Intracellular Parasitism of Chlamydiae: Specific Infectivity of Chlamydiaphage Chp2 in Chlamyphila abortus

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The obligate intracellular nature of chlamydiae presents challenges to the characterization of its phages, which are potential tools for a genetic transfer system. An assay for phage infectivity is described, and the infectious properties of phage Chp2 were determined.

Chlamydia and Chlamyphila spp. are pathogenic obligate intracellular parasitic bacteria. The lack of a DNA transfer system has hindered the use of genetic approaches which could elucidate the molecular mechanisms of pathogenesis, microbail control, and cellular physiology. Although chlamydiaphages could be adapted for this purpose (3, 5, 6), host intracellular replication presents technical challenges to phage characterization. Several novel assays were developed to determine the specific infectivity of chlamydiaphage Chp2 and its host Chlamyphila abortus. The phage is highly infectious, with specific infectivity of 0.5 to 1.0. In contrast, the specific infectivity of C. abortus is 2 orders of magnitude lower than that of its phage.

Infectious virion assays rely on host cell viability and, in this case, the ability of the bacterial host to infect a eukaryotic cell. To infect Chlamyphila abortus with phage Chp2, a member of the Microviridae, the bacterial host must first be propagated and isolated from eukaryotic cells. Although preparations typically yield a mixture of elementary bodies (EBs) and reticulate bodies (RBs), the latter of which are not infectious (8), RBs are osmotically fragile (4) and were lysed during preparation. However, not all EBs are infectious in laboratory procedures.

Thus, to determine the specific infectivity of Chp2, the specific infectivity of Chlamyphila abortus B577 (inclusion-forming units [ifu]/total cell count) had to be determined. The titer ofifu represents the number of infectious and/or viable EBs in a sample under the chosen laboratory conditions.

C. abortus forms densely packed inclusions during infection (Fig. 1A), which makes it amenable for the development of an accurate immunostaining titration protocol. EBs of the host, a bacteriophage-free strain of C. abortus (B577), were titrated in BGMK cells seeded at 5.0 × 10⁴ cells/well on 96-well microtiter tissue-culture-quality plates. At 72 h postinfection, the time to completion of the C. abortus developmental cycle, cells were fixed with methanol for 20 min at −20°C before incubation with a chlamydia-specific monoclonal antibody at 4.0°C for 16 h. After unbound antibody was removed, samples were incubated with a second-affinity anti-mouse antibody conjugated with β-galactosidase (Calbiochem). β-Galactosidase was chosen as the conjugate over fluorescent conjugates to facilitate the assay for a variety of reasons, including ease of use and cost. Moreover, stained-inclusion procedures can be conducted with a standard light microscope, allowing observation of an entire well, as opposed to fields, which could yield more reliable counts. For staining, 100 µl of a staining solution [5.0 mM K₃Fe(CN)₆, 5.0 mM K₄Fe(CN)₆·3H₂O, 2.0 mM MgCl₂·6H₂O, 0.25 M 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)] was added per well and incubated for 4.0 h at 37°C. The chromogenic substrate X-Gal generates blue-stained C. abortus inclusions, which were easily and reliably recognized using the light microscope (Fig. 1B and C). The titer of infectious EBs in this preparation was approximately 1.7 × 10⁷ ifu/ml.

To determine the total number of bacterial cells in samples regardless of infectivity, the concentration of C. abortus genomes was calculated by quantitative PCR. By this assay the number of genomes was found to be 6.9 × 10⁹ genomes/ml (Fig. 2). Thus, approximately 1 out of 400 C. abortus bacterial cells was infectious in this preparation, assuming one genome per bacterium. A similar specific infectivity for a C. trachomatis strain that also requires centrifugation for cell infection has been previously reported (7). To determine whether free chlamydial genomes were influencing the quantitative PCR results, a particle count of EBs (infectious and noninfectious) was also performed in the presence of concentration-quantified 100-nm-diameter latex spheres (data not shown). The EB concentration from this assay was approximately 7.3 × 10⁸ bacterial cells/ml, a value in good agreement with the results of the quantitative PCR assay. While a small percentage of aggregates must exist in any chlamydial preparation, none were observed in multiple fields assayed by electron microscopy (EM) during EB particle counting. Thus, eukaryotic cells infected by an aggregate of EBs are unlikely to significantly affect the results.

Partially purified chlamydiaphage preparations contain infectious virions, procapsids, and packaging intermediates (1). The presence of packaging intermediates, with unpackaged replicative-form DNA, complicates infectious particle titer determination by quantitative PCR. To circumvent this problem, the titer of Chp2 virions was determined by EM counting with latex spheres of a known concentration. Ratios of virions and...
 procapsids to latex spheres can be calculated, as each particle has a distinctive morphology (Fig. 3). Latex spheres are relatively large structures (diameter, ~100 nm) compared to procapsids and virions (diameter, ~20 nm). Virions and procapsids can be further differentiated by their appearance. Procapsids, devoid of genomes, stain with a darkened center, while stain does not penetrate virions (Fig. 3B). The titers determined by this technique were approximately $3.0 \times 10^{12}$ virions/ml and $1.5 \times 10^{12}$ procapsids/ml.

To determine the number of virion-like staining particles that were infectious, a phage-specific primary monoclonal antibody was used to stain phage-infected C. abortus inclusions. For these assays, BGMK cells were infected with C. abortus B577 at a multiplicity of infection of 1.0 ifu/eukaryotic cell. A 10-fold dilution series of semipurified Chp2 was preincubated with C. abortus for 30 min prior to infecting BGMK cells. Cells were stained with a phage-specific primary monoclonal antibody and then with a β-galactosidase-conjugated second-affinity antibody as described above. Inclusions formed by bacteriophage-infected C. abortus stained as blue compact structures, which had a sharply defined outline and therefore were easy to count directly by light microscopy (Fig. 1D).

By this assay, which was performed multiple times with little variation, the titer of infectious particles was approximately $5.8 \times 10^9$ chlamydiaphage-stained inclusions/ml, a value significantly at variance with the EM-determined particle count of approx-
imately $3.0 \times 10^{12}$ virion-staining particles/ml. However, only 1 in 400 (0.25%) C. abortus B577 EBs is infectious. The presence of noninfectious EBs affects the determination of viable phage titers. Chp2 is known to bind noninfectious RBs and killed EBs with the same efficiency with which it binds to live EBs (2). Thus, the low specific infectivity of the host ($2.5 \times 10^{-3}$ ifu/bacterial cell) should be taken into account when determining the true specific infectivity of the phage. For every event of attachment to an infectious EB, there are approximately 400 events involving replication-incompetent bacterial host cells. Thus, a titer of approximately $5.8 \times 10^9$ chlamydiaphage-stained inclusions/ml reflects approximately $2.3 \times 10^{12}$ infectious particles/ml, a value close to the EM-determined titer of approximately $3.0 \times 10^{12}$, indicating that Chp2 is highly infectious.

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