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Higher Concentrations of Bacterial Enveloped Virus Phi6 Can Protect the Virus from Environmental Decay

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ABSTRACT Phage Phi6 is an enveloped virus considered a possible nonpathogenic surrogate for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and other viral pathogens in transmission studies. Larger input amounts of bacteriophage Phi6 are shown to delay and protect the phage from environmental decay, both when the phages are dried in plastic tubes and when they are stored in saline solution at 4°C. In contrast, when bacteriophage Phi6 is placed in LB (Luria-Bertani) growth medium (instead of saline) prior to placement on the plastic surface, the influence of the starting concentration on viral recovery is negligible. Protection is reflected in the phage half-lives at higher concentrations being longer than the half-lives at lower concentrations. Because experiments supporting the possibility of fomite transmission of SARS-CoV-2 and other viruses rely upon the survival of infectious virus following inoculation onto various surfaces, large initial amounts of input virus on a surface may generate artificially inflated survival times compared to realistic lower levels of virus that a subject would normally encounter. This is not only because there are extra half-lives to go through at higher concentrations but also because the half-lives themselves are extended at higher virus concentrations. It is important to design surface drying experiments for pathogens with realistic levels of input virus and to consider the role of the carrier and matrix if the results are to be clinically relevant.

IMPORTANCE During the coronavirus disease 2019 (COVID-19) pandemic, much attention has been paid to the environmental decay of SARS-CoV-2 due to the proposed transmission of the virus via fomites. However, published experiments have commenced with inocula with very high virus titers, an experimental design not representative of real-life conditions. The study described here evaluated the impact of the initial virus titer on the environmental decay of an enveloped virus, using a nonpathogenic surrogate for the transmission of SARS-CoV-2, enveloped bacteriophage Phi6. We establish that higher concentrations of virus can protect the virus from environmental decay, depending on conditions. This has important implications for stability studies of SARS-CoV-2 and other viruses. Our results point to a limitation in the fundamental methodology that has been used to attribute fomite transmission for almost all respiratory viruses.

KEYWORDS SARS-CoV-2, bacteriophage Phi6, fomite transmission

Early in the coronavirus disease 2019 (COVID-19) pandemic, there was an intense focus on fomites (i.e., inanimate objects and surfaces) as possible conduits for the transmission of the causative agent, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This was because of a widely repeated contention that a person touching a freshly contaminated surface, not washing hands, and then quickly touching their mouth, nose, or eyes would lead to self-inoculation of this respiratory virus. Consequently, considerable effort has been made to determine how long the virus remains infectious after

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being deposited on various surfaces and what conditions favor or disfavor the viability of the virus on these surfaces (1–11).

In parallel with these studies, workers tested for the presence of viral RNA on surfaces in hospitals treating COVID-19 patients (12–16). These reverse transcription-PCR (RT-PCR) tests found viral RNA to be present on many surfaces (but did not test for infectious virus, with one exception) and reinforced the perception that fomites were indeed a significant risk factor for the transmission of the disease.

In July 2020, a comment was published (online) arguing that the risk of transmission of SARS-CoV-2 by fomites was exaggerated (17). New information that appeared since then has strengthened this conclusion (18). The basis for this argument was that the amounts of virus used in experiments for determining how long infectious virus remains viable on surfaces were orders of magnitude too large compared to what someone would actually encounter in a real-world situation. Since the virus decays with a defined half-life depending on the surface, the larger the inoculum, the more half-lives have to be gone through before there is <1 infectious virus particle remaining on the surface. Smaller, more realistic inocula would survive through fewer half-lives, and therefore, much less time would pass before the surface would be free of infectious virus.

Among conditions favoring virus survival on surfaces was the observation that bovine serum albumin (BSA) protected the virus from environmental decay and extended the time that the virus remained viable (8). Similar observations with bovine serum albumin were also noted in experiments assessing the viability of bacteriophage MS2 and enveloped bacteriophage Phi6 in droplets (19). Bacteriophage Phi6 has been considered a potential nonpathogenic transmission surrogate for enveloped viral pathogens like SARS-CoV-2 and Ebola virus (20, 21). This is largely based on the fact that Phi6 is an enveloped RNA virus, like coronavirus, and therefore, its stability in the environment provides a legitimate comparison to the behaviors of other enveloped RNA viruses such as SARS-CoV-2.

The fact that bovine serum albumin protected at least three viruses (SARS-CoV-2, phage MS2, and phage Phi6) from environmental decay made us wonder if higher concentrations of a virus itself might similarly protect the virus from decay. Indeed, Lin and coworkers suggested the value of "investigating the role of viral titer, which might affect aggregation and other characteristics, on virus survival" (19).

There was already a suggestion that this might be the case for SARS-CoV-1. Lai et al. (see Table 1 in reference 22) indicated that the survival of SARS-CoV-1 on paper, cotton gowns, and disposable gowns was much greater for a 10^6 inoculum of infectious virus particles than for a 10^4 inoculum. At a 10^4 inoculum, infectious virus was not detectable after 5 min, but with a 10^6 inoculum, infectious virus remained detectable for 24 h. This result suggested that the virus half-life was greatly extended with larger amounts of input virus.

In the work reported here, we have investigated the role of the initial virus concentration in the environmental decay of phage Phi6. We assayed the survival of virus samples dried in plastic tubes for various lengths of time after drying. We show that higher input virus concentrations indeed exhibit significantly higher percent survivals and longer half-lives than a lower virus input; however, this effect is influenced by the inoculating matrix. The protective effect of higher virus concentrations was also observed for virus samples kept in solution at 4°C. This protective effect was not found for virus placed in Luria-Bertani (LB) growth medium (which contains tryptone and yeast extract) before being placed into plastic tubes for drying.

RESULTS AND DISCUSSION

Survival of Phi6 dried on plastic is increased at higher phage concentrations. Table 1 shows the survival of Phi6 dried in plastic tubes and left for various lengths of times as a function of the initial virus concentration. The simple act of drying the phage led to the loss of nearly all viable phage at the lower phage input concentrations (samples 3 and 4) while having no significant effect (93% recovery) on the highest phage

TABLE 1 Extent of survival of Phi6 dried on plastic is increased at higher phage concentrations[a]

Sample	Dry time (min)	Phage amt		% recovery	Half-life (min)
		Inoculum	Recovered		
1	0	6×10^3	5.6×10^3	93	
2	0	6×10^2	4.2×10^2	70	
3	0	60	1	2	
4	0	6	0	0	
5	15	1.2×10^4	1×10^4	83	57
6	15	1.2×10^3	3.2×10^2	27	8
7	15	1.2×10^2	13	11	5
8	15	12	0	0	
9	30	4.7×10^4	2×10^4	43	24
10	30	4.7×10^3	7.6×10^2	16	11
11	30	4.7×10^2	18	4	6
12	30	47	0	0	
13	60	3.2×10^4	1.2×10^4	38	42
14	60	3.2×10^3	1.4×10^2	4	13
15	60	3.2×10^2	0	0	
16	60	32	0	0	

[a]Amounts of phage Phi6 as shown in the "Inoculum" column were dried in polypropylene tubes. Samples were reconstituted in 100 μl saline at the times after drying shown in the table. The amounts of viable phage remaining in the reconstituted samples were determined and are shown in the "Recovered" column. The half-life was calculated as described in Materials and Methods.

concentration tested (sample 1) and a small effect (70% recovery) on a 10-fold-lower initial phage concentration (sample 2).

Similar patterns of protection by higher initial phage concentrations were also seen for all subsequent lengths of time that the phage remained dry in the tube. At 15 min of dry time, we began to see some loss of survival from the most concentrated initial virus input (sample 5, 83% recovery) compared to a 10-fold-lower virus input (sample 6, 27% recovery). As was seen for the samples dried and assayed immediately, the lowest virus inputs led to the loss of almost all viable phage (sample 7, 11% recovery; sample 8, none recovered).

After 30 or 60 min of dry time, the highest initial phage inputs began to show significant environmental decay, with just 43% recovery after 30 min (sample 9) and 38% recovery at 60 min (sample 13). But even more substantial environmental decay was observed for the lower-input virus samples (samples 10 to 12 for the 30-min dry time and samples 14 to 16 for 60 min).

For those samples with measurable virus survival, we were able to calculate the half-lives of virus in those samples (Table 1), which were commensurate with the percent survival observed. That is, at higher initial phage input levels, the half-lives were much longer than the half-lives at lower initial phage input levels; e.g., compare sample 5 (57-min half-life) to sample 6 (8-min half-life), sample 9 (24-min half-life) to sample 11 (6-min half-life), or sample 13 (42-min half-life) to sample 14 (13-min half-life).

The results in Table 1 show that the higher phage input concentrations delay and protect dried phage from environmental decay compared to lower phage input concentrations. This effect is also visualized in Fig. 1A. The black circles in Fig. 1A show the relationship between the log PFU inoculated onto the surface and the level recovered. The dashed line represents the best-fit regression line. The solid line represents a relationship where all inoculated viruses would be recovered (i.e., 100% recovery). It is clear from the difference between the slopes of the two lines that the rate of recovery is progressively lower as the inoculation level declines. In those experiments where inoculated virus was not recovered, no recovery (0 PFU) is visualized in Fig. 1 at -1 log PFU.

Survival of Phi6 in saline at 4°C is increased at higher phage concentrations. We decided to test whether there was an effect of the phage concentration on virus

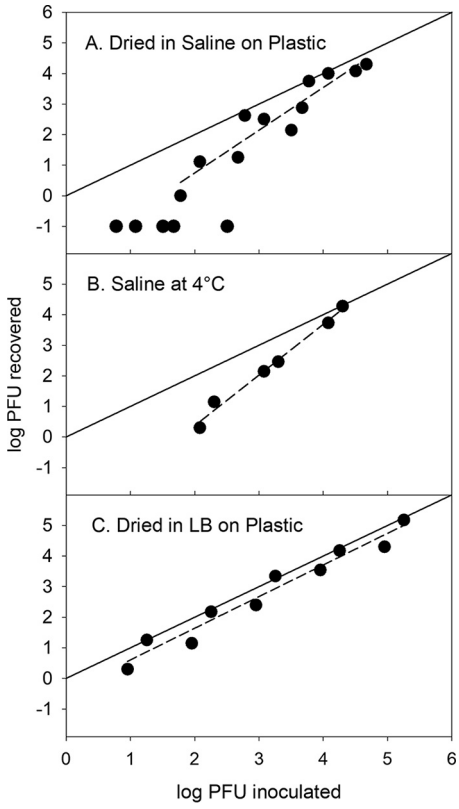

FIG 1 Relationship between the log PFU inoculated and the level recovered (black circles) for bacteriophage Phi6 on plastic in saline at room temperature (A), bacteriophage Phi6 in saline at 4°C (B), and bacteriophage Phi6 on plastic in Luria-Bertani broth at room temperature (C). The dashed lines represent the best-fit regression line for each data set. The solid lines represent the line of perfect recovery where all inoculated viruses would be recovered (i.e., 100% recovery). Samples in those experiments where inoculated virus was not recovered (0 PFU) are visualized at −1 log PFU.

stability in solution, as was seen for phage dried on surfaces. Table 2 shows little decay of phage in solution at 4°C at the highest concentrations tested after 20 days (sample 1) or about half decay after 56 days (sample 4). But as the input concentration is reduced, decay increases at both time points (samples 2 and 5), with the percent recovery in single digits for the lowest-input phage samples (samples 3 and 6). These results can also be visualized in Fig. 1B. As in Fig. 1A, the black circles show the relationship between the log PFU inoculated onto the surface and the level recovered. The dashed line represents the best-fit regression line, and the solid line represents a relationship where all inoculated viruses would be recovered (i.e., 100% recovery). As in Fig. 1A, it is clear from the difference between the slopes of the two lines that recovery

TABLE 2 Survival of Phi6 in saline at 4°C is increased at higher phage concentrations[a]

		Phage amt			
Sample	Time (days)	Initial input	Recovered	% recovery	Half-life (days)
1	20	2×10^4	1.9×10^4	95	270
2	20	2×10^3	2.9×10^2	15	7
3	20	2×10^2	14	7	5
4	56	1.2×10^4	5.4×10^3	45	49
5	56	1.2×10^3	1.4×10^2	12	18
6	56	1.2×10^2	2	2	9

[a]Amounts of phage Phi6 in saline shown in the "Initial input" column were placed in tubes on day 1 and kept at 4°C for the times indicated, at which point the amounts of viable phage remaining in the tubes were determined and are shown in the "Recovered" column. The half-life was calculated as described in Materials and Methods.

TABLE 3 Protection of Phi6 from decay in LB medium dried on plastic[a]

Sample	Dry time (h)	Phage amt		% recovery	Half-life (h)
		Inoculum	Recovered		
1	0.5	1.8×10^5	1.5×10^5	83	
2	0.5	1.8×10^4	1.5×10^4	83	
3	0.5	1.8×10^3	2.2×10^3	122	
4	0.5	1.8×10^2	1.5×10^2	83	
5	0.5	18	18	100	
6	24	9×10^4	2×10^4	22	11
7	24	9×10^3	3.5×10^3	39	18
8	24	9×10^2	2.5×10^2	28	13
9	24	90	14	16	9
10	24	9	2	22	11

[a]Samples were generated as described in Table 1, except that the serial dilutions of the phage stock were in LB medium instead of saline. The half-life was calculated as described in Materials and Methods.

is progressively lower as the inoculation level declines. These experiments had no trials where inoculated virus was not recovered.

Protection of Phi6 from decay by LB medium. In our earliest experiments, we used the "initial phage stock" (see Materials and Methods), which showed protection from decay at higher phage concentrations. But we realized that this stock also had some level of LB medium present, derived from the soft top agar layer containing the lysate in the phage preparation. Therefore, dilutions of the initial phage stock also diluted part of the medium that the phage had been grown in, which is what prompted us to develop the filtration method described in Materials and Methods. This allowed the phage to be tested without residual components of the growth medium, which could affect phage survival.

Nevertheless, we wondered whether the growth medium itself might also protect the phage from decay, similar to observations with BSA (19). Table 3 shows that this is indeed the case. When phage samples were diluted in LB medium instead of saline, phages were essentially completely protected from decay following a 1/2-h dry time (samples 1 to 5). This is in marked contrast to the results when phages were diluted in saline (Table 1). After extending the dry time for LB medium-containing samples to 24 h, environmental decay was now evident in the samples, with rates of recovery of phage ranging between 16 and 39% (samples 6 to 10). There was no effect of varying the amount of the initial input phage, showing that LB medium delays and protects even lower concentrations of phage from environmental decay. This is evident in Fig. 1C, which shows that as the level of log PFU inoculated declines, the log PFU recovered declines proportionally, and the slope of the regression line (dashed line) is essentially parallel to the line of 100% recovery (solid black line). The regression lies slightly under the line of 100% recovery, which indicates that most but not all of the virus inoculated was recovered with the same rate of environmental decay, but this varied slightly from experiment to experiment. These experiments also had no trials where inoculated virus was not recovered.

Our data demonstrate that bacteriophage Phi6, an enveloped virus that has been considered a potential nonpathogenic surrogate for SARS-CoV-2 transmission studies, exhibits a slower loss of infectivity from environmental decay by higher initial virus concentrations, depending upon the carrier. This slower loss of infectivity is true both when the phages are dried on plastic surfaces and when the phages are left in saline solution in a refrigerator. The protection at higher phage concentrations is reflected in longer half-lives than with lower phage concentrations.

Phage survival on dried surfaces can be greatly affected by the type of surface, by temperature and humidity, and by the medium containing the phage (19–21). Indeed, we observed dramatic delay and protection of phage in LB growth medium, which superseded the effects of the initial phage concentration. Thus, an important limitation

of our results is that we do not know what effect (if any) on phage survival would result from other natural additions, such as mucous, for example, which has been tested for SARS-CoV-2 (23).

A recent study compared the environmental stabilities of dried SARS-CoV-2 for two different initial virus inocula (4×10^5 versus 4×10^3) on stainless steel and did not observe additional protection in the rate of decay at the higher concentration (9). The SARS-CoV-2 samples used in this study were in growth medium, including fetal bovine serum, which may be more comparable to our results for Phi6 in LB medium since we know that a protein-containing matrix stabilizes enveloped viruses and protects against decay (8). Also, their lower tested concentration (4×10^3) may still have been too high to observe accelerated decay at lower virus concentrations.

Our results have implications for experiments measuring SARS-CoV-2 survival on surfaces as purported sources of transmission. Large initial amounts of input virus on a surface may generate artificially inflated survival times compared to the realistic lower levels of virus that a subject would normally encounter, not only because there are extra half-lives to go through at the higher concentrations but also because the half-lives themselves are extended at higher virus concentrations.

The implications of our findings are also relevant for experiments supporting the transmission of respiratory viruses by fomites in general. With the exception of respiratory syncytial virus, the belief that most, if not all, of these viruses can be spread by fomites is based solely on dried-virus stability experiments (24). In the case of rhinovirus, the major cause of the common cold, an early report demonstrating experimental fomite transmission used unrealistic conditions (25). A subsequent study closer to real-life conditions disproved this route of transmission, at least to a first approximation (26). The amount of influenza virus in droplets has been measured by both RNA content and virus viability; the RNA content was consistent with 10 to 100 viral particles in a droplet, while the amount of virus capable of forming plaques was considerably smaller (27). These levels of virus are likely far too low to support putative fomite transmission. Furthermore, mask wearing during the COVID-19 pandemic correlates with a 99% drop in influenza virus infections this past year compared to the previous year (28). This enormous decline would likely not have been observed if fomites were a significant route of transmission. The apparent low efficiency of virus transfer by fingers also needs to be considered when assessing the possibility of fomite transmission (29, 36), as contact with hands might inactivate some viruses (30).

We do not know why higher concentrations of input Phi6 protect the virus from decay or why it is influenced by the carrier. It could just be a result of higher protein levels in the phage solution buffering the phage particles from the effects of drying out. Lin and coworkers pointed out that as liquid evaporates, the concentration of solutes (in our case, salt) increases in the microenvironment (19), which may affect survival. Furthermore, virus aggregation at higher concentrations is likely, and such aggregates may be protective (31) or simply manifest as protective because clusters of viruses will be counted as single PFU but behave kinetically as more resistant viruses. Whatever the cause of this protection, it is even more imperative to design surface drying experiments for pathogens with realistic levels of input virus and account for the effects of the carrier and matrix. We expect that a realistic quantity of virus for such experiments would be in the range of 50 to 100 virus particles, and the matrix would not contain bovine serum albumin, which is not found in human secretions. In addition to a reduced viral inoculum, the parameters of a realistic matrix need to be explored for virus stability experiments to be clinically relevant.

MATERIALS AND METHODS

Phage preparation. Bacteriophage Phi6 and a bacterial host strain in which it grows, *Pseudomonas syringae* pv. *phaseolicola* HB10Y, were generous gifts of Lenny Mindich (now retired) of the Public Health Research Institute of Rutgers University. Cells grown overnight in Luria-Bertani (LB) medium in tubes shaken at 25°C were used in plaque assays with serial dilutions of virus to obtain countable numbers of plaques. Plaque assays were performed as described previously by Goldman (32), except that the plates

were incubated at room temperature (approximately 20°C). LB medium contained 10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl, and NaOH to adjust the pH to 7.0 (33).

Phage stocks were obtained by harvesting the top agar (6.5 g/liter in LB medium) from one or two petri dishes (containing 10 g/liter agar in LB medium) exhibiting confluent lysis of the bacterial lawn. A saline solution (9 g/liter NaCl), at 1 ml per plate, was added to the top agar, which was transferred to centrifuge tubes and centrifuged at 20,000 $\times$ *g* for 5 min to remove agar and debris. This supernatant, stored at 4°C, comprised the initial phage stock.

Preparation of phage stock in saline solution. An Amicon Ultra 100K filter device from Millipore was prerinsed with 4 ml of distilled water, followed by subsequent washes with 70% ethanol and sterile saline solution, using centrifugation at 4°C in a fixed-angle rotor at 5,000 $\times$ *g*. Up to 4 ml of the Phi6 initial phage stock was loaded onto this filter unit and centrifuged such that a 200-μl volume remained above the filter (typical spin time of 15 to 20 min). The fluid below the filter was discarded, the filter unit was refilled with 3.8 ml of sterile saline solution, and the same centrifugation steps were repeated 5 times. The sample (saline stock) was recovered in a 200- to 400-μl volume, and the virus titer was determined by a plaque assay.

Phage survival following drying in polypropylene tubes. An aliquot from the saline stock was subjected to a sequential series of 10-fold dilutions in saline. Five microliters of the stock and 5 μl of each of the 10-fold dilutions were placed near the bottom of 1.5-ml-capacity conical polypropylene Eppendorf microcentrifuge tubes (Corning). In our early experiments, we allowed samples to air dry but switched to desiccation to save time. Samples were desiccated under a house vacuum (approximately 20 mm Hg) and removed from the desiccator when visually dry. Generally, this took between 15 and 20 min, with higher concentrations of phage exhibiting shorter drying times, except for samples dried in LB medium, where all samples took 19 to 20 min to dry. We observed that there were no significant differences in the patterns of phage survival between air drying and desiccation. The data reported here were obtained from desiccated samples. After the samples were dried, they were allowed to stand at room temperature with open lids for the time intervals indicated.

Ambient room humidity was not controlled and varied in the building within a range between 10% and 45% over a period of 6 months, depending on the weather. However, for most of the experiments reported here, the humidity was around 15 to 25%. Humidity was monitored on a Holmes HHG-150 comfort check hygrometer and thermometer. Although humidity is known to significantly affect the environmental decay of Phi6 (19–21), all samples within a given experiment were subjected to the same humidity, and our interest was only to ascertain the effects of the initial viral concentration. Also, experiments measuring virus survival under real-world conditions, as has been done for SARS-CoV-2 (e.g., see references 34 and 35), do not control for humidity, which is variable. Ambient room temperature was also not controlled but generally was maintained at around 20°C.

Dried samples were reconstituted with 100 μl saline added to the Eppendorf tubes and vortexed. The time points at which dried samples were reconstituted included 0, 15, 30, and 60 min for samples in saline and 30 min and 24 h for samples in LB medium, as indicated. The titer of viable virus remaining in each tube was then determined by a plaque assay. Percent survival was simply the amount of phage recovered after reconstitution divided by the amount of input phage placed into the tube at the beginning of the experiment, times 100. For the higher-dilution samples (i.e., samples that received smaller amounts of input phage), the entire reconstituted volume was tested in plaque assays, and in some cases, no phage was recovered, as indicated. The half-life for the virus in a particular sample was calculated using the tool at https://www.calculator.net/half-life-calculator.html?type=1&nt=25&n0=2300&t=60&t12=&x=45&y=11.

All experiments were repeated 1 to 3 times; data from representative experiments are shown in the tables. Because of variations in conditions from experiment to experiment (such as humidity and the initial titer of the phage stock), we are not showing pooled results to obtain averages. However, because the relative percent survival in each experiment was generally consistent for the same time points, and the patterns were reproducible within experimental limits, we present pooled percent survival results with averages and standard deviations in Tables SA1 to SA3 in the supplemental material.

For experiments with LB medium, the initial phage stock was subjected to a sequential series of 10-fold dilutions in LB medium. The remainder of the protocol was the same as the one described above.

Phage survival in solution. The serial dilutions used for the dry time experiments were stored at 4°C in the dark for later testing. After the number of days shown in the tables, the titer of the phage in each dilution was determined by a plaque assay and compared to the initial titers as measured on day 1.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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R.B. performed the dry time experiments. E.G. performed the solution experiments with assistance from R.B. M.C. assisted in the preparation of the materials used and developed the filtration method for generating saline solutions of phage from LB stocks. The first draft of the manuscript was prepared by E.G., with input from a report by R.B. R.B. reformatted the revised manuscript to the journal's specifications, with the assistance of E.G. D.W.S. generated the figure and assisted in the interpretation of the results of experiments and revisions of the manuscript. All authors reviewed the manuscript, made corrections, and approved the final version. Overall supervision of the project was under the direction of E.G.

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