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Use of Small Fluorescent Molecules to Monitor Channel Activity

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The Mechanosensitive channel of Large conductance (MscL) allows bacteria to rapidly adapt to changing environmental conditions such as osmolarity. The MscL channel opens in response to increases in membrane tension, which allows for the efflux of cytoplasmic constituents. Here we describe the cloning and expression of *Salmonella typhimurium* MscL (St-MscL). The amino acid sequence encoding for this MscL exhibits a high degree of similarity to *Escherichia coli* MscL (Eco-MscL). Using a fluorescence efflux assay, we demonstrate that efflux through the MscL channel during hypoosmotic shock can be monitored using endogenously produced fluorophores. These fluorophores are synthesized by a cotransformed gene, *cobA*. In addition, we observe that thermal stimulation, i.e., heat shock, can induce efflux through MscL.

Key Words: **MscL; *cobA;* fluorescence; heat stress.**

The Mechano sensitive channel of Large conductance (MscL) is an inner-cell membrane protein that allows bacteria to sense and respond to changing environmental conditions. This 15-kDa protein is found in the membrane as homopentameric units. Each monomer contains two transmembrane domains, a periplasmic loop, and a cytoplasmic helix (1–3). MscL is gated by tension in the cell membrane. MscL senses and responds to changes in osmotic balance by permitting the efflux of ions, small molecules, or proteins into the periplasm during hypoosmotic stress. Efflux of small proteins like thioredoxin, DnaK, and elongation factor Tu into the periplasmic space has been observed in wild-type cells during hypoosmotic shock and is dependent on MscL function (4, 5).

MscL has been extensively studied as a model for how a protein senses and responds to changes in membrane tension. MscL function has been measured by patch clamp techniques, the ability to allow growth of cells in low osmotic conditions, and by the detection of proteins released into the periplasmic space (1, 4–7). These methods are too cumbersome and time consuming to perform on a large scale, and therefore are impractical to use in a genetic screen. Here we explore the use of fluorescent molecules to monitor efflux during hypoosmotic shock as a more convenient method of studying MscL function. The bacterial *cobA* gene has been used as a reporter system in bacteria, yeast, and mammalian cells (8). *cobA* encodes for uroporphyrinogen III methyltransferase, a key enzyme involved in vitamin B_{12} synthesis. Overproduction of *cobA* results in the accumulation of sirohydrochlorin and trimethylpyrrocorphin, which emit a red fluorescence when excited with ultraviolet light (λ = 360 nm) (8, 9).

Here we describe the cloning and characterization of *Salmonella typhimurium* MscL. More importantly, we demonstrate the use of these fluorescent molecules as markers for monitoring MscL-based channel efflux. As previously mentioned, the response of MscL to hypoosmotic shock has been demonstrated. To our knowledge, we present the first evidence of thermally activating MscL efflux by heat shocking cells expressing the *S. typhimurium* protein variant. This finding has significant biosensor implications, especially for investigators exploring the use of channel proteins in biosensor applications. Thermal biosensors are relatively unexplored, but would have considerable commercial and military utility.

MATERIALS AND METHODS

Bacterial strains and growth conditions. E. coli strain Top10 (Invitrogen, Carlsbad, CA) was used for routine cloning. *E. coli* MscL-knockout strain, PB104 (a kind gift from Dr. C. Kung, University of Wisconsin), was used for expression, growth, hypoosmotic, and temperature shock studies. *E. coli* parental strain BL21 was used to determine wild-type MscL activity. Strains were routinely grown in Luria–Bertani (LB) broth under aerobic conditions at 37°C. 100 μg/ml ampicillin and/or 50 μg/ml kanamyacin was added for

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MSCL_STYPHI       MSFIKEFREFAMRGNVVDLAVGVIIGAAFGKIVSSLVADIIMPPLGLLIGGIDFKQFAVT
MSCL_ECOLI        MSIIKEFREFAMRGNVVDLAVGVIIGAAFGKIVSSLVADIIMPPLGLLIGGIDFKQFAVT
MSCL_HAEIN        MNFIKEFREFAMRGNVVDMAVGVIIGSAFGKIVSSLVSDIFTPVLGILTGGIDFKDMKFV
MSCL_PECTCAR      MSIIKEFREFAMRGNVVDLAVGVIIGALFGKIVSSLVSDIIMPPLGLLIGGVDFKQFALF

MSCL_STYPHI       LRDAQGDIPAVVMHYGVFIQNVFDFLIVAFAIFMAIKLINKLNRKKE-EPAAVPAPSKEE
MSCL_ECOLI        LRDAQGDIPAVVMHYGVFIQNVFDFLIVAFAIFMAIKLINKLNRKKE-EPAAAPAPTKEE
MSCL_HAEIN        LAQAQGDVPAVTLNYGLFIQNVIDFIIIAFAIFMMIKVINKVRKPEE----K-KTAPKAE
MSCL_PECTCAR      LRNAQGGIPAVVMNYGAFIQNIFDFIIVAFAIFIAIKLMNKMRCKQEDTPAAPPKPSAEE

MSCL_STYPHI       VLLTEIRDLLKEQNNRP 136
MSCL_ECOLI        VLLTEIRDLLKEQNNRS 136
MSCL_HAEIN        TLLTEIRDLLKNK---- 128
MSCL_PECTCAR      KLLAEIRDLLKEQQTRQ 137
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FIG. 1. Alignment of the MscL amino acid sequences from *S. typhimurium, E. coli, H. influenzae, and P. carotovorum*. Amino acid differences are denoted in bold type.

plasmid selection. MscL expression was induced with 100 μM isopropylthio-β-d-galactoside (IPTG; Gibco, Rockville, MD).

DNA manipulations. Polymerase chain reaction (PCR) was performed using standard methods in a 2400 Perkin–Elmer thermocycler (Foster City, CA). Restriction enzymes and ligase were purchased from Roche Biochemicals (Indianapolis, IN). Gel purification was performed with the Qiaquick Gel Extraction II kit (Qiagen, Valencia, CA). Plasmid DNA isolation was achieved by use of the Spin Column kit (Qiagen, Valencia, CA).

Cloning. The *S. typhimurium* MscL gene was amplified from genomic DNA using degenerate oligonucleotides designed against the Eco-MscL gene. The amplified product was digested with restriction enzymes and cloned into the corresponding sites of the pHAT12 expression vector (Novagen, Madison, WI). The plasmids were then sequenced using Big Dye Reaction chemistry (ABI Prism, Foster City, CA) in conjunction with an ABI 310 genetic analyzer (Perkin–Elmer, Foster City, CA). The *B. megaterium cobA* gene and Shine–Delgarno sequence were amplified by PCR and subsequently cloned into a modified pCR II-Topo vector that contains only a kanamycin resistance marker for plasmid selection (Invitrogen, Carlsbad, CA).

In vitro transcription and translation. Eco-MscL and St-MscL were cloned into PCRT7/NT-TOPO vector (Invitrogen, Carlsbad, CA) for *in vitro* expression studies. *In vitro* transcription and translation reactions with ^{35}S were performed as described in the S30 kit (Promega, Madison, WI). Products were electrophoresed on SDS–PAGE gels and detected on autoradiographic film.

Immunoblots. *E. coli* strain PB104 (MscL null mutant) was transformed with vector, Eco-MscL, or St-MscL. The transformant strains were grown to log phase in LB media with 100 μg/ml ampicillin and induced for 3 h with 100 μM IPTG at 37°C. Cell pellets were suspended in SDS–PAGE buffer and electrophoresed on a 16% SDS–PAGE gel (Bio-Rad, Hercules, CA). Separated proteins were electroblotted onto Immobilon P membrane (Millipore, Bedford, MA). Membranes were stained briefly with Ponceau S (Amresco, Solon, OH) to detect equivalent loading prior to immunoblotting with anti-HAT (Clonetech, Palo Alto, CA). For DnaK immunoblot analysis, cells were grown to saturation in LB medium and then diluted into M9 medium with 100 μg/ml ampicillin and 100 μM IPTG. Cells were allowed to grow for about 4.0 h at 37°C, resuspended to $OD_{650} = 5$ (10 mM Tris, 2.5 mM EDTA, and 20% sucrose solution) and equilibrated for 10 min. Cells were diluted 5-fold into sterile water. After 20 min of incubation at room temperature, samples were centrifuged. Supernatants were subjected to 20% trichloroacetic acid (TCA) precipitation for 48 h at −20°C. Samples were then subjected to immunoblot analysis as described above.

Cell growth assay. PB104 strain (MscL null) transformed with empty vector, Eco-MscL, or St-MscL was grown overnight. An equal number of cells, as determined by OD_{600}, were transferred into M9 medium (10) with 100 μg/ml ampicillin and 100 μM IPTG. OD_{600} was measured at 45-min intervals for 4.5 h postinoculation using a Lambda 20 spectrophotometer (Perkin–Elmer, Foster City, CA).

Fluorescence efflux assay. The kan^r *cobA* construct was cotransformed into BL21 and PB104 *E. coli* strains with or without St-MscL. Cells were grown to saturation in LB medium and then diluted into M9 medium with 100 μg/ml ampicillin and/or kanamyacin (50 μg/ml). Cells were allowed to grow for 6.0 h at 37°C in the presence of 100 μM IPTG. Cells were resuspended in 10 mM Tris, 2.5 mM EDTA, and 20% sucrose solution and equilibrated for 20 min. Cells were diluted 5-fold into sterile water. After 20 min of incubation at room temperature, samples were centrifuged. Supernatants were subjected to fluorometric analysis with a Perkin–Elmer luminescence spectrometer LS50B instrument (Foster City, CA). Excitation was performed at 360 nm and emission measured at 610 nm. Values (% Efflux) are reported as a fraction of the fluorescence present in the supernatant after hypoosmotic shock over the total fluorescence.

Heat and cold shock experiments. For heat shock experiments, overnight cultures were diluted 10-fold into fresh LB medium with 100 μM IPTG, 100 μg/ml ampicillin, and/or 50 μg/ml kanamyacin. Cells were allowed to grow for 6 h at 30°C before suspension and equilibration in 10 mM Tris and 2.5 mM EDTA. Cultures were incubated at 37°C for 20 min, centrifuged, and supernatants were subjected to fluorometric analysis as above. For cold shock experiments, strains were grown overnight in LB medium and diluted into fresh LB medium with 100 μM IPTG, 100 μg/ml ampicillin and/or 50 μg/ml kanamyacin. Cells were grown for 6 h at 37°C; cell pellets were equilibrated in 10 mM Tris and 2.5 mM EDTA prior to incubation in an ice bath for 20 min. Cell pellets were separated from the supernatants, which were subjected to fluorometric analysis as previously described.

RESULTS AND DISCUSSION

Sequence Analysis

MscL genes from *E. coli* and several prokaryotes were previously cloned (11). BLAST alignment of the MscL protein sequences for *S. typhimurium* (GenBank Accession No. AF 272843) showed a high degree of homology to *E. coli, H. influenzae*, and *P. carotovorum* MscL's. Figure 1 shows that the *S. typhimurium* MscL (St-MscL) sequence has a high degree of similarity to the *E. coli* MscL (Eco-MscL); St-MscL is 97% identical to the Eco-MscL protein product.

In Vitro and in Vivo Expression

In vitro transcription and translation reactions were performed to confirm expression of the recombinant

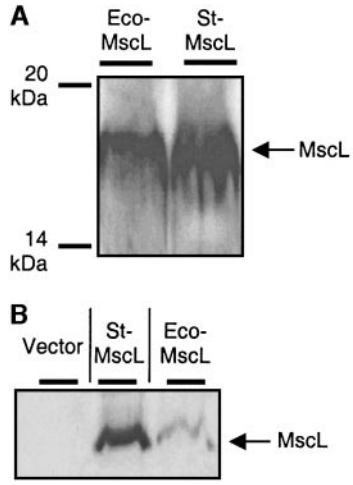

FIG. 2. *In vitro* and *in vivo* expression of St-MscL. (A) Eco-MscL and St-MscL expressed in *in vitro* expression studies. (B) *In vivo* expression studies of St-MscL and Eco-MscL.

Eco-MscL and St-MscL genes. In Fig. 2A, we show that *in vitro* translation products migrated around 18 kDa on a SDS–PAGE gel. This is in agreement with the estimated size of Eco-MscL plus the addition of an N-terminal amino acid tag (1). Next, we tested the expression of Eco-MscL and St-MscL *in vivo*. The MscL null mutant was transformed with vector, Eco-MscL or St-MscL. Whole cell lysates from vector only, Eco-MscL, and St-MscL induced cultures were separated by SDS–PAGE gels and electroblotted onto a nylon membrane for immunoblot analysis with anti-HAT antibody. Figure 2B shows the ~18-kDa band in the Eco-MscL and St-MscL samples. Higher molecular weight bands have been noticed in some immunoblots indicating homopentameric structures (data not shown). We consistently observed higher steady-state levels of St-MscL compared to Eco-MscL when expressed in the MscL null mutant. However, the *in vitro* translation experiments showed that St-MscL and Eco-MscL are expressed at similar levels. Although the amino acid sequence shows few differences, it is possible that St-MscL may be more stable than native Eco-MscL *in vivo*.

St-MscL complements a MscL null strain: MscL has been shown to be important in maintaining cellular osmotic balance and sustaining normal cell growth rates in media with low osmolarity (7). MscL null mutants exhibit decreased growth rates and can be rescued by transforming the mutant with wild-type MscL (7). We performed growth assays to determine the functionality of recombinant St-MscL. Equivalent number of cells from an overnight saturated culture of MscL null mutant transformed with vector only, Eco-MscL, or St-MscL were inoculated into fresh medium and growth was measured by monitoring the optical density at 600 nm (OD_{600}; Fig. 3). The null mutant transformed with St-MscL showed improved growth compared to the MscL null mutant harboring empty vector alone. Surprisingly, we repeatedly observed that the growth rate of the MscL null strain transformed with St-MscL was much higher compared to the null mutant transformed with Eco-MscL. This is most likely due to the higher steady-state levels of St-MscL protein (see Fig. 2B). This data shows that St-MscL rescues the slow growth phenotype of the MscL knockout strain and is functioning equivalently to Eco-MscL.

Certain proteins (thioredoxin, elongation factor Tu, and DnaK) are released to the periplasmic space during hypoosmotic shock via the MscL channel (4, 5). To further provide evidence that St-MscL is able to complement a MscL null mutant, we tested DnaK release from EDTA-treated cultures of vector, Eco-MscL or St-MscL in a MscL null mutant. Cultures were grown in M9 minimal medium and resuspended in a high osmolarity buffer prior to 5-fold dilution in sterile water. Cell pellets and supernatants were harvested, separated by SDS–PAGE, and electroblotted to a nylon membrane. As shown in Fig. 4, the MscL null mutant expressing St-MscL or Eco-MscL exhibited DnaK efflux into the supernatant during hypoosmotic shock. In contrast, no efflux of DnaK into the supernatant fraction of a MscL-deficient strain during hypoosmotic shock was observed. This indicates that the efflux defect during hypoosmotic shock in a MscL null mutant can be rescued by St-MscL.

Use of Fluorescent Molecules to Monitor Efflux

The efflux of small fluorescent molecules during hypoosmotic shock is a useful tool to monitor MscL func-

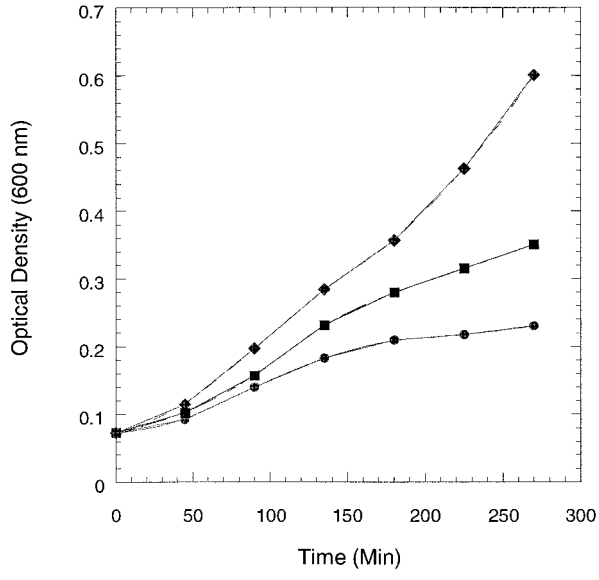

FIG. 3. St-MscL rescues the growth defect of the MscL null mutant in M9 minimal media. MscL null mutant transformed with vector only (circles), Eco-MscL (squares), or St-MscL (diamonds).

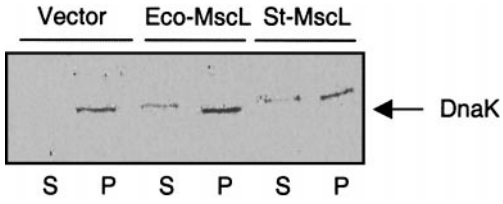

FIG. 4. Hypoosmotic shock induced efflux of DnaK. DnaK was detected by immunoblotting of the supernatant (S) and pellet (P) fractions of MscL-null strain transformed with vector only, Eco-MscL, or St-MscL.

tion. We first asked whether *cobA* could be used as a reporter in studying MscL efflux. Overexpression of *cobA* results in the intracellular accumulation of sirohydrochlorin and trimethylpyrrocorphin [which we will refer to as red fluorescent molecules (RFM)], which emit a strong red fluorescence at 605 nm when excited with near-UV light (8). We first determined whether hypoosmotic shock would induce the efflux of RFM into the periplasmic space of wild type cells. As shown in Fig. 5, wild-type cells exhibited an efflux of RFM into the supernatant—approximately 44% of the total fluorescence was recovered in supernatant after hypoosmotic shock. In contrast, in the MscL null mutant, only 12% (3.6-fold decrease) of the total fluorescence was released to the supernatant during hypoosmotic shock. These results indicate that RFM is released into the periplasmic space during hypoosmotic shock and efflux is in part regulated by MscL. This is further supported by the fact that the MscL null mutant transformed with St-MscL exhibits almost wild-type levels of fluorescence in the supernatant following hypoosmotic shock. It is quite possible that other channels or mechanisms may contribute to the efflux of RFM during hypoosmotic shock since the null mutant exhibits RFM release, albeit at much lower levels than in wild type. Using this fluorescent assay, we are in the process of

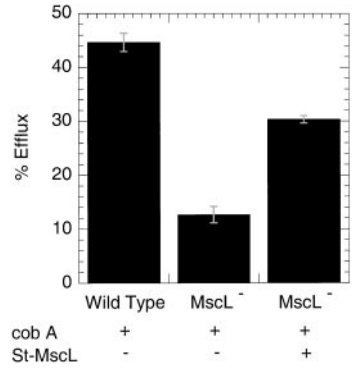

FIG. 5. Hypoosmotic shock induced efflux of red fluorescent molecules (RFM). Wild-type and MscL null mutant were transformed with indicated plasmids and tested for efflux of RFM into the supernatant. The emission spectra at 605 nm were measured with the excitation wavelength fixed at 360 nm.

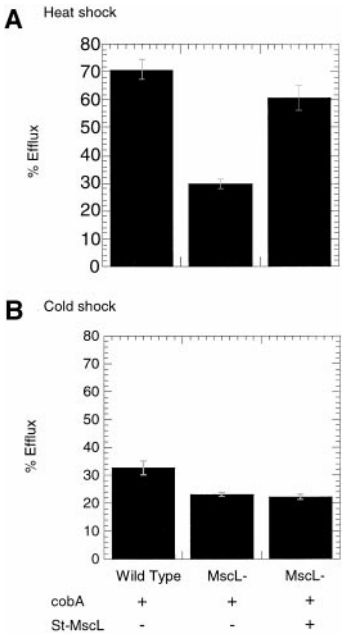

FIG. 6. Release of RFM during heat shock but not cold shock. (A) Fluorescence detected in the supernatant of wild type and MscL null mutant transformed with indicated plasmids. (B) Wild-type, MscL null mutant were transformed with indicated plasmids and tested for efflux of RFM into the supernatant. The emission spectra at 605 nm were measured with the excitation wavelength fixed at 360 nm.

identifying mutations within MscL that have altered gating activity.

Heat shock but not cold shock induces efflux: Since MscL is activated under conditions where tension is applied to the plasma membrane, we hypothesized that heat might increase membrane tension resulting in the opening of the channel allowing efflux of small molecules. To test this hypothesis, we resuspended cells grown at 30°C in Tris–EDTA and heat shocked the cells at 37°C for 20 min. Fluorescent analysis of the supernatants showed that the wild-type strain released 70% of the total fluorescence during heat shock (Fig. 6A). However, the MscL null mutant released only 29% of the total fluorescence after heat shock. This indicates that heat shock is capable of inducing the efflux of RFM, and the majority of RFM efflux was dependent on MscL. This was further confirmed by the restoration of RFM efflux in the MscL null mutant transformed with St-MscL (Fig. 6A, last column). Experiments performed with growth at 37°C followed by heat shocked at 42°C showed a very similar effect (data not shown). We also tested the effect of cold shock on RFM efflux in wild-type and MscL null mutant cells. Cells were grown under normal conditions, suspended in Tris–EDTA, and incubated on ice for 20 min. In this case, we observed no efflux of RFM into the supernatant in all the three strains (Fig. 6B). These results suggest that heat shock perturbs cell membrane integrity which in turn causes opening of the MscL channel and allows

efflux of molecules. This indicates that heat shock also leads to efflux of small molecules through MscL, although other channels may contribute to this effect as evidenced by low levels of RFM efflux in the null mutant.

In conclusion, we have cloned and expressed the MscL homologue from *S. typhimurium*. St-MscL functionally complements a MscL null mutant by restoring normal growth rates and by releasing DnaK from cells undergoing hypoosmotic shock. More importantly, fluorescent molecule efflux can be used to monitor MscL function during hypoosmotic shock. Furthermore, we have demonstrated that heat stress can induce MscL channel activity.

ACKNOWLEDGMENT

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