Enhancement of Bacteriophage ϕ X-174 Plaques by Homoionic Clay Minerals

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The incorporation of selected particulates or charged polymers into qualitative and quantitative assay systems has been found to increase virus specific infectivity. The purpose of this study was to identify a mechanism(s) to explain the effect of charged particulates upon plaquing efficiency. The clay minerals kaolinite (K), montmorillonite (M), with the bacteriophage ϕ X-174 and its host *Escherichia coli*, were used as a model system. Enhanced bacteriophage infectivity (expressed as plaque-forming units) occurred at K and M concentrations in the agar overlay of 0.14–0.50 and 0.01–0.15 mg ml⁻¹, respectively. A maximum increase of bacteriophage titre (to 180–230% of the control value) occurred at 0.18–0.20 and 0.06–0.08 mg ml⁻¹ K and M, respectively. Increased infectivity was related to the type of clay mineral and to the cation saturating the exchange complex in the order K homoionic (i.e. saturated with a single cation type) to Na⁺ > Mg²⁺ > K⁺ and M homoionic to Mg²⁺ > Na⁺ > K⁺. Enhanced infectivity of the bacteriophage was not related to the surface area of K nor to the external or total surface area of M. The increase of bacteriophage titre above that of the control was only minimally related to cation-exchange capacity, but was associated with the anion-exchange capacity of the clay. The increased plaquing efficiency is ascribed to the formation of high-density bacteria–clay microcosms which, in turn, modify bacteriophage contact and adsorption to the host cell.

INTRODUCTION

The incorporation of particles with a net negative charge (e.g. montmorillonite) and certain cationic and anionic polymers into overlay (liquid) medium has been found to increase virus infectivity more than threefold. Similar effects brought about by the incorporation of charged particles and polymers have also been observed with various non-related virus groups in qualitative assay systems (Bishai & Labzoffsky, 1974; Craighead & Lane, 1969; Kaplan *et al.*, 1967; Lipson & Stotzky, 1985; Nguyen *et al.*, 1987; Shirobokov, 1973; Takemoto & Liebhaver, 1962; Wallis & Melnick, 1968). The enhancement of virus infectivity by charged particles and molecular polymers has been ascribed to several mechanisms, including alteration of electrostatic forces at the virus–cell interface, increased diffusion efficiency through the overlay gel, the removal of inhibitors from the overlay medium, dispersive and carrier effects, abrasion of the host cell (in plant systems), and the inhibition of nucleases (Bishai & Labzoffsky, 1974; Craighead & Lane, 1969; Crandell & Gomes, 1970; Dunn & Hitchborne, 1965; Lipson & Stotzky, 1987; Nguyen *et al.*, 1987; Kaplan *et al.*, 1967; Shirobokov, 1973; Takemoto & Liebhaver, 1962; Toyoshima & Vogt, 1969; Vaheri *et al.*, 1967; Wallis & Melnick, 1968; Yarwood, 1966, 1972).

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Abbreviations: AEC, anion-exchange capacity; CEC, cation-exchange capacity; K, kaolinite; M, montmorillonite.

Negatively-charged particles and cationic polymers in general increase virus infectivity. Polyanions, on the other hand, commonly suppress virus adsorption and growth. However, numerous exceptions, albeit ill-defined, have been reported (Craighead & Lane, 1969; Nguyen *et al.*, 1987; Takemoto & Liebhaver, 1962; Toyoshima & Vogt, 1969; Wiggins & Alexander, 1985; Wunner *et al.*, 1984). In an effort to investigate this phenomenon, a model system was constructed wherein the plaquing efficiency of the bacteriophage ϕ X-174 was studied in the presence of two highly reactive aluminosilicate clay minerals, namely kaolinite and montmorillonite. The physicochemical characteristics of these clays are not only well defined, but they differ sufficiently to permit a clear analysis of those reactions which might occur at the bacteriophage-clay-cell interface.

METHODS

Bacteriophage and host cell. Bacteriophage ϕ X-174 and its host, *Escherichia coli* C, were purchased from Carolina Biological Supply Co. [cat. no. 12-4425 (ATCC no. 13706-B1, 1982) and 12-4400, respectively]. Bacteriophage stock was 6.7×10^8 plaque-forming units (p.f.u.) ml^{-1} in tryptone broth medium (10 g Bacto-tryptone, 5 g KCl, 2 ml 1 M- CaCl_2 l^{-1}). The stock was diluted in sterile distilled H_2O to obtain titres appropriate for the experiments. Preliminary experiments revealed that dilution of the bacteriophage in either H_2O or saline (0.9%) resulted in similar plaque counts.

Clay minerals. Colloidal kaolin (kaolinite; K) (cat. no. K-6, lot no. 734748) and bentonite (montmorillonite; M) (cat. no. B-235, lot no. 730257B) were purchased from Fisher Chemical Co.

Preparation of homoionic clays. Twelve grams of K or M was suspended in 400 ml 0.2 M- Na_2CO_3 , and stirred for 3 h. The resultant mixture was allowed to settle overnight at 4 °C. The upper third of each suspension was collected by aspiration, followed by centrifugation at 10000 g for 10 min at 4 °C. Sediments were washed three times (20000 g for 10 min at 4 °C) with 0.5 M- NaCl , 0.5 M- KCl or 0.25 M- MgCl_2 . These clays, in turn, were washed seven times with distilled water (20000 g) to obtain a chloride-free system.

Titration of bacteriophage and experimental design. Bacteriophage ϕ X-174 was assayed by the double agar layer technique (Adams, 1959). Briefly, 0.1 ml stock virus (diluted in sterile distilled H_2O) was inoculated into 4.5 ml overlay medium, viz. a tryptic soy broth (Difco, cat. no. 49800B0, consisting of 17 g Bacto-Tryptone, 3 g Bacto-Soytone, 2.5 g Bacto-dextrose, 5 g NaCl , 2.5 g dipotassium phosphate and 7.5 g Bacto-agar l^{-1}) immediately followed by seeding with 0.5 ml exponential-phase bacterial host cells (about 10^8 cells ml^{-1}) and 0.1 ml of each homoionic clay mineral to give final concentrations of 0.01, 0.02, 0.04, 0.06, 0.08, 0.10, 0.15 and 0.20 mg M or 0.14, 0.16, 0.18, 0.20, 0.22, 0.50 and 1.0 mg K ml^{-1} . The underlay consisted of tryptic soy broth, but containing 15 g agar l^{-1} . Inoculated plates were permitted to gel at room temperature, then inverted and incubated at 37 °C for 48 h. Plaques were counted on an optical cell counter and are reported as p.f.u. per 0.1 ml. Controls, without the clay minerals, were run in parallel.

Statistics. Experiments were done in triplicate, and bacteriophage titrations were done at least in quadruplicate. The arithmetic mean $\pm$ standard error of the mean (SEM) of control and experimental results were compared by Student's *t*-test. $P < 0.05$ was taken to be statistically significant.

RESULTS AND DISCUSSION

Wherever surfaces occur, especially in the microenvironment, an opportunity for adsorption, either non-biological (e.g. due to hydrogen bonding, van der Waals forces) or biological (e.g. receptor site specificity) exists (Brock, 1966; Lipson & Stotzky, 1987). The clay mineral-bacteriophage assay system constructed in the current study typifies such an environment.

Within the range of K and M concentrations studied, enhanced infectivity of bacteriophage ϕ X-174 occurred at K and M concentrations in the agar overlay of 0.14–0.50 and 0.01–2.0 mg ml^{-1} (except M homoionic to K^+ ions), respectively (Fig. 1). The maximum increase in titre occurred at 0.18 and 0.20 mg K ml^{-1} (180–210%), and at 0.06 and 0.08 mg M ml^{-1} (180–230%). The increased infectivity of the bacteriophage was related to the type of clay mineral and to the cation saturating the exchange complex in the order K homoionic to $\text{Na}^+ > \text{Mg}^{2+} > \text{K}^+$, and M homoionic to $\text{Mg}^{2+} > \text{Na}^+ > \text{K}^+$.

Infectivity of the bacteriophage in the presence of each homoionic clay was plotted as a function of the surface area of K (15 $\text{m}^2 \text{g}^{-1}$) and the external (82 $\text{m}^2 \text{g}^{-1}$) and total (750 $\text{m}^2 \text{g}^{-1}$) surface area of M (Lipson & Stotzky, 1983). Infectivity of the phage was not related to the surface area of either clay mineral (data not shown).

Fig. 1. Effect of clay minerals on the plaquing efficiency of bacteriophage ϕ X-174. (a) Montmorillonite (M) homoionic to potassium (M/K⁺), magnesium (M/Mg²⁺) and sodium (M/Na⁺). Bacteriophage added: $1.41 \pm 0.47 \times 10^4$ p.f.u. per 0.1 ml. (b) Kaolinite (K) homoionic to potassium (K/K⁺), magnesium (K/Mg²⁺) and sodium (K/Na⁺). Bacteriophage added: $1.30 \pm 0.53 \times 10^4$ p.f.u. per 0.1 ml. The data are normalized on the basis of bacteriophage titres in the control (i.e. in the absence of either clay mineral), and are shown $\pm$ SEM.

Fig. 2. Effect of CEC of K and M on the plaquing efficiency of bacteriophage ϕ X-174. CEC of M, 97.7 meq per 100 g clay; CEC of K, 5.8 meq per 100 g clay. The results are shown $\pm$ SEM.

Infectivity was further investigated as a factor of each clay mineral's cation- and anion-exchange capacities. Cation/anion-exchange capacity refers to the ability of a substrate, such as a clay mineral, to adsorb, retain, and then release the ions in question. Enhanced infectivity of bacteriophage ϕ X-174 was minimally associated with the cation-exchange capacity (CEC) of K and M (CEC = 5.8 and 97.7 meq per 100 g clay, respectively: Chaikum *et al.*, 1981; Grim, 1968): increased plaquing efficiency was observed at the upper and lower concentration range of the K and M utilized in this study (Fig. 2). The reported anion-exchange capacity (AEC) of K remains ill-defined; values obtained in different laboratories reflect different exchange capacities due to the assays being performed in aqueous and non-aqueous (95% ethanol)

Fig. 3. Effect of AEC of K and M on the plaquing efficiency of bacteriophage ϕ X-174. AEC of M, 23 meq per 100 g clay; AEC of K, 6.6–13.4 meq per 100 g clay. The results are shown $\pm$ SEM.

systems, and in varying electrolyte concentrations at different pH values. These factors, coupled with the exposure of Al^{3+} , Si^{4+} or Fe^{3+} ions resulting from breakage at the edges of the clay, probably account for the reported large range of AEC values (from about 6.6 to 20 meq per 100 g clay: Ferris & Jepson, 1975; Lipson & Stotzky, 1987). Under controlled conditions, however, the AEC of K was found to be markedly reduced at neutral pH (Ferris & Jepson, 1975). Such neutral conditions prevailed in the ϕ X-174 plaque assay utilized in the current study. Consequently, ϕ X-174 plaquing efficiency was plotted as a function of the lower AEC range of K, and compared with the AEC value commonly recognized for the homoionic M (i.e. 23 meq per 100 g clay) (Grim, 1968). Our data show a strong association between increased bacteriophage plaquing efficiency and the AEC of the clays tested (Fig. 3).

Sorptive interactions between bacterial cells (Marshall, 1968, 1969*a,b*; Santoro & Stotzky, 1967*a*, 1968), the tail-less bacteriophages (i.e. f2, ϕ X-174) (Burge & Enkiri, 1978; Goyal & Gerba, 1979; Moore *et al.*, 1975; Schaub *et al.*, 1980), clay minerals and soil systems have been studied extensively. These investigations, as relevant to our study, indicate that bacterial cells adsorb primarily to positively-charged or anion-exchange sites on clay minerals. Adsorption of the tail-less phages at neutral pH by kaolinite, montmorillonite, sewage-containing water and numerous soil types also occurred, but at reduced levels (Boyce *et al.*, 1981; Burge & Enkiri, 1978; Drewry & Eliassen, 1968; Koya & Chaudhuri, 1977; Moore *et al.*, 1975). A recent report by Armon & Cabelli (1988), for example, confirmed that adsorption of tail-less bacteriophages (i.e. f2) by K and M (homoionic to Na^+ ions) was minimal at the effective clay concentrations (0.18–0.20 and 0.06–0.08 mg ml^{-1} , respectively) utilized in the current investigation. Goyal *et al.* (1980) reported a unique behavioural characteristic of the bacteriophage ϕ X-174. Adsorption of the bacteriophage suspended in secondary sewage, reservoir or bayou waters (at neutral pH) to a Series S Zeta Plus filter was significantly reduced compared with that of phage types MS-2, T2 or T4.

These earlier findings, in conjunction with our data (Figs 2 and 3), suggest the following mechanism to account for the plaque-enhancement effect in this system. A preferential adsorption of *E. coli*, but not bacteriophage ϕ X-174, by K or M occurred in the overlay medium of the phage assay system, resulting in an increase in cell density on the clay surface and thereby creating localized high density bacteria–clay mineral microcosms. This enabled more efficient contact between the bacteriophage and its host cell. An apparent preferential adsorption by M of *E. coli* rather than bacteriophage T4 or M13 has been reported (Roper & Marshall, 1974, 1978*a*; Wiggins & Alexander, 1985). Significantly, the reported preferential adsorption of *E. coli* by M (Roper & Marshall, 1974) occurred at low (0.15 mg ml^{-1}) clay concentrations. The clay concentrations utilized by Roper & Marshall (1974) were similar to those used in the current study.

Fig. 4. Diagrammatic representation of the mechanism involved in the plaque enhancement effect of bacteriophage ϕ X-174 by K and M. Addition of appropriate concentrations of K (about 0.18–0.20 mg ml^{-1}) and M (about 0.06–0.10 mg ml^{-1}) to the overlay enhances the plaquing efficiency of ϕ X-174 by producing high-density bacterial-clay mineral microenvironments. These aggregates of microbial cells in turn permit a more efficient contact (and adsorption) of the phage to its host cell. High clay concentrations physically block attachment of the phage to its host cell and thus reduce plaquing efficiency.

Higher clay concentrations (more than 0.50 mg K ml^{-1} or 0.15 mg M ml^{-1}) reduced plaquing efficiency in our study. This result may be ascribed to the formation of a clay envelope around the cell, thereby preventing contact between the bacteriophage and its host. Similar protective effects of *E. coli* by M from phage attack and, to a lesser extent, from microbial predation, have been reported (Roper & Marshall, 1974, 1978b). In a similar study, bacterial growth and, in turn, phage T4 production, may have been inhibited by the addition of a high concentration (5 mg ml^{-1}) of M (Wiggins & Alexander, 1985). However, the mechanism of cell or phage growth inhibition by M is difficult to explain, as suboptimal bacterial inocula and a single clay concentration were utilized by Wiggins & Alexander (1985). Low K or M concentrations (i.e. trace or microgram levels) would have been expected to have no effect on phage plaquing efficiency, as clay-*E. coli* aggregates would not occur. A diagrammatic representation of our proposed mechanism is presented in Fig. 4.

An association between plaquing efficiency and a clay-bacteriophage sorptive reaction is also suggested by the differential effects on infectivity among the homoionic clays tested (i.e. K homoionic to $\text{Na}^+ > \text{Mg}^{2+} > \text{K}^+$ and M homoionic to $\text{Mg}^{2+} > \text{Na}^+ > \text{K}^+$) (Fig. 1). Differences in plaquing efficiency relative to the type of cation saturating the exchange complex are consistent with the differences observed in the adsorption of reovirus type 3 and actinophage f6 to K and M or to K alone, respectively (Lipson & Stotzky, 1983; Sykes & Williams, 1978). It would not be unreasonable to suggest that K and M served, in part, as carrier vehicles of the phage to its host (Lipson & Stotzky, 1985). It should be pointed out that the cation-exchange reactions referred to in the current study did not occur or occurred only minimally at the clay-bacterial cell interface, as adsorption of bacterial cells to clay minerals occurs primarily at anion-exchange sites of inorganic colloids (Marshall, 1975). Factors responsible for the differential adsorption of viruses to homoionic clays have been previously described in detail (Lipson & Stotzky, 1987).

Removal of bacterial metabolites from the agar overlay by K or M is unlikely as an explanation of the results. In general, adsorption of microbial metabolites by clays appears to be related to the valency of the cation saturating the clay complex (i.e. tetravalent > trivalent > divalent) (Santoro & Stotzky, 1967*b*). Our data, in contrast, show that plaque enhancement is associated with the AEC of the clays.

The effects of polymers or particulates on infectivity cannot be inferred from their electric charge alone. Differences between virus groups, types, strains within the same type, the host cell and the size of the charged agent itself, must be addressed prior to the utilization of an extrinsic substance to enhance infectivity (Craighead & Lane, 1969; Crandell & Gomes, 1970; Lipson & Stotzky, 1984, 1985; Nguyen *et al.*, 1987; Shirobokov, 1968; Takemoto & Liebhaver, 1962; Toyoshima & Vogt, 1969; Wallis & Melnick, 1965; WuDunn & Spear, 1989). In the current study, for example, one may not discount those unique physicochemical characteristics of K and M and, in turn, the differential enhancement effects of these clays on the plaquing efficiency of bacteriophage ϕ X-174 (Fig. 4).

In summary, the incorporation of K or M into the overlay during the titration of bacteriophage ϕ X-174 resulted in an increase in the number of plaques to some 180–230% of the control value. In our system, this effect was probably due to the formation of localized high-density bacteria–clay mineral microcosms which, in turn, permitted an increased contact of the bacteriophage with its host cell. Some adsorption of the phage to cation-exchange sites on the clay mineral surface may have occurred as well, resulting in a transport (and enhanced contact) of the virion to its host cell. The results of this study suggest that the addition of clay minerals to other bacteriophage assay systems, or the use of these specific clays in the clinical setting (i.e. addition of K or M into maintenance medium), may be advantageous for the detection of viral titres below minimal levels capable of producing plaques or cytopathic effects.

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