

Lactococcus lactis Uses MscL as Its Principal Mechanosensitive Channel*

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Joost H. A. Folgering‡, Paul C. Moe§¶, Gea K. Schuurman-Wolters‡, Paul Blount§¶, and Bert Poolman‡¶

From the ‡Department of Biochemistry, Groninger Biomolecular Sciences and Biotechnology Institute and Material Science Centre-plus, University of Groningen, Nijenborgh 4, 9747 AG, Groningen, The Netherlands and the §Department of Physiology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9040

The functions of the mechanosensitive channels from Lactococcus lactis were determined by biochemical, physiological, and electrophysiological methods. Patch-clamp studies showed that the genes yncB and mscL encode MscS and MscL-like channels, respectively, when expressed in Escherichia coli or if the gene products were purified and reconstituted in proteoliposomes. However, unless yncB was expressed in trans, wild type membranes of L. lactis displayed only MscL activity. Membranes prepared from an mscL disruption mutant did not show any mechanosensitive channel activity, irrespective of whether the cells had been grown on low or high osmolarity medium. In osmotic downshift assays, wild type cells survived and retained 20% of the glycine betaine internalized under external high salt conditions. On the other hand, the mscL disruption mutant retained 40% of internalized glycine betaine and was significantly compromised in its survival upon osmotic downshifts. The data strongly suggest that L. lactis uses MscL as the main mechanosensitive solute release system to protect the cells under conditions of osmotic downshift.

Mechanosensitive channels play an important role in prokaryotic cell volume regulation (1). In small, single-cell organisms, this regulation can mean the difference between life and death under extreme osmotic downshift conditions. By diffusion over the semipermeable membrane and/or aquaporins embedded in the membrane, water can enter and leave the cell until equilibrium is established between internal and external osmolality. This allows microorganisms to adapt to changes in external osmolyte concentrations. When the external osmolyte concentrations increase (hyperosmotic stress), water will leak out of the cell, causing loss of turgor, and ultimately the cell may plasmolyse. Bacteria respond to this hyperosmotic stress by rapid uptake of ions (K⁺) and/or compatible solutes or increasing the intracellular osmolyte concentration through synthesis of compatible solutes. The increase in internal compatible solute concentration compensates for the high external osmolality, allowing water to diffuse back and the cell to regain its original volume and turgor (2–4).

Conversely, when the external osmolyte concentration suddenly decreases, water will diffuse into the cell, causing it to swell and, in extreme conditions, lyse. This is where the mechanosensitive channels are thought to play a role by opening in response to the increased membrane tension effected by the rapid increase in cell volume. The best known example of a channel in this role is the mechanosensitive channel of large conductance from Escherichia coli (MscLEc), but homologues are present in most eubacteria (5–7). MscL opens near the lytic tension limit of the bacterial membrane. A second mechanosensitive channel, that of small conductance, MscS, has been characterized in only a few organisms (8, 9). MscS opens at lower membrane tensions and has a smaller conductance than MscL, making it useful for fine regulation of internal compatible solute concentration. Crystal structures of MscL and MscS are available (10, 11). The electrophysiological characteristics of MscS from E. coli (12) and of a number of MscL homologues (5) have been described. So far, their physiological roles in osmoprotection are not fully understood.

Previously, we have established the mechanism of the osmotic regulation of the upshift-activated glycine betaine transporter OpuA from Lactococcus lactis (13, 14). Although the physicochemical properties of the membrane and lipid-protein interactions also play a critical role in the osmotic activation of OpuA, the mechanism is entirely different from that underlying the gating of MS1 channels. We have shown that increasing internal ionic strength, a consequence of osmotic upshift, activates OpuA by altering the electrostatic interactions between anionic lipids and charged residues at the cytoplasmic face of the protein (15). For a better understanding of the total osmoregulatory response of L. lactis, we present here the electrophysiological and biochemical characterization of L. lactis MscL (MscL1, 45.4% identity with MscLEc; Fig. 1A) and YncB (hereafter referred to as MscS1, 24.0% identity with MscS from E. coli; Fig. 1B) and show that MscL1 is critical for protection of L. lactis against osmotic downshifts. In fact, the majority of glycine betaine, accumulated upon upshift activation of OpuA, seems to exit the cell via MscL1 upon subsequent osmotic downshift. We also examined the expression of the oppA, mscL, and yncB genes during cell growth under low and high salt conditions.

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‡ To whom correspondence should be addressed. Tel.: 31-50-3634190; Fax: 31-50-3634165; E-mail: B.Poolman@rug.nl.

¶ The abbreviations used are: MS, mechanosensitive; GUV, giant unilamellar vesicle; MTSET, [2-(trimethylammonium)ethyl] methanesulfonate; CFU, colony-forming units; CDM, chemically defined medium; RT, reverse transcription; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.
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MATERIALS AND METHODS

Strains, Plasmids, and Growth Conditions
Experiments were performed using the strains listed in Table I and plasmids listed in Table II. E. coli strains were grown in Luria broth, with 100 μg/ml ampicillin when required. L. lactis cells were grown in M17 broth (Difco) or chemically defined medium (27) supplemented with 25 μM glucose and 365 mM KCl for high salt conditions (1050 mM KCl). Chloramphenicol (5 μg/ml) or erythromycin (5 μg/ml) was added when the cells were transformed with plasmids. For growth on solid medium, 1.5% (w/v) agar was added to the broth.

Cloning of mscL and yncB
The primers that were used for the amplification of mscL and yncB are listed in Table III. Since the yncB gene product is homologous to MscE from E. coli and functions similarly, the YncB protein is referred to as MscSL1. The superscripts L1 and L4 will be used to denote genes or proteins from L. lactis and E. coli, respectively. For PCR amplifications, Expand High-Fidelity DNA polymerase (Roche Applied Science) was used, and reactions were performed according to the manufacturer’s instructions. Chromosomal DNA of L. lactis IL1403 was used as template, the annealing temperature was 50 °C for mscL1 and 53 °C for yncB, and the elongation times were 45 s and 60 s, respectively. For the histidine-tagged version of MscL1, a two-step PCR was performed using primers LL SD5’ and LL SD4Hi (see Table III) to introduce a HindIII site, and then a second step was used to engineer another two histidines, a stop codon and a restriction site for cloning.

The mscL1 and mscL1-6H genes were first subcloned in pBluescript, isolated from E. coli JM110, using the XhoI-XbaI restriction sites. This construct was used to transform JM110 and, after plasmid isolation, the genes were excised from the Bluescript vector and ligated into pNZ8020 and pBl10b again using the XhoI-XbaI restriction sites. The resulting plasmids pNZ8020mscL16H and pB10bmscL16H were then used to transform L. lactis JIM7049 and E. coli PB104, respectively. YncB104H was ligated directly after PCR into the vectors pNZ8048, pET32a, and pBAD and used to transform L. lactis IL1403 and E. coli JM465, respectively. Finally, all constructs were midi-prepped (Qiagen), and the DNA sequence was analyzed to confirm fidelity.

Construction of Single Cysteine Mutants and a L. lactis mscL Disruption Strain
The primers used for the construction of the mutants of MscL1 are listed in Table III. The cysteine mutants were constructed using pBl10bmscL1-6H as template. First, LL SD5 was combined with the primer for the specific mutant to create a megaprimer, which in a second amplification step was combined with LL SD5’ to obtain the complete mscL sequence with the desired cysteine modification. All other conditions were as described above for the cloning of MscL6H.

For the construction of the L. lactis JIM7049 mscL disruption strain, an internal gene fragment, from codon 12 to codon 86, was amplified. Primers used with truncation mutants of E. coli MscL were designed to introduce a XhoI and BamHI restriction site at the 5’ and 3’ end of the gene, respectively. For this construct, the DNA sequence was analyzed by ultracentrifugation at 270,000 × g for 20 min at 4 °C. This supernatant was then loaded onto nickel-nitrilotriacetic acid affinity resin pre-equilibrated with solubilization buffer. The column was washed with 20–30 volumes of solubilization buffer containing 0.5% Triton X-100, and the proteins were eluted with 50 mM KPi, pH 7.0, plus 0.2% Triton X-100, plus 0.2% Triton X-100 with a step gradient of imidazole. The purified proteins were mixed with Triton X-100-stabilized preformed liposomes (10 mg/ml lipid); the lipid mixtures were composed of dioleoylphosphatidylcholine, dioleoylphosphatidylethanolamine, and/or dioleoylphosphatidylserine), typically at 30 °C for 20 min and under continuous stirring of the suspension. The solubilized proteins and remaining membrane material were separated by ultracentrifugation at 270,000 × g for 20 min at 4 °C. The supernatant was filtered to decrease electronic noise, using Clampfit 8.0 software (Axon Instruments) with the low elongation time was 60 s. The products were analyzed on a 1.5% agarose gel after 15 and 22 cycles. As a control, the PCR was also performed on chromosomal DNA of L. lactis; as a negative control, the RNA samples were not reverse-transcribed, except that avian leukosis virus reverse transcriptase was omitted in the reaction with RNA as template.

Preparation of MS Channel-containing Membranes for Patch Clamp Analysis

Hybrid Proteo-GUVs—L. lactis NZ9000 containing pNZ8020mscL16H was grown in M17-glucose medium to an Absof of 0.8, after which mscL expression was induced for 3 h with a 1:1000 dilution of the supernatant of a culture from the nisinA-producing L. lactis NZ700 strain (20). For growth of single membrane vesicles were prepared by lysing the bacteria (20 mg/ml protein) with a high pressure homogenizer (Kindler type NN2002; single passage at 10,000 p.s.i.), following (partial) digestion of the cell wall with 10 mg/ml lysozyme for 30 min at 30 °C. After removing the cellulose matrix and cell wall debris by centrifugation at 20,000 × g, the membrane vesicles were washed once by centrifugation at 150,000 × g and then resuspended in 50 mM KPi, pH 6.5. Aliquots of 0.5 ml were frozen in liquid nitrogen and stored at −80 °C. The membrane vesicles were mixed with liposomes (2:25 (w/v) total membrane protein/lipid ratio), centrifuged at 270,000 × g, and resuspended in 5% ethylene glycol and dehydrated on a glass slide. To obtain fused proteo-GUVs, rehydration was performed by placing rehydration buffer (200 mM KCl, 0.1 mM EDTA, 0.01 mM CaCl2 plus 5 mM HEPES, pH 7.2) on top of the vesicles and membranes to obtain a final lipid concentration of 100 mg/ml as described (30). Alternatively, after drying on glass slides coated with indium tin oxide, hybrid proteo-GUVs were obtained by rehydration in a flow chamber with 300 μl of 10 mM KCl, 2 mM MgCl2, 0.25 mM HEPES, pH 7.2, plus 320 mM sucrose (the sucrose was added to make the rehydration medium equiosmolar to the buffer for patch clamp analysis). The flow chamber was closed with a second indium tin oxide-coated glass slide. A voltage of 1.2 V at 10 Hz was applied for at least 3 h through electrodes sealed on the glass plates. The resulting giant unilamellar vesicles (GUWs), 5–50 μm in diameter, were used in patch clamp experiments (31).

Proteo-GUVs—Membrane vesicles, containing overexpressed channel protein (MscL1 or MscSL1), were solubilized in 50 mM KPi, 35 mM imidazole, 300 mM NaCl, pH 7.0, plus 5% octyl-β-D-glucoside (Anatrace) at 4 °C for 20 min and under continuous stirring of the suspension. The solubilized proteins and remaining membrane material were separated by ultracentrifugation at 270,000 × g for 20 min at 4 °C. The supernatant was loaded onto nickel-nitrilotriacetic acid affinity resin pre-equilibrated with solubilization buffer. The column was washed with 20–30 volumes of solubilization buffer containing 0.5% Triton X-100, and the proteins were eluted with 50 mM KPi, pH 7.0, plus 0.2% Triton X-100 with a step gradient of imidazole. The purified proteins were mixed with Triton X-100-stabilized preformed liposomes (10 mg/ml lipid); the lipid mixtures were composed of dioleoylphosphatidylcholine, dioleoylphosphatidylethanolamine, and/or dioleoylphosphatidylserine), typically at protein/lipid ratios of 1:2000–20,000 (mol of monomeric channel protein/ mol of lipid), and reconstitutions were performed as described (32). The proteoliposomes were converted to proteo-GUVs by dehydorisation and rehydration in the presence or absence of an electrical field, as described under “Hybrid Proteo-GUVs.”

Spheroplasts—For determination of pressure ratios, relative to MscS−, of MscL1 and the individual cysteine mutants, giant spheroplasts were prepared from E. coli JM110 containing pET32aΔMscL16H or its derivatives. For other channel characteristics, giant spheroplasts were prepared from E. coli MJF465 containing pBl10bmscL12, pBl10bmscL16H, or pET32Δ4yncB104H. All spheroplasts were prepared as described (30).

Patch Clamp Experiments
Experiments were performed as described previously (30). After preparation, an aliquot of proteo-GUVs or 1–5 μl of a spheroplast sample was transferred to a sample chamber containing a ground electrode and 300 μl of pure water. A 200 μl pipette buffer: 5 mM KCl, 40 mM MgCl2 for proteo-GUVs; 5 mM HEPES, pH 7.2, 200 mM KC1, 90 mM MgCl2, 10 mM CaCl2 for spheroplasts. Channel activity was recorded using an Axopatch 200A amplifier together with a digital converter and Axoscope software (Axon Instruments, Foster City, CA). Data were acquired at a sampling rate of 33 kHz and filtered at 10 kHz. The presented traces were additionally filtered to decrease electronic noise, using Clampfit 8.0 software (Axon Instruments) with the low
pass Boxcar filter at smoothing point 7. Offline analysis was performed using PClamp 6.0 software (Axon Instruments).

**Glycine Betaine Efflux**

Glycine betaine efflux was performed as described \(^{(13)}\) with some modifications. Cells were grown overnight to late log phase in high osmolality medium (1050 mosmol/kg) and washed three times in their original volume with an equimosmolar buffer (50 mM KPi, 500 mM KCl, pH 6.5). The cells were then resuspended to a protein concentration of 10 mg/ml and stored on ice. For uptake of glycine betaine, the cells were diluted 10-fold in 50 mM KPi, pH 6.5, 500 mM KCl, supplemented with 10 mM glucose and 1.88 mM of [14C]glycine betaine. After 40 min of uptake at 30 °C, aliquots of the cells were subjected to no dilution or a 3-, 5-, 10-, 20-, 50-, and 100-fold dilution into 50 mM KPi, pH 6.5. This resulted in final osmolalities of 1050, 420, 295, 200, 155, 125, and 115 mosmol/kg, respectively. Samples were taken in 5-fold to determine the steady state internal glycine betaine concentrations prior to the osmotic downshift (final internal glycine betaine content) and at different time intervals (at 1 (in duplicate), 5, 10, and 30 min) after each of the downshifts. At least three independent uptake and downshift experiments were performed on three independent cultures of *L. lactis* IL1403 and JIM7049ΔMscL. The steady state levels of internalized glycine betaine after uptake were ∼900 nmol/mg protein for both strains. Release is plotted as a percentage of retained glycine betaine.

**Analysis of Cell Viability**

Survival of *E. coli* MJF465, carrying pB10bmscLLl6H, pET324yncB10H, or empty plasmid controls, under osmotic downshift conditions, was analyzed as described \(^{(12)}\). Because *E. coli* MJF465 is not able to use arabinose as an energy source and glucose represses the expression of the arabinose-inducible system, the isopropyl-1-thio-D-galactopyranoside-inducible construct pET324yncB10H was used here. The cysteine mutants were screened for survival with, and without, MTSET as described \(^{(33)}\). For survival under osmotic downshift conditions of *L. lactis* IL1403 or JIM7049ΔMscL, cells were grown overnight in chemically defined medium \(^{(27)}\), supplemented with 25 mM glucose and 5 μg/ml erythromycin (where applicable), and diluted 1:100 to the same medium supplemented with 365 mM KCl (1050 mosmol/kg). Cells were allowed to grow to an A\(_{600}\) of 0.4 and then diluted 100-fold into 50 mM KPi, pH 6.5, plus 500 mM KCl (1050 mosmol/kg) or into 50 mM KPi, pH 6.5 (final osmolality of 115 mosmol/kg). Prior to plating onto agar-containing media, the cells were diluted serially with sterile
equimolar buffers, all prewarmed to 30 °C. To determine cell viability, 20-μl samples were spotted onto equimolar CDM agar plates and incubated for 36 h at 30 °C before the number of colony-forming units (CFU) was determined.

Metabolic Activity after Osmotic Downshift

Because *L. lactis* grows in chains, the number of CFU is not necessarily a quantitative indicator of the survival of cells after downshift. Therefore, metabolic activity based on the production of acid by *L. lactis* during fermentation of glucose was also determined. The method was adapted from Ref. 34. In brief, cells were cultured, washed, and osmotically stressed as described above for the glycine betaine efflux assay, but with 5 mM KPi, pH 6.5, and 65 mM KC1 (105 mosmol/kg) instead of 50 mM KP, buffer (105 mosmol/kg) to reduce the buffering capacity. Acidification rates per mg of total protein in the presence of 10 mM glucose were determined for all samples and compared with the acidification rates of the unshocked sample (100% value).

Other Procedures

**Protein Biochemistry**—Purified proteins were analyzed on 15% acrylamide SDS-PAGE (35). Protein concentration was determined by Amido Black 10B staining as described (36). MscL was also identified...
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RESULTS

Cloning, Expression, and Purification

Cloning and expression of mscL\(^{L1}\) and yncBL\(^{L1}\) was achieved in both E. coli (not shown) and L. lactis (Fig. 2A). For amplification of MscL\(^{L1}\) and MscS\(^{L1}\) in L. lactis, the nisin-inducible expression system was used (38). In E. coli, the lacUV5 promoter system was used for amplification of MscL\(^{L1}\), and the arabinose-inducible system was used for amplification of MscS\(^{L1}\). MscL\(^{L1}-6\)H and MscS\(^{L1}-10\)H were purified by nickel-nitritotriacetic acid chromatography. Based on the yield of purified protein, both channels were found to be amplified in L. lactis to levels between 5 and 10% and in E. coli between 2 and 5% of total membrane protein.

As can be seen in Fig. 2, A and B, MscL\(^{L1}\) protein amplified in and purified from L. lactis NZ29000 migrated over a wide range in SDS-PAGE and gave rise to a “fuzzy” appearance. To establish the nature of the differently migrating species, various fractions from the gel were analyzed by MALDI-TOF mass spectrometry. Four distinct slices of the gel were digested with CNBr and trypsin, and the resulting peptide fragments were identified. The fragments of the protein, after cleavage, accounted for 70% of the total protein (data not shown), including both the N and C termini, indicating that the heterogeneity observed on the gel was not due to proteolytic degradation of the amplified protein. We suggest that the broad and fuzzy band reflects incomplete unfolding of the protein in the presence of SDS or a very strong association between individual subunits, which is only dissociated during SDS-PAGE analysis.

In Vivo Complementation of E. coli MJF465 (MscL\(^{L1}\)/MscS\(^{L1}\))

For initial characterization of MscL\(^{L1}\) and MscS\(^{L1}\), the corresponding genes were expressed in E. coli MJF465 (MscL\(^{L1}\)/MscS\(^{L1}\)), using plasmids pB10mscL\(^{L1}\)-6H and pET24yncB10H. This allowed a comparison of the ability of the L. lactis and E. coli channels to rescue the osmotic-fragile phenotype of E. coli MJF465 (12). Clearly, both MscL\(^{L1}\) and MscS\(^{L1}\) were able to rescue MJF465 in osmotic downshift experiments (Table IV), albeit not as well as the endogenous E. coli channels.

It has been demonstrated that MscL\(^{Ec}\) can be activated by MTSET modification of a cysteine introduced at position 22 (33, 39, 40). The equivalent residue, Gly\(^{20}\), in MscL\(^{L1}\) and its flanking residues (Val\(^{21}\), Ile\(^{22}\), and Ile\(^{23}\); boxed region in Fig. 1) were replaced by cysteines, and the phenotype of the mutants, upon labeling with MTSET, was determined (Table IV). The cysteine positions that showed a clear gain-of-function phenotype as a result of increased channel activity after reaction with MTSET under iso-osmotic conditions, G20C and V21C (equivalent to positions G22C and V23C in MscL\(^{Ec}\)), were further characterized in patch clamp experiments.

Electrophysiological Characterization

MscL\(^{L1}\) in E. coli—Channel activity of an excised patch from E. coli MJF465 expressing MscS\(^{L1}\)-10H (from pBADyncB10H) is shown in Fig. 3A. The slope of the current-voltage relationship in Fig. 4A indicates that MscS\(^{L1}\) allows a conductance of 0.67 ± 0.07 nanosiemens. The fact that the data in Fig. 4A could fit to a straight line reveals that the channel shows no rectification between −50 and 50 mV. Open dwell times of one to several hundred ms were observed, and the channel appeared to desensitize when pressure was maintained for longer periods of time (n = 5). Overall, the channel characteristics of MscS\(^{L1}\) are analogous to those described previously for MscS from E. coli (12).

MscL\(^{L1}\) in E. coli—Channel activity of an excised patch from E. coli MJF465, expressing MscL\(^{L1}\)-6H (from pB1DyncB10H) is shown in Fig. 3B. The current-voltage relationship for MscL\(^{L1}\) (Fig. 4A) shows that MscL\(^{L1}\) has a conductance of 2.00 ± 0.29 nanosiemens. Also, MscL\(^{L1}\) did not show rectification between −50 and 50 mV. Two short but distinct dwell times were observed in 34 independent recordings (both from lipid fusions and spheroplasts; Fig. 4B). One dwell time could be determined at 3.6 ± 0.29 ms (mean and S.E.); the second was shorter than 1 ms and could not be determined more accurately given the experimental parameters. These dwell times are comparable with the dwell times of MscL from Staphylococcus aureus and are shorter than the dwell times observed for MscL from E. coli (5). The pressure at which MscL\(^{L1}\) fully opened for the first time was determined and compared with the pressure at which MscS\(^{Ec}\) opened in giant spheroplasts from E. coli PB104. The pressure ratio of MscL\(^{L1}\)/MscS\(^{Ec}\) (i.e., the pressure at which MscL first opens fully relative to the pressure at which MscS \(^{Ec}\) opens in giant spheroplasts of E. coli PB104) was 1.81 ± 0.09 (n = 20). This means that there might be a small difference in the pressure sensitivity of MscL\(^{L1}\) compared with MscL\(^{Ec}\),
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Survival upon osmotic downshift of E. coli MJF465 (MscL\textsuperscript{-}/MscS\textsuperscript{-}) carrying plasmids for MscL\textsubscript{Ec} (pB10bmscL\textsubscript{Ec}6H), MscL\textsubscript{Li} (pB10bmscL\textsubscript{Li}6H), MscL\textsubscript{Ec} (pyggb2), MscS\textsubscript{Li} (pET324yncB10H), or empty plasmid controls (pB10B, pET324). Survival upon modification with MTSET (1 mM, final concentration) of E. coli PB104 (MscL\textsuperscript{-}) carrying pB10bmscL\textsubscript{Li}6H or one of the derived cysteine mutants pB10bmscL\textsubscript{Li6H} G20C, pB10bmscL\textsubscript{Li6H} V21C, pB10bmscL\textsubscript{Li6H} I22C, or pB10bmscL\textsubscript{Li6H} I23C. 100% survival was set for unchallenged cells. For all values, n \geq 3.

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Data from Ref. 12.

which was found to have a MscL\textsubscript{Ec}/MscS\textsubscript{Ec} pressure ratio of 1.64 \pm 0.08 (41). The open probability of MscL\textsubscript{Li} was determined by the current average (measured at a given pressure) divided by the current if all channels in the patch were open. The open probability was plotted against the pressure ratio MscL/MscS\textsubscript{Ec} and fitted to a Boltzmann distribution (Fig. 4C). Both MscL\textsubscript{Ec} and MscL\textsubscript{Li} show initial openings around the same relative pressure. However, further opening of MscL\textsubscript{Li} required a higher energy input than opening of MscL\textsubscript{Ec} as reflected by the different slopes of the sigmoidals (42). Finally, MscL\textsubscript{Li} appeared to have distinct substates at 0.26, 0.53, and 0.75 of full conductance (Fig. 4D), which compares with 0.22, 0.45, 0.70, and 0.93 of full opening for MscL\textsubscript{Ec} (43).

Table IV

![Figure 3](image-url)  
**Fig. 3.** Typical electrophysiological recordings at 20 mV (pipette) of patches excised from MJF465 spheroplasts expressing pBADyncB10H (A), MJF465, pB10bmscL\textsubscript{Li6H} spheroplasts (B), IL1403 membranes fused with liposomes (C), JIM7049, pNZ8048yncB10H membranes fused with liposomes (D), and JIM7049A/MscL\textsubscript{Li} membranes fused with liposomes (E). The top traces show current (bar corresponds to 50 pA for all traces), and the bottom traces show applied suction pressure (bar corresponds to 50 mm Hg for all traces). The horizontal bars below the traces indicate 200 ms for each trace. All traces start at 0 mm Hg pressure and 0 pA current and were filtered in Clampfit 8.0 software (Axon Instruments) with the low pass Boxcar filter at smoothing point 7. * first openings of MscS\textsubscript{Li}, # first full openings of MscL\textsubscript{Li}, # partial opening MscL\textsubscript{Li}.

Cysteine Mutants of MscL\textsubscript{Li} in E. coli—The cysteine mutants that had a gain-of-function phenotype in response to MTSET labeling were analyzed with respect to the pressure ratio MscL\textsubscript{Li}/MscS\textsubscript{Ec}, using E. coli PB104 expressing the channel from pB10bmscL\textsubscript{Li6H} (G20C) or pB10bmscL\textsubscript{Li6H} (V21C). Both G20C and G21C had a high pressure ratio of 2.6 \pm 0.3 (n = 8) and 3.7 \pm 0.2 (n = 4), respectively, in the absence of MTSET, but their activity became pressure-independent upon the addition of MTSET (data not shown).

**Expression of mscL, yncB, and OpuAA**

Since electrophysiological characterization of L. lactis IL1403 and JIM7049A/MscL membranes, fused with liposomes, showed no MscL\textsubscript{Li} activity (Fig. 3C), it seemed possible that yncB was not transcribed in these cells. RT-PCR was performed on RNA extracted from L. lactis IL1403 cells grown in CDM or CDM plus 0.5 mM NaCl, and the resulting amplified DNA fragments were analyzed on gel (Fig. 5). After 15 PCR cycles, it is clearly visible...
that the transcription of opuAA (the first gene of the opuA operon) was increased in the cells grown at high salt (Fig. 5A, compare lane A3 with lane B3), which is in agreement with a higher glycine betaine uptake capacity under these conditions (13). RT-PCR DNA corresponding to mscL and yncB fragments were visible after 22 PCR cycles. Both mscL and yncB are transcribed in wild type L. lactis IL1403 cells, albeit to a low level compared with the opuAA gene of the opuA operon; the expression of the mscL and yncB was not significantly influenced by the osmolality of the medium (Fig. 5B, compare lanes A1 and B1 and compare lanes A2 and B2). The negative controls showed that the extracted RNA was not contaminated with significant amounts of DNA, since no product was obtained when reverse transcriptase was omitted from the reaction mixture (Fig. 5B, lanes A1/H11002 and B1/H11002); the lanes marked C1, C2, and C3 correspond to PCR products of mscL, yncB, and OpuAA, respectively, using chromosomal DNA as template.

**Physiological Characterization of JIM7049ΔMscL**

Plasmid pOri280ΔMscL is not replicated in L. lactis JIM7049 unless it integrates into the chromosome, which preferentially takes place at the homologous position of the chromosomal mscL gene. By selecting for clones that grow in the presence of erythromycin, a disruption strain of mscL was obtained. MscL1 could not be detected by Western blot analysis in membranes of these cells, using antibodies raised against MscL1 (Fig. 2B).

To determine the role of MscL1 under conditions of hypo-osmotic stress, L. lactis IL1403 and JIM7049ΔMscL cells were grown on CDM supplemented with 365 mM KCl. After washing in 50 mM KPi plus 500 mM KCl, pH 7.0, the cells were incubated with [14C]glycine betaine for 40 min to allow internalization of the compatible solute and thereby compensate for the high external osmolality. A steady state level of uptake was attained, corresponding to an internal concentration of about 900 nmol of glycine betaine/mg of total protein. The cells were then diluted into various hypotonic media, and the fate of the internal glycine betaine content was determined. In Fig. 6A the internal concentrations are expressed as a percentage of the internal glycine betaine before osmotic downshift. The L. lactis IL1403 cells released 80% of their glycine betaine when diluted 5-fold or more. The release of glycine betaine from L. lactis JIM7049ΔMscL was significantly lower; around 40% of the glycine betaine was retained by the cells. Taken as a whole, the data suggest that at least a fraction of the internalized glycine betaine is released via MscL1.

Next, cell survival was examined using protocols developed for E. coli. Under osmotic downshift conditions, 98% (n = 3) of wild type CFU survived and 80 ± 5% (n = 3) of the disruption
strain. Because \textit{L. lactis} grows in chains of mostly two or four cells, this could mean that in reality between 33 and 55\% of the cells survive; to obtain a CFU from a chain of cocci, it is sufficient if only a single cell is viable (also see “Discussion”).

To obtain a quantitative measure of the consequences of an osmotic downshift on the metabolic activity of \textit{L. lactis} IL1403 and JIM7049ΔMscL, the capacity to ferment glucose was determined before and after osmotic downshift. Cells were grown in high salt medium and preloaded with glycine betaine and subjected to osmotic downshifts as described above. For \textit{L. lactis} IL1403, only the metabolic activity after 100-fold dilution was determined, since even under this extreme downshift condition 100\% of glucose metabolism was retained. In contrast, the rate of acidification of the buffer around \textit{L. lactis} JIM7049ΔMscL decreased with -fold dilution (Fig. 6B). The decrease in metabolic activity is quantitatively similar to the release of glycine betaine from JIM7049ΔMscL, suggesting that the release of glycine betaine in the disruption mutant might be caused by loss of cell integrity rather than efflux through the MscL\textsuperscript{L1} channel.

**DISCUSSION**

\textbf{MS Channels of \textit{L. lactis}}—MscL and MscS from \textit{L. lactis} form functional mechanosensitive channels when expressed in \textit{E. coli} or \textit{L. lactis}. Both channels provided \textit{E. coli} MJF465 (MscS/MscL\textsuperscript{+}) with protection against osmotic downshifts as demonstrated in plate assays, and both channels were functional in patch clamp experiments using membrane patches from \textit{E. coli} spheroplasts, membrane patches from hybrid proteo-GUVs prepared from \textit{L. lactis} membrane vesicles, or membrane patches from proteo-GUVs prepared from purified and membrane-reconstituted protein. Importantly, in wild type \textit{L. lactis} only MscL\textsuperscript{L1} seems to be functional, and this MS channel appears critical for survival of the organism under conditions of hypo-osmotic stress.

The unitary conductances of MscS\textsuperscript{L1} and MscL\textsuperscript{L1} were 0.87 and 2.0 nano Siemens, respectively, which are within the range previously reported for MscS and MscL from other organisms (8, 9, 39). Open dwell times for MscL\textsuperscript{L1}6H were short compared with MscL\textsuperscript{L2} and most other MscL homologues studied to date, giving rise to a “flickery” appearance in channel recordings. The activation thresholds of both \textit{L. lactis} channels were comparable with those of the \textit{E. coli} homologues, as were the channel substates observed for MscL\textsuperscript{L1} (43). We also showed that MscL\textsuperscript{L1} could be activated by modifying cysteine residues with MTSET (G20C or V21C) at the constriction site of the pore, demonstrating that MscL from \textit{L. lactis} functions very much like MscL from \textit{E. coli}.

**Survival and Metabolic Activity after Osmotic Downshift**—Cell survival of \textit{E. coli} MJF465 after osmotic downshift conditions was determined after heterologous expression of MscS\textsuperscript{L1} and MscL\textsuperscript{L1}. The experiments showed that both channels were able to partially rescue the MscL /MscS\textsuperscript{−} phenotype of the MJF465 strain. It can therefore be concluded that, in \textit{E. coli}, both proteins form functional MS channels. In \textit{L. lactis}, it was harder to determine the effect of an osmotic downshift on cell survival because the cells grow in chains. The number of CFU of the disruption strain JIM7049ΔMscL decreased by 20\% when the cells were subjected to an osmotic downshift. If a binomial distribution of cell death in a chain of four cells is assumed, then the distribution of live and dead cells would be \(L^4 + 4L^3D + 6L^2D^2 + 4LD^3 + D^4\) (where \(L\) represents the fraction of live cells and \(D\) is the fraction of dead cells). Since \(D^4\) (i.e. all cells in one chain are dead) is the only case that gives rise to a decrease in the number of CFU, the fraction of dead cells can be calculated as the fourth root of the fractional decrease in CFU. In this case, the fraction of dead cells is \((0.20)^{0.25} = 0.67\), implying that only 33\% of the cells survived the osmotic downshift. Assuming a chain length of two cells and a normal distribution, the survival would be 55\%. With most cells growing in chains of 2 and 4, the observed decrease in cell viability of 20\% thus corresponds to an actual decrease in viability of 45–67\%.

The glucose fermentation capacity of \textit{L. lactis} JIM7049ΔMscL decreased by 60\% after osmotic downshift.
Since the IL1403 wild type strain (which normally expressed MscL Ll) was not affected by the osmotic downshift, a fraction of the JIM7049 MscL MscL may have lysed, as a consequence of the inability to rapidly release osmolytes upon osmotic downshift. Strikingly, the decrease in glucose fermentation capacity of 60% compares well with the actual decrease in cell viability of 45–67%.

**MscS Activity Is Not Detectable in Wild Type L. lactis**—Based on the following observations, *L. lactis* IL1403 does not seem to possess detectable levels of functional MscS, although the yncB gene is transcribed. First, MscS Ll activity was not observed in patches excised from hybrid proteo-GUVs of IL1403, which displayed MscL activity. Second, MscS Ll activity was not observed in patches excised from hybrid proteo-GUVs of JIM7049, which lacked any MS activity even when prepared from cells grown under different osmotic conditions. Finally, efflux of glycine betaine upon osmotic downshift was significantly decreased in JIM7049. The residual 60% of efflux can be explained by cell lysis, as could be inferred from the decrease in CFL in the drop plate assay and the decrease in metabolic activity upon osmotic downshift. In contrast to *L. lactis*, an *E. coli* knock-out strain released the same amount of glycine betaine as the wild type *E. coli* upon osmotic downshift (44), suggesting that in *E. coli* another MS channel compensates for the absence of MscL or that in the wild type strain MscL is not activated under the conditions used.

However, when *yncB* was expressed in *E. coli* MJF465 (using pBADyncB10H) or in *L. lactis* JIM7049 (using pNZ8048yncB10H), MS channel activity was observed. RT-PCR showed that the *yncB* gene is transcribed. In patches excised from hybrid proteo-GUVs of *L. lactis* IL1403, which displayed MscL activity, second, MscS Ll activity was not observed in patches excised from hybrid proteo-GUVs of JIM7049, which lacked any MS activity even when prepared from cells grown under different osmotic conditions. Finally, efflux of glycine betaine upon osmotic downshift was significantly decreased in JIM7049. The residual 60% of efflux can be explained by cell lysis, as could be inferred from the decrease in CFL in the drop plate assay and the decrease in metabolic activity upon osmotic downshift. In contrast to *L. lactis*, an *E. coli* knock-out strain released the same amount of glycine betaine as the wild type *E. coli* upon osmotic downshift (44), suggesting that in *E. coli* another MS channel compensates for the absence of MscL or that in the wild type strain MscL is not activated under the conditions used.

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