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Research Paper

Robust High Throughput Real-time Monitoring Assay for the Specific Screening of Bacterial Cell Envelope Inhibitors

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Fluorescent dyes have been widely used for the assessment of the microbial and eukaryotic cell viability. However, the available methods require special protocols to be used in specific methods such as fluorescence microscopy and fluorescence-activated cell sorting (FACS). Although simpler methods have been developed using these fluorescent dyes that directly provide a "read-out" for immediate estimation of cell survival, these require constant monitoring and manual intervention. To circumvent all of these problems, we have developed a robust automated high-throughput real-time cell viability monitoring assay using Sytox green dye. Our assay requires no manual intervention during bacterial cell growth. Furthermore, the assay is very specific for the cell membrane perturbing agents. As a proof-of-concept, we show *E. coli* growth in the presence of three different antibiotics that inhibit three different processes in the cell – Ampicillin, for cell wall; Kanamycin, for protein translation; Ofloxacin, for DNA replication; Nisin, for cell membrane. We show that the assay is very specific for Ampicillin and Nisin and does not respond to Kanamycin and Ofloxacin. This assay is performed in a 96-well microtitre plate which makes it possible to analyze, at the same time, several antibiotics and chemical compounds that have the potential to specifically disrupt the cell envelope. Our method is thus very rapid and specific, and can be efficiently used to screen a library of compounds. The assay was further tested on the D29 mycobacteriophage holin protein and its mutant HolG28D. The assay provided very distinct functional differences between the two proteins.

Keywords: Antibiotic; Bacteriophage; Rapid Method; Membrane; Cell Injury

Introduction

An assessment of bacterial cell viability by classical microbiological methods include time consuming and labour intensive techniques such as growth assay by monitoring OD_{600} and counting of colonies on the agar plate in the form of colony forming unit (CFU). However, fluorescence-based methods have been developed as efficient tools to determine bacterial cell viability (Boye and Lobner-Olesen, 1991; Pore, 1994; Breeuwer and Abee, 2000). These methods involve the use of fluorescent dyes that label the bacteria by penetrating the membrane and emitting fluorescence upon excitation thus reporting the physical state of the bacterium (Boulos *et al.*, 1999; Breeuwer and Abee, 2000). Some of these dyes can enter live cells while others are blocked by the intact cellular barriers (Boulos *et al*., 1999). Therefore, fluorescence-based methods not only can be used to quantitatively assess bacterial cell viability, but also can be developed as a high throughput assay to carry out a large scale screening of viability inhibiting agents (Feng *et al.*, 2014). The bacterial system can be perturbed by many chemical agents, including antibiotics, which hinder growth or kill bacterial cells by negatively affecting any of the cellular processes. The bacterial cell viability in the presence of these chemicals can be analysed by making use of fluorescent dyes. Fluorescent probes such as propidium iodide and sytox green are nucleic acid staining dyes, which can only enter damaged cells or cells with compromised cell envelopes (Roth *et al.*, 1997; Lebaron *et al.*, 1998).

In this article, we have presented a high throughput microtitre plate method for the real-time monitoring of bacterial cell viability in the presence of

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growth inhibitors. The assay reported here employs sytox green dye, which selectively binds to the nucleic acid of bacterial cells with damaged cell envelopes *viz*., cell wall or cell membrane. We report that out of the three antibiotics tested, only the cell wall disrupting antibiotic Ampicillin could demonstrate an increased fluorescence, which correlates with the bacterial viability. We also demonstrate the utility of this assay by expressing a mycobacteriophage D29-encoded membrane pore forming protein holin.

Material and Methods

Minimum Inhibitory Concentration (MIC) Determination

The MIC was determined by growing *E. coli* MG1655 bacteria in the presence of various concentrations of antibiotics and measuring the OD_{600} at 0 h (i.e. at the time of inoculation) and after 14 h of growth. The ratio of the final and the initial OD_{600} values was calculated; a value that equals to 1 was considered the MIC for that antibiotic. The MIC determined in these experiments is reported in Table 1.

Table 1: List of antibiotics with their measured MIC values

Antibiotic	Measured MIC
Ampicillin	15.0 μg ml^{-1}
Kanamycin	3.0 μg ml^{-1}
Ofloxacin	1.5 μg ml^{-1}

Fluorescence-based Real-time Cell Viability Monitoring Assay

E. coli MG1655 was cultured in minimal medium supplemented with glucose at 37ºC till OD_{600} reached ~0.3. 200 μl of the culture was then transferred in each well of a 96-well black plate (Thermo) followed by the addition of 5 μM of sytox green dye (Invitrogen). Required antibiotics ampicillin (Amp), kanamycin (Kan), ofloxacin (Ofl), were then added into the respective wells at the following concentrations: 1xMIC, 2xMIC, and 3xMIC. The plate was incubated at 37°C within the Spectramax M5 plate reader (Molecular Devices), followed by shaking for 3 s before every measurement. Sytox dye was excited at 488 nm and the emission was recorded at 523 nm after every 1 min interval continuously for 2 h. Culture without antibiotic was used as negative control, whereas 2% chloroform or 300 μg ml^{-1}nisin along with 30 mM EDTA were used as positive controls, since both of these treatments permeabilize the bacterial cell membrane (Ruhr and Sahl, 1985).

Real-time monitoring of the expression of D29 mycobacteriophage holin and its mutant

The expression of D29 mycobacteriophage holin and its mutant was achieved as described previously (Kamilla and Jain, 2016). Briefly, *E. coli* BL21(DE3) cells carrying expression plasmids were grown at 37°C in LB-broth supplemented with 100 μg ml^{-1} ampicillin. After the culture reached an OD_{600} ~0.6, cells were harvested and re-suspended in minimal medium supplemented with glucose and 100 μg ml^{-1} ampicillin. 200 μl of the culture was then transferred to each well of a 96-well black plate followed by the addition of 5 μM of sytox green dye and 1 mM IPTG. The incubation of the plate and the fluorescence recording were performed essentially as described above.

Results and Discussion

Determination of the MIC for Antibiotics

Three different antibiotics *viz*. ampicillin (Amp), kanamycin (Kan), and ofloxacin (Ofl), which target three different cellular processes *viz*. cell wall synthesis (Marcus *et al.*, 2012), bacterial protein translation (Shrestha *et al.*, 2014) and bacterial DNA replication (Cui *et al.*, 2011), respectively, were used for the assay. We first estimated the MIC of all of these antibiotics for *E. coli* MG1655. The MIC for each antibiotic is listed in Table 1.

Real-time Sytox Green Assay for Cell Viability

We next attempted to develop an assay that could be used for the real-time monitoring of cell death by the antibiotics and other chemical compounds. Sytox green fluorescence dye has been used earlier for determining the membrane integrity in both eukaryotes and bacteria (Jernaes and Steen, 1994; Lopez-Amoros *et al.*, 1995; Roth *et al.*, 1997; Feng *et al.*, 2014). However, a high throughput assay that allows a real-time monitoring of cell viability is not yet available. Therefore, in order to develop such a method, we cultured *E. coli* MG1655 in the presence of sytox green dye and recorded the fluorescence in a 96-well black plate on a fluorescence plate reader. Sytox green

is known to bind to double stranded DNA and gives bright fluorescence (λ_{Ex} = 488 nm; λ_{Em} = 523 nm). On the other hand, the background fluorescence from this dye is minimal in the absence of DNA. Additionally, sytox green does not enter live cells due to the intact membrane. However, when the membrane integrity is compromised, cells are easily stained with this dye (Roth *et al*., 1997; Lebaron *et al*., 1998). In our experiments, we utilized both of these properties of sytox green *viz*. no entry in live cells and an ability to bind to DNA, to develop an assay where bacterial cell death could be monitored in real-time.

The results obtained from the dye binding assay in *E. coli* culture demonstrate an increase in fluorescence that is suggestive of the membrane (plasma membrane or cell wall) damage. The samples that do not contain antibiotics show no change in fluorescence for up to 120 min of recording, whereas samples treated with chloroform and nisin show a substantial increase in the fluorescence values (Fig. 1); here EDTA was used along with nisin to allow the latter to enter the bacterial cell. Interestingly in these experiments, only Amp shows sytox fluorescence suggesting that Amp treatment results in a compromised membrane (Fig. 1). It should also be noted that an increase in concentration of Amp not only results in an increase in sytox green fluorescence, but also takes lesser time to kill the cells. Kan and Ofl treatments with as high as 3-fold excess of the MIC do not result in enhanced fluorescence, although they cause cell death (as observed during MIC determination). Since the increase in fluorescence corresponds to the cell death due to damage of the cell wall or the cell membrane, our results clearly suggest that cells exposed to Kan and Ofl retain their intact and undamaged cell envelopes. This is most likely because the mode of action of these antibiotics does not compromise with the membrane permeability.

In these experiments, the intact cell envelope (present in sample with no antibiotics and in samples treated with Kan and Ofl) does not allow the entry of sytox green dye into the cell and, therefore, does not show fluorescence. The positive controls used here are known to disrupt the cell membrane and, therefore, result in high fluorescence from sytox. We thus conclude that the assay presented here can be used for selective screening of membrane and cell wall disrupting antibiotics and other synthetic chemical compounds.

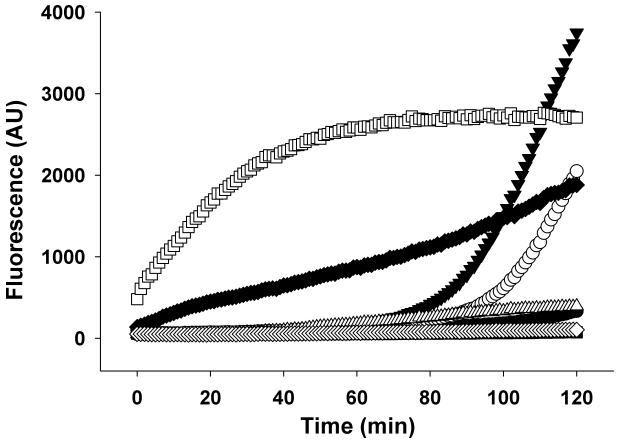

Fig. 1: Real-time fluorescence monitoring of the effect of antibiotics on the bacterial cell envelope. *E. coli* MG1655 was grown in the presence of different concentration of various antibiotics in a 96-well black plate. The plot shows the fluorescence of the sytox green dye recorded on a plate reader every minute by keeping the excitation and the emission wavelengths at 488 and 523 nm, respectively. The concentrations of antibiotics used were with respect to their MIC values given in Table 1. Three concentrations of Amp are shown – 1x MIC (●), 2x MIC (o), and 3x MIC (▼). The latter two concentrations show a significant increase in the fluorescence. Kan (△) and Ofl (■) were used at 3x MIC concentration value. Here, only Amp shows an increase in the fluorescence; both Kan and Ofl do not show such phenomenon. Chloroform (□) and Nisin + EDTA (◆) are used as positive controls. The culture carrying no antibiotic (◊) acts as negative control. The experiment was repeated at least thrice; only one representative experiment is shown

Sytox green assay to compare D29 mycobacteriophage holin and its mutant

We also extended this assay to study the effect of a mycobacteriophage D29-encoded membrane pore forming protein holin. Upon its production, holin is known to form holes in the cell membrane of bacteria (O'Donnell *et al.*, 1992; Kamilla and Jain, 2016). Holins along with the peptidoglycan hydrolases are required by the phages to lyse the cells to release their progeny from the host. We evaluated the membrane perturbing ability of mycobacteriophage D29 holin, when it is expressed in *E. coli* BL21(DE3) cells from a T7 promoter. We have earlier shown that such expression of D29 holin leads to rapid cell

death. Upon holin expression in *E. coli* BL21(DE3), we notice an increase in sytox green fluorescence (Fig. 2) that strongly suggests that the D29 holin forms holes in the cell membrane that is sufficient to cause sytox dye diffusion into the cell. Similar increase in fluorescence is not observed when an inactive holin mutant, HolG28D, is expressed (Fig. 2); we have previously shown that HolG28D is incapable of causing bacterial cell death (Kamilla and Jain, 2016). These studies thus confirm that the sytox green assay can be used to examine the properties of membrane pore forming proteins.

In conclusion, we have developed a fluorescence-based high throughput assay that requires no manual intervention and allows for the real-time monitoring of bacterial cell viability in the presence of various cell envelope inhibitors. Furthermore, this assay can be adopted for the automated protocols where rapid screening of the cell wall and cell membrane disrupting chemicals is desirable. Of the three antibiotics that inhibit three different pathways, only the cell wall disrupting Ampicillin shows increase in fluorescence indicating cell death due to disrupted cell envelope. We also observe similar phenomenon with chloroform, nisin, and mycobacteriophage D29 holin. This assay is performed in a 96-well microtitre plate, which makes it possible to analyze, at the same time, several antibiotics and chemical compounds that have the potential to specifically disrupt the cell envelope. We are also tempted to add that the proposed assay can be used to screen for the membrane pore forming proteins, peptidoglycan hydrolases, and other cell wall disrupting enzymes from bacteria, bacteriophages, and eukaryotic cells. We are certain that this method will find its use in large scale screening of proteinaceous and non-proteinaceous anti-bacterial molecules for specifically targeting the cell membranes.

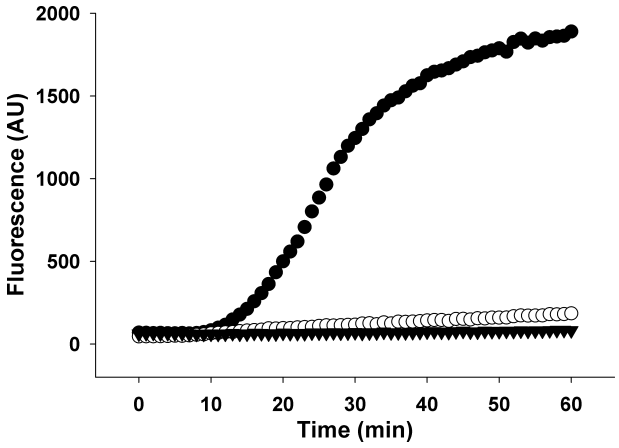

Fig. 2. Effect of the expression of wild-type and mutant holin from mycobacteriophage D29 on *E. coli*. Bacterial cells expressing the wild-type holin (HolFL) and its mutant HolG28D were processed for sytox green dye binding assay. HolFL production in *E. coli* results in hole formation in the cell membrane that allows sytox dye to enter the cell resulting in an increase in the fluorescence (●). On the other hand, the mutant protein HolG28D is incapable of causing hole formation and therefore, sytox green fluorescence is not observed in this case (○). The experiment was carried out in a 96-well black plate with both uninduced and induced cultures; induction of protein was achieved by the addition of IPTG. Sytox green was excited at 488 nm and the fluorescence emission was recorded every minute at 523 nm. The uninduced culture acted as negative control (▼). The experiment was repeated at least thrice; only one representative experiment is shown

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