid pDM4 (Milton *et al.*, 1996), previously digested with the same enzymes, to form plasmid pST-4. This plasmid was conjugated from S17-1 (Simon *et al.*, 1983) into MJF379 (Frag1 Δ*kefA::kan*), where it recombined into the chromosome to obtain strain MJF428 (Frag1, Δ*kefA::kan, yggB*::pDM-4). These mutants were confirmed by PCR with a combination of pDM4 specific primers (NQcat and NQrev) and chromosomal primers that lie adjacent to the flanking regions cloned into the plasmid (Y5F and YM2R) (Table III). The suicide plasmid was then excised by a second recombination event by

Table III. Primers used for strain construction and analysis

Name	Sequence (5′ to 3′)
YggB	
Y1F	AACCTGGGAAGGCGTTCTAGACTAC
Y2R	TACACGTTCAGCAGCGCCTGGTTAGCTAC
Y3F	CGCTGCTGAACGTGTACTGGGATGTGCTG
Y4R	TTTCCATCTGGCATGCTTCATGAC
Y5F	TGACTCCGACCATCCTGCG
YM2R	GTTTTGGATCCACATCAAGTTGCCC
YM1F	CTAGGATCCGCGCGTATGGCTGC
YjeP	
KD8F	CTGCGTGAGATGATCTAGATGCCGG
KD9R3	GTGACCGATTTTGCCTGCTCCAGTTCCTG
KD10F3	AGGCAAAATCGGTCACGCGCGTGGTGTTG
KD11R	CATCACTTCCGCGCGCATGCAAGAGTGG
D2SR	ACGAGCGCCTTAAGACGTGC
DS6F	TAACGGCCACCGTGCCGCTG
pDM4	
NQcat	TAACGGCAAAAGCACCGCCGGACATCA
NQrev	ACATGTGGAATTGTGAGCGGATAACAA

growth on Luria–Bertani (LB) medium containing sucrose (5% w/v) (Milton *et al*., 1996), and Cm^S mutants were obtained and screened by PCR for the chromosomal *yggB* deletion. Strains carrying a deletion of *yjeP* were constructed by a similar strategy using primers listed in Table III.

The *yggB* gene was amplified using primers that flank the promoter and terminator regions (YM1F/YM2R; Table II) and cloned into a moderate copy number plasmid (pHG165; Ap^R; Stewart *et al.*, 1986) or a low copy number plasmid (pHSG575; Cm^R; Takeshita *et al.*, 1987) to create pMAJ8 and pyggB1, respectively. To facilitate complementation studies in strains carrying *mscL*::*cm*, which were resistant to chloramphenicol, pyggB1 was modified by introduction of a kanamycin resistance cassette (Pharmacia) into the multiple cloning site to create pyggB2. The accuracy of all PCR products was verified by DNA sequencing. Sequence comparisons were performed via BLASTP 2.0.5 (Altschul *et al.*, 1997) and using CLUSTAL programme (DNASTAR, Madison, WI).

Electrophysiology

Single-channel analysis (Hamill *et al.*, 1981) was performed on giant protoplasts (Martinac *et al.*, 1987). Recordings were undertaken in the cell-attached configuration. Electrodes were pulled from borosilicate capillary tubes (Drummond Scientific Company) on a micropippette puller (Sutter Instrument Company) to give a pipette resistance of ~3 MΩ in bath solution (200 mM KCl, 90 mM $MgCl_2$, 10 mM $CaCl_2$, 5 mM HEPES, 300 mM sucrose, pH 7). It was observed that mutants lacking KefA had altered osmotic properties, and to stabilize the protoplasts a higher osmolarity solution was used for all experiments. The pipette solution was identical except that it lacked sucrose. Negative pressure was applied using a syringe, assessed by a pressure monitor (World Precision Instruments) and calibrated using a transducer (Micro Switch, Newark Electronics). Currents were acquired with an Axopatch 200 A amplifier (Axon Instruments), filtered at 5 kHz, and recorded either digitally (Biologic DTR-1205 DAT recorder) and analysed in PAT (Dempster) or recorded and analysed using pCLAMP6 software. All measurements have been conducted on patches derived from at least two protoplast preparations.

Growth conditions and analysis of cell viability

Cells were grown aerobically at 37°C in conical flasks in citrate-phosphate defined medium (pH 7), with an osmolality of 220 mOsM and containing (per litre) 8.58 g Na_2HPO_4, 0.87 g K_2HPO_4, 1.34 g citric acid, 1.0 g (NH_4SO_4), 0.001 g thiamine, 0.1 g $MgSO_4{\cdot}7H_2O$ and 0.002 g $(NH_4)_2SO_4{\cdot}FeSO_4{\cdot}6H_2O$. A single colony was used for the overnight inoculum into medium containing 0.04% (w/v) glucose and supplemented the following morning with glucose to 0.2% (w/v). After one doubling, the culture was diluted into medium of identical composition and grown to exponential phase, before diluting into medium with 0.5 M NaCl to OD_{650} = 0.05. This culture was incubated to OD_{650} = 0.35 before 20-fold dilution into pre-warmed medium with (control) or without (downshock) NaCl (0–0.5 M). To determine cell viability, 50 µl samples were taken at the times indicated, serial-diluted and four 5 µl samples were spotted onto LB agar plates. The osmolarity of the dilution medium and plates was maintained constant during the recovery. Plates were incubated overnight at 37°C and the CFUs were enumerated the following morning. For determination of viability after combined acid and osmotic downshock, cells of Frag1 were grown to exponential phase in minimal medium (pH 7) supplemented with 0.3 M NaCl and then diluted 20-fold into minimal medium (pH 3.6) supplemented with a range of NaCl concentrations. After 30 min, samples were taken for serial dilution in minimal medium (pH 7).

Potassium efflux

Cells were grown as described above. When cells were in steady state exponential growth in the presence of 0.5 M NaCl, an aliquot (5 ml) was filtered (Whatmann 2.5 cm, 0.45 µm pore size) and the filter immediately transferred to a Falcon tube containing 5 ml of pre-warmed minimal medium with the required NaCl concentration. The tube was vortexed for 15 s and 2× 1 ml samples transferred to Eppendorf tubes. The cells were collected by centrifugation (Jouan A14 full speed, 30 s), the pellet was re-suspended in 1 ml of distilled water and the potassium content was determined as described previously (Ferguson *et al.*, 1993).

Measurement of cell lysis

Cells were grown as described above in the presence of 0.5 M NaCl to OD_{650} = 0.35, filtered and re-suspended in an equal volume of medium in the presence or absence of 0.5 M NaCl. At intervals, 3 ml samples were removed, the OD_{650} measured and samples centrifuged (Jouan A14 full speed, 30 s), and the $A_{260/280}$ determined.

Acknowledgements

The authors thank their colleagues Gwenola Gouesbet and Debra McLaggan for their advice, Jayne Glover for development of the combined pH and downshock assay, and Ching Kung, Paul Blount, Andrej Kubalski, Sergei Sukharev (University of Wisconsin, Madison, WI) and Paco Herson (University of Aberdeen, UK) for their assistance in the development of patch–clamp techniques. Plasmid pHSG575 was obtained from Erhard Bremer (University of Marburg, Germany). The work was supported by The Wellcome Trust (040174), BBSRC grant (F01296), a research leave fellowship to I.R.B. and by a BBSRC Food Directorate studentship to N.R.S.

References

Ajouz,B., Berrier,C., Garrigues,A., Besnard,M. and Ghazi,A. (1998) Release of thioredoxin via the mechanosensitive channel MscL during osmotic downshock of *Escherichia coli* cells. *J. Biol. Chem.*, **273**, 26670–26674.

Altschul,S.F., Madden,T.L., Schaffer,A.A., Zhang,J., Zhang,Z., Miller,W. and Lipman,D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programmes. *Nucleic Acids Res.*, **25**, 3389–3402.

Berrier,C., Coulombe,A., Szabo,I., Zoratti,M. and Ghazi,A. (1992) Gadolinium ions inhibit the loss of metabolites induced by osmotic shock and large stretch-activated channels in bacteria. *Eur. J. Biochem.*, **206**, 559–565.

Berrier,C., Besnard,M., Ajouz,B., Coulombe,A. and Ghazi,A. (1996) Multiple mechanosensitive ion channels from *Escherichia coli*, activated at different thresholds of applied pressure. *J. Memb. Biol.*, **151**, 175–187.

Blount,P., Sukharev,S.I., Moe,P.C., Schroeder,M.J., Guy,H.R. and Kung,C. (1996a) Membrane topology and multimeric structure of a mechanosensitive channel protein of *Escherichia coli*. *EMBO J.*, **15**, 4798–4805.

Blount,P., Sukharev,S.I., Schroeder,M.J., Nagle,S.K. and Kung,C. (1996b) Single residue substitutions that change the gating properties of a mechanosensitive channel in *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **93**, 11652–11657.

Blount,P., Sukharev,S.I., Moe,P.C., Nagle,S.K. and Kung,C. (1996c) Towards an understanding of the structural and functional properties of MscL, a mechanosensitive channel in bacteria. *Biol. Cell*, **87**, 1–8.

Blount,P., Schroeder,M.J. and Kung,C. (1997) Mutations in a bacterial mechanosensitive channel change the cellular response to osmotic stress. *J. Biol. Chem.*, **272**, 32150–32157.

Booth,I.R., Pourkomailian,B.P., McLaggan,D. and Koo,S.P. (1994) Mechanisms controlling compatible solute accumulation: a

consideration of the genetics and physiology of bacterial osmoregulation. In Fito,P., Mulet,A. and McKenna,B. (eds), *Water in Foods*. Elsevier, Amsterdam, The Netherlands, pp. 381–388.

Calamita,G., Bishai,W.R., Preston,G.M., Guggino,W. and Agre,P. (1996) Molecular cloning and characterization of AqpZ, a water channel from *Escherichia coli*. *J. Biol. Chem.*, **270**, 29063–29066.

Chang,G., Spencer,R.H., Lee,A.T., Barclay,M.T. and Rees,D.C. (1998) Structure of the MscL Homologue from *Mycobacterium tuberculosis*: a gated mechanosensitive ion channel. *Science*, **282**, 2220–2226.

Csonka,L.N. and Epstein,W. (1996) Osmoregulation. In Curtis,R.,III, Ingraham,J.L., Lin,E.C.C., Low,K.B., Magasanik,B., Reznikoff,W.S., Tiley,M., Schaechter,M. and Umbarger,H.E. (eds), *Escherichia coli and Salmonella typhimurium Cellular and Molecular Biology*. ASM Press, Washington, DC, pp. 1210–1224.

Cui,C. and Adler,J. (1996) Effect of mutation of potassium-efflux system, KefA, on mechanosensitive channels in the cytoplasmic membrane of *Escherichia coli*. *J. Memb. Biol.*, **150**, 143–152.

Cui,C., Smith,D.O. and Adler,J. (1995) Characterization of mechanosensitive channels in *Escherichia coli* cytoplasmic membranes by whole cell patch clamp recording. *J. Memb. Biol.*, **144**, 31–42.

Epstein,W. and Kim,B.S. (1971) Potassium transport loci in *Escherichia coli* K-12. *J. Bacteriol.*, **108**, 639–644.

Ferguson,G.P., Munro,A.W., Douglas,R.M., McLaggan,D. and Booth,I.R. (1993) Activation of potassium channels during metabolite detoxification in *Escherichia coli*. *Mol. Microbiol.*, **9**, 1297–1304.

Hamill,O.P., Marty,A., Neher,E., Sakmann,B. and Sigworth,F.J. (1981) Improved patch–clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.*, **391**, 85–100.

Ho,S.N., Hunt,H.D., Horton,R.M., Pullen,J.K. and Pease,L.R. (1989) Site-directed mutagenesis using polymerase chain reaction. *Gene*, **77**, 51–59.

Holtje,J.-V. (1995) From growth to autolysis: the murein hydrolases in *Escherichia coli*. *Arch. Microbiol.*, **164**, 243–254.

Kaniga,K., Delor,I. and Cornelis,G.R. (1991) A wide-host-range suicide vector for improving reverse genetics in Gram negative bacteria: inactivation of the *bla* gene of *Yersinia enterocolitica*. *Gene*, **109**, 137–141.

Koch,A.L. (1983) The surface stress theory of microbial morphogenesis. *Adv. Microbiol. Physiol.*, **24**, 301–336.

Kyte,J. and Doolittle,R.F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.*, **157**, 105–132.

Le Dain,A.C., Saint,N., Kloda,A., Ghazi,A. and Martinac,B. (1988) Mechanosensitive ion channels of the archeon *Haloferax volcanii*. *J. Biol. Chem.*, **273**, 12116–12119.

Martinac,B., Buechner,M., Delcour,A.H., Adler,J. and Kung,C. (1987) Pressure-sensitive ion channel in *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **84**, 2297–2301.

Milton,D.L., O'Toole,R., Horstedt,P. and Wolf-Watz,H. (1996) Flagellin A is essential for virulence of *Vibrio anguillarum*. *J. Bacteriol.*, **178**, 1310–1319.

Moe,P.C., Blount,P. and Kung,C. (1998) Functional and structural conservation in the mechanosensitive channel MscL implicates elements crucial for mechanosensation. *Mol. Microbiol.*, **28**, 583–592.

Ou,X., Blount,P., Hoffman,R.J. and Kung,C. (1998) One face of a transmembrane helix is crucial in mechanosensitive channel gating. *Proc. Natl Acad. Sci. USA*, **95**, 11471–11475.

Poolman,B. and Glaasker,E. (1998) Regulation of compatible solute accumulation in bacteria. *Mol. Microbiol.*, **29**, 397–407.

Saint,N., Lacapere,J.-J., Gu,L.-Q., Ghazi,A., Martinac,B. and Rigaud,J.-L. (1998) A hexameric transmembrane pore revealed by two-dimensional crystallization of the large mechanosensitive ion channel (MscL) of *Escherichia coli*. *J. Biol. Chem.*, **273**, 14667–14670.

Sanders,J.W., Venema,G., Kok,J. and Leenhouts,K. (1998) Identification of a sodium chloride-regulated promoter in *Lactococcus lactis* by a single copy chromosomal fusion with a reporter gene. *Mol. Gen. Genet.*, **257**, 681–685.

Schleyer,M., Schmid,R. and Bakker,E.P. (1993) Transient, specific and extremely rapid release of osmolytes from growing cells of *Escherichia coli* K-12 exposed to hypoosmotic shock. *Arch. Microbiol.*, **160**, 424–431.

Simon,R., Priefer,U. and Puhler,A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Biotechnology*, **1**, 787–796.

Stewart,G.S.A.B., Lubinsky-Mink,S., Jackson,C.G., Cassel,A. and Kuhn,J. (1986) pHG165: A pBR322 copy number derivative of pUC8 for cloning and expression. *Plasmid*, **15**, 172–181.

Sukharev,S.I., Blount,P., Martinac,B., Blattner,F.R. and Kung,C. (1994) A large-conductance mechanosensitive channel in *E.coli* encoded by MscL alone. *Nature*, **368**, 265–268.

Sukharev,S.I., Blount,P., Martinac,B. and Kung,C. (1997) Mechanosensitive channels of *Escherichia coli*: The MscL gene, protein and activities. *Annu. Rev. Physiol.*, **59**, 633–657.

Szabo,I., Petronelli,V. and Zoratti,M. (1993) A patch–clamp study of *B. subtilis*. *J. Memb. Biol.*, **131**, 203–218.

Takeshita,S., Sato,M., Toba,M., Masahashi,W. and Hashimoto-Gotoh,T. (1987) High copy number and low copy number plasmid vectors for *lacZ* α complementation and chloramphenicol- or kanamycin-resistance selection. *Gene*, **61**, 63–74.

Zoratti,M. and Petronelli,V. (1988) Ion-conducting channels in Gram positive bacteria. *FEBS Lett.*, **240**, 105–109.

Received January 18, 1999; revised February 15, 1999; accepted February 18, 1999