CHARACTERIZATION OF THREE BACTERIOPHAGES OF *XANTHOMONAS ORYZAE* (UYEDA ET ISHIYAMA) DOWSON[1]

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Introduction

Bacterial leaf blight of rice plant caused by *Xanthomonas oryzae* (Uyeda et Ishiyama) Dowson is a wide-spread disease in Taiwan. For forecasting the occurrence of the disease, the population of bacteria in the rice field has been indirectly measured by the phage titer. The study on this relationship it is necessary to understand the strains of phage in the rice field. The phages collected from different part of this island were isolated and classified into several strains based on their plaque morphology and host specificity. Among these isolates three morphologically distinctive strains were obtained. Since they are different from other strains of *X. oryzae* phage deserибed by other workers (Wakimoto 1960a, Wakimoto 1960b), their properties and relationship are extensively studied and reported in this paper.

Materials and Methods

Bacterium, phage and medium: Xanthomonas oryzae (Uyeda et Ishiyama) Dowson used in these studies was strain 500 of our institute. Phages Xp10, Xp20 and Xf were isolated on the 500 of *X. oryzae* from the water of rice field. The isolation and propagation of phages from a single plaque assay were carried out as described by Adams (1959). The medium (PS) consisting of potato, 200g; $Ca(NO_3)_2 \cdot 4H_2O$, 0.5g; $Na_2HPO_4 \cdot 12H_2O$, 2.0g; peptone, 5.0g; sucrose, 15.0g in 1 L of distilled water was used for routine cultivation. This medium was satisfactory for the isolation and propagation of phages. Phage adsorption and one step growth experiment were also conducted in the same medium.

Growth and partial purification of phage: For electron microscopic examination

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and nucleic acid determinations, high-titer preparations of phage, free from bacterial debris were required. All phages were propagated in bacteria which were grown exponentially in PS medium and resuspended in the same medium at final concentration of 3×10^8/ml. The bacteria were inoculated with a phage multiplicity of 2 and the infected cultures were continuously shaken at 30°C for 5 to 8 hours. Then the phage lysate were centrifugad at 8000 × G for 20 minutes to remove the bacteria and debris. The supernatants were centrifuged at 20,000 rpm (Spinco L No. 30 rotor) for 60 minutes. The resulting pellets were resuspended in 0.1*M* phosphate buffer (pH6.5), The same procedure was repeated once for further clarification. Highly purified preparation of Xf phage could be obtained by passing lysate through bacterium-proof millipore filter. This procedure eliminated large amount of bacterial debris.

Assay: The standard two-layer agar technique for plaque forming units (PFU) was used (Adams 1959). The bottom layer was PS medium plus 1.5% agar, and the top layer was PS medium plus 0.8% agar. Indicator bacteria were on overnight culture grown in PS medium.

Phage antisera: Antisera for the phages were prepared in rabbits by the use of three subcutanceous injection of partial purified phage. Each time each animal was injected with 2 ml of antigen containing 10^{10} PFU/ml of phage in 0.01 *M* saline phosphate buffer (pH7.0). injections were given at 5 day intervals. Trial antiserum was collected at one week intervals after the final injection. The K value of the antisera and the cross neutralization was examined according to Adams' method (1959).

Thermal inactivation: Phage suspension (3×10^7 PFU/ml in PS) was heated in a water bath at 60°C for various periods and assayed for survival. The heat stability of phages at 40° to 100°C for 10 minutes was also examined under the same condition.

Adsorption of phages to bacteria: A method modified from that described by Adams (1959) was used to determine adsorption rates. Logarithmically growing bacteria were mixed with phage in a ratio of less than one phage per bacterium. Samples were withdrawn from the aerated mixture at intervals, freed from bacterial cells by centrifugation at 8000 × G and assayed for free phage.

One step growth experiment: Growth characteristics were determined by one-step experiment (Adams 1959). The host bacteria were grown with aeration to a final concentration of about 5×10^8 colony-forming units per ml. The multiplicity of infection in the adsorption tubes was always less than one. Adsorption was allowed to proceed for 5 minutes, a sample of the adsorption mixture was then exposed to antiserum action for 7 minutes and dilutions were made into growth tubes. The growth tubes were shaken in a water bath at

30°C. Samples were withdrawn and assayed for plaque count until 200 minutes had elapsed at which time lysis of infected bacterial cells was essentially completed.

Electron microscopy: Partial purified phages were resuspended in 0.1 *M* ammonium acetate to approximately 10^{10} PFU/ml and mixed with an equal volume of 2% phosphotungstic acid (pH7.2) and observed in a Hitachi HU-11A type electron microscope.

Thermal denaturation of phage DNA: Solution containing 10–20 μg DNA/ml were heated in Backman DU spectrophotometer fitted with thermal spacers (Marmur and Doty 1962) and the increase in extinction on denaturation of the DNA (hyperchromic effect) observed. Since the GC% appeared very high, the Tm determination in the usual solvent SSC (Standard saline citrate; 0.15 M Na_3 citrate pH7.0) required temperatures higher than 100° (Marmur and Doty 1962) therefore solvent (Na_2HPO_4, 0.2 M 2.5 ml; NaH_2PO_4, 0.2 M, 2.5 ml: EDTA, 0.01, 10 ml diluted to 100 ml. pH 6.8) was used which gave Tm value less than SSC solvent.

Nucleic acid determination: The pellets of partial purified phage were suspended in 0.01 *M* phosphate buffer and incubated with DNase (50μg/ml) and RNase (50 μg/ml) in the presence of $MgCl_2$ 0.002 *M* for 3 hours. The suspension was dialyzed against the phosphate buffer and clarified by centrifugation at 8,000×G for 20 minutes, then the phage was precipitated by centrifugation at 20,000 rpm for 1 hour. The pellets were suspended in SSC buffer, equal volume of water-saturated phenol was added and shaken for 30 minutes at 4°C. The layers were separated by centrifugation, and the aqeous layer containing the nucleic acids was removed. Residual phenol was removed by dialysis against several change of SSC. DNA was isolated by the use of procedure of Marmur (1961), starting with the last 95% ethanol precipitation step. DNA preparations were dialyzed for 16 hours at 4°C against SSC.

Diphenylamine and orcinol reactions were used for the qualitative determination of DNA and RNA. For differentiation between single-stranded DNA, double-stranded DNA and RNA, the Bradley's method (1965) was also used. Purified phage preparations stained with acridine orange in the presence of Na_2HPO_4 and citrate, and subsequently examined under ultraviolet irradiation.

Results

The plaque morphology of the phages: The plaque morphology of three phages was different from one another. The plaque of Xf on the indicator strain was turbid and extremely minute, with an average diameter which was less 0.5 mm. The appearance of plaque was very slow, it took about 24 hours.

The phages Xp10 and Xp20 produced clear plaques. The plaque size of Xp10 was 6 mm in diameter and Xp20 was about 2 mm. The plaque formation of both Xp10 and Xp20 was about 8 hours. These plaque morphologies are shown in Fig. 1, 2, and 3.

Electron microscopy of phages: All isolates of phage were examined, and they appeared to be morphologically different. The phage Xp10 was shown in Fig. 4a and 4b. It possessed a regular hexagonal head and a long tail. The diameter of intact phage head was 53 mμ. The dimension of tail was 147 mμ in length and 7 mμ in width. Sometime short spikes and tail fibers could be observed at the proximal end of the tail. The overall picure of phage Xp20 was shown in Fig. 5. Morphologically it resembled T-even phage of *E. coli*. The head was the hexagonal shape which was 62 mμ in diameter. The head attached by a straight tail about 70 mμ long and 20 mμ wide. The upper part of tail became contracted. From the distal end of tail short spikes and tail fibers were projected. The Xf phage was a filamentous phage, which is shown in Fig. 6. The unit length was determined at 858 mμ. The diameter was about 6 mμ.

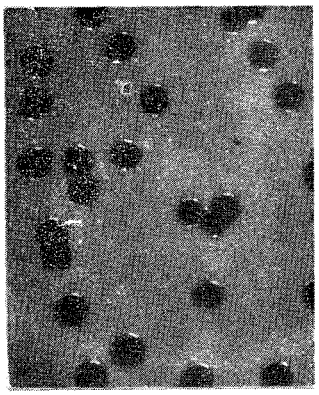
Thermal inactivation of three phages: Results of heating the phages at 60°C were shown in Fig. 7. Xp10 was very sensitive to heating, when it was heated at 60°C their survival decreased sharply after 2 minutes. However, Xf and Xp20 were completely resistant to heating at 60°C for 80 minutes. The inactivation temperatures of Xp10, Xp20 and Xf were 55°, 75° and 85° for 10 minutes respectively.

Serological relatedness: Homologous neutralization rate constants K value was 84 for Xp20 antiserum, 44 for Xp10 antiserum and 26 for Xf antiserum. Cross-neutralization was tested by reacting each antiserum with three phage. The result appears in Table 1, three phages were serologically different from one another.

Table 1. *K value of antiphage sera against homologous and heterologous phage*

Phage / Antiphage sera	Xp10	Xp20	Xf
Xp10	44	0	0
Xp20	0	84	0
Xf	0	0	26

1



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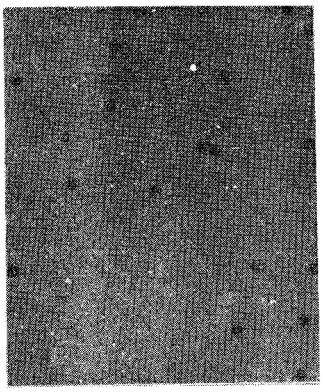

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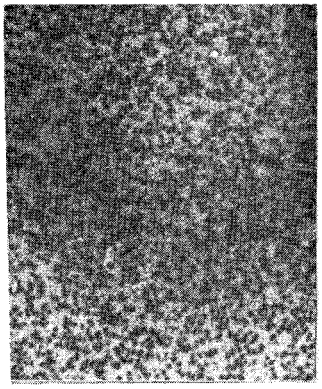
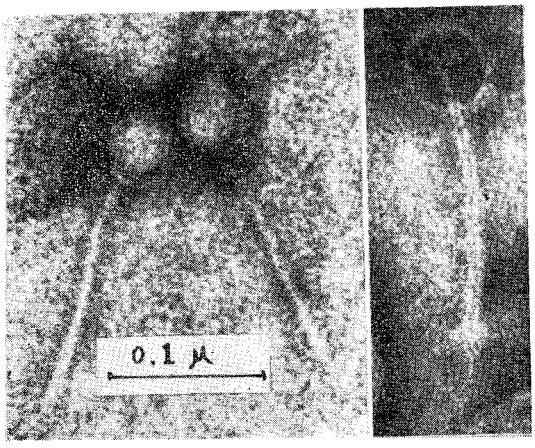


4a, 4b



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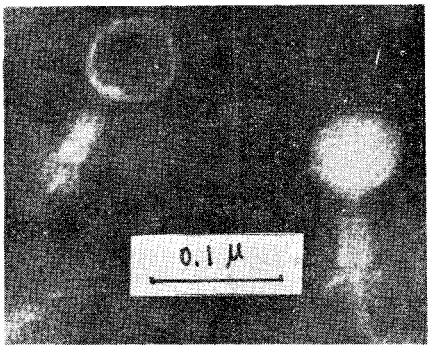

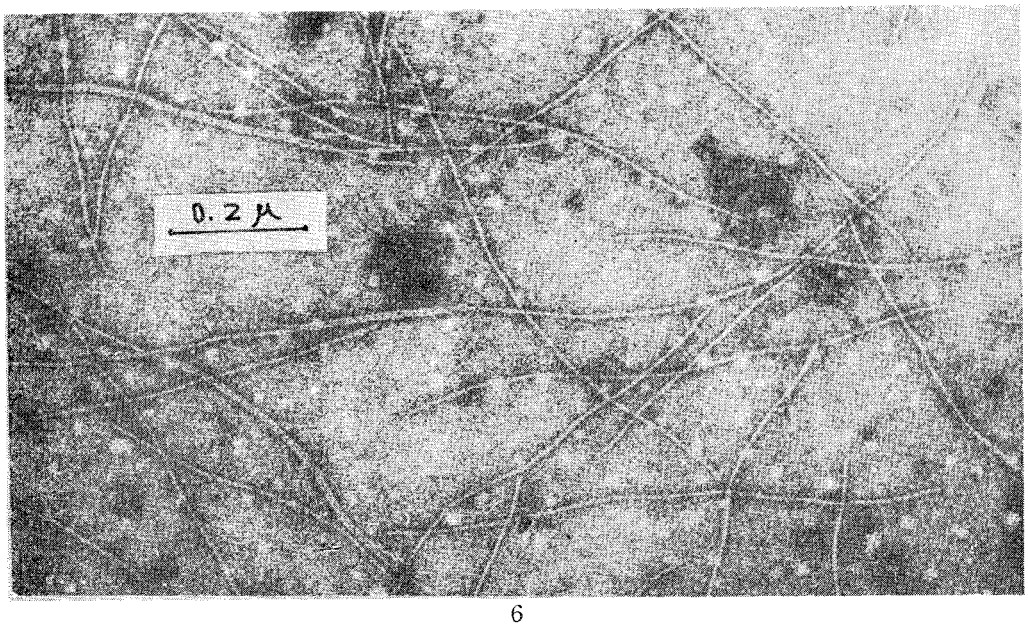

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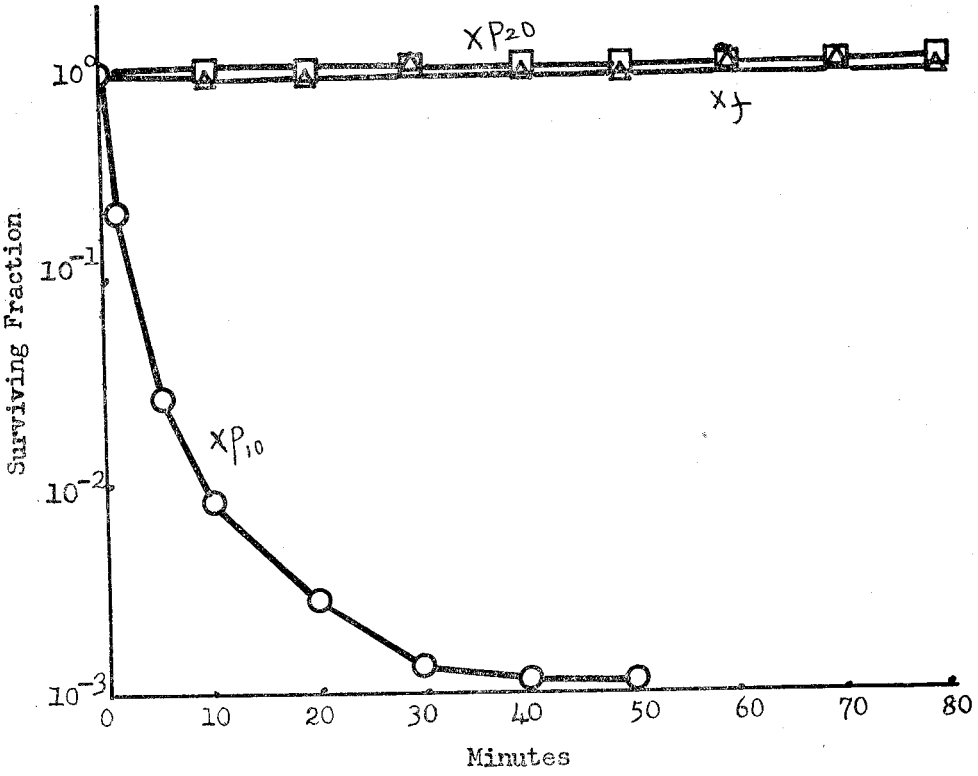

Fig. 7. Inactivation of three phages at 60°C

Adsorption rates: The kinetics of adsorption of Xp10 and Xp20 by indicator strains were determined. Adsorption rates of phage Xp20 and Xp10 were very similar. Bocterial cells adsorbed 95% of input phages of both Xp10 Xp20 in 5 minutes.

One step growth characteristics: One step growth and burst size experiments were done with phages Xp10 and Xp20 in PS medium. The latent periods for Xp10 and Xp20 were 50 and 60 minutes and the burst sizes of Xp10 and Xp20 were 58 and 40 respectively.

Nucleic acid determination: Positive dephenylamine reaction by the nucleic acids extracted from the three phages revealed that the phages contain DNA. Orcinol reactions of nucleic acids were negative. From the results of the

Explanation of the plate figures

Fig. 1. Plaque morphology of Xp10. (original siza)
Fig. 2. Plaque morphology of Xp20. (original size)
Fig. 3. Plaque morphology of Xf. (original size)
Fig. 4a. Phage Xp10.
Fig. 4b. Empty phage Xp10 with a tail having a diac at the proximal end.
Fig. 5. Phage Xp20.
Fig. 6. Phage Xf.

acridine orange-staining technique it showed that phage Xp10 and Xp20 gave a yellow green color for double-stranded DNA and phage Xf gave flame-red for single-stranded DNA.

Thermal denaturation of phage DNA: The ultraviolet absorption of Xf DNA was marked function of temperature over a wide range between 25° and 90°C. With double-stranded DNA heated under the same condition there was very little influence of temperature upon the ultraviolet absorption until the temperature reached about 70–75°C then absorption increase abruptly (Fig. 8). The temperature dependence of the ultraviolet absorption of Xf DNA can be interpreted to mean that the DNA has a single stranded structure, as shown for $\phi \times 174$ DNA. (Sinsheimer 1959)

The hyperchromicity as a function of temperature for Xp20 and Xp10 DNA was shown in Fig. 8. The absorption of Xp10 and Xp20 DNA remained almost constant until 70°C and then increased abruptly. The results suggested that DNA of these two phages was a double-stranded nature.

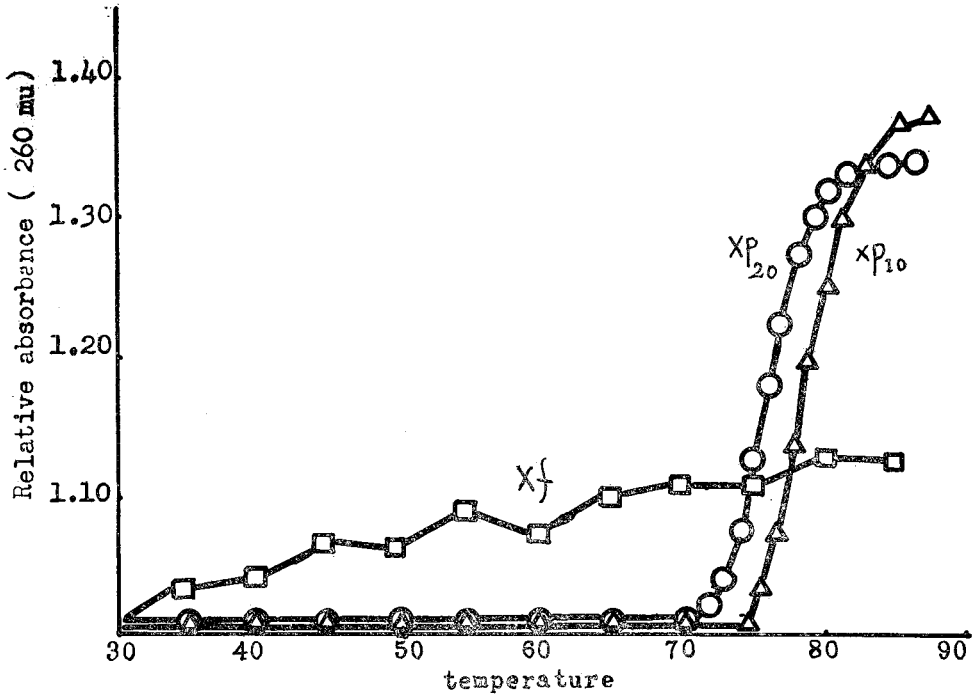

Fig. 8. Temperature dependence of the ultraviolet absorption of DNA of three phages

Discussion

Based on the plaque shape, electron-microscopic morphology, serological relationship and other properties, it can be concluded that three phages are completely different from one another. Phage Xp10 has a head which is 54 mμ

in diameter and a tail of the size of 7×147 mμ. It is similar to the Opl type of *X. oryzae* phage reported by Wakimoto (1960b). Xp20 has a head which is 62 mμ in diameter and shorter tail of the size of 20×70 mμ. Accordingly the head of Xp20 is bigger than that of Xp10 and the tail is shorter and wider. In addition, the upper part of tail is contracted. It is similar to the T-even. phage of *E. coli*. Besides the difference in morphology they are also easily distinguished by their thermal inactivation. Xp10 is very sensitive to heating whereas Xp20 is resistant. Their plaque morphology and hyperchromicity of DNA are similar.

Xf is a special type of phage. Electron micrograph of Xf shows that it is a flexible rod-shape. It is reminiscent of certain plant virus. The rods are about 858 mμ in length and 6.0 mμ in diameter. The results of diphenylamine reaction, acridine orange staining and hyperchromicity of Xf nucleic acid demonstrate that the nucleic acid of phage Xf is a single-stranded DNA. Recently this type of phage has been reported on *E. coli*. (Zinder *et al* 1963, Marvin and Hoffmann-Berling 1963, Hofschneider 1963) and *Pseudomonas aeruginosa* (Takeya and Amako 1966). They are filamentous rod shaped phage with a single-stranded DNA. They produce high titer of phage in lysate without lysis of host cells. These properties are quite similar to our Xf phage. However, the host range of the rod-shaped phages of *E. coli* and *Pseudomonas aeruginosa* is extremely narrow. In contrast all rod-shaped coliphages are male-specific and Pf of *Pseudomonas aeruginosa* infects the K strain only whereas our Xf phage has a wide host range which can infect the bacterial strains which are resistant to Xp10 and Xp20.

Summary

Three morphologically distinctive phages of *Xanthomonas oryzae* were isolated and their properties and relationships were extensively studied. Electron microscopic study revealed that both Xp10 and Xp20 were tadpolelike having a polyhedral head and a tail. Xp10 phage possessed a head which was 53 mμ in diameter, and a tail of the size of 7×147 mμ, and Xp20 had a head which was 62 mμ in diameter and tail of the size 20×70 mμ. Xf was a filamentous rodshaped phage which was 6 mμ in diameter and 858 mμ in length. These three phages were unrelated in their serological relationship. The thermal inactivation temperature for phage Xp10, Xp20 and Xf were 55°, 75° and 85°C respectively. One step growth studies showed that latent periods for Xp10 and Xp20 were 50 and 60 minutes, and burst size of Xp10 and Xp20 were 58 and 40 respectively. Based on diphenylamine reaction, acridine orange staining and hyperchromicity of phage nucleic acid showed that the DNA of Xp10 and Xp20 was double-stranded, and Xf was a single-stranded DNA.

三種水稻白葉枯病病原細菌之噬菌體之特性

郭宗德 黃檀溪 吳榮洋 楊晴美

自臺灣水稻田中很容易分離出白葉枯病病原細菌之噬菌體，自已分離之品系中得到三種外部形態完全不同之噬菌體。我們將其命名爲 Xp10, Xp20 和 Xf. 本實驗就此三種噬菌體分析其特性及其互相間之關係。Xp10 和 Xp20 爲蝌蚪形之噬菌體，有一多角形之頭與尾巴。Xp10 其頭之直徑爲 53 mu, 尾巴之大小爲 147 × 7 mu. Xp20 其頭之直徑爲 62 mu, 尾巴大小爲 70 × 20 mu, 同時在頭與尾巴連接處有縊縮，Xf 爲長桿狀噬菌體長 858 mu 而直徑爲 6mu, 此三種噬菌體其血清學之性質均無關係，十分鐘處理之不活性溫度對 Xp10, Xp20 和 Xf 各爲 55°, 75° 和 85°C。自噬菌體侵入細菌至將細菌溶解放出噬菌體所需之時間對 Xp20 爲 50 分鐘，而對 Xp10 爲 60 分鐘。一個細菌細胞所能放出之噬菌體數目對 Xp10 爲 58, 對 Xp20 爲 40。根據 diphenylamine reaction 之反應，acridine orange 之染色，和 核酸之 hyperchromicity 證明 Xp20 和 Xp10 爲雙條之 DNA，而 Xf 爲單條之 DNA。

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