Amoebae as Battlefields for Bacteria, Giant Viruses, and Virophages

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When amoebae are simultaneously infected with *Acanthamoeba polyphaga Mimivirus* (APM) and the strictly intracellular BABL1 bacterium, the latter is always lost after serial sub culturing. We showed that the virophage Sputnik 1, by reducing APM fitness, preserved BABL1 growth in acute and chronic models. This capability of a virophage to modulate the virulence of mimiviruses highlights the competition that occurs between them during natural host infection.

Amoebae are predators that are able to phagocytize particles that are over 0.5 μm in size without distinction, and they can also act as hosts to several different microorganisms that may coexist simultaneously (1). Unlike allopatric specialized intracellular pathogens, microorganisms that survive phagocytosis by an amoeba experience a sympatric lifestyle with species from different phyla (2). They are also faced with constraints similar to those of other environmental bacteria, including the competition for survival and multiplication and access to nutrients. In our previous studies assessing the isolation of new microorganisms using amoebic coculturing, we have commonly discovered amoebae with multiple infections, such as infection with a giant virus member of the *Megavirales* (3) and an amoebal pathogenic bacterium that is lytic in pure coculture, which we provisionally named BABL1 (unpublished data). Based on its 16S rRNA gene sequence, this bacterium is a member of the delta-epsilon group of *Proteobacteria* (GenBank access number GQ495224), and the analyses of its genome sequence and physiology are under way. Following the discovery of *Acanthamoeba polyphaga Mimivirus* (APM) (4), we isolated a mamavirus, a small virus named Sputnik, that infected not amoebae but the APM virus factory (5). Based on the characteristics of bacteriophages, this new type of virus was termed a “virophage” (6). Because the virophage modulates the growth of APM, we aimed to determine whether the lytic effects of APM could be tempered by a virophage and thus promote coinfection with other bacterial species, such as BABL1. Indeed, when APM and BABL1 are isolated together, BABL1 is lost after several subcultures.

The microorganisms that were used in this work included the *Acanthamoeba polyphaga* strain Linc-AP1, the original APM strain, the first strain of Sputnik virophage isolated (5), the BABL1 bacterium, and an isolate of *Legionella anisa* (7). A schematic representation of the procedures used to perform coinfections is presented in Fig. 1. We first studied the growth of the BABL1 bacterium, APM, and Sputnik-infected APM alone or in coinfections with the amoeba in 10 ml of proteose peptone-yeast extract-glucose medium (PYG). After 1 h of incubation, the supernatants were removed to remove nonphagocytized microorganisms, and the amoebae were rinsed in Page’s amoeba saline (PAS) buffer and resuspended in 10 ml of fresh PYG. After 8 h of incubation, the cocultures were centrifuged for 10 min at 2,000 rpm, and the pellets of BABL1-infected amoebae were resuspended in 10 ml of fresh PYG and inoculated with APM or Sputnik-infected APM as described...
above. These 8 h of preincubation with the BABL1 bacterium were necessary to perform coinfection studies, because if they are inoculated simultaneously, APM multiplication is so rapid that it would mask BABL1 bacterium multiplication (4). Sampling was performed every 8 h to determine the concentration of the infectious agents in addition to the amoeba counts, and samples were stored at −20°C. The concentrations of infectious agents were measured using real-time PCR. DNA from the frozen samples was extracted using the QIamp DNA minikit from Qiagen (Hilden, Germany) according to the manufacturer’s instructions. The real-time PCR experiments incorporated specific primers for each agent being detected (Table 1). These molecular tests were performed in triplicate. The results are expressed as logarithms of the microorganism concentration (logC) based on a quantification scale that correlated the cycle threshold (C_T) to the number of DNA copies. All of the tests were performed in triplicate.

The concentration of the amoeba A. polyphaga alone showed an increase of approximately 1.5 logC after 32 h (data not shown). The growth kinetics of the BABL1 bacterium showed an increase of 2.5 logC. Regarding APM, after a short increase, the amoebic population began to decrease, with a loss of approximately 1 logC, whereas APM showed an increase of 3.5 logC (data not shown). It was also confirmed that the virophage alone had no effect on amoebal growth (data not shown). When combined with APM, the concentration of Sputnik increased markedly by 4 logs over 32 h, the growth of APM was less efficient (2.5 logC), and the population levels of the amoebae remained stable (data not shown), confirming the negative effects of the virophage on APM. When the BABL1 bacterium-infected A. polyphaga was superinfected with APM, the level of BABL1 bacteria remained stable until 24 h and then decreased, whereas APM multiplication was comparable to that observed when alone (Fig. 2a). When they were superinfected with Sputnik-infected APM, the BABL1 and A. polyphaga population changes were comparable to those observed with BABL1 alone (Fig. 2b) and thus “preserved” compared with those of APM superinfection alone (Fig. 2a). Taken together, these results were in agreement with our observation of the loss of BABL1 bacterium in subcultures when isolated together with APM. To confirm this, we performed chronic coinfection with BABL1 bac-

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**TABLE 1 Primers used in real-time PCR**

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mimivirus</td>
<td>R322 L</td>
<td>5’-AAACAGGTGCACCAACATCA-3’</td>
</tr>
<tr>
<td></td>
<td>R322 R</td>
<td>5’-GGTCTTCATTTTGACCCAG-3’</td>
</tr>
<tr>
<td>Sputnik</td>
<td>Sputcap F</td>
<td>5’-GAGATGCTGATGGGAGCCAT-3’</td>
</tr>
<tr>
<td></td>
<td>Sputcap R</td>
<td>5’-CATCCCACAAGGAGAGGA-3’</td>
</tr>
<tr>
<td>BABL1 bacterium</td>
<td>B-sp4F</td>
<td>5’-GCAATGAGTTATAGTTGCTAAAAA-3’</td>
</tr>
<tr>
<td></td>
<td>B-sp4R</td>
<td>5’-TGATGCCAAAAATGTACACCTGA-3’</td>
</tr>
</tbody>
</table>

*PCR was performed using a LightCycler instrument (Roche Biochemicals, Mannheim, Germany) in a final volume of 30 μl with 15 μl of the SYBR green kit (LightCycler fast-start DNA Master SYBR green I; Roche, Germany), 9.4 μl of distilled water, 1.6 μl of MgCl₂, 1 μl of each primer, and 2 μl of extracted DNA. The amplification conditions were as follows: initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation for 15 s at 95°C, annealing for 5 s at 60°C, and elongation for 15 s at 72°C, with fluorescence detection in single mode. The specificity of PCR amplification was assessed by testing all of the primer pairs on the BABL1 bacterium, APM, Sputnik-infected APM, and uninfected A. polyphaga. In each specific quantification reaction, sterile distilled water and DNA extracted from A. polyphaga were used as negative controls.

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**FIG 2** The growth kinetics of BABL1 and Acanthamoeba polyphaga Mimivirus (APM) infected by the Sputnik virophage within A. polyphaga. The amoebic concentration was measured by counting amoebae in culture and expressed as the log of the concentration in amoebae/ml. The BABL1 bacterium and virus concentrations, which were measured by quantitative real-time PCR, are represented as logC. (a) The growth kinetics of APM and the BABL1 bacterium in A. polyphaga; (b) the growth kinetics of APM, the Sputnik virophage, and BABL1 bacterium in A. polyphaga; (c) the chronic coinfection of A. polyphaga with APM and the BABL1 bacterium; (d) the chronic coinfection of A. polyphaga with APM, the BABL1 bacterium, and the Sputnik virophage.
terium and APM or Sputnik-infected APM (Fig. 1). Inoculations were performed as described above and were performed in duplicate. At 72-hour intervals up to 23 days, the culture was vortexed, and 1 ml was removed and stored at −20°C. The remainder of the culture was centrifuged at 2,000 rpm for 10 min, and the resulting pellet was resuspended in 10 ml of fresh PYG medium. After 5 subcultures, the BABL1 bacterium became undetectable, while the concentration of APM remained stable (Fig. 2c). In contrast, coinfection with the BABL1 bacterium and Sputnik-infected APM resulted in the concentration of BABL1 bacterium remaining stable until the end of the experiment (Fig. 2d). In this model, the APM concentration was lower than in the previous experiment. However, in a similar model using *L. anisa*, we observed an equilibrium between the virus and bacteria, even in the absence of a virophage to modulate APM (data not shown), suggesting that the mechanism observed with BABL1 is not common to all amoeba-associated microorganisms and not related to the initial APM concentration.

The ability of the virophage to modulate APM virulence provides evidence against the theory that Sputnik is a simple satellite virus type 4, have been shown to interfere with their helper virus (11). Our study reveals a model of how different microorganisms within amoebae modulate the population of their hosts and the populations of other microorganisms that coexist within these hosts. For example, we observed that the serial subculturing of APM in the absence of competitor microorganisms led to the emergence of a bald form of the virus that lacked surface fibers and replicated in a morphologically different type of viral factory (12). These changes were associated with dramatic genome reductions, suggesting that APM had lost unnecessary genes that were likely involved in surviving alongside competitors. In our work, we showed that the virophage has the ability to regulate, through the control of APM, its amoebic host and competing bacteria. This type of regulation of giant viruses and their eukaryotic hosts by virophages has also been suggested as a model of alga-virus-virophage interactions leading to an increased frequency of blooms during polar summit light periods in a hypersaline meromictic lake in Antarctica (13). In fact, obligate intracellular agents have long been considered to exist alone in their hosts without any contact with other agents. Recent data that have been obtained from the genome analysis of amoeba-associated agents, in accordance with the results that are presented here, show competition between microorganisms with regard to their host-infecting abilities.

**REFERENCES**