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Rapid report

A high-throughput screen for MscL channel activity and mutational phenotyping

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Abstract

A novel fluorescence-based screen for bacterial mechanosensitive ion-channel activity has been developed. This assay is capable of clearly distinguishing the previously observed gain of function and loss of function phenotypes for the *Escherichia coli* mechanosensitive channel of large conductance (Ec-MscL). The method modifies Molecular Probes' Live/Dead® *Bac*Light™ bacterial viability assay to monitor MscL channel activity as a function of bacterial survival from osmotic downshock. © 2001 Published by Elsevier Science B.V.

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For all organisms, mechanosensitive ion-channels play critical roles in sensation and regulation. Such channels have been implicated in everything from osmotic regulation in bacteria and proprioception in plants to hearing, pain sensation, and blood pressure regulation in humans [1–3]. Despite these important roles very little is known about the molecular mechanisms by which membrane tension can be transduced through a lipid bilayer to open an ion channel. As such, the crystal structure of the mechanosensitive channel of large conductance from *Mycobacterium tuberculosis* (Tb-MscL) [4] provides unique opportunities to study structure-function relationships for ion channels in general and mechanosensitive ion channels specifically. This structure, along with that of the bacterial K^+ channel, KcsA [5], opens new doors for computational studies and modeling of ion channels. Recently, both open state modeling and molecular dynamics simulations have been applied to MscL [6–8]. Despite the great potential of these methods, experimental evidence for the behavior predicted by these methods is essential if these tools are going to play a role in modern ion-channel structure–function analysis. Without extensive experimental testing, the models and simulations, which seek to explain in atomic detail the function of MscL, are of little value.

Current methods for assaying MscL function are either difficult, tedious, or both. As such, obtaining the large amounts of functional data required for a full understanding of the molecular mechanism by which MscL gates seems at this point in time unrealistic. Adler and Kung's adaptation of patch-clamp electrophysiology to the study of mechanosensitve

Abbreviations: MscL, mechanosensitve channel of large conductance; Ec, *Escherichia coli*; Tb, *Mycobacterium tuberculosis*; GOF, gain of function; LOF, loss of function; MscS, mechanosensitive channel of small conductance; HOEM, high osmolyte enhanced M9

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channels in bacteria has proved very valuable for gaining detailed information about MscL gating [9]. Electrophysiological characterization of *Escherichia coli* MscL (Ec-MscL) in both bacterial spheroplasts and reconstituted lipid vesicles has demonstrated that MscL is opened by tension from the lipid bilayer [10]; shown quantitatively the tension required to open MscL [11]; predicted the pore size of the open channel [12]; and suggested that there are several discreet steps on the opening pathway [11]. This technique has provided exquisite information about both the wild-type channel and may point mutations [13–16]. However, such data are difficult to obtain and are not amenable to large-scale mutational screening. A screen for large-scale mutational analysis would be advantageous in efforts to understand the specific functions of the different regions of the MscL channel.

Since patch-clamp analysis is infeasible for mutational screening, growth based methods have been developed to screen MscL mutations. Two phenotypical screens were designed and correlated with electrophysiological data [13,15,17–20]. These screens are summarized in Fig. 1.

The first screen looks for what has been termed gain of function (GOF) mutations. These are mutations that either decrease the tension of MscL opening, increase the probability of spontaneous MscL openings, or both. Phenotypically, these mutations are characterized by decreased bacterial growth, presumably because the leaky channel compromises membrane integrity. Quantitatively, the assay has been performed both in liquid media and on solid media. In all cases the studies are carried out in a MscL null bacterial strain that contains the MscL gene of interest on an inducible plasmid. For liquid media, bacterial growth after induction is monitored by changes in the optical density of the bacterial culture. GOF mutants show both a slight change in the shape of the growth curve and a decrease in the steady-state optical density [13,15,19,20]. If a solid medium is used, a comparison is made between bacteria grown on the medium in the presence and absence of an inducing agent. Gain of function mutations are identified either by poor growth or no growth in the presence of the inducer [13,15,18,20].

The second screen looks for what has been termed loss of function (LOF) mutations. These are mutations that cause MscL to open either with greater tension or cause MscL not to open at all. Phenotypically, these mutations are characterized by bacterial death upon exposure to hypotonic shock. These experiments must be carried out in bacteria that lack both the MscL gene and the MscS (mechanosensitive channel of small conductance) gene, since MscS alone has been demonstrated to rescue bacteria from osmotic downshock [21]. As with the GOF assay the MscL gene of interest is introduced to the bacterial strain on an inducible plasmid. Typically, these experiments are carried out by downshocking both induced and uninduced bacterial cultures, calculating the number of colony forming units following downshock, and determining the relative survival rate for a particular mutation [19,22].

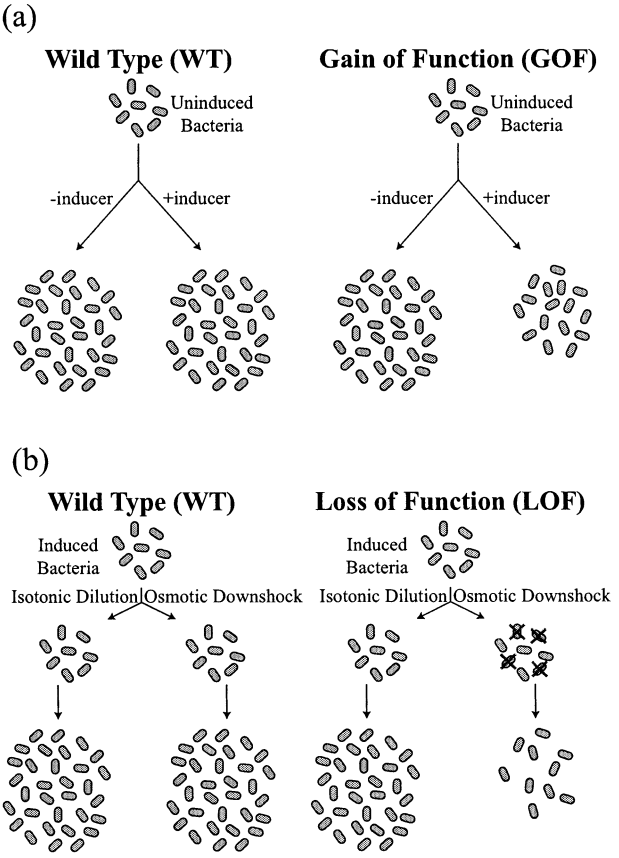

Fig. 1. A schematic representation of the classical growth-based assays for MscL mutational phenotyping. (a) Gain of function mutations, in an inducible plasmid, show decreased growth in the presence of the inducer when compared to wild type. (b) Loss of function mutations expressing MscL show a decreased survival from osmotic downshock when compared to wild type.

Despite being much easier than patch clamp electrophysiology, these methods are still tedious and time consuming. To obtain accurate data, in both assays, great care must be taken to properly adjust the optical density of the bacterial cultures prior to the analysis. This is necessary to ensure that all samples contain an equal number of bacteria. Additionally, each mutation must be screened in two separate assays, which require different sample preparations. In general, these methods are unattractive for screening the large number of mutations needed to fully understand MscL function.

We now report that phenotypical screening for both LOF and GOF MscL mutants can be accomplished in a single step by modifying Molecular Probes' Live/Dead *Bac*Light[1] bacterial viability assay. The assay uses two fluorescent dyes: propidium iodide and SYTO 9. SYTO 9 is a permeable green fluorescent dye that exhibits an increase in fluorescence upon binding to DNA. Propidium iodide is a cell-impermeable, red dye that binds DNA without a fluorescence change. In this assay live bacteria exhibit green fluorescence due to the binding of SYTO 9. Bacteria with compromised cell membranes exhibit red fluorescence, since SYTO 9 is competed off the DNA by propidium iodide.

For probing MscL function, two different comparisons of wild-type vs. mutant channels are made. The first is a comparison of the relative amounts of bacteria after growth in the presence of an inducing agent; the second monitors the effects of osmotic downshock. A gain of function mutant should grow to a lower density than wild-type MscL, producing a weaker signal in an absolute sense with the Live/Dead *Bac*Light assay. For loss of function mutations, one would expect increased bacterial death upon application of osmotic downshock, and thus a decrease in the green to red fluorescence ratio. These expected phenotypical differences are summarized in Fig. 2. The great potential of this approach is its compatibility with a fluorescent plate reader. This allows both the GOF and LOF phenotype to be probed quickly and simultaneously.

It was necessary to perform this assay in defined

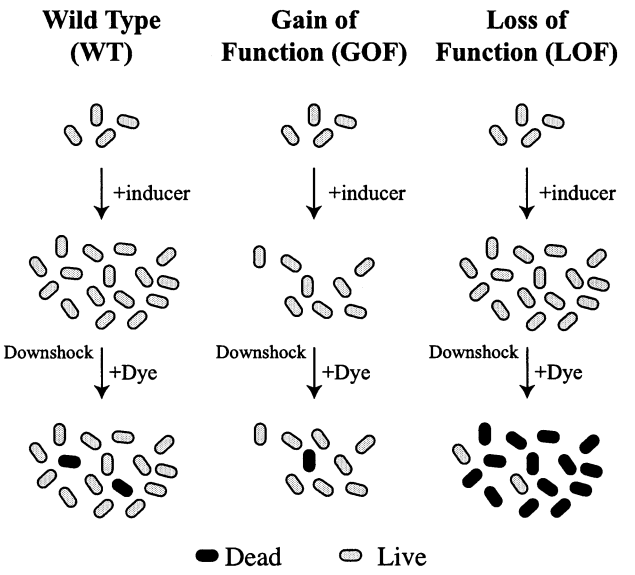

Fig. 2. A schematic representation of the modified Live/Dead *Bac*Light assay. Gain of function mutations show a decreased green fluorescence both in the presence and absence of an osmotic shock due to the reduced number of bacteria. Loss of function mutations show a decreased green fluorescence following osmotic downshock.

media to prevent the interference observed for DNA binding dyes in the presence of complex media. A high osmolyte enhanced M9 (HOEM) medium consisting of 12.8 g/l $Na_2HPO_4 \cdot 7H_2O$, 3.0 g/l KH_2PO_4, 29.8 g/l NaCl, 1.0 g/l NH_4Cl, 0.4% glucose, 1 mM $MgSO_4$, 100 μM $CaCl_2$ 10 mg/l thiamine, and 100 mg/l each of the twenty natural amino acids was used. The MJF465 bacterial strain, which is MscL, MscS (YggB), Kef A null provided an *E. coli* strain that was free of mechanosensitive ion channels [21]. The MscL constructs used were under the control of an IPTG-inducible promoter, in the pB10b vector, which carries the ampicillin resistance gene [23]. Fluorescence measurements were performed using a SpectroMax Gemini XS fluorescent plate reader with dual monochrometers from Molecular Devices.

Bacterial cultures in LB medium (2 mL) with ampicillin (100 μg/ml) were seeded either from freshly streaked agar plates or frozen permastocks. The cultures were grown at 37°C for 14 h without induction. Despite slight differences in the starting concentrations of bacteria used, at steady state the concentration of bacteria in all of the cultures were determined to be essentially equal. The LB cultures (5 μl) were then used to seed cultures in HOEM medium (1 ml)

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containing ampicillin (100 μg/ml) and IPTG (1 mM). The HOEM cultures were grown for 7.5 h at 37°C. In a 96-well plate the HOEM cultures (10 μl) were diluted twenty fold by addition of solutions (190 μl) of various osmotic strengths containing propidium iodide and SYTO 9 (1× concentration). Each HOEM culture was subjected to eight different downshock solutions. The downshock solutions were prepared by mixing HOEM media with water in the following ratios: 1:0, 4:1, 13:7, 1:1, 3:5, 1:3, 1:7, and 0:1. After mixing, the 96-well plate was incubated at 37°C in a plate incubator for 75 min. The plates were then read with excitation wavelengths of 480 and 490 nm and emission wavelengths of 500 and 635 nm, respectively. The assay was also performed using 384-well plates where the total volume was reduced from 200 to 100 μl.

The ability of the modified Live/Dead *Bac*Light assay to screen MscL mutational phenotypes was assessed using wild-type Ec-MscL and a series of well characterized mutations. Mutations at G22 in Ec-MscL have been shown to dramatically alter channel gating. Substitution with hydrophilic residues at this position results in gain of function mutations, while substitution of hydrophobic residues results in loss of function mutations [17,20]. The modified Live/Dead *Bac*Light assay was verified using wild-type Ec-MscL, G22C MscL, G22I MscL, G22N MscL, and G22S MscL. G22C MscL and G22I MscL are loss function mutations, while G22N MscL and G22S MscL are gain of function mutations. In addition, we probed bacteria transfected with the empty vector pET 14b, which provided ampicillin resistance to bacteria lacking all mechanosensitive channels.

Fig. 3 shows the results obtained for the modified Live/Dead *Bac*Light assay. Each line on the plot represents an average of six trials, starting from different initial bacterial cultures. For each culture the ratio of green to red fluorescence is plotted as a function of the extent of osmotic downshock. Standard errors among trials are shown, although they are often comparable in size to the plot marker.

The assay clearly differentiates the various MscL mutational phenotypes. Wild-type Ec-MscL shows initial upward curvature followed by a down turn at only the greatest osmotic shock. The initial upward curvature is reproducible and may be an effect of osmotic strength on the DNA binding ability of propidium iodide and/or SYTO 9. The loss of function mutations show little or no upturn, followed by a down turn with intermediate downshocks. Bacteria not expressing any mechanosensitive channels show a sharp down turn at still lower osmotic shocks. In clear contrast, the gain of function mutations show a substantially reduced initial fluorescence that is not significantly impacted by downshock.

The data in Fig. 3 establish that the modified Live/Dead *Bac*Light assay provides a quick fluorescent screen for Ec-MscL function and the differentiation

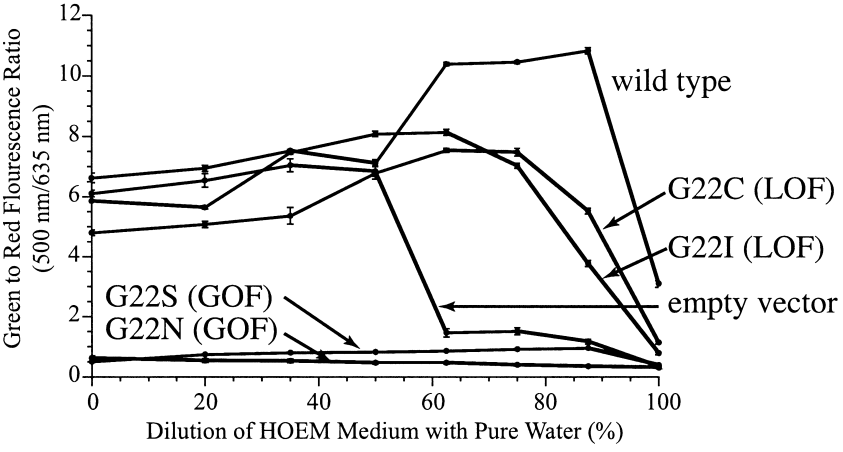

Fig. 3. Modified Live/Dead *Bac*Light assay for Ec-MscL mutants of known phenotype. Wild type Ec-MscL shows an increase in the green to red fluorescence ratio with moderate osmotic downshocks followed by a decrease with severe osmotic downshocks. The loss of function mutants and the empty vector control show a decrease in the green to red fluorescence ratio for less severe osmotic downshocks than for wild type. The gain of function mutations show a decreased green to red fluorescence ratio compared to wild type for all downshock conditions.

of mutational phenotypes. The three patterns – upturn, followed by downturn only at very high osmotic shock; downturn at significantly less severe osmotic shock; and globally diminished growth – are completely reproducible, such that simple visual inspection allows phenotyping. In practice, though, a parallel study with wild-type MscL is always advisable.

In a 96- or 384-well plate format the assay is convenient and rapid. This assay generates both gain and loss of function data in significantly less time than required to run our previous GOF assay alone [18]. The total time required for the assay is reduced from approximately 63 h to approximately 23 h. Furthermore, the active working time per sample is reduced over 20-fold when a moderate number of samples is examined. With this assay a single person can easily screen over 75 different mutations in a 23-h period. Recently, Jones and co-workers have developed a fluorescent method for analyzing Ec-MscL and St-MscL (*Salmonella typhimurium*) activity by monitoring the release of the fluorescent cobA protein [24]. However, this method is not capable of differentiating mutational phenotypes and requires extensive sample preparation prior to fluorescence analysis. Additionally, recent work suggests that small proteins may not actually efflux through MscL upon osmotic downshock; instead these proteins may leak through the transiently damaged bacterial envelope [25].

This assay has the potential of generating the large amounts of functional data needed to understand the molecular mechanisms behind MscL gating. Libraries of randomly generated MscL mutants can now be quickly screened for both gain and loss of function phenotypes. Combination of these data with molecular dynamics simulations and molecular modeling will hopefully lead to an accurate picture of the mechanism of opening for MscL. The modified Live/Dead *Bac*Light assay may also be useful in looking for open channel blockers, which could potentially help to elucidate the open state structure of MscL.

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