

# Minicells of *Bacillus subtilis*: a New Bacteriophage-Blocking Agent

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Bacteriophage SP01, SP17, and  $\phi$ 29 rapidly absorb irreversibly to minicells of *Bacillus subtilis* but do not produce a lytic cycle in minicells.

Minicells are small, DNA-less cells produced by cell division close to the pole of rod-shaped bacteria (1, 2, 4). Minicells represent a unique region of bacterial surface, namely, the cell pole region. We have investigated the use of minicells as hosts for several different *Bacillus subtilis* phages. The data indicate that phage adsorb to but do not replicate in minicells.

*B. subtilis* CU403 div IV-B1 was the source of minicells used in all experiments (4). Late-exponential-phase cultures (absorbancy at 660 nm = 1.0) grown in minimal medium were harvested by centrifugation at  $4,000 \times g$  at 4 C (4). The pellet was resuspended in a minimal volume of growth medium and sonicated at 0 C for seven 1-min exposures, interspaced by 1-min cooling periods. The suspension was diluted to 50 ml with growth medium and sedimented by centrifugation at  $6,000 \times g$ , and the pellet was resuspended in 1 to 2 ml of growth medium. The suspension was again sonicated until no parental cells could be observed by phase contrast microscopy. The suspension was diluted to 50 ml with growth medium and sedimented by centrifugation at  $6,000 \times g$ , and the pellet was washed twice by resuspension and centrifugation. The final pellet was suspended in a minimal volume of growth medium (30-60  $\mu$ liters) and placed in capillary tubes (75 mm in length, 1.2 mm internal diameter). One end of each capillary tube was plugged, and the capillaries were centrifuged at 11,500 rpm ( $13,000 \times g$ ) in an International microcapillary centrifuge, model MB, for 10 min. After centrifugation two bands were evident in the capillaries: an upper band of debris and a lower band of minicells. The lower band was removed, and the minicells were suspended in nutrient broth (8 g/liter; Difco Laboratories, Detroit, Mich.) containing NaCl (5 g/liter),  $MgSO_4 \cdot 7H_2O$  (0.2 g/liter),  $MnSO_4 \cdot H_2O$  (0.05 g/liter),  $CaCl_2 \cdot 2H_2O$  (0.15 g/liter), and thymine (0.02 g/liter). Parental CU403 div IV-B1 cells were also suspended in

nutrient broth with the above additions for phage adsorption. Phage adsorption to cells and minicells was carried out at 30 C with vigorous aeration in this medium. Standard overlay plating techniques were used. Exponentially growing *B. subtilis* 168 cells ( $10^8$  CFU/ml) were used as bacteriophage indicator bacteria. All dilutions were made in 1% peptone at 0 C.

SP01 was added to cells or purified minicells, and samples were removed at intervals, diluted 1:100, and centrifuged to sediment the bacteria. The supernatants were assayed for unadsorbed phage (Fig. 1). SP01 adsorbs rapidly to cells and minicells. The difference in initial adsorption rates shown in Fig. 1 was probably due to the approximately fourfold higher concentration of minicells. Minicells were counted directly in a Hawksley counting chamber (Lancing, Sussex, England) by using a Wild

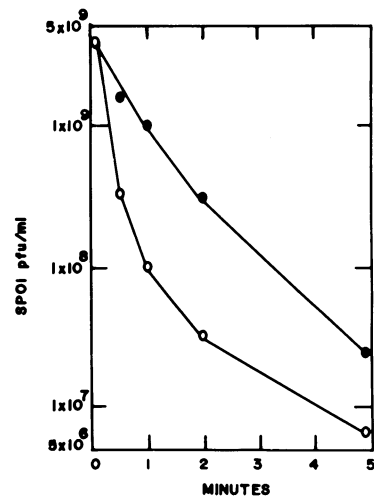


FIG. 1. Adsorption of SP01 to CU403 div IV-B1 cells and minicells. SP01 was mixed with CU403 cells (●) at a multiplicity of infection of 3 and with purified minicells (○) at a multiplicity of infection of 0.5. Samples were removed and centrifuged, and the supernatants were assayed for free SP01 as described in the text.

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phase contrast microscope. However, the small size of minicells (about 0.1 the size of parent cells) makes accurate visual quantitation of minicell numbers difficult.

We have examined the adsorption of SP01 to minicells by direct plating of samples from the phage-minicell mixture. The number of PFU per milliliter rapidly decreased, just as in the samples from which the cells had been removed by sedimentation (Fig. 2). These data indicate that SP01 adsorbs irreversibly to minicells but does not produce infectious centers. Similar results were found when minicells were heated at 100 C for 15 min before phage addition.

The kinetics of SP01 adsorption to varying concentrations of minicells are shown in Fig. 3. The initial linear regions of the curves give an adsorption rate constant of about  $7.8 \times 10^{-12}$  when calculated from the Schlesinger equation (5). Unlike the data published for coliphages, our data indicate two rates of adsorption at various minicell concentrations: an initial rapid rate followed by a second slower rate. Similar findings have been reported recently for adsorption of *Acholeplasma* virus (D. Fraser, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 67, 1973).

In addition to SP01, we have found that SP17 and  $\phi 29$  also adsorb rapidly and irreversibly to minicells but cannot replicate in them. It appears, therefore, that minicells could confer an evolutionary advantage as phage-blocking agents to those bacterial mutants which produce them. Since phage adsorbed to minicells

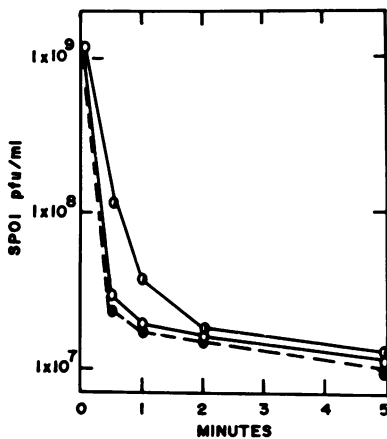


FIG. 2. Adsorption of SP01 to minicells and heated minicells. SP01 was mixed with minicells at a multiplicity of infection of 0.1. Unadsorbed phage were assayed before (O) and after (●) removal of minicells by centrifugation. SP01 were mixed with the same concentration of minicells which had been held at 100 C for 15 min, and unadsorbed phage were assayed without removal of minicells (●).

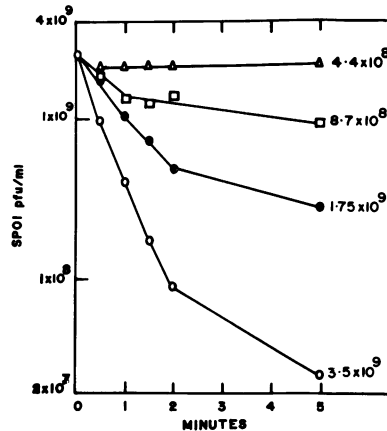


FIG. 3. Adsorption of SP01 to different concentrations of minicells. SP01 ( $2.5 \times 10^9$  PFU) was mixed with minicells at the following final concentrations per ml:  $4.4 \times 10^8$  ( $\Delta$ );  $8.7 \times 10^8$  ( $\square$ );  $1.75 \times 10^9$  ( $\bullet$ ); and  $3.5 \times 10^9$  ( $\circ$ ). The unadsorbed phage were assayed at various times in each suspension without removal of the minicells.

are inactivated, parental cells are protected to the extent that phage happen to collide with minicells. To date no other properties of minicells have been found which can be interpreted as a possible selective advantage to minicell-producing bacterial mutants.

Experimentally, minicells could prove useful (i) in studying quantitative and other aspects of phage adsorption, (ii) in searching for phage with pole-specific or lateral wall-specific adsorption sites, and (iii) perhaps even in removing unadsorbed phage from an experimental system since minicells can be rapidly and easily separated from normal-size cells (3, 4).

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