Chapter 3: A High-Throughput Screen for MscL Channel Activity and Mutational Phenotyping

3.1 Introduction

The crystal structure of the mechanosensitive channel of large conductance from *M. tuberculosis* (Tb-MscL) (Chang et al., 1998) provides unique opportunities to study structure-function relationships for ion channels in general and mechanosensitive ion channels specifically. This structure 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 (Elmore and Dougherty, 2001; Gullingsrud et al., 2001; Sukharev et al., 2001a; Sukharev et al., 2001b). 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 channels in bacteria has proved very valuable for gaining detailed information about MscL gating (Martinac et al., 1987). Electrophysiological characterization of *E. coli* MscL (Ec-MscL) in both bacterial spheroplasts and reconstituted lipid vesicles has demonstrated that MscL is opened by tension from the lipid bilayer (Sukharev et al., 1994); shown quantitatively the tension required to open MscL (Sukharev et al., 1999); predicted the pore size of the open channel(Cruickshank et

al., 1997); and suggested that there are several discreet steps on the opening pathway (Sukharev et al., 1999). This technique has provided exquisite information about both the wild-type channel and many point mutations (Blount et al., 1997; Blount et al., 1996; Ou et al., 1998; Yoshimura et al., 1999). 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.

3.1.1 Phenotypical Characterization

Electrophysiological characterization of MscL has lead to two distinct mutational phenotypes. These phenotypes have been termed gain of function (GOF) and loss of function (LOF). Gain of function mutations exhibit either a decrease in the tension required to open MscL, increase the probability of spontaneous MscL openings, or both. Loss of function mutations cause MscL to open either with greater tension or cause MscL not to open at all. Idealized electrophysiology as a function of applied tension is shown in Figure 3.1 for MscL forms that are phenotypically wild type, GOF, and LOF.

3.1.2 Classical Growth-based Assays

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 electrophyiological data (Batiza and Kung, 2000; Blount et al., 1997; Maurer et al., 2000; Moe et al., 2000; Ou et al., 1998; Yoshimura et al., 1999). The first screen for gain of function mutations simply observes bacterial growth, while

the second screen for loss of function mutations observes the consequences of osmotic downshock on bacterial growth.

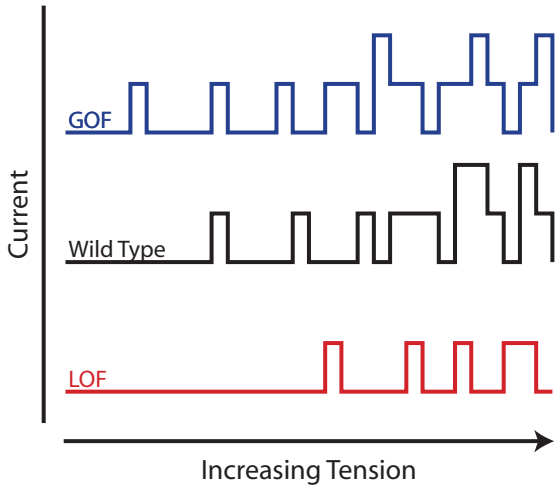

Figure 3.1: Idealized electrophysiological traces showing phenotypically wild type, GOF and LOF MscL mutants.

Gain of function mutations have been phenotypically 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 (Blount et al., 1997; Moe et al., 2000; Ou et al., 1998; Yoshimura et al., 1999). If a solid medium is used, a comparison is made between bacterial 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.(Blount et al., 1997; Maurer et al., 2000; Ou et al., 1998; Yoshimura et al., 1999) Figure 3.2 shows a schematic representation of the classical gain of function assay.

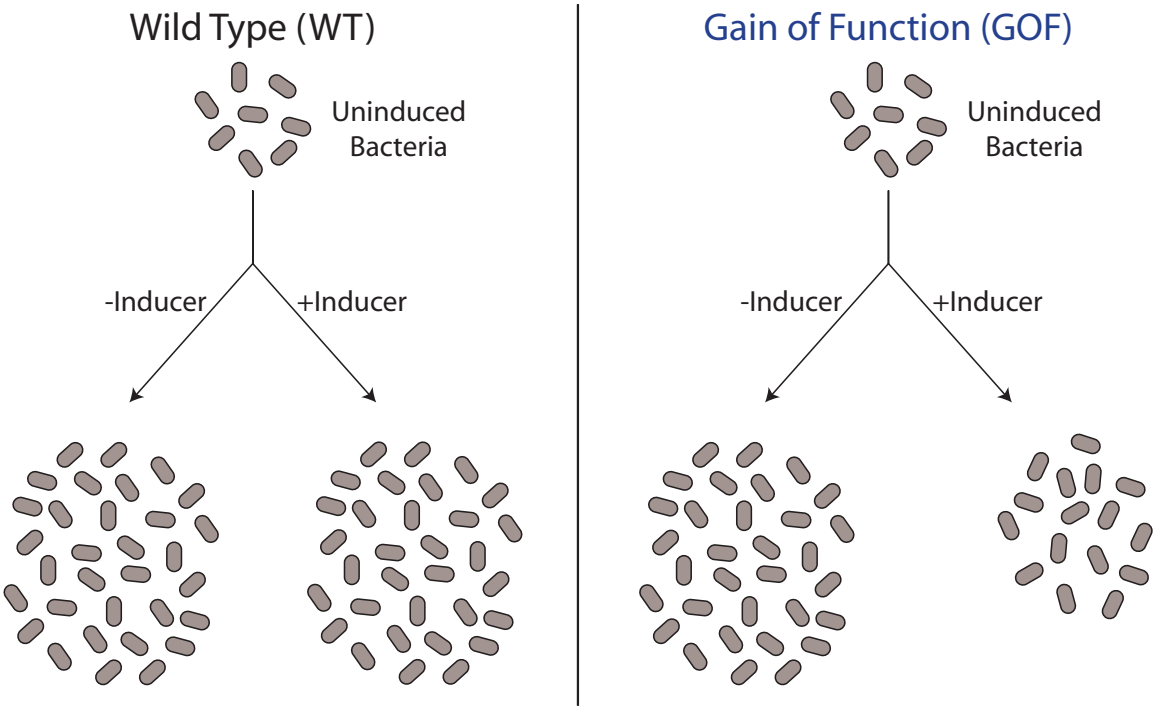

Figure 3.2: Schematic representation of the classical gain of function assay. Gain of function mutations, in an inducible plasmid, show decreased growth in the presence of the inducer when compared to wild type.

Loss of function mutations have been phenotypically 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 (mechanosenesitive channel of small conductance) gene, since MscS alone has been demonstrated to rescue bacteria from osmotic downshock (Levina et al., 1999). 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 (Batiza and Kung, 2000; Moe et al., 2000). Figure 3.3 shows a schematic representation of the classic loss of function assay.

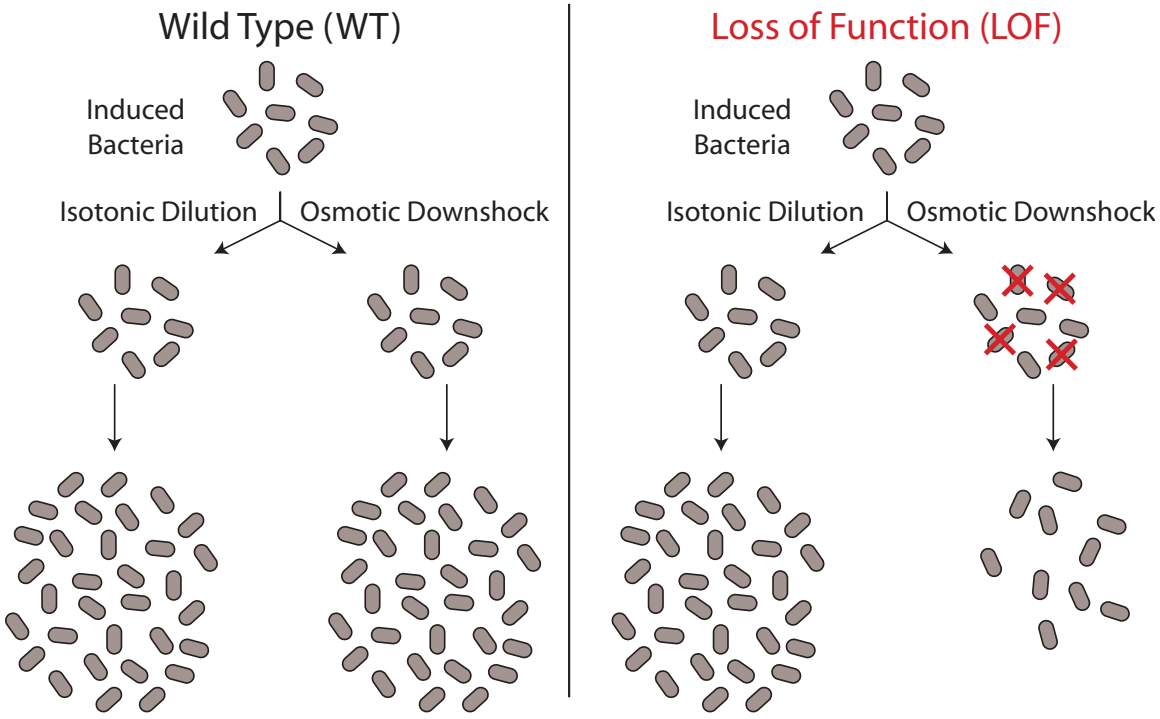

Figure 3.3: Schematic representing that classic growth-based loss of function assay. Loss of function mutations expressing MscL show a decreased survival from osmotic downshock when compared to wild type.

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.

3.2 Results and Discussion

Phenotypical screening for both LOF and GOF MscL mutants can be accomplished in a single step by modifying Molecular Probes' Live/Dead® *Bac*Light™ 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. Figure 3.4 shows a schematic representation of the Live/Dead *Bac*Light system and an image of bacteria stained with SYTO 9 and propidium iodide.

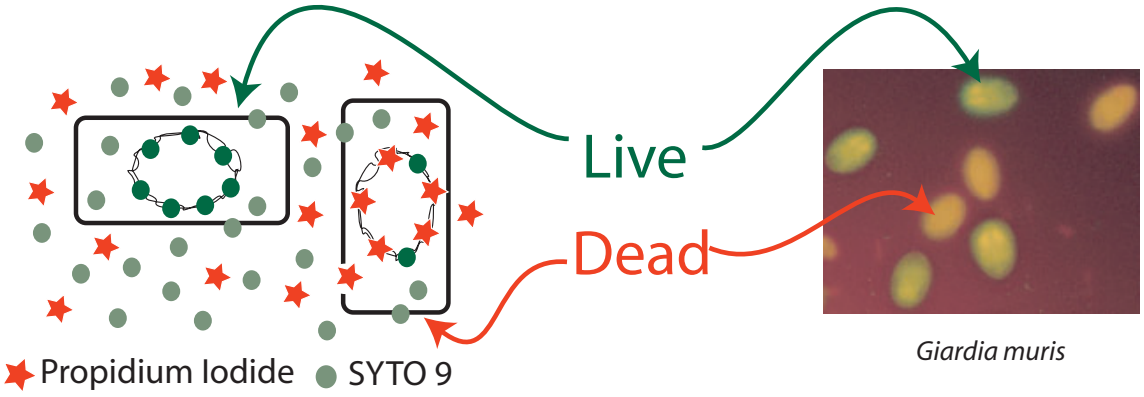

Figure 3.4: Schematic representation of the Live/Dead *Bac*Light bacterial viability assay and a fluorescent microscope image of bacteria stained with the SYTO 9 and propidium iodide (Molecular Probes, Eugene OR).

Typically, what is measured in the Live/Dead® *Bac*Light™ is the ratio of green to red fluorescence. Although this ratio can be measured using a fluorescence microscope, the measurements presented here were obtained on bacterial suspensions using a fluorescence plate reader. The resulting signal is sensitive to both the ratio of live to dead bacteria and the total number of bacteria. Sensitivity to the number of bacteria arises from the presence of excess SYTO 9.

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 Figure 3.5. 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 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 $Na_2HPO_4 \bullet 7H_2O$, 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 μg/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.(Levina et al., 1999) The MscL constructs used were under the control of an IPTG inducible promoter, in the pB10b vector, which carries the ampicillin resistance gene.(Moe et al., 1998) Fluorescence measurements were performed using a SpectroMax Gemini XS fluorescent plate reader with dual monochrometers from Molecular Devices.

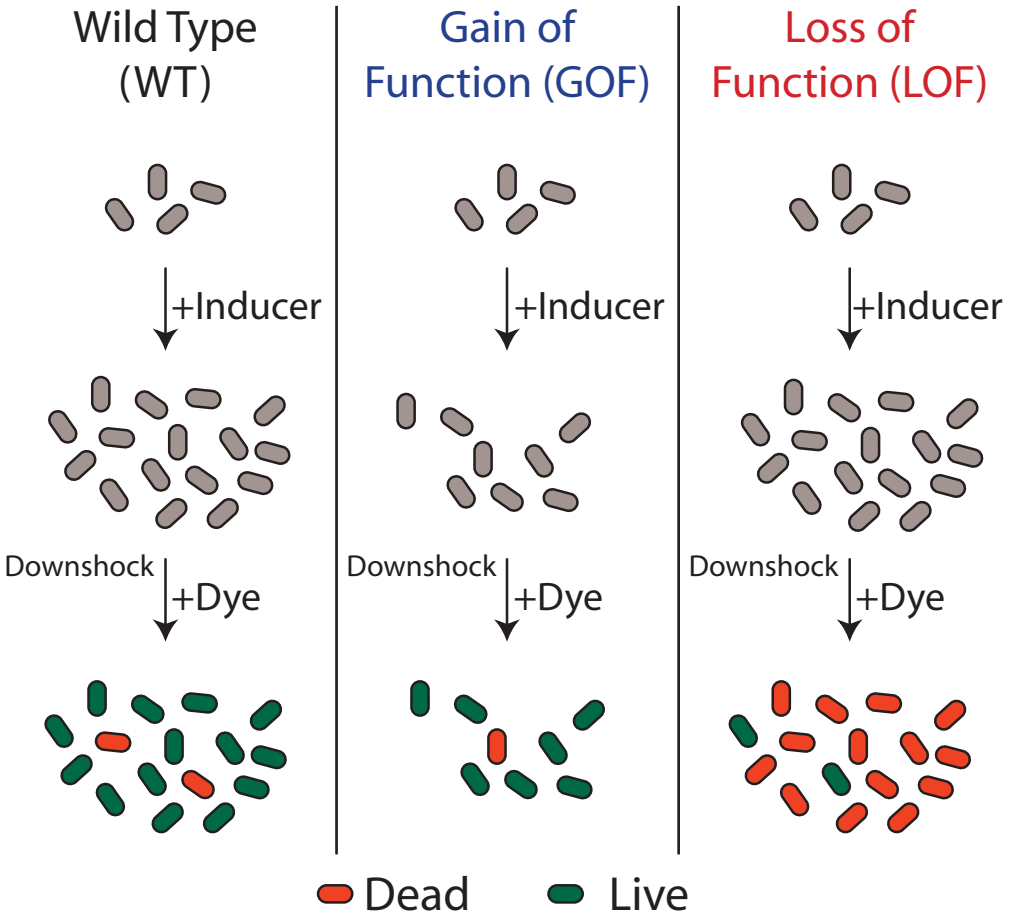

Figure 3.5: A schematic representation of the modified Live/Dead BacLight assay. Gain of function mutations show a decreased green fluorescence both in the presence and absence of an osmotic shock due to the reduced number of bacteria. Loss of function mutations show a decreased green fluorescence following osmotic downshock.

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 hours 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) containing ampicillin (100 μg/mL) and IPTG (1mM). The HOEM cultures were grown for 7.5 hours at 37°C. In a 96-well plate the HOEM cultures (10 μL) were diluted twentyfold by addition of solutions (190 μL) of various osmotic strengths containing propidium iodide and SYTO 9 (1x 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 seventy-five minutes. The plates were then read with excitation wavelengths of 480 nm and 490 nm and emission wavelengths of 500 nm and 635 nm, respectively. The assay was also performed using 384-well plates where the total volume was reduced from 200 μL 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(Batiza and Kung, 2000; Yoshimura et al., 1999). 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.

Figure 3.6 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.

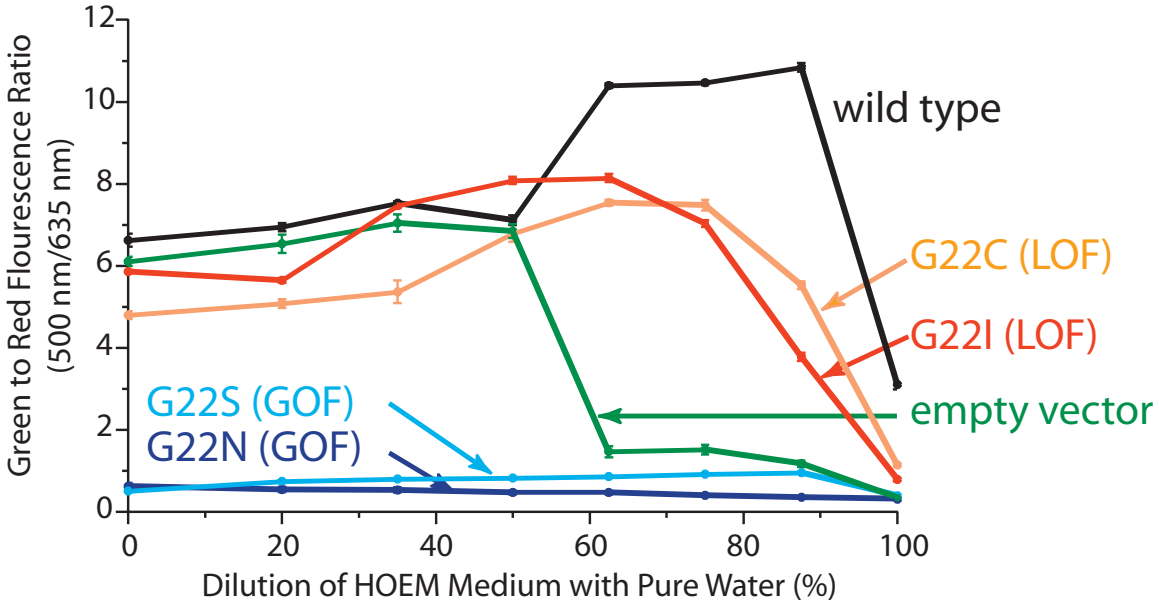

Figure 3.6: Modified Live/Dead BacLight assay for Ec-MscL mutants of known phenotype. Wild-type Ec-MscL shows an increase in the green to red fluorescence ratio with moderate osmotic downshocks followed by a decrease with severe osmotic downshocks. The loss of function mutants and the empty vector control 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.

The data in Figure 3.6 establish that the modified Live/Dead® *Bac*Light™ assay provides a quick fluorescent screen for Ec-MscL function and the differentiation of mutational phenotypes. The three patterns–upturn, followed by down turn only at very high osmotic shock; down turn 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 (Maurer et al., 2000). The total time required for the assay is reduced from approximately 63 hours to approximately 23 hours. 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 hour period.

Recently, Jones and co-workers have developed a fluorescent method for analyzing Ec-MscL and St-MscL (*S. typhimurium*) activity by monitoring the release of the fluorescent cobA protein (Jones et al., 2000). However this method is not capable of differentiating mutational phenotypes and requires extensive sample preparation prior to fluorescence analysis. Additionally, recent work suggest that small proteins may not actually efflux through MscL upon osmotic downshock; instead these proteins may leak through the transiently damaged bacterial envelope (Vazquez-Laslop et al., 2001).

3.3 Conclusions and Future Directions

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; the results of such a screening will be described in Chapter 4. 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.

The largest drawback of the current method is that it is not compatible with Tb-MscL, since the gating tension of Tb-MscL is similar to that of an Ec-MscL loss of function mutation. Expansion of this method to a *Mycobacterium* strain may allow for analysis of Tb-MscL constructs, since some current evident suggests that membrane environment of the channel is related to gating tension (Hamill and Martinac, 2001). To that end, *Mycobacterium Smegmatis,* mc^2 155, has been identified as a potential candidate for assay development. This strain has the desired property of being non-pathogenic and a vector, pJAM2, containing an inducible promoter has been developed for protein expression (Triccas et al., 1998). Initial efforts should focus on expansion of the current assay system. However, if the live/dead *Bac*Light system is incompatible with this strain, viability can be determined using alamarBlue™ as previously described (Page et al., 1993).

3.4 Literature Cited

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