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Propagation of Fluorescent Viruses in Growing Plaques

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ABSTRACT: To study virus propagation, we have developed a method by which the propagation of the Lambda bacteriophage can be observed and quantified. This is done by creating a fusion protein of the capsid protein gpD and the enhanced yellow fluorescent protein (EYFP). We show that this fusion allows capsid formation and that the modified viruses propagate on a surface covered with host bacteria thus forming fluorescent plaques. The intensity of fluorescence in a growing plaque determines the distribution of phages. This provides a new tool to study the propagation of infection at the microscopic level.

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KEYWORDS: bacteriophage Lambda; plaque growth; fluorescence microscopy

Introduction

Over the last 90 years, a continuous effort has been made to characterize and understand the propagation of viruses in growing plaques. When bacteriophages are mixed in a layer containing agar-immobilized host bacteria, each virus can infect a host and initiate a spreading infection, leading to a visible plaque within the bacterial population. Such macroscopic observations led to the discovery of bacteriophages in 1915 by Frederick W. Twort (Twort, 1915) and have become a common technique used to titer a phage solution or to isolate mutant phages. It is also an ideal tool to study the propagation of viruses because of its simplicity.

In the last decades, studies focused on the propagation of T7 phage (Lee and Yin, 1996; Yin, 1991; Yin and McCaskill, 1992; You and Yin, 1999) and the evolution of the phage during the propagation of infection (Yin, 1993). This led to several theoretical developments that describe the propagation of infection by a reaction-diffusion model (Fort and Méndez, 2002; Yin and McCaskill, 1992).

In the field of viral infection, fluorescence microscopy has been exploited in several ways. A viral DNA has been tagged with a fluorescent particle to identify bacterial species (Mosier-Boss et al., 2003). A gene expressing the Enhanced Green Fluorescent Protein has been integrated in the genome of measles virus (Duprex et al., 1999, 2000) allowing studying infection in real time. The viral DNA can also be modified to express a protein fusion between a viral protein and a fluorescent protein. For example, this has been used to visualize the trafficking of a viral structural component of Herpes Simplex Virus in the cytoplasm (Elliott and O'Hare, 1999).

Along the same lines, we constructed a fluorescent Lambda bacteriophage to follow the propagation of infection of microcolonies of host agar-immobilized *Escherichia coli* on Petri dishes. Thus, Lambda phage population during infection can be observed in real time, which should allow to study infection at the microscopic level and test models of virus propagation.

We report how we modified the lambda genome to code for a protein fusion of the gpD capsid protein (11.4 kDa) and the enhanced yellow fluorescent protein (EYFP, 27 kDa). We find that this genetically modified fluorescent phage is able to propagate on a solid substrate containing immobilized host bacteria, and estimate the 2D phage density in plaques by measuring fluorescence intensity. This demonstrates the suitability of the fusion to study the infection of host bacteria.

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Materials and Methods

Strains

Strains *E. coli*: HS996 (F-*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G *fhu*A::IS2), NM538 (*supF*, *hsdR*(r_K^-,m_K^+), *trpR, lacY*), and YMC (*sup*F *lac*Y) were used.

We worked with Lambda *cI857 Sam7*, called Lambda WT (wild-type), in the following for convenience. Lambda *cI b2* and Lambda *cI h80 Δ(b2-int)*, selection phages (provided by Mathias Springer), were used to select for lysogens.

Recombination

First, the lambda genome was integrated into the chromosome of the bacterium with Lambda WT (Silhavy et al., 1984), leading to a lysogen (HS996λ). The Counter Selection Kit from Gene Bridges was then used to genetically modify the integrated lambda genome by homologous recombination. Briefly, a counter-selection cassette carrying resistance to kanamycin and sensitivity to streptomycin is first inserted immediately upstream of the stop codon of gene D. This cassette is then replaced by a non-selectable DNA insert, which contains the *eyfp* gene. This two steps approach allows for both steps to be selectable since the strain is first selected for kanamycine resistance and then for streptomycin resistance. The DNA insert (*eyfp* gene sequence) was amplified by PCR from plasmid pEYFP from Clontech (Mountain View, CA) and introduced just before the stop codon of *gpD* (leading to HS996λY strain); this added the linker sequence: GGTCT CGGCTCTGGTGGAGGAAG-TGGTGGAGGTTCTGGTGGCACT GCC, between the two genes. The linker codes for the amino acid sequence $GL(GSGG)_3TA$, which is composed of neutral amino acids and was previously used in fusions of gpD with various proteins (Yang et al., 2000).

The WT Lambda DNA is 48,502-bp long, the DNA of the fluorescent Lambda (λeyfp, see text) is 49,264-bp long, and the DNA of the b2 mutant fluorescent Lambda (λeyfpb2, see text) is 43,267-bp long.

Lytic Cycle Induction

The lysogen HS996λY was first grown in LB medium including Streptomycin (50 μg/mL) at 30°C, until an OD at 600 nm of 0.1 has been reached. Lambda *Sam7 cI857* has a thermo-sensitive mutation in the gene *cI* which regulates lysogeny. Lytic cycle was induced by rising temperature up to 42°C for 15 min, and bacteria were then incubated at 37°C with good aeration for at least 45 min. As HS996 is not a *sup*F strain, it cannot suppress the *Sam7* mutation, leading to loss in activity of the lysis protein gpS. In order to recover the phages, we centrifuged the induced bacteria, resuspended them in a smaller volume (about 1 mL) of Phage Buffer (20 mM Tris-HCl, 10 mM $MgSO_4$, 100 mM NaCl), added about 100 μL of chloroform, incubated 10 min at 37°C with vigorous shaking, centrifuged at 4,000*g* for 10 min, and recovered the supernatant containing the phage particles.

Continuous Propagation of Phage

All titers were performed using strain NM538. The protocol was adapted from Maniatis et al. (1989) using top agar (10 g peptone, 5 g NaCl, and 4.8 g of agar per liter) and bottom agar (LB and 15 g agar per liter) which were both autoclaved; 10 mM $MgSO_4$ was added both in bottom and top agar after cooling down to 60°C. Protocols typically use 7 g of agar in the top agar but lower concentrations of agar allows faster diffusion of the virus in the gel which results in larger plaque size. Before mixing with top agar, plating bacteria with phages were incubated for 22 min at 37°C; plates were incubated at 37°C overnight (>16 h). When needed, the phages were diluted in Phage Buffer.

Lysogenization

Individual fluorescent plaques were resuspended in Phage Buffer. Bacterial strains YMC and NM538 were lysogenized by incubating bacteria with fluorescent phages, followed by spreading on plate covered with selection phages. The genome of the lysogens was then used as template for PCR to identify b2 mutants: PCR were led in the attR region and the amplified sequence was checked by sequencing.

Lysogens were also induced in order to produce stocks of fluorescent viruses.

One-Step Growth Experiments

Protocol was adapted from those described earlier (Wegrzyn et al., 1995). NM538 were grown at 37°C in 50 mL LB supplemented with 0.2% Maltose and 10 mM $MgSO_4$ to $OD_{600} = 0.1$, sedimented at 2,500 g/10 min and suspended in 5 mL of pre-warmed 3 mM NaN_3 and 10 mM $MgSO_4$ in LB. After 5 min at 37°C, the phages were added to multiplicity of infection of 2.5; phage adsorption at 37°C was carried out for 10 min. The infected bacteria were then diluted sixfold in warm (37°C) LB with 3 mM NaN_3 and 10 mM $MgSO_4$. Instead of using anti-λ serum to remove free phages, the infected bacteria were centrifuged three times at 2,500*g* for 6 min and suspended each time in 30 mL LB with 3 mM NaN_3 and 10 mM $MgSO_4$. Bacteria were then diluted 1,000 times in LB with 10 mM $MgSO_4$ and incubated at 37°C; this corresponds to $t = 0$.

Fluorescence

Fluorescent growing plaques on Petri dishes were observed with an Olympus BX51WI microscope equipped with a

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common epifluorescence set-up. Images were captured with a Micro-max camera (Roper Scientific, Duluth, GA) and controlled by a PC with Metamorph software. A 100×, oil immersion objective (Zeiss, Jena, Germany) was used to observe bacteria after lytic cycle induction and a 4× air objective (Olympus, Tokyo, Japan) was used to observed plaques.

Fluorescence intensity profile of a plaque was determined by image analysis. We calculated the intensity mean value with its standard error for each concentric ring to the center of the plaque. The center of the plaque was calculated using the intensity from each pixel in the image as a statistic weight.

$$\overrightarrow{R_c} = \frac{\sum_{i,j} I_{i,j} \cdot \overrightarrow{(i,j)}}{\sum_{i,j} I_{i,j}}$$

I is for the intensity and i, j are the coordinates for each pixel in the image.

Fluorescence Recovery After Pattern Photobleaching Experiments

Measurement of the diffusion coefficient of free gpD-EYFP protein fusion in agar plates has been done using fluorescence recovery after pattern photobleaching (FRAPP). The technique has been theoretically described (Davoust et al., 1982) and recently applied in superdiffusion experiments (Gambin et al., 2005)

Basically, the gpD-EYFP fusion proteins are bleached with a high intensity interference pattern of laser light. Fluorescent molecules diffuse in the sample (agar) and the diffusion coefficient can be measured by analyzing the progressive recovery of fluorescence in the bleached regions.

Results and Discussion

gpD is a head protein of Lambda and forms a trimer, which binds to the capsid (405–420 gpD per capsid). gpD is believed to stabilize the head since it is essential to stably package the full-length DNA. As fusions between capsid protein gpD and several proteins have been earlier performed successfully, leading to functional gpD fusions (Forrer and Jaussi, 1998; Yang et al., 2000), we chose to fuse EYFP with gpD.

In a first step, we lysogenized HS996 with wild-type Lambda in order to obtain HS996λ. Homologous recombination was then used to insert *eyfp* gene at the very end of gene *D*, leading to HS996λY, and HS996λY strains were induced (all details are described in Materials and Methods). Because HS996 is not *sup*F, the phages cannot escape from the inside of the bacteria after all compounds of the viruses have been assembled (DNA packaging, capsid assembly). This allows a qualitative control of the efficiency of induction by looking at the bacteria under microscope (see Fig. 1). Using HS996λY chromosome as template, a PCR was performed to amplify the sequence in the region surrounding the *gpD* gene. The PCR product was deposited on agarose gel and led to only one band corresponding to the gene fusion *D-eyfp*. The product was sequenced and it revealed that the *eyfp* gene had been correctly inserted. Thus gpD and EYFP cannot be expressed alone; this implies that the fluorescence can only originate from gpD-EYFP and not from single EYFP. Moreover, as the Lambda capsid is not stable in the absence of gpD proteins, if infection spreads, the Lambda viruses carry gpD-EYFP fusion proteins and fluoresce (the possibility of the apparition of a non-fluorescent phage during infection is discussed later).

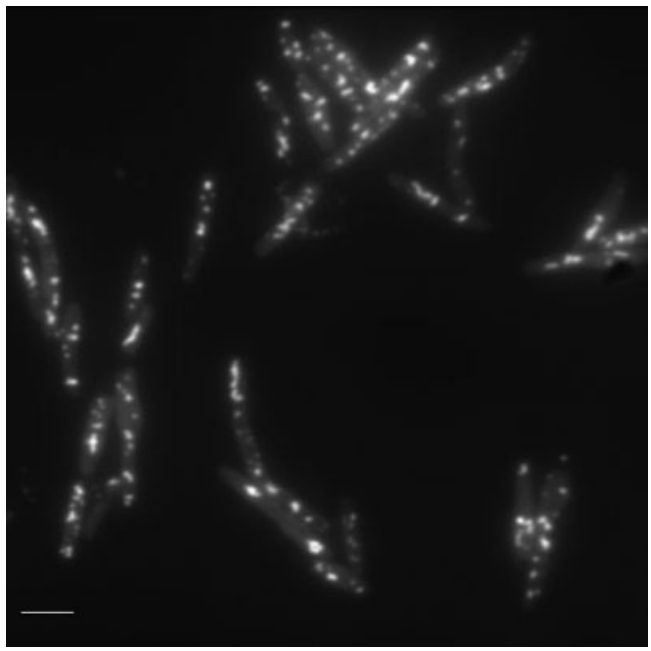

Figure 1. Strain HS996λY after lytic cycle induction. Bacteria were deposited on microscope slide and observed with 100× objective. The bar represents 5 μm. The punctuated pattern is interpreted as the distribution of the fluorescent phages (each carrying about 400 gpD-EYFP proteins) produced in the cell, which is different from single fluorescent fusion proteins whose contribution is a fluorescent background. The number of fluorescent points observed by microscopy (10–20) matches well with the yield of 18 (+/−3) phages per cell obtained in the one-step growth experiment (see Fig. 3).

Lambda Viruses Containing the gpD-EYFP Fusion Produce Fluorescent Plaques

After lysis of HS996λY bacteria, fluorescent bacteriophages (λeyfp) were recovered (see Materials and Methods section). The phages λeyfp propagate in growing plaques as shown in Figure 2. The two images are of the same plaque after an overnight infection using bright and epifluorescence

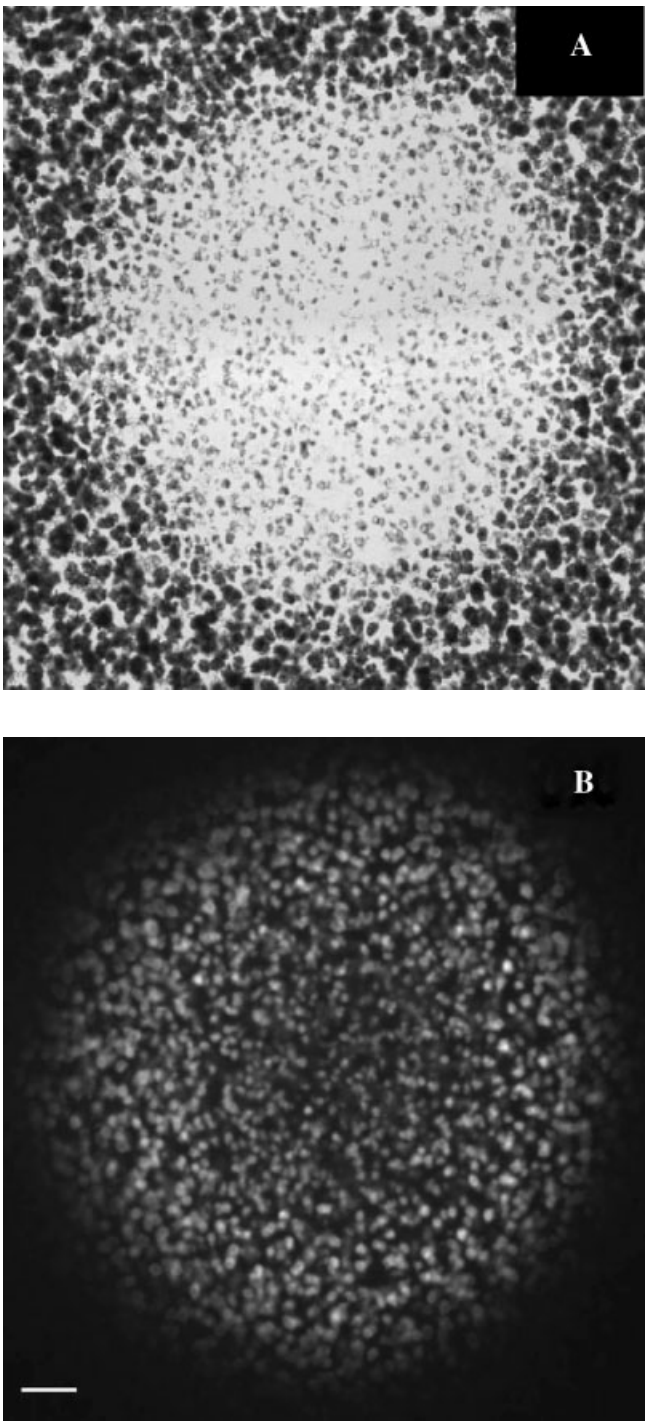

Figure 2. Plaque produced by bacteriophages after incubation of 17 h at 37°C, in bright (**A**) and epifluorescence illumination (**B**). The bar represents 150 μm.

illumination. The plaque (lysed cells) appears in bright areas in Figure 2a and matches the fluorescence in Figure 2b. By titering λeyfp with a higher top agar density (8 g/L) and lower Mg^{2+} concentration (~1 mM $MgSO_4$; lowering magnesium destabilizes phage particles), we selected a fluorescent plaque which turned out to only contain a mutant fluorescent phage (λeyfpb2) with its DNA deleted in the b2 non-essential region (deletion from $i = 21{,}743$ to $i = 27{,}738$ in the Lambda WT index). The b2 mutant was only obtained in this condition (low magnesium and high top agar density). It was not surprising because b2 mutants are commonly selected by titering phages in absence of Mg^{2+} (Silhavy et al., 1984).

Lambda Viruses Containing the gpD-EYFP Fusion Form Stable Capsids

We wanted to determine how the gpD-EYFP fusion changed stability of the capsid. One way to test the stability of the capsid is to measure the sensitivity of the capsid to EDTA. We did this by infecting bacteria in growing plaques with either fluorescent viruses (λeyfp and λeyfpb2) or wild-type Lambda that were each pre-incubated in 10 mM Tris and 10 mM EDTA for 15 min at 25°C. We found that EDTA decreased the titer by <10 for λeyfpb2, about 10^2 for Lambda WT, and about 10^3 for λeyfp. The gpD-EYFP fusion destabilizes the capsid since λeyfp is more sensitive to EDTA than WT phage. Interestingly, λeyfpb2 is more resistant than WT phage. This is likely due to a reduction of stress on the capsid because the b2 deletion reduces the genome size by about 12%. The gain in stability held by the deletion seems to balance the loss in stability due to the protein fusion. In addition, we find that the fluorescent phages (λeyfp and λeyfpb2) can be stored at 4°C for several months without a significant reduction in the titer, like Lambda WT.

Fluorescence Quantification of the Virus Distribution Within Plaques

The plaques are about the same size for λeyfp and λeyfpb2, but we noted that Lambda WT made larger plaques than fluorescent phages (about twice the diameter of fluorescent plaques after overnight infection). This indicates that fusion somehow inhibits propagation. The stability of the capsid does not correlate with plaque size, because λeyfpb2 plaques are smaller than Lambda WT plaques, whereas λeyfpb2 capsid is more stable than the capsid of Lambda WT, as shown above.

To understand this difference in plaque size, we performed one-step growth experiments (see Materials and Methods) on the three phages (see Fig. 3). It was found that Lambda WT produces about 3.5 times more phages per cell than λeyfpb2 and 8.8 times more phages per cell than λeyfp. One can also notice that the mutant phages are produced more slowly than Lambda WT (see Fig. 3). How can we explain these differences? A burden of biosynthesis can come from protein translation but it is difficult to estimate this effect. A burden could also come from capsid assembly due to the fusion protein gpD-EYFP. The burden due to DNA replication seems to be not relevant: the λeyfp DNA is about 2% longer than Lambda WT DNA; in the case

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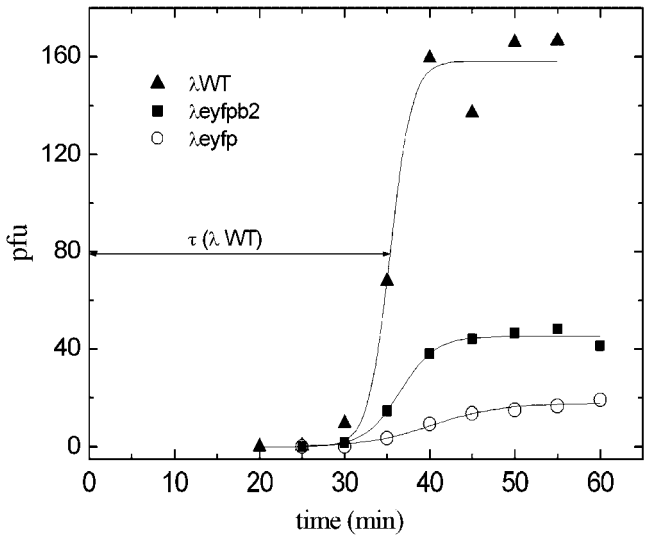

Figure 3. One-step growth of Lambda WT, λeyfp, and λeyfpb2 versus the time elapsed after adsorption ($t=0$). The solid lines are fits of data points to equation: $y = a/[1+\exp(-k(t-\tau))]$. This leads to $(\tau, k) = (35.3\text{ min}, 0.78\text{ min}^{-1})$ for Lambda WT, $(\tau, k) = (36.5\text{ min}, 0.48\text{ min}^{-1})$ for λeyfpb2, and $(\tau, k) = (40.2\text{ min}, 0.26\text{ min}^{-1})$ for λeyfp. The parameter a gives the number of phages per cell: 158 +/− 33 for Lambda WT, 45 +/− 5 for λeyfpb2, and 18 +/− 3 for for λeyfp.

of λeyfpb2, the DNA is about 10% smaller than Lambda WT DNA.

Since phages with fusions form smaller plaques, there may be selection for phages that lose fluorescence. To make sure that all of the phages from a plaque make fluorescent phages, we picked some of the plaques and plated them. This experiment has been performed several times, and each resuspended fluorescent plaque made 100% fluorescent plaques in subsequent titers.

As the modified phages form growing plaques, they can be used to map the distribution of viruses in plaques by measuring fluorescence. Which other molecules contribute to fluorescence? Indeed free gpD-EYFP fusion proteins could contribute to fluorescence. This was verified by FRAPP experiments (see Materials and Methods) performed on several samples.

FRAPP was performed on a fluorescent plaque after overnight infection (an example of such measurements is shown in Fig. 4). Two diffusing fluorescent species were observed which we identified as the free gpD-EYFP fusion protein and the fluorescent phage. Inside a plaque, the diffusion coefficient of the phage in the center was observed to be about two times the diffusion coefficient for the phage in the border. The diffusion coefficients for the virus and for the gpD-EYFP fusion protein were respectively measured to be $D_{vir} = 0.025$ (+/−0.004) $\mu m^2/s$ in the center and 0.013 (+/−0.001) in the border; and $D_{prot} = 0.29$ (+/−0.03) $\mu m^2/s$ in the center and 0.17 (+/−0.01) in the border. The diffusion coefficients are very low: the viruses and the proteins are expected to diffuse respectively on about 15–20 μm/h and 60–80 μm/h, which is small compared to the spread of infection (about 100 μm/h). As the thickness of the top Agar layer is about 500 μm, it is thus expected that only a few part of the phages and proteins diffuse in the bottom Agar layer. The low values of the diffusion

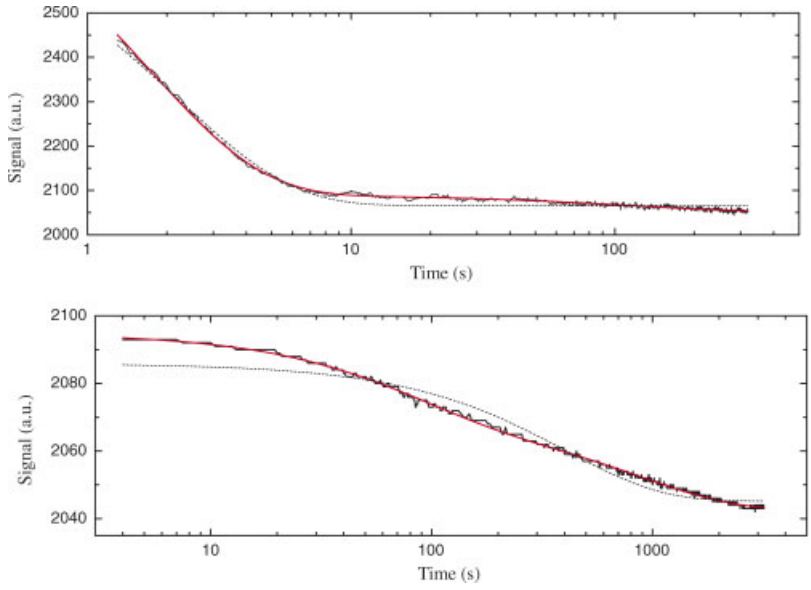

Figure 4. Example of fluorescence signals recovered during FRAPP experiments versus time. The upper curve shows signal (in arbitrary units) recovered in a layer of bottom agar under a plaque after photobleaching. The lower curve shows signal recovered in the center of a plaque. Solid lines are a double exponential fits of the data and dashed lines are single exponential fits of the data. The fit parameters of double exponential give the diffusion coefficients [Color scheme can be viewed in the online issue, which is available at http://www.interscience.wiley.com].

coefficients are explained by the presence of the bacteria which act as a barrier (Yin and McCaskill, 1992). We concluded that when microcolonies of bacteria occupy the major part of the medium, the spread of infection does not significantly depend on diffusion. In this case the free gpD-EYFP fusion proteins only slightly diffuse and are expected to match then the phage density, since free EYFP-gpD leave a lysed bacterium at the same time as the viruses.

FRAPP was also performed on a layer of bottom agar under the plaque. Initially bottom agar density is 15 g/L, but the density was not controlled in the layer during the FRAPP experiment and evaporation could contribute to lower the diffusion coefficient. It was observed that the phage diffused at 0.42 (+/−0.04) $\mu m^2/s$ in this layer, a value consistent with the diffusion coefficient of 4 $\mu m^2/s$ in 10 g/L Agar for the P22 phage (Yin and McCaskill, 1992), which is different in shape from Lambda. The protein diffused at 33 (+/−3) $\mu m^2/s$. The values of the diffusion coefficient obtained in bottom Agar (15 g/L) are much higher than those obtained in the plaque (about 5 g/L Agar), pointing that bacteria substantially hinder diffusion.

The ratio obtained between the intensity of the viruses and the intensity of the proteins gave us an estimation of the phages:proteins ratio, given that one phage carries 405–420 gpD-EYFP. We estimated 1 phage:250 fusion proteins in the plaque, and 1 phage:3,000–8,000 fusion proteins in the bottom agar under the plaque (in the later case, the ratio depends on the distance from the plaque on the axis perpendicular to the plaque).

The depth of field of the objective is about 20 μm. As the focus is made on the surface of the top agar layer, the fluorescent species under the focus plane are expected to mainly contribute to a uniform background of fluorescence. The free proteins and the phages which diffuse in the bottom agar are thus not expected to contribute significantly to neither the fluorescence intensity nor the fluorescence profile. Moreover, a phage diffusing in the bottom agar takes about 30 h to diffuse about 0.5 mm and cannot reproduce due to the absence of host, whereas the infection spreads forming a plaque with a radius of 0.5–1 mm in half that time (i.e., 15 h). Although the diffusion coefficient of the virus is larger in the bottom agar than in the plaque after overnight infection, the spread of infection is more rapid than the diffusion in the layer. For this reason, viruses diffusing in bottom agar are not expected to contribute to the fluorescence intensity.

At last, could capsid without DNA fluoresce? An empty head is not assumed to fluoresce because the binding sites for gpD are created or exposed only after the prohead expands (Imber, 1980; Wurtz, 1976). This implies that an empty head does not carry gpD-EYFP.

Therefore, we assumed that fluorescence intensity was a good estimation of the distribution of the Lambda phages in the plaque.

Data were acquired by measuring fluorescence intensity in several plaques following an overnight infection (see Fig. 5). Assuming that the phage density is proportional to

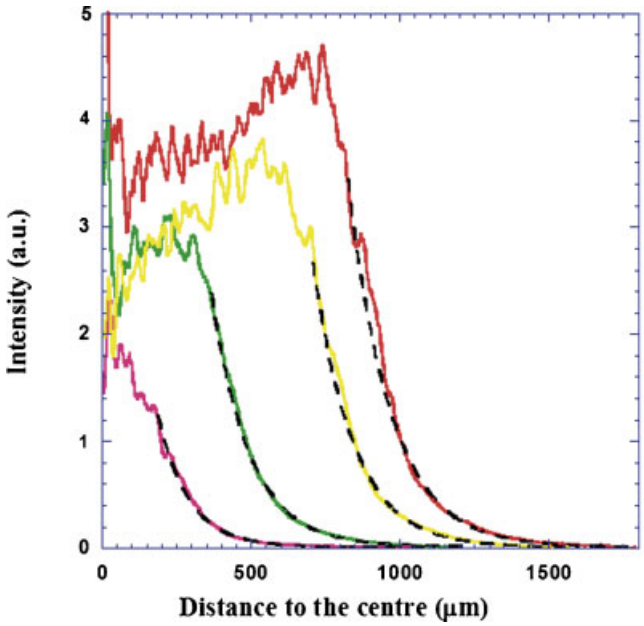

Figure 5. Intensity profile for four different plaques after 17 h of incubation at 37°C. Dashed lines are simple exponential fits of data (see text) [Color scheme can be viewed in the online issue, which is available at http://www.interscience.wiley.com].

the fluorescence intensity, it is observed that the distribution of Lambda in a growing plaque rises slowly from the center of the plaque until the front of infection; there is a peak of fluorescence at the front, and a large decrease after it. This has been already observed with the T7 phage by another method which only allowed for a coarse estimate of the phage concentration at few distances from the plaque center (Yin, 1991). This distribution profile is not well understood. It can be argued that the living bacteria at the front of infection act as a barrier, an argument supported by the difference between the diffusion coefficient measured in the center of the plaque and the one measured in the border. Although diffusion is not expected to be responsible of the spread of infection, the bias in diffusion could contribute to the distribution profile. This issue could be enlightened thanks to time lapse experiments under microscope we will perform.

In Figure 5, four plaque profiles are shown, which give the range of the plaque sizes after overnight infection: two profiles correspond respectively to one of the smallest and to one of the largest plaques, and the two other profiles correspond to size-intermediate plaques. The difference in size in not well understood but (i) it is also observed with Lambda WT, and (ii) it is not a phenotypic character as resuspended tiny plaques provided a large range in plaque size after a subsequent titer.

The decrease in fluorescence intensity at the front of infection fits well to a single exponential decay, as shown in Figure 5. We systematically fitted the decrease in

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fluorescence by $\exp(-r/\xi)$ for each plaque from several plates and find the decay length, ξ, to be about 0.1 mm.

Conclusion

In this work, we presented a new tool suitable to study different properties of infection propagation at microscopic level, by producing a fluorescent phage owing to fusion between a capsid protein and a fluorescent protein.

The next step will be to measure the 2D repartition of viruses during the first hours of infection by performing time lapse experiments under microscope. This will determine the propagation of infection in real time, and measure the velocity of propagation. A more precise understanding of the dynamics of infection will then be achieved and could allow investigation of more complex situations such as the case of the infection of motile bacteria; such an approach could also help understanding how viral infection is affected by the growing state of the host cells, quorum sensing effects, or lysogenic dependence.

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