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Stabilization of T4 bacteriophage at acidic and basic pH by adsorption on paper

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ABSTRACT

Bacteriophages find applications in agriculture, medicine, and food safety. Many of these applications can expose bacteriophages to stresses that inactivate them including acidic and basic pH. Bacteriophages can be stabilized against these stresses by materials including paper, a common material in packaging and consumer products. Combining paper and bacteriophages creates antibacterial materials, which can reduce the use of antibiotics. Here we show that adsorption on paper protects T4, T5, and T7 bacteriophage from acidic and basic pH. We added bacteriophages to filter paper functionalized with carboxylic acid (carboxyl methyl cellulose) or amine (chitosan) groups, and exposed them to pH from 5.6 to 14. We determined the number of infective bacteriophages after exposure directly on the paper. All papers extended the lifetime of infective bacteriophage by at least a factor of four with some papers stabilizing bacteriophages for up to one week. The degree of stabilization depended on five main factors (i) the family of the bacteriophage, (ii) the charge of the paper and bacteriophages, (iii) the location of the bacteriophages within the paper, (iv) the ability of the paper to prevent bacteriophage-bacteriophage aggregation, and (v) the sensitivity of the bacteriophage proteins to the tested pH. Even when adsorbed on paper the bacteriophages were able to remove *E. coli* in milk. Choosing the right paper modification or material will protect bacteriophages adsorbed on that material against detrimental pH and other environmental challenges increasing the range of applications of bacteriophages on materials.

1. Introduction

Bacteriophages offer an alternative to antibacterial compounds in very diverse areas, including agriculture, medicine, and food safety [1–6]. Production, storage, and utilization can inactivate bacteriophages by exposing them to stresses from temperature, radiation, desiccation, and changes in pH [7–9]. For example, bacteriophages can encounter changes in pH (i) during bacteria detection (e.g., in surface waters with pH between 6.5 and 9), (ii) during oral drug delivery to the stomach (pH 1.5–3.5) or intestine (pH 5.7–7.4), and (iii) as food packaging (e.g., food pH ranges from acidic, in fruit juices and cheeses, to basic, in crackers and eggs). There is need for the stabilization of bacteriophages in conditions spanning a wide range of pH.

Bacteriophages are intrinsically sensitive to both acidic and basic environments. Temperature and the nature of the bacteriophage both determine the pH at which it will inactivate [9]. Generally speaking, bacteriophages are stable close to neutral pH (between pH 5–9). For example, T4 bacteriophage were found to be most stable from pH 6–7.4, while they were unstable below pH 5 or at pH 9.2 [9]. Similarly, T2 and T7 bacteriophages were stable between pH 5–9, losing infectivity faster the further the pH was outside of this range [10,11]. Bacteriophages were also found to aggregate and precipitate at acidic pH due to a decrease in their surface charge [11,12].

The stability of bacteriophages against changes in pH has been improved by encapsulating them in a material [9]. For example, alginate hydrogels stabilized encapsulated bacteriophages against acidic solutions, especially when combined with a neutralizing agents such as calcium carbonate [13–15]. Bacteriophages encapsulated within films of cellulose acetate showed activity for up to two weeks [16].

A material often used to immobilize bacteriophages or other biological entities is paper [17–20]. For example, paper or cellu-

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lose with bacteriophages has been used to capture *E. coli* [21], and to reduce the number of *E. coli* on raw beef [22] and *Listeria monocytogenes* on cut cantaloupe [23]. Bacteriophages on paper functionalized with amine groups reduced the bacterial load on cooked turkey, ready-to-eat meat, or sprouts [22–24]. The number of amine groups was tentatively correlated to the number of bacteriophages on the paper [25].

Different functional groups on the paper will lead to different charge on the fibers depending on the pH of the solution and the pK_a of the material (Fig. 1). Cellulose has a pK_a between 13.4–13.7 [26,27], carboxylmethyl cellulose (CMC)-functionalized paper has a pK_a of 4 [26], and chitosan-functionalized paper has a pK_a between 6.3–7 [27]. Bacteriophages are also charged with an isoelectric point for T4 bacteriophage at pH 2 or pH 4–5 depending on the measurement method [28]. The charge on the T4 bacteriophage is not equally distributed leading to a more negatively charged capsid (-5.31 ± 0.67 mV) and a more positively charged tail and tail fibers (1.80 ± 0.19 mV) [22]. This charge of the bacteriophage is determined by its proteins. The tail and tail fibers consist of six major proteins: the tail sheath protein (gene product (gp)18), the tail tube protein (gp19), and the long tail fiber proteins (gp34–gp37) [29]. The capsid consists of four main proteins the major capsid protein (gp23), the head vertex protein (gp24), the smaller outer capsid protein (gpsoc) and the highly immunogenic outer capsid protein (gphoc). Three of these proteins are negatively charged at neutral pH (gphoc pI ≈ 4, gp23: pI = 6.2, gp24: pI = 5.5) [30,31], while gpsoc is considered neutral [32]. There is less information about the charge for T5 and T7 bacteriophage available, however their similar protein structures suggests a similar charge distribution [33–37]. These different charges on the paper and bacteriophages can influence both the strength of adsorption and orientation of bacteriophages on the surface of the paper, as well as the material's ability to remove hydrogen and hydroxyl ions locally from solution.

In this study, we explored if and how the charge of the different functional groups on paper and T4, T5, and T7 bacteriophage lead to different degrees of stabilization of bacteriophages at different pH. We added the bacteriophages to filter paper and filter paper functionalized with carboxylic acid groups (CMC) or primary amines (chitosan) before exposing them to solutions with pH between 2 and 14 for up to one week. After neutralizing the papers and removing any excess liquid, we placed them directly on an overlay containing *Escherichia coli* K12 or *E. coli* B and measured the size of the lysis area around the papers after incubation. The size of the lysis area was correlated to the number of infective bacteriophages on the paper. All papers improved the length of time that infective bacteriophages were present at extreme pH by at least a factor of four compared to bacteriophages in solution at the same pH. The degree of stabilization was dependent on the functional groups of the paper, the type of bacteriophage, and the pH. We attribute these differences to the different strength of adsorption, orientation and denaturation of the bacteriophages.

2. Materials and method

2.1. Materials

Magnesium sulfate anhydrous ($MgSO_4$),sodium hydroxide (NaOH), hydrochloric acid (HCl), calcium chloride ($CaCl_2$) and chloroform were purchased from Fisher Chemicals (Denver, CO). Carboxylmethyl cellulose and chitosan were purchase from Sigma-Aldrich (St. Louis, MO). Glycerol, 99+% and citrate buffer (pH 5, 0.5 M) were purchased from Alfa Aesar (Ward Hill, MA). Gelatin was purchased from AMRESCO (Solon, OH), while 4H 1 M Tris pH 7.5, sodium chloride (NaCl) and Whatman grade 3 filter paper were purchased from VWR (Wayne, PA). Difco Luria Bertani (LB) Broth Lennox and Bacto Agar were purchased from Becton-Dickinson (BD) (Franklin Lakes, NJ). T4 bacteriophage (ATCC#11303-B4), T5 bacteriophage (ATCC#11303-B5), T7 bacteriophage (ATCC# BAA-1025-B2), *E. coli* K12 (ATCC#25404) and *E. coli* B (ATCC#11303) were purchased from American Type Culture Collection (Manassas, VA). Milk 2% fat content from Anderson Erickson lot#19053 was bought on Iowa State University campus.

2.2. Preparation of E. coli and T-bacteriophage

We used T4, T5 and T7 bacteriophage and their hosts *E. coli* K12 and *E. coli* B for all experiments. Fresh overnight cultures of *E. coli* were prepared in 5 ml Luria Bertani (LB) broth at 37 °C under shaking (300 rpm) and maintained in LB broth at 4 °C. The cultures were enumerated on LB agar (1.5% agar) showing 10^{10} colony forming units (CFU) per milliliter. T-bacteriophage were propagated on *E. coli* K12 overlays [38]. In short, single plaques of T-bacteriophage from overlays were incubated in 200 μl of lambda buffer (100 mM of NaCl, 16.6 mM of $MgSO_4$, 50 ml of 4H 1 M Tris pH 7.5, and 1.8 μM of gelatin in 1 l of millipure water) for two hours at room temperature. The solution was then spread on overlays (100 mm diameter) consisting of 10 ml LB bottom agar (1.5% agar) and 5 ml LB top agar (0.5% agar) with 10^8 CFU *E. coli* K12. After overnight incubation at 37 °C, 3 ml sterile deionized water was added to each plate and the top agar and water were placed into a sterile 50 ml centrifuge tube. Chloroform (2 ml) was added and the mixture was vortexed (20 s) and then centrifuged. The aqueous layer was filtered through a 0.45 μm filter membrane and the concentration of bacteriophage in plaque forming units (PFU) was enumerated on overlays. For enumeration, the bacteriophage dispersions were diluted serially, 10-fold each time – and four 5 μl droplets of each dilution were spotted on two separate overlays with 10^8 CFU *E. coli* in each top layer. After overnight incubation at 37 °C spots with 3–30 plaques were counted and numbers of plaques for the same dilution were averaged. The concentrations per milliliter were calculated by multiplying the average number of plaques by the reciprocal dilution and a factor of 200.

2.3. Modification of papers with carboxyl methyl cellulose and chitosan

Circular Whatman grade 3 filter papers with 38.5 mm^2 surface area were used in all experiments. Larger filter papers were cut with a 3-hole punch before autoclaving (120 °C, 20 min). Sterile papers were treated with carboxylmethyl cellulose (CMC) or chitosan using protocols for the adsorption to cellulose films with modifications [39–41]. In detail, the papers were immersed for one hour under shaking (300 rpm) at room temperature in either 200 μl of 2.0 g/L of CMC in 10 mM aqueous $CaCl_2$, or 0.5 w/w% of chitosan at pH 3.6. After immersion, the papers were removed and allowed to dry overnight in the biosafety cabinet. Modification was confirmed by mechanical and physical changes of the papers.

2.4. Exposure of bacteriophages in solution to different pH

T-bacteriophage at 10^8 PFU/ml (200 μl) were mixed with equal volumes of pH 2 or pH 14 aqueous solution for a final pH of 5.6 or 14. The pH of the water was changed by adding the appropriate volume of 1.0 M HCl or 1.0 M NaOH and determined by a pH meter. At different time points (5 min to 300 min), 20 μl of solution were removed and neutralized in 50 μl lambda or citrate buffer. The concentration of bacteriophage was determined after serial dilution using overlays.