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In vitro Biofilm Formation by *Bacillus subtilis* and AR9 Phage Infection: SEM Study

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Bacteriophages, the viruses that infect bacteria, hold enormous potential for the treatment of multidrug-resistant bacterial infections and other applications due to their unmatched diversity and recent advances in genetic engineering. However, the fundamental understanding of the molecular mechanisms driving phage-host interactions is mostly restricted to a small number of conventional model systems and has not kept pace with the recent tremendous expansion in this field [1]. For example, bacteria employ a number of defense strategies such as Adsorption inhibition, CRISPR-Cas systems, restriction-modification systems, abortive infection (Abi), and other to combat invading phages, meanwhile to counter the bacterial defense systems, phages use intelligent tactics such as accessing host receptors, evading restriction-modification systems, and express anti-CRISPR proteins [2]. The full potential of molecular biology encoded by these viruses has largely gone unrealized, and the selection of phages for therapeutic or other purposes is still frequently done empirically.

Bacteriophage AR9 is a recently sequenced giant phage that encodes two multisubunit RNA polymerases and infects *Bacillus subtilis* (host). We therefore systematically explore phage interactions with *Bacillus spp* biofilms using microscopy techniques and further determine the molecular mechanisms of these interactions through transcriptome profiling and molecular biology. In-depth knowledge of these mechanisms will create new frontiers for broad applications of phages in various fields of microbiology, and biotechnology. The strain *Bacillus subtilis* 168 was grown in Luria-Bertani (LB) at 37°C, and at 25°C to induce biofilm/pellicle formation. For colony formation and swarming essays, 1.5% and 0.75% agar were appropriately added into the media above. The colony images are obtained using the Zeiss stereo light microscope, biofilm structure and the AR9 phage-biofilm interactions were monitored by KYKY EM6200 Scanning Electron Microscope.

Preliminary results indicated that the strain had optimal growth at 37°C, meanwhile 25°C was optimal for biofilm formation as shown in Fig. 1.

Our findings suggested a gradual stepwise action of the bacteriophage on the *Bacillus subtilis* biofilms. Indeed after 30 minutes of interaction, the cell density composition of the biofilm was significantly high, and the bacterial cells greatly conjugated together. However, after an hour of interaction, the cell density reduced to a certain extent with cells visibly gradually separating from each other. After 2 hours of incubation, the cell density of the biofilm reduced significantly and further less cells conjugated together (Fig. 2A- 2C). This observation could be associated with the lytic effect of the phage on the biofilm. It should be noted that very few cells were observed with their structure disoriented and we hypothesize that this was due to the resistance to lytic activity provided by the bacterial biofilm [3]. On the other hand, several lytic phages have also been reported to inhibit lysis such that chronically infected phage cells give rise to progeny that either slowly leave the cell or are passed on to daughter cells without causing lysis or integrating the phage genome into the host genome, a condition known as "carrier state". It is hypothesized that this tactic aids phage persistence in the host when nutrients are scarce and cannot sustain the proliferation of pathogens or the production of high amounts of virions in the extracellular environment [4].

In conclusion, the current results highlight a gradual stepwise interaction activity of the phage on bacterial biofilms. However, further work is required to optimization the reaction conditions and to understand the underlying mechanisms of the phage-host interactions [5].

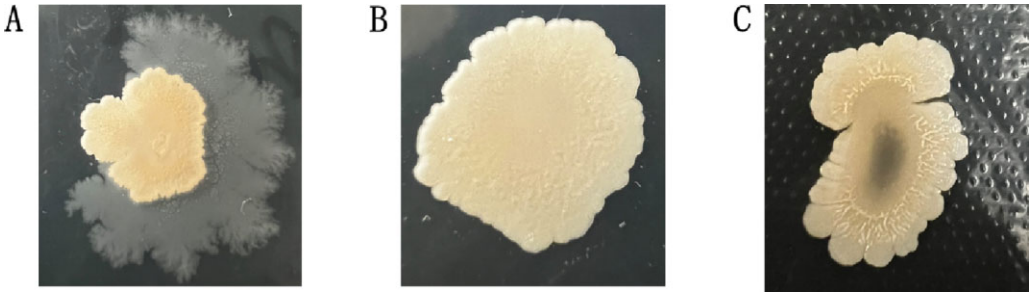

Fig. 1. Colonies after 5 days incubation. A *Bacillus subtilis* 168 on 0.75% LB agar at 25°C with tendrils B *Bacillus subtilis* 168 on 1.5% LB agar at 25°C without tendril growth C *Bacillus subtilis* 168 on 1.5% LB agar at 30°C.

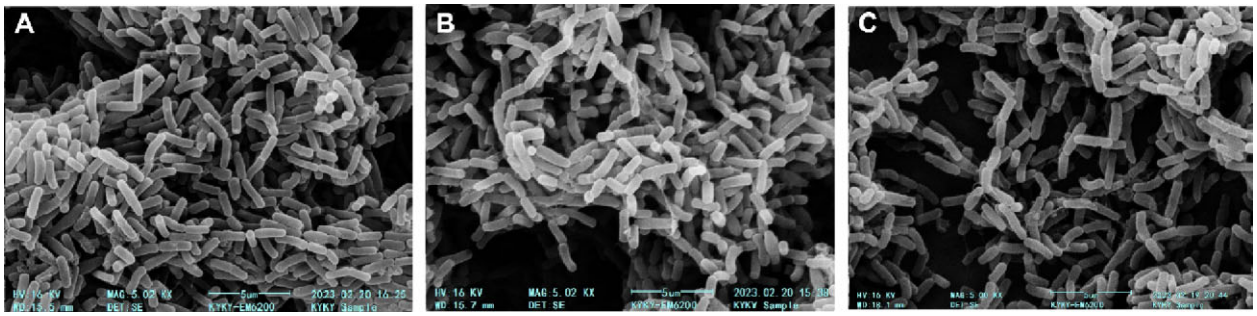

Fig. 2. Biofilms after 3 days incubation: A *Bacillus subtilis* 168- phage AR9 interaction at 25 °C for 30 minutes. B *Bacillus subtilis* 168- phage AR9 interaction at 25 °C for 1 hour. C *Bacillus subtilis* 168- phage AR9 interaction at 25 °C for 2 hours.

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