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4.1.1. Determination of optimal phage-host ratio

In order to investigate the effect of mycobacteriophage on mycobacterial growth it was necessary to first investigate the optimum conditions necessary for phage infection. Mycobacteriophage D29 along with similar other phages of the family require CaCl₂ for infection at 37°C and moderate aeration condition (120 rpm). However another issue that is critical in the phage-host interaction mechanism is the MOI (Multiplicity of Infection). To find out the MOI at which maximum number of infectious centers can be formed, we performed theoretical as well as experimental investigations.

The number of phages that adsorb on the bacterial surface is determined by the probability with which collision occur between bacteria and phage. In this context the Poisson equation (Shabram and Aguilar-Cordova, 2000) may be adapted which gives the probability of the number of bacteria that will be infected by a single phage at different MOI. It is assumed that all virions are infectious, all cells are susceptible to infection and that the number of phage infecting each bacterium is a random distribution. Based on these assumptions, the number of phage infecting each bacterium could be calculated from the Poisson equation which is as follows:

\[ P(n) = e^{-m} \frac{m^n}{n!}, \]

where \( P(n) \) is the probability of finding cells infected by ‘n’ phage(s) and ‘m’ is the average number of phage present per cell (MOI). Putting \( n=0 \) the expected number of uninfected cells can be computed. The equation then simplifies to:

\[ f_{un} = e^{-m} \text{ or } f_{in} = (1 - e^{-m}). \]

Where \( f_{un} \) and \( f_{in} \) represent the fraction of uninfected and phage infected cells respectively.

By incorporating different MOIs into the equation, it was possible to derive the fraction of cells infected at each MOI. The theoretical values thus obtained were plotted against the MOI (figure 4.1.1). The resulting curve as expected was hyperbolic in nature indicating thereby that at higher MOIs, the fraction infected will not change much. Clearly it is not possible to achieve 100% infection even at the highest possible MOI (figure 4.1.1). Since 100% infection is not theoretically possible therefore we chose an MOI at which substantial fraction of cells (defined arbitrarily as 60%) can be infected. Theoretical prediction states that such a benchmark can be achieved at the MOI of 1. Experimentally this was verified by performing an experiment where the host, *Mycobacterium smegmatis* was allowed to be infected by phage D29 at various phage concentrations by varying the MOI (figure 4.1.1 bar graph, inset). The number of cells being infected was calculated by plating the cells before phage addition at a suitable dilution (10⁶) in MB7H9 medium. After phage addition, 1 ml of the cell-phage suspension was isolated and centrifuged at 13,000 rpm for 5 min and
the supernatant and pellet were separately assayed for plaque formation (PFU). The MOI was calculated by dividing the total PFUs (supernatant + phage) by the total number of cells (CFU). The experiment was performed at various MOIs. However, for the sake of simplicity the result of the MOI, at which 60% infection is expected to occur is shown (Black and white bars, figure 4.1.1). The results of these experiments show that at the MOI of 1, the fraction of cells infected as experimentally determined is about 60% (white bar), which is consistent with the value that was predicted. In the experiments to follow an MOI of 1 was used in most experiments. Addition of higher MOI was not done, as an increase in MOI beyond 1 is not expected to have a major effect on the fraction of cells infected. Moreover, use of high MOI can lead to nonspecific cell death and lysis.

4.1.2. Adsorption kinetics

In a phage host interaction system it is expected that a certain number of susceptible bacteria (S) will be engaged by a certain number of phages (P). The ratio of P to S may be considered as the MOI. In a model such as this, one can expect the bacteria to grow at a constant rate and with the phage growth dependent on its host. The dynamic process of phage-bacterial interaction can be described by using a set of differential equations. Considering that at time ‘t’ the number of bacteria present S(t) and P(t) respectively, then the equation describing time rate of bacterial growth can be written as follows

\[
\frac{dS(t)}{dt} = S(0)e^{\alpha t} \tag{1}
\]

Where \(\alpha\) is the growth rate constant.

Similarly the rate of phage growth is expected to follow a second order reaction which will be proportional to the number of susceptible bacteria present at any instant and the number of phages present at that instant S(t) and P(t) respectively. Based on this argument the equation representing phage growth can be written as

\[
\frac{dP(t)}{dt} = -rS(t)P(t) \tag{2}
\]

Where \(r\) is a constant, which is the adsorption coefficient. The negative sign indicates that the free phage population goes down with time as they are adsorbed on its surface.

Solving the equation 1 and 2 we get the equation:

\[
\ln\left(\frac{Pt}{P0}\right) = -r \frac{S(0)}{\alpha} (e^{-\alpha t} - 1) \tag{3}
\]

Since we know P(0), S(0) and P(t) therefore if \(\ln(Pt/P0)\) is plotted against time, the constants \(\alpha\) and \(r\) can be derived. Figure 4.1.2 shows the plot which we derived using the following general equation.
\[ y = y_0 + \frac{a}{b} (e^{-bt} - 1) \]  

(4)

Now, comparing the equations 3 and 4, we obtained the value the bacterial growth rate, \( \alpha \) and the phage adsorption rate \( r \) to be 0.0103 min\(^{-1}\) and 4.32E-11 cell\(^{-1}\)phage\(^{-1}\)min\(^{-1}\)ml\(^{-1}\) respectively (Shao and Wang, 2008) (table 4.1).

### 4.1.3. Determination of burst size and latent period

For one step growth experiment, host cells were mixed with phage (MOI of 0.1). Adsorption was allowed for a limited period of time (20 min) after which the cells were diluted 10\(^4\) times, to prevent further adsorption. The diluted cells were incubated at 37\(^\circ\)C and samples withdrawn at regular intervals. Time course of phage release was monitored by estimating the titers at regular intervals. The period during which no change in phage titer was observed was considered to be the latent period. For burst size determination an aliquot of the cells withdrawn immediately after infection was centrifuged. The pellet was then re-suspended in SM (suspension medium) buffer and plated. The number of PFUs obtained corresponds to infected centers. The PFUs obtained at the saturation point of a one step growth experiment which was obtained following the rise period was divided by the number of infectious centers present in the pellet to obtain a value for the burst size (figure 4.1.3). The values of the various parameters derived experimentally for phage infection are summarized in table 4.1.

**Figure 4.1.3:** One step growth curve of mycobacteriophage D29. Latent period is followed by the rise period after which the burst size can be calculated

**Table 4.1. List of experimentally derived parameters associated with D29-host interactions**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha ) (bacterial growth rate constant)</td>
<td>0.01 min(^{-1})</td>
</tr>
<tr>
<td>( r ) (phage-adsorption rate)</td>
<td>4.32E-11 cell(^{-1})phage(^{-1})min(^{-1})ml(^{-1})</td>
</tr>
<tr>
<td>( \tau ) (latent period)</td>
<td>60 min</td>
</tr>
<tr>
<td>( b ) (average burst size)</td>
<td>219</td>
</tr>
</tbody>
</table>
4.1.4. Microscopic investigations

Bacteriophages are known to bind to the receptors present on the surface of target bacteria. In some cases the adsorbed bacteriophages bring about lysis from outside, resulting in cell death. We suspected that the mycobacteriophage adsorption on the cell surface of mycobacteria would lead to changes in the morphology of the affected cells. In order to investigate this we performed microscopic studies on mycobacteria which were infected with mycobacteriophage at an optimal MOI of 1.

Figure 4.1.4. Acid fast staining and fluorescence staining profile of *M. smegmatis* mc² 155 cells upon D29 phage infection. For acid fast staining cells incubated for 4h in presence of either wild type (wt D29) or mutant (D29ts 10) phage and phage untreated cells as control were taken. The samples after staining were viewed either at 20X or 100X (Oil immersion) magnification (indicated on the left). Cells after similar treatment with the phages were stained with FDA (Battin, 1997). The number of fluorescent cells and their intensity were measured using the green filter (fluorescence) and the morphological changes of the cells after cell lysis (4 h) was monitored through DIC (differential interference contrast) in parallel using confocal microscopy.
Microscopic studies were performed after staining the cells using acid fast staining techniques and alternatively by performing confocal microscopy using FDA staining (figure 4.1.4). When the acid first stained cells were visualized at low resolution (20X), a substantial reduction in the number of stained cells was observed after phage D29 infection (figure 4.1.4, 20X, D29 compared to control). A few cellular particles that were visible were much smaller in size. These observations were specific to the wild type. In the case of the mutant D29Ts10, which grows slowly at 37°C, it was observed that the cells retain their integrity, but are stained poorly. Similar observations were made when the cells were examined at a higher resolution (figure 4.1.4, 100X). In the higher resolution images it can be seen that following phage treatment, cording was diminished significantly in case of wild type D29, but not the mutant (figure 4.1.4 fluorescence). The confocal images reveal that following phage infection the FDA staining intensity increased which indicates that cellular metabolism increased following infection. However the corresponding phase contrast picture (figure 4.1.4, DIC) shows that, cells were smaller in size and isolated from each other. When the mutant phage was used, the FDA intensity did not change significantly although in this case too altered morphology was observed. The results indicate that adsorption of phage on mycobacteria leads to dramatic changes in cell morphology and staining pattern. The changes in staining pattern are most likely due to alteration in the lipid content of the cells. The altered morphology indicates that cells are possibly in a nonviable state (figure 4.1.4).
4.1.5. Developing strategies to estimate the total bacterial count

For quantitative analysis of the effect of phage on the host cells it is necessary to develop methods to accurately count the number of cells present in the system, either viable or non-viable or both. There are a number of methods available to estimate the number of bacteria present in a suspension. For example the bio-physical property of bacteria to scatter light has been exploited to measure the optical density (O.D.) using a spectrophotometer. The bacterial cell surface absorbs light at a wavelength of 600 nm hence by measuring O.D.\textsubscript{600} the average cell density can be obtained. But O.D.\textsubscript{600} does not give the exact number of cells. To obtain an estimate, of the number of viable cells present CFU counting procedure may be used. In this procedure, the cell suspension is serially diluted and a known volume of each dilution is plated on nutrient agar. After incubation the colonies that are formed are counted. The cells, those that are able to sustain their capability of doubling (DNA replication and cell division), only give rise to a colony and therefore each colony represents a viable cell. The CFU counting method only gives us an idea about the number of viable cells present but not the non viable or viable but not replicating (VBNCS) ones. In this context, haemocytometer can be used to count the number of cells present in a particular volume of the suspension. However, haemocytometer method is a cumbersome one which relies on visual counting under a microscope. Only a limited number of cells can be counted at a time and the results are based on obtaining the averages. The haemocytometer also cannot be used for other purposes such as investigating the cell’s DNA content, metabolic state, size and shape. We therefore considered the possibility of using a cell sorter for the purpose of counting. The cell sorter has several advantages.
Firstly, it can give a precise estimate of the number of cells present in the suspension within a short period of time. Thus a large cohort of cells can be counted accurately, within minutes. Moreover, one can get significant information regarding the size and shape of each bacterium present in the population. Also the metabolic status of the cells can be understood by staining the cells with various dyes. Hence overall, the cell sorting using a FACS machine can potentially lead to lot of information about cells, both quantitative and qualitative. However, prior to using cell sorting as a method to analyze bacterial cells quantitatively several basic tests had to be performed. The most important issue was to know the dynamic range of the counting procedure. In other words, it was necessary to know the limits within which the counts derived may be considered accurate. Prior to using FACS to count mycobacterial cells it was necessary to standardize the PMT voltage that could be used for detecting the electrical signals emanating from the bacterial particles. Being small the light scattering in case of bacterial cells is expected to be less in comparison to eukaryotic cells. If the PMT voltage is too high, then the signals emanating from bacteria would be so low in intensity that the dots would be consigned to the origin of a scatter plot. Thus it was necessary to reduce the PMT voltage to a lesser value. However, in doing so, we stand the risk that the particles detected may not be cellular in nature, but debris. It was therefore necessary to examine whether the dots that are registered on the scatter diagram truly represent, bacterial particles. In order to confirm this, we took a suspension of mycobacterial cells at a given density of \(10^7\) cells ml\(^{-1}\), injected it into a cell sorter and measured the extent of scattering both forward and side. By performing this experiment we obtained scatter plots (figure 4.1.6A and B) in which each spot is expected to represent a single bacterial cell. Now to make sure that the spots do represent cellular entities, the suspension was treated with two cell specific fluorescent dyes, one of which was SYTO13, a DNA staining dye that stains both live and dead cells, and the other Fluorescent di-acetate (FDA) that stains only live cells. The stained cells were then subjected to cell sorting. The results (figure 4.1.6 C) indicate that almost 99% of the cells were stained with SYTO13, which confirms that each dot represents a cellular entity and not debris. At the same time 97% of the cells stained with FDA, which means almost all the cells were in the viable state (figure 4.1.6D). Therefore, it is proved beyond doubt that, the dots represent cellular entities.

Having being convinced that the cells sorted were indeed cellular entities, our next objective was to investigate whether the sorting method could be used to count the number of cells accurately. To achieve this, the cell suspension was serially diluted and a fixed volume (2.4 µL) from each dilution was injected into a FACS machine and the number of dots that appear on a scatter plot (SSC vs FSC) was recorded. The numbers thus obtained were converted to counts ml\(^{-1}\), by considering that each dot represents one cellular unit, irrespective of viability. The counts ml\(^{-1}\), were found to vary linearly over a broad range of dilutions from 1 (undiluted) to \(10^{-3}\) (figure 4.1.5A). A similar experiment was performed by monitoring OD\(_{600}\). In this case too a linear co-relation between the OD\(_{600}\) values and relative concentration was evident (figure 4.1.5B). Finally a co-relation was concluded between the relative counts determined by FACS and the OD\(_{600}\) values (figure 4.1.5C). The results obtained validate the use of the above mentioned techniques for monitoring changes in cell densities over a broad range.
References


4.2. Interaction dynamics of mycobacteriophage D29-\textit{M. smegmatis} phage-host system.

4.2.1. Kinetics of the host cell depletion following phage addition.

In order to examine the effect of phage addition on \textit{M. smegmatis} cells, they were added in such a way so as to generate two different MOIs (1 and 0.1). The time dependent changes in cell and phage numbers were then analyzed by removing aliquots at regular intervals and plating for CFUs and PFUs. The results (figure 4.2.1 A and B) show that at both the MOIs, the number of CFUs declined in a time dependent manner, and that there was a concomitant increase in the number of PFUs. However if the results are carefully analyzed several intriguing aspects emerge (figure 4.2.1A). Thus we see that the decline in cell counts commences, not immediately but after a definite interval of time. During this interval, the phage counts increase steadily in such a way that at a particular time point (about 3 hrs. indicated by an arrow, figure 4.2.1a), the number of phage particles become equal to that of the viable cells present in the suspension. It is only when, the phage counts become equal to cell counts, that decline in viability of the cells was observed. Thus a critical phage-cell ratio is necessary for host cell death to take place. From the results presented in figure 4.2.1a it is evident that the ratio must be 1:1. We then argued that if the phage : host ratio is 1:1, to begin with then the cell death should commence at an earlier time point. To test this possibility, phage was added to the cells at an MOI of 1. The time dependent increase in phage titer, as well as decrease in CFUs, was then monitored. The results indicate that, when the starting MOI is 1, cell death commences earlier as compared to that when the MOI was 0.1. Thus the phage-host ratio has an important role to play in determining the fate of the cell.

4.2.2. Evidence for non-lytic cell death

The results presented, above indicate that in a phage-host interaction system, there is an almost $10^3$ fold decline in the viability count of the host cells following incubation for a period of 6 hours. The question that may be raised at this stage is whether this drastic reduction of cell counts is truly due to lysis following infection, or due to some other
reasons. To address this issue we decided to monitor the decline in cell numbers using three methods in parallel: a) FACS b) Optical density (OD$_{590}$) and c) viability counts CFU/ml. The expectation was that, if the cell counts decrease due to lysis, then the particle counts as determined by FACS should decrease proportionately. The same is expected in case of OD$_{590}$, which measures the turbidity of the suspension due to the presence of cellular entities. To address this question, the host cell culture was infected at an MOI of 1, and the cell counts, as measured by the three methods mentioned above, were determined at regular intervals for a period of 4 hrs. Within this interval, and at this MOI, it is expected that at least 50% of the cells will be infected (as described earlier). The results indicate that the decrease in total cell numbers, as determined either by flow cytometry or spectrophotometry was to the extent of about 70% of the original value, which is not even one order of magnitude (figure 4.2.2 A and B), whereas in case of viable counts the decrease was about three orders of magnitude (figure 4.2.2C). Thus we observe that total cell counts as determined by either FACS or optical density measurement decreased only marginally whereas viability counts decreased to a much larger extent.

4.2.3 The effect of phage-host ratio on cell death kinetics

The above experiment was performed using a fixed MOI. To investigate the disparity in the results obtained by FACS and viability counting, the above experiment was repeated by varying the MOIs, from values below 1 to those above. The experiment was performed by adding phage to cells at an intended MOI. Prior to addition of phage an aliquot of the cells was removed, and plated for CFUs. In order to determine precisely the number of virus particles that were added to the cells, the viral titers was estimated immediately after addition of phage to the cells. The ratio derived, viral titers to cell count, is the effective MOI. For the determination of MOI dependent killing of host cells, estimation of the number of cells either viable (CFUs) or total (FACS) was determined and plotted against time. The experiments were performed over a range of MOIs (0.1-2.47) and the direct cell count and cell viability was measured in parallel (figure 4.2.3A and B). The results obtained indicate that all the MOIs the decrease in cell counts as observed by CFU counting was much more than that compared to FACS count. Finally the extent of killing observed after 6 h was not linearly correlated with MOI (figure 4.2.3, C and D but was hyperbolic. Thus by using MOIs higher than 1 no significant change in the extent of cellular depletion was observed. This is consistent with the Poisson’s principle, which, as discussed in the previous chapter states, that adsorption efficiency will saturate if phage host ratio exceeds 1.
4.2.4 Cell death requires phage growth

The results presented above imply that as phage grows in number host cell numbers get depleted. Thus it may be assumed that phage growth is essential for cell death. However such an assumption needs to be tested. To address this question, we took advantage of the availability of a mutant phage (D29ts10) in our laboratory that is known to grow in a temperature sensitive manner. We asked the question as to whether cell killing will occur or not if the multiplication of phage is stopped by increasing the growth temperature from 32°C to 42°C. Prior to performing this experiment it was necessary to analyze the extent to which the growth of the mutant (D29ts10) is restricted by temperature shift to 42°C. The results show that at 37°C both the wild type and mutant grow at almost the same rate (figure 4.2.4C, squares compared to circles), however at 42°C the growth of the mutant was completely inhibited (figure 4.2.4C). When the cell fate of phage treated cells was assessed it was found that the mutant fails to kill the cells at 42°C (figure 4.2.4B, diamonds compared to squares). At 37°C (figure 4.2.4A) cell killing was observed although to a lesser extent as compared to wild type. The results indicate that the cell death ensues only in case of the wild type but not the mutant phage, and hence the phenomenon of cell death is specifically dependent on phage proliferation.
4.2.5. Viability assays using fluorescent dyes

The results presented in the previous section indicate that a sizable fraction of the cells which have not lysed have been rendered nonviable. To explore the metabolic status of the cells that appeared to have been rendered non-viable following phage treatment, we decided to stain the cells with propidium iodide (PI). Prior to performing the experiment with PI, we tested whether this dye can be used to monitor cell death in case of mycobacteria. To standardize the protocol, we treated the cells with either phage or the antibiotic kanamycin. The decline in viability was examined by monitoring the number of CFUs present in the cell suspension following treatment with one or the other of the two anti-bacterials at various time points after their addition. The results indicate that whereas the untreated cells grew normally (figure 4.2.5A green trace), there was a decline in the number viable cells, within 50 min of the addition of the antibiotic (figure 4.2.5A blue trace). However, from 50 min the viable cell count did not change significantly. In the case of phage, the pattern was different (figure 4.2.5A brown). In the initial stage, no significant difference was observed, but after a...
period of 150 min a sharp decline was evident. The results indicate that the kinetics of cell killing was different in the two cases. Next we attempted to investigate the extent to which the cells take up PI. The results of this investigation revealed that whereas in the exponentially growing cells, PI staining did not change significantly with time (figure 4.2.5B and graphical representation C, green lines). An increase in PI uptake was however observed in the case of antibiotic and phage treated cells (figure 4.2.5B and graphical representation C, blue and brown lines for antibiotic and phage respectively). This indicates that cell death of mycobacteria can be monitored using PI staining. An interesting observation however, is that the increase in PI uptake was much more in case of phage treated cells as compared to kanamycin.

By using PI staining one can figure out whether the cells are dead or not. However PI staining does not give us any idea about the number of live cells present. To obtain an insight into the fraction of cells that remain alive following phage treatment, we performed FDA/PI (live-dead) staining of the phage treated cells. The experiment was carried out by infecting host cells at an MOI of 1 and incubating them for 4 h. The staining profiles of the cells either treated (T) with phage for 4 h or untreated (U) was then monitored. Fraction of the cell population that was FDA positive was clearly same (figure 4.2.6A and C, green dots) irrespective of whether the cells were phage untreated (U) or treated (T). However, there was a distinct difference in the fraction of FDA stained cells that accepted PI stain between (U) and (T) (figure 4.2.6B and D). In case of (T) nearly all (96%) of the FDA positive cells were also positive for PI whereas for (U) the corresponding value was only 22% (figure 4.2.6D, red dots overlaid on green). The results indicate that almost all the cells that did not undergo lysis following 4 h treatments with phage, stain heavily with PI and are therefore likely to be in a non-viable state which is consistent with the drastic reduction in viable counts as reported in the earlier sections.

Figure 4.2.6: Live-dead staining of host cells treated with phage at an MOI of 1. Cells either untreated (U) or phage treated (T) were stained after 240 min (4 h) of phage addition. The FDA staining (A and C) and overlay of the PI staining (B and D) are shown. (B and D) The percentage of FDA stained cells that also can be stained with PI (red dots overlaid on green) is indicated for untreated as well as treated cells.

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4.3. The mechanism of phage induced mycobacterial cell death.

4.3.1 Evidence for a secondary killing factor that acts in trans.

To find an answer to the question as to how these cells were being rendered non-viable, theoretical modeling studies were done (refer supplementary information, Chapter 7). Such studies indicated that there must be a secondary factor involved in the process. The hypothesis put forward was that the host cells might release certain products after phage infection which act extra-cellularly on other cells in trans and kill them. To test this possibility, cells not infected with phage, ‘fresh cells’ (FC), were added to those that were infected, in the same proportion, either at time 0, or at various time points subsequently (figure 4.3.1, colored arrows). The rate of decay of the combined cell population (initial and freshly added) was then examined. If death of the freshly added cells (FC) required phage infection and lysis then a delay – a period corresponding to at least the latent phase, was expected. However, this was not observed. The results indicate that all cells including the FCs decayed (figure 4.3.1, traces corresponding to + FC) at the same rate indicating that the secondary factor did not discriminate between the primary and secondary populations.

4.3.2 Superoxide production in mycobacteria following infection.

The results presented above indicate that, a trans acting factor released from phage infected cells is responsible for the observed reduction in viable count. In several earlier studies it has been pointed out that one of the reasons why cells die following antibiotic treatment is due to the release of superoxide radicals. We suspected that a similar phenomenon must be happening here too. Hence we considered the possibility that following phage infection superoxide radicals may be generated. In order to examine this possibility we decided to use the superoxide radical specific dye dihydroethidium (DHE).

The experiment was performed by treating the cells with phage, followed by the staining of the cells by DHE. For comparison we performed a control experiment in which superoxide generation was induced by the addition of hydroxyurea (HU), a well-known inhibitor of DNA replication and cell division. HU treatment on bacteria rapidly induces a set of protective responses to manage genomic instability. Continued HU stress activates iron uptake and toxins MazF and RelE, whose activity causes the synthesis of incompletely translated proteins and stimulation of envelope stress responses (Kohanski et al., 2007) causing an increase in superoxide production after a series of events. The increased superoxide production subsequently increases the formation of hydroxyl radicals that contribute to HU-induced bacterial cell death (Davies et al., 2009). Results show that as expected HU treatment does result in production of superoxide at a higher level as compared to the untreated control as indicated by the increased DHE fluorescence observed after 150
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min of treatment (figure 4.3.2A and graphical representation B). Treatment with phage D29 also led to superoxide production nearly to the same extent as HU. Thus the results indicate that D29 treatment of host cells result in superoxide production.

Figure 4.3.2: Control experiment to show the superoxide production with HU and compare the level of generation after D29 treatment of the cells. (A) A FACS scatter dot plot showing cells that were treated either with HU or phage (D29) for superoxide production and amount of fluorescence was measured in PI channel at 2.5 h after treatment. Quadrant gate was done to analyze the shift in the cells showing increased amount of superoxide through DHE accumulation. (B) Bar diagram showing the mean DHE fluorescence of the cells in arbitrary units (a.u.) after 2.5 h of treatment. Control represents the untreated cells. The colour of the dots are representative and are not directly related to the fluorescence of the test samples considered.

4.3.3 Superoxide production and cell death.

It is currently believed that bacterial cell death induced by external agents such as antibiotics is a complex process (Kohanski et al., 2007). The interaction between a lethal agent and a target leads to secondary events such as generation of reactive oxygen species (ROS). In order to investigate whether there is any connection between cell death and ROS formation in the context of the present investigation, phage was added to host cells followed by staining either by PI (for cell death) or DHE (for ROS) (Robinson et al., 2006). The results indicate that there was a time dependent increase in the population of PI stained cells in the case of phage treated cells (figure 4.3.3A, T(PI) panel, red dots) whereas in case of the control (untreated) no significant increase was observed (figure 4.3.3A, U(PI) panel). This confirmed that following phage treatment the cells were facing death. When another aliquot taken from the same phage infected culture was stained with DHE, an increase in the fluorescent population was observed (figure 4.3.3A, T(DHE) panel, red dots). As before the increased DHE staining was observed only in case of the phage infected cells, but not the uninfected ones (figure 4.3.3A, U(DHE) panel). While performing these experiments it was observed that the maximum increase in DHE staining activity happened at an earlier time point compared to that of PI. To confirm this further the PI and DHE staining experiments were repeated, this time in triplicate, and the average values obtained ± standard deviation were plotted against time of infection. The results show that indeed DHE staining activity peaks prior to that of PI (figure 4.3.3B, thin arrow compared to thick). The result suggests that ROS formation is possibly the cause and cell death, the effect.
Figure 4.3.3: Superoxide generation associated with phage growth. (A) Cell death and generation of superoxide were monitored at different stages of phage growth using PI and DHE as indicated. The cells were either treated with phage (T) or left untreated (U). The images are described as U(PI), T(PI), U(DHE) and T(DHE) depending on their treatment status. The numbers in boldface at the top indicate the time points, in minutes, at which monitoring was done. Thick (PI) and thin (DHE) circles highlight the presence of the maximum number of cells in the Q1 quadrant (highly stained population). (B) The experiment was repeated three times, and the mean population density ± standard deviation was plotted against time. Thick (PI) and thin (DHE) arrows point to the stage where maximum Q1 population density was achieved during the course of the experiment.
4.3.4 Effect of ROS antagonist on survival of phage treated cells.

Manganese (Mn²⁺) ions have the ability to scavenge ROS (Imlay, 2008). Therefore, it was argued that if formation and release of superoxide radicals is the reason behind the death of cells, then by the addition of Mn²⁺, a superoxide radical scavenger (Coassin et al., 1992), it should be possible to prevent cell death. To perform this experiment, phage was added to cells at an MOI of 1. After a period of 2 h, the time taken for adsorption to reach saturation and the latent phase to start, the infected cells were divided into two fractions. Mn²⁺ was added to one fraction at a final concentration of 5 mM, while the other fraction was left alone. Both fractions then were allowed to grow. As a control, a culture which was not infected was monitored in parallel (figure 4.3.4A). At regular intervals, the release of extracellular superoxide radicals was measured using the NBT assay (Beauchamp and Fridovich, 1971). The results show that although in both uninfected and infected cases superoxide levels increased in a time-dependent manner, in the case of the latter (host plus phage), the increase was significantly greater than that of the former (host only). Moreover, as observed in the previous set of experiments, the activity was found to peak at an intermediate time point. Thus, the kinetics of extracellular ROS accumulation appears to be of the same pattern as that of its intracellular formation. When phage-infected cells were treated with the ROS blocker Mn²⁺, the superoxide level was found to decrease to a low level. The results indicate that phage-infected cells release more ROS than the uninfected ones; moreover, the released superoxide radicals can be neutralized by the addition of Mn²⁺. The effect of Mn²⁺ addition on the survival of host cells subjected to phage attack then was monitored (figure 4.3.4B). The results show that the viability of the cells improved significantly. The observed increase in cell survivability in the presence of Mn²⁺ was not due to any inhibitory effect of Mn²⁺ on phage growth. This is evident from the observation that the phage amplification was not affected significantly by the addition of Mn²⁺ (figure 4.3.4C).

Figure 4.3.4: Effect of Mn²⁺ on cell survival and phage stability. (A) Time-dependent monitoring of ROS in the supernatant of phage-treated (host plus phage) or untreated cells (host) using NBT. In the case of the phage treated cells, the experiment also was performed in the presence ofMn²⁺ (host plus phage plus Mn²⁺, which was added at the 2-h time point (arrow). (B) Survival of host cells following phage treatment performed either in the absence (-) or presence (+) of Mn²⁺ at the indicated time points. Each data point represents the mean ± standard deviation derived from three biological replicate experiments.
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References


