Interactions of Actinophage and Clays

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Over 60% of streptomycete phage, isolated from soil, were adsorbed by unsubstituted or sodium-treated kaolin, when suspended in nutrient broth, but less phage were adsorbed by sodium and calcium montmorillonite or by calcium- and aluminium-treated kaolin. The montmorillonites did, however, partially protect one of the phage from damage during experimental manipulations. Adsorption to kaolin was not influenced by pH and most of the adsorbed phage retained their infectivity. Attempts to obtain free infectious phage by desorption were unsuccessful. The pH stabilities of adsorbed and free phage differed: 50% inactivation of phage adsorbed to kaolin occurred at pH 6.1 whereas for free phage 50% inactivation occurred at pH 4.9. This suggested that ΔpH ($\text{pH}_b - \text{pH}_s$) was about 1.2 units, i.e. similar to previous estimates based on the activity of enzymes adsorbed to kaolin.

INTRODUCTION

Most studies of phage have been made in laboratory cultures and little is known of their behaviour or significance in natural habitats. Phage are usually readily isolated from soil, although their titres are often very low (Casida & Liu, 1974; Tan & Reanney, 1975). The genus *Streptomyces* is widespread in soil and phage attacking these actinomycetes are similarly distributed. It is not easy to simulate conditions in soil but the presence of solid surfaces is an obvious and important difference between the soil environment and broth cultures. Clay minerals and other surface-active particles can adsorb or coat microbial cells (Marshall, 1968, 1969), enhance microbial growth (Stotzky & Rem, 1966) and influence enzyme activity (Skujins *et al.*, 1974). Although phage may be adsorbed to a variety of surfaces, there is little information on their interaction with clays.

In a recent study, Bystricky *et al.* (1975) used electron microscopy to demonstrate adsorption of phage to kaolinite. In the investigation reported here, the interactions of streptomycete phage with a range of clay minerals were studied, and the effects of clays on certain properties of the phage particles were assessed.

METHODS

Clays. The three clays used were light kaolin (Hopkin & Williams), calcium montmorillonite (B.D.H.) and sodium montmorillonite (B.D.H.). Homoionic clays were prepared by washing a known weight of clay three times in a solution of either 0.5 M-NaCl, 0.25 M-CaCl₂ or 0.17 M-AlCl₃. The sample was then washed with distilled water until the supernatant was free of chloride. The treated clay was centrifuged to a thick slurry and stored at 4 °C.

Adsorption of phage. The two phage used were designated f6 and f13. They were isolated from soil and propagated on two soil isolates classified as *Streptomyces* f6 and *Streptomyces* f13. Samples of unsubstituted or homoionized clay were added to flasks of nutrient broth (Oxoid CM1) to give concentrations of 0.001 to 200 mg ml⁻¹. An accurately titrated phage suspension was added to the flasks to give a titre of 10⁶ plaque-forming units (p.f.u.) ml⁻¹. The flasks were incubated at 25 °C on an orbital shaker at 200 rev. min⁻¹ and

Fig. 1

Fig. 2

Fig. 1. Adsorption of phage f6 (●) and f13 (○) by kaolin in nutrient broth at 25 °C. Standard errors of the mean of three replicates are shown.

Fig. 2. Time course of adsorption of phage f6 by kaolin at 2 mg ml⁻¹ (●) and 20 mg ml⁻¹ (○) in nutrient broth at 25 °C.

samples were removed at intervals. These were centrifuged at 2000 *g* for 5 min at room temperature and then passed through a cellulose acetate filter (pore size 0.45 µm). Free phage were counted by the double layer method. Serial 10-fold dilutions were prepared and 0.1 ml samples were pipetted on to a base layer of nutrient agar (Oxoid, CM3) supplemented with 0.5 % (w/v) glucose and 4 mM-Ca(NO₃)₂. These plates were overlaid with 2.5 ml of soft agar (0.8 %, w/v) at 46 °C seeded with the host spore suspension. Plaques were counted after 18 h at 25 °C. All counts were expressed as a proportion of the titre in control flasks without clay.

Infectivity and pH stability of adsorbed phage. Whole sample counts were made to determine the infectivity of adsorbed phage. Phage were added to flasks of nutrient broth containing 20 mg clay ml⁻¹ to give an initial concentration of 10⁸ p.f.u. ml⁻¹. Samples were removed at intervals and assayed directly for phage.

The effect of pH on phage stability was assessed in a similar manner, the adsorption mixtures being adjusted with 0.1 M-HCl or 0.1 M-NaOH to cover a range from pH 3.5 to 10.0. Phage were assayed after 10 min in the flask mixtures.

Desorption of phage. Numbers of phage adsorbed on clay at 20 mg ml⁻¹ were determined by difference from the numbers of free phage in the supernatant. The clay with attached phage was resuspended in 10 ml nutrient broth, agitated for 1 min and centrifuged. The supernatant was assayed for phage and the clay pellet was resuspended. After several such cycles, the pellet itself was finally plated out for phage assay. Phage suspension in nutrient broth without kaolin was treated and assayed in the same manner.

RESULTS

Adsorption of phage

Both phage were apparently adsorbed by kaolin, with a maximum removal of 65% of f6 and 75% of f13 (Fig. 1). Adsorption values were calculated as a proportion of counts in control flasks without clay; phage f13 was sensitive to experimental manipulation, up to 65% being inactivated in the control flasks. No significant adsorption occurred at clay concentrations below about 1 mg ml⁻¹. Adsorption increased steadily above this concentration, levelling off between 10 and 20 mg ml⁻¹.

The effect of varying phage concentrations on adsorption was studied using f6 with 20 mg kaolin ml⁻¹. A constant proportion of phage (50 to 60%) was adsorbed throughout the concentration range tested. The adsorption of phage f6 by kaolin at 20 mg ml⁻¹ was also tested at different pH values. Within the limits of phage stability (pH 6.5 to 8.5), adsorption was unaffected.

Fig. 3

Fig. 4

Fig. 3. Adsorption of phage f6 by sodium montmorillonite (○) and calcium montmorillonite (●) in nutrient broth at 25 °C.

Fig. 4. Adsorption of phage f6 by sodium kaolin (○), calcium kaolin (●) and aluminium kaolin (■) in nutrient broth at 25 °C.

Adsorption of phage f6 by kaolin at 2 and 20 mg ml⁻¹ was tested after different times (Fig. 2). Little change in numbers of adsorbed phage occurred after 5 min at either concentration.

The montmorillonites were less effective in removing phage from suspension. Sodium montmorillonite adsorbed 25 to 30% of phage f6 at equilibrium, while calcium montmorillonite removed 20% (Fig. 3). Little adsorption of phage f13 occurred with these clays, but increasing concentrations of clay decreased the losses due to the sensitivity of this phage to the experimental procedures.

Adsorption of phage f6 to kaolin treated with Na⁺, Ca²⁺ and Al³⁺ ions was investigated (Fig. 4). Sodium kaolin proved to be a more efficient adsorbent than the unsubstituted kaolin, with as little as 0.1 mg ml⁻¹ adsorbing significant proportions of the phage. Maximum adsorption again removed about 60% of the phage, but this occurred at lower clay concentrations (5 to 10 mg ml⁻¹). Adsorption by calcium and aluminium kaolin was less than with the unsubstituted clay, the maximum removal being 35 to 40% with no significant adsorption below 1 mg ml⁻¹.

Infectivity of adsorbed phage

The efficiencies of plating of phage f6 mixed with 20 mg kaolin ml⁻¹ and with the same concentration of calcium montmorillonite were compared with that in broth without clay. With the poor adsorbent, calcium montmorillonite, the mean loss was $6.14 \pm \text{S.E.M. } 1.75\%$. This was not significantly higher than that in broth without clay ($4.80 \pm 0.93\%$). The loss with kaolin ($9.74 \pm 1.60\%$) was significantly higher than that obtained with calcium montmorillonite or the control. However, as the percentage loss was much less than the percentage adsorption (60%), it was concluded that the majority of adsorbed phage were still infective.

Desorption of phage

The centrifugation–washing procedure resulted in the recovery of only 1 to 2% of the adsorbed phage (Table 1). Furthermore, plating of the final clay pellet after washing produced few or no plaques. Similar treatment of the control broth resulted in recovery of about 80% of the phage.

Table 1. *Desorption of phage f6 adsorbed by kaolin (20 mg ml⁻¹) (expressed as p.f.u.)*

	Phage stock	Phage adsorbed	Phage recovered					Total phage recovered	Percentage recovery
			1st wash	2nd wash	3rd wash	4th wash	Final pellet		
With kaolin	1.38×10^6	6.60×10^5	6.80×10^3	4.00×10^3	0	0	7.00×10^2	7.90×10^3	1.2
	1.34×10^6	4.40×10^5	1.00×10^4	4.00×10^3	0	ND	0	1.04×10^4	2.4
	1.42×10^6	8.08×10^5	1.80×10^4	1.60×10^3	0	ND	0	1.96×10^4	2.5
Control without kaolin	1.30×10^6	—	—	—	—	—	—	1.05×10^6	80.8

ND, Not determined.

Fig. 5. Effect of pH on the stability of phage f6 with 20 mg kaolin ml⁻¹ (○), 20 mg calcium montmorillonite ml⁻¹ (■) and without clay (●) in nutrient broth at 25 °C.*Effect of clay on pH stability of phage*

The pH stabilities of phage f6 after 10 min in the presence of kaolin, calcium montmorillonite and in broth without clay were compared (Fig. 5). In nutrient broth, the limits of phage stability were pH 9.5 to pH 5.5 and 50% inactivation (ID_{50}) occurred at pH 4.9. With calcium montmorillonite, the limits were extended to pH 10.0 to pH 5.3 and the ID_{50} was at pH 4.8. In contrast, the phage stability range with kaolin was reduced to pH 9.5 to pH 7.0 and the ID_{50} was at pH 6.1. In all cases, total phage inactivation occurred after 10 min at pH 4.0.

DISCUSSION

Kaolin adsorbed more phage than montmorillonite and adsorption to the former was greater when it was unsubstituted or homoionized with Na⁺ than when it was treated with Ca²⁺ or Al³⁺. As the experiments were carried out in nutrient broth which contains 0.5% (w/v) NaCl, 'unsubstituted' kaolin presumably contained the sodium form. Our results only partially agree with those of Bystricky *et al.* (1975) who reported strong adsorption of phage and other viruses to kaolinite and montmorillonite.

Both clay and phage particles carry a net negative charge under most conditions. Adsorption of negatively charged microbial cells to clays and other surfaces depends on the presence, concentration and valency of associated cations. Zvyagintsev (1962) suggested that adsorption of bacteria to negatively charged resins depended on cation valency and saturation. Adsorption of actinomycete spores to anion exchange resins and montmorillonite was greatest in the presence of polyvalent cations (Ruddick & Williams, 1972). Similarly, Stotzky & Bystricky (1969) found that adsorption of bacteria in clays homoionic to Al³⁺ was greater than in those saturated with Na⁺. However, our results with actinophage and

homoionized kaolin suggest that interactions between clay-phage surfaces and cations do not provide an explanation for adsorption.

Different degrees of adsorption of microbial cells to kaolin and montmorillonite have been reported. Streptomycete spores were strongly adsorbed by kaolin over a wide pH range but adsorption to montmorillonite was slight above pH 4 (Ruddick & Williams, 1972). A similar effect was obtained with *Bacillus* cells by Santaro & Stotzky (1968) who suggested that the difference could be due to the greater number of positively charged edge sites on kaolinite compared with montmorillonite (Van Olphen, 1963). Our results indicate that direct attachment of phage particles to positively charged edge sites on the clays is a possible mechanism of adsorption.

Adsorption of phage to kaolin was rapid and, like that of streptomycete spores (Ruddick & Williams, 1972), was unaffected by pH. Most of the adsorbed phage retained their infectivity, as did the viruses studied by Bystricky *et al.* (1975). At kaolin concentrations of 20 mg ml⁻¹ and above, a fixed proportion of the phage was adsorbed irrespective of the concentration of phage particles. This was possibly due to heterogeneity in the surface properties of the phage population. Interestingly, populations of *Bacillus subtilis* mixed with clay colloids contained both adsorbing and non-adsorbing cells (Lahav, 1962). Phage f13, which was quite sensitive to the experimental procedures, was progressively protected by increasing concentrations of montmorillonites, although little adsorption occurred. The phage was presumably protected against factors such as increased temperature or mechanical damage during the experimental procedure.

Results of the desorption experiments were surprising. Although washing removed only 1 to 2% of the adsorbed phage, little or no plaques resulted when the clay pellet was plated out. As previously mentioned, direct plating of clay-phage mixtures indicated that most adsorbed phage were still infective. Therefore, it is possible that either the process of desorption or the successive suspension and centrifugation in the presence of clay led to inactivation of the phage.

Clay particles are strongly electronegative and therefore, in an aqueous phase, they require a cloud of cations, including hydrogen ions, for overall neutrality. This results in a difference (ΔpH) between the pH at or near the particle surface (pH_s) and that in the bulk phase (pH_b) which is usually higher. pH cannot be measured directly but can be estimated in a number of ways. One method is to compare the pH optimum of an enzyme in the presence of clay particles with that in a solution without clay, the difference representing the approximate ΔpH (McLaren & Skujins, 1968). The pH optimum for activity of chymotrypsin was about 2 units higher when adsorbed to kaolin than when in solution (McLaren & Estermann, 1957). Similar results have been reported for other enzymes, including phosphatase (Ramirez & McLaren, 1966) and chitinase (Skujins *et al.*, 1974). Comparison of the pH stability of phage f6 in broth, in the presence of a poor adsorbent and a strong adsorbent (kaolin) also provides an estimation of ΔpH . The ID_{50} values in broth and calcium montmorillonite were at pH 4.9 and pH 4.8, respectively. However with kaolin the ID_{50} was at pH 6.1, suggesting that pH_s encountered by adsorbed phage was about 1.2 units lower than pH_b . Thus phage stability, like enzyme activity, can be used as a 'molecular pH meter' (McLaren, 1975). ΔpH for kaolin with chymotrypsin was 2.0 (McLaren & Estermann, 1957) and with chitinase was 1.2 (Skujins *et al.*, 1974). The results also supported the conclusion that many of the phage particles were in close association with kaolin particles.

Soil colloids may therefore have a major influence on phage in soil. Adsorbed phage can remain infective, possibly be protected and be in close proximity to host spores which can also be adsorbed. On the other hand, adsorbed phage will be more susceptible to inactivation by low pH in moderately acidic soils.

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