JOURNAL OF VIROLOGY, Mar. 1977, p. 1223-1227
Vol. 21, No. 3

Printed in U.S.A.

Adsorption of Bacteriophages φ29 and 22a to Protoplasts of *Bacillus subtilis* 168

ELIZABETH D. JACOBSON[1]* AND OTTO E. LANDMAN
Department of Biology, Georgetown University, Washington, D.C. 20007

Received for publication 1 September 1976

Adsorption of bacteriophages φ29 and 22a to protoplasts of *Bacillus subtilis* 168 is described. The number of binding sites on bacilli and protoplasts is determined for each phage. Bacilli and protoplasts possess roughly the same number of sites per unit area for φ29, i.e., approximately 700 sites per bacillus. There are also approximately 700 sites per bacillus for 22a, but only about one-third as many sites per unit area on the protoplast surface. A model for φ29 adsorption is proposed.

We reported previously that bacteriophage can productively infect wall-less forms of *Bacillus subtilis* (7). Here we compare quantitatively the adsorption of phages φ29 and 22a to protoplasts with their adsorption to bacilli. Phages 22a and φ29 were chosen for this comparison because earlier work showed that they recognize different adsorption sites: adsorption of φ29 is blocked in bacilli deficient in the glucosylation of their wall teichoic acid (7, 15), whereas adsorption of 22a is normal in such mutants (7).

The bacterial strain used was *B. subtilis* 168 *trpC2* (3), given by J. Spizizen. Protoplasts were prepared by subjecting suspensions of bacilli to the action of lysozyme in osmotically stabilized medium (7, 8, 14). Phages φ29 and φ25, isolated by B. Reilly (Ph.D. thesis, Western Reserve University, Cleveland, Ohio, 1965), were also obtained from J. Spizizen. Phage 22a was isolated by R. Zsigray (Ph.D. thesis, Georgetown University, Washington, D.C., 1968).

Data from three kinds of experiments are reported: (i) the rates of adsorption of the two phages to protoplasts and to bacilli, (ii) the reversibility of adsorption, and (iii) the number of adsorption sites on protoplasts and on bacilli, determined for the two phages.

Adsorption and adsorption-reversal experiments were done at 0 and 30°C to differentiate the initial step in adsorption from succeeding steps in the phage life cycle (2, 5, 10, 13). The adsorption of φ29 to both bacilli and protoplasts is slow, even at 30°C (Fig. 1A). Adsorption of this phage to protoplasts at 0°C is reversible by dilution, but adsorption at 30°C is irreversible (Fig. 2A). The evidence for the reversibility of the attachment of φ29 to bacilli is not clearcut in the intact-cell experiments (Fig. 2A); however, in experiments with isolated cell walls, φ29 attachment was shown to be completely reversible (Table 1). The latter data agree with earlier observations that φ29 adsorbed to isolated cell walls, but that intact infectious phage were released upon autolysis of the walls (15). We also confirmed the finding of Young (15) that phage φ25 is inactivated irreversibly by adsorption to cell walls (Table 1).

The adsorption of 22a to bacilli and protoplasts (Fig. 1B) is more rapid than that of φ29. Binding to bacilli at 30°C is irreversible by dilution; binding at 0°C is partly reversible (Fig. 2B).

To calculate the number of phage adsorption sites on protoplasts and bacilli, we plotted adsorption data by the formulations of Scatchard (11): $PC/P = k(C_0 - PC)$, where PC represents the adsorbed phage (expressed as counts per minute in the cell pellet), P represents the free phage (expressed as counts per minute in the supernatant), C_0 represents the total number of receptor sites, and k is a constant.

Phage labeled in their coat proteins were added to cells at concentrations ranging from levels where the cellular receptor sites were almost unoccupied up to levels where they were completely saturated with phage. Adsorption was allowed to proceed for 30 min in an ice bath. The phage-bacterium complexes were then sedimented, and the supernatant and pellet were assayed for radioactivity. The resulting data were plotted in Scatchard plots (Fig. 3A and B).

Since many rods contain more than one cell, there were approximately 2.4 times as many

[1] Present address: Division of Biological Effects, Bureau of Radiological Health, Rockville, MD 20852.

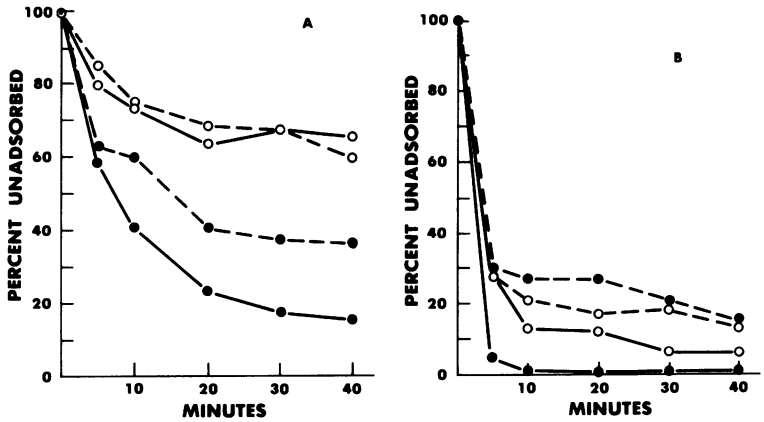

FIG. 1. *(A) Kinetics of φ29 adsorption to bacilli and protoplasts at 0 and 30°C. (B) Kinetics of 22a adsorption to bacilli and protoplasts at 0 and 30°C. Phage (3 × 10⁷ PFUs) were added to host cells (3 × 10⁸ bacillary colony-forming units or approximately 7.2 × 10⁸ protoplasts resulting from lysozyme treatment of the same bacillary suspension) in a total volume of 2.0 ml of CGS sorbitol broth (7). The mixtures were incubated at 0°C (○) and 30°C (●). At the times shown, samples were removed and centrifuged in the cold at 10,000 × g for 10 min. The supernatant fractions were assayed for percent input PFU unadsorbed to bacilli (——) or protoplasts (- - -).*

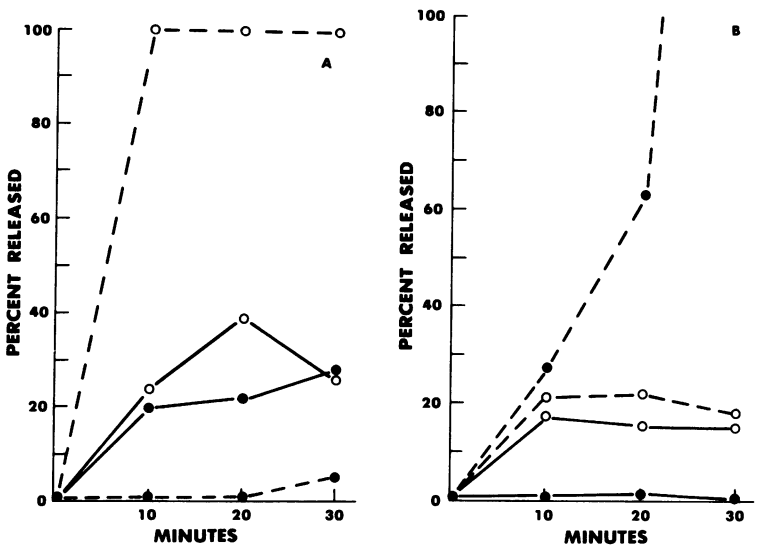

FIG. 2. *Dissociation of bacterium-phage complexes by dilution. (A) Complexes of φ29 with bacilli or protoplasts. (B) Complexes of 22a with bacilli or protoplasts. Phage (3 × 10⁷ PFUs) were added to host cells (3 × 10⁸ bacillary colony-forming units or approximately 7.2 × 10⁸ protoplasts resulting from lysozyme treatment of the same bacillary suspension) in a total volume of 2.0 ml of CGS sorbitol broth (7). The mixtures were incubated at 0°C (○) and 30°C (●) for 20 min. At this time, "zero time," 1 ml was removed and centrifuged in the cold at 10,000 × g for 10 min, and the supernatants were assayed for percent input PFU unadsorbed to bacilli (——) and protoplasts (- - -). The remaining cells were diluted 1:100 in prewarmed or precooled broth. Samples (1 ml) were removed 10, 20, and 30 min after dilution and assayed for unadsorbed phage. All values were corrected by subtracting the percentage of input phage remaining unadsorbed at zero time. The total time elapsed in this experiment was 60 min (20 min of initial incubation and dilution at zero time, followed by an additional 10 to 30 min of incubation and 10 min of centrifugation). This was sufficient for a liquid burst to occur in the protoplasts infected with 22a and held at 30°C. Phage recovered from the supernatant after 30 min of additional incubation totaled 291% of input PFUs. Bacilli infected with 22a did not yield phage within this time period. The data from protoplasts infected with 22a at 30°C thus cannot be interpreted as showing release of attached phage.*

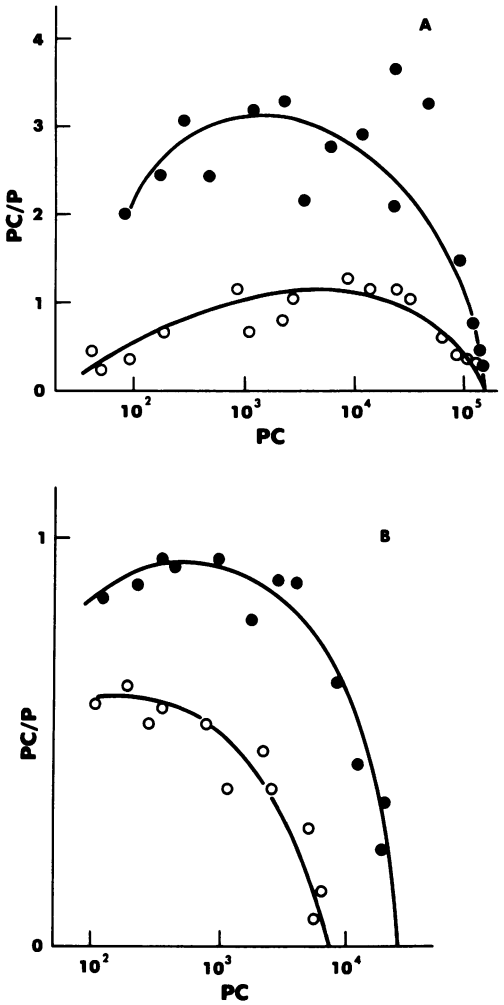

FIG. 3. *(A) Scatchard plot for the binding of φ29 to bacilli and protoplasts. To 0.1-ml cell suspensions in CGS sorbitol broth (7) (3 × 10⁷ bacillary colony-forming units [CFU], or approximately 7.2 × 10⁷ protoplasts resulting from lysozyme treatment of the bacillary suspension) were added 0.1-ml samples of ³⁵S-labeled φ29 (1 count per minute [cpm]/10⁵ PFUs), for a total volume of 0.2 ml. Phage concentrations (in PFUs) ranged from a multiplicity of infection of 0.5 to 2,000. Adsorption medium was CGS sorbitol broth (7). The mixtures were incubated in an ice bath for 30 min, and the phage-bacterium complexes were centrifuged in the cold at 10,000 × g for 10 min. Both the supernatants and the pellets were assayed for radioactivity to determine the quantity of (adsorbed) phage in the pellet (PC) and the quantity of phage free in the supernatant (P). Nonspecific adsorption, or trapping of labeled phage in the interstices of the pellet, was estimated as follows. Cells were exposed to saturating concentrations of unlabeled phage at 0°C for 20 min. Labeled phage was then added, and the mixture was centrifuged immediately. The percentage of added radioactivity retained in this pellet (i.e., that could not be excluded by cold phage) was considered as occluded label, and a similar percentage was subtracted from pellet counts and added to supernatant counts. In different experiments the values ranged from 4 to 18% of the total input cpm. This was in fair agreement with the data of Hershey and Chase (6), who obtained a value of 5% in a similar system with phage T2 of Escherichia coli. The amount of phage bound to bacilli (●) and to protoplasts (○), PC, is expressed in cpm. To determine the number of phage-binding sites per cell, the labeled phage were titered and a value of cpm/PFU was determined. Total binding capacity of the system (in cpm) was estimated by extrapolating the data curves to the abscissa. The total binding capacity (in cpm) was then divided by cpm per PFU to give the total number of PFU bound. This number, in turn, was divided by the total number of bacillary CFU and (estimated) protoplast CFU to give the number of PFU bound per bacillus and per protoplast, respectively. (B) Scatchard plot for the binding of 22a to bacilli and protoplasts. To 0.1-ml cell suspensions (3 × 10⁷ bacillary CFU, or approximately 7.2 × 10⁷ protoplasts resulting from lysozyme treatment of the bacillary suspension) were added 0.1-ml samples of [³H]tryptophan-labeled 22a (1 cpm/10⁶ PFU), for a total volume of 0.2 ml. Phage concentrations ranged (in PFU) from a multiplicity of infection of 5.0 to 2,000. The remainder of procedure is same as for Fig. 3A.*

protoplasts in the phage-protoplast mixtures as there were bacilli in the phage-bacilli mixtures (7). These protoplasts are, on the average, smaller than their parent bacilli and, per cell, have a smaller surface area. (The total surface area of 2.4 protoplasts is roughly similar to the surface area of the rod from which they derive, provided the radii of the two bodies are assumed to be the same.) For phage 22a, bacilli appear to have about 700 binding sites, and protoplasts appear to have about 90. This difference cannot be accounted for solely on the basis of the differences in surface area; we estimate that there are roughly one-third as many 22a sites per unit surface area of protoplasts as there are per unit surface area of bacillus. Bacilli also possess approximately 700 binding sites for phage φ29, and there are about 300 binding sites per protoplast for this phage. Thus, the number of φ29 attachment sites per unit surface area is about the same in bacilli and protoplasts.

In studies of ligand binding to receptor, a Scatchard plot that is convex to the abscissa is interpreted to indicate that positive cooperativity occurs among receptors. Our data indicate apparent positive cooperativity (Fig. 3).

Factors that might lead to complications in the analysis of Scatchard plots when applied to

TABLE 1. *Adsorption and release of ϕ29 from isolated cell walls*[a]

Phage	PFU (%)	
	Left in supernatant	Recovered on plate
ϕ25	<1	<1
ϕ29	35	100

[a] Method modified from that of Doyle and Birdsell (4). A 0.9-ml volume of SP3 buffer (7) containing 0.2 mg of cell wall was incubated at 30°C for 10 min. Then 2×10^7 PFU (in 0.1 ml) were added. The mixture was incubated at 30°C for an additional 15 min and then split in half. One-half was centrifuged at 45,000 × *g* for 10 min, and the supernatant fraction was assayed for unadsorbed PFUs on CYS agar plates (7). The other half was diluted directly and assayed for PFUs on CYS agar plates.

phage adsorption include: (i) the presence of two or more types of binding sites per cell, or (ii) the presence of two or more classes in the phage population, each having its own binding characteristics.

Situations i and ii could be likely explanations for concave Scatchard plots but do not easily account for our convex plots. A possible explanation for positive cooperativity is that the adsorbing phage modify the bacterial envelope (enzymatically?), thereby facilitating further adsorption. Although all phage preparations were carefully purified (including banding in a CsCl equilibrium density gradient), a substantial portion of the counts remained in the supernatant even at low multiplicities (Fig. 3). We do not know whether these counts include noninfectious particles, corresponding, for example, to the 43% noninfectious particles observed in an electron microscope by Anderson et al. (1) for ϕ29.

Putting together earlier results with our present quantitative results on ϕ29 adsorption, we have developed a tentative model for the interaction of ϕ29 with the bacterial envelope. The model is consistent with the following observations. (i) The phage binds only reversibly to isolated cell wall (Table 1) (15). (ii) ϕ29 binds irreversibly to protoplasts at 30°C (Fig. 2A). (iii) ϕ29 recognizes the same number of binding sites per unit surface area in protoplasts and in bacilli (Fig. 3A). (iv) ϕ29 plaques normally on L-form lawns of wild-type *B. subtilis* and L-form lawns of the mutant *gtaA* (wall teichoic acid nonglucosylated [15]) but does not adsorb to *gtaA* bacilli (7). (v) ϕ29 possesses a neck, consisting of two collars, 12 appendages, and a tail (1). Tosi and Anderson (12) suggested that the neck appendages play a role in the adsorption of ϕ29 to its host as demonstrated by serum blocking and apposition to cell wall in thin sections.

Our model postulates a reversible attachment of ϕ29 to glucosylated teichoic acid in the wall, perhaps with the neck appendages. This reversible binding enables the phage tail to attach to a membrane component, first reversibly and then irreversibly (Fig. 2A). The membrane component is exposed on the surface of protoplasts, and ϕ29 binds to it freely. In *gtaA* bacilli, the teichoic acid of the wall is nonglucosylated, and the phage is unable to complete the initial reversible adsorption to the wall. Protoplasts of *gtaA* mutants, however, present ϕ29 with a surface essentially like that of wild-type protoplasts. With the barrier to the membrane component removed (i.e., the cell wall), the phage is free to adsorb. (A similar system may exist in *Micrococcus lysodeikticus*. Lovett and Shockman [9] reported that phage N1 binds only reversibly to isolated cell walls of *M. lysodeikticus*. They postulated that the reversible binding might be followed by a step involving some other component, possibly a membrane component, to achieve irreversible binding.)

We thank John Spizizen for gifts of strains, and Kenneth Krell and Melvin Blecher for helpful discussions.

While this work was in progress, our laboratory was supported by Public Health Service grant AI-05972 from the National Institute of Allergy and Infectious Diseases and grant GB-27606 from the National Science Foundation to O.E.L. E.D.J. was supported by University Fellowships from Georgetown University.

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