Full Paper

Isolation, identification and characterization of a glyphosate-degrading bacterium, *Bacillus cereus* CB4, from soil

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A bacterial strain named CB4, with highly effective glyphosate degradation capability, was isolated from soil after enrichment. On the basis of the Biolog omniLog identification system (Biolog) and 16S ribosomal RNA (rRNA) gene sequencing methods, strain CB4 was identified as *Bacillus cereus*. Further experiments were carried out to optimize the growth of strain CB4 and the glyphosate degradation activity by high performance liquid chromatography (HPLC). The optimal conditions were found as follows: initial pH 6.0, incubation temperature 35°C, glyphosate concentration 6 g L⁻¹, inoculation amount 5% and incubation time 5 days. Under the optimal conditions, strain CB4 utilized 94.47% of glyphosate. This is the first report on *B. cereus* with a capacity to utilize herbicide glyphosate, and it can degrade glyphosate concentrations up to 12 g L⁻¹. Metabolization of glyphosate by strain *B. cereus* CB4 was studied. Results indicated that two concurrent pathways were capable of degrading glyphosate to AMPA, glyoxylate, sarcosine, glycine and formaldehyde as products. Glyphosate breakdown in *B. cereus* CB4 was achieved by the C-P lyase activity and the glyphosate oxidoreductase activity.

Key Words—*Bacillus cereus*; biodegradation; glyphosate; herbicide; metabolism

Introduction

Glyphosate (isopropylamine salt of *N*-phosphonomethyl-glycine) is the active ingredient in Roundup, a herbicide manufactured by Monsanto. It is a broad-spectrum postemergent used to control both perennial and annual weeds (Kent-Moor et al., 1983). Glyphosate is a potent inhibitor of the enzyme 3-enol-pyruvylshikimate-5-phosphate synthase (EPSP synthase, EC 2.5.1.19), which is involved in the biosynthesis of the aromatic amino acids phenylalanine, tyrosine, and

tryptophan (Liu et al., 1991). Glyphosate is also a competitive inhibitor of EPSPS with respect to phosphoenolpyruvate and has been shown to block import of the EPSPS preprotein into chloroplast, the subcellular location of the shikimic acid pathway (Penalosa-Vazquez et al., 1995).

China is a big exporter of glyphosate. During the production of glyphosate, the discharge of wastewater can cause serious water pollution problems and environmental damage (Xu et al., 2007). This is because the pollutant in wastewater mainly contains glyphosate, methanol, formaldehyde and so on (Amoros et al., 2007). Microorganism treatment has been widely used in the wastewater treatment process. Two pathways for the breakdown of glyphosate have been documented. One involves cleavage of the C–N bond by the enzyme known as glyphosate oxidoreductase with

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formation of glyoxylate and aminomethylphosphonic acid (AMPA) (Kishore and Barry, 1992). The second pathway is via initial cleavage of the C-P bond to give sarcosine, glycine and formaldehyde by a C-P lyase activity (Sviridov et al., 2011). Therefore, to isolate and characterize effective glyphosate-degrading strains shows great significance for application in bioremediation and wastewater treatment.

The study presented here reports on the isolation, identification and characterization of a new bacterial strain capable of degrading high concentrations of glyphosate. The study aims at elucidating a possible application for biodegradation of glyphosate in wastewater.

Materials and Methods

Chemicals and media. Glyphosate standard (99.4%) was obtained from Beier Group, Ltd. Solvents for HPLC were purchased from Chengdu Forest Science and Technology Development Co., Ltd. (Chengdu, China). All other chemicals used were of analytical grade. Mineral salts medium (MSM) was used for isolation and characterization of glyphosate-degrading bacteria. The MSM had the following composition (per liter): $(\text{NH}_4)_2\text{SO}_4$ 2 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g, $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ 0.6 g, $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ 0.15 g, K_2HPO_4 0.625 g, with pH 7.0 (Pan, 2001). In MSM, glyphosate was used as the sole carbon source.

Enrichment and isolation. Four soil samples were collected from the glyphosate-polluted soil in the herbicide plant located in Chengdu, Sichuan Province, China. For each sample, approximately 10 g soil was added to an Erlenmeyer flask (100 ml) containing 30 ml MSM supplemented with 3 g L^{-1} glyphosate as the sole carbon source, and incubated on a rotary shaker at 35°C for 5 days. Then 3 ml of the culture was inoculated into 30 ml of fresh MSM containing 6 g L^{-1} glyphosate as the sole carbon source. The enrichment was done for approximately 4 weeks, by gradually increasing the initial glyphosate concentrations up to 12 g L^{-1} at the incremental step of 3 g L^{-1} every 5 days. Finally, the culture was serially diluted, and the dilutions (10^{-3} to 10^{-6}) were plated on MSM agar. The separated single colonies were picked up and stored at 4°C for further experiments.

Biodegradation studies. The strains were inoculated in 30 ml of MSM containing 6 g L^{-1} glyphosate as the sole carbon source (pH 7.0) and incubated at 35°C

for 2 days until the OD_{600} reached 0.5. The culture was inoculated into fresh MSM, incubated under the same conditions for 7 days, and then centrifuged (Sigma Centrifuge, model 3K15, Germany) (15 min, 10,000 rpm). The degradation rate was determined by measuring the residual glyphosate using high performance liquid chromatography (HPLC) (LC-20A Shimadzu, Japan) (ODS-SP Chromatography column $4.6 \text{ mm} \times 150 \text{ mm}$) at 240 nm using NaNO_2 as the derivation agent (Wu and Yang, 2006). The degradation rate was calculated by comparing the peak area with that of standard glyphosate according to the glyphosate standard curve.

Identification of the glyphosate-degrading strain. The Biolog OmniLog Identification System (Biolog) and 16S ribosomal RNA (rRNA) gene sequencing methods were applied to the glyphosate-degrading strain. A Biolog system using GP2 microplate (Biolog Inc., Hayward, CA, USA) was adopted to identify the strain. The morphology of strain CB4 was first determined by light microscopy (CH Olympus, Japan) for preliminary characterization of the bacterium and then the isolate was prepared according to Biolog instructions. After incubation (16–24 h), the optical density was read using a Biolog Microplate reader in conjunction with the MicroLog (Version 4.20.04). A correct identification was attained when the similarity probability (PROB), similarity index (SIM) and genetic distance (DIS) values were >0.500 , >0.500 and <5.00 , respectively. DNA was extracted from pure cultures. The 16S rRNA gene was amplified by PCR using the following primers: Eu27F (5'-GAGAGTTTGATCCTG GCTCAG-3') and 1492R (5'-CTACGGCTACCTTGT TACGA-3'). The PCR conditions were 94°C for 5 min, 94°C for 60 s, 50°C for 60 s, followed by 30 cycles of 72°C for 90 s, with a final step at 72°C for 10 min (Yang et al., 2010). The purified PCR products were then sequenced by Invitrogen Biotechnology Co., Ltd. (Shanghai, China). The sequencing result was submitted to GenBank for BLAST analysis. Phylogenetic analysis was performed using MEGA version 4.0 software packages (Tamura et al., 2007). The neighbor-joining (NJ) method (Saitou and Nei, 1987) was used for phylogenetic analysis.

Growth and biodegradation characterization of the glyphosate-degrading strain. A series of experiments were carried out to study the bacterial growth and biodegradation activity. Effects of media pH (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0), incubation temperature (25, 30, 35 and 40°C), initial glyphosate

concentrations (4, 6, 8, 10 and 12 g L⁻¹), inoculation amounts (1, 3, 5, 10, 20, 30 and 40%) and incubation time (12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 136, 144 and 156 h) on bacterial growth and glyphosate biodegradation were studied. Bacterial growth was determined at 600 nm in a spectrophotometer (Pgeneral, model TU-1800, Beijing, China), and the degradation was measured by HPLC.

Preparation of cell-free extracts. Cell-free extracts (CFEs) were prepared following the modified procedure of Camargo and Pal (Camargo et al., 2003; Pal et al., 2005). Cells from overnight-grown culture were harvested by centrifugation (15 min, 8,000 rpm), and washed twice with phosphate buffer (0.05 M; pH 7.0). Cells in an ice bath were sonicated in the same buffer using an ultrasonic probe (Scientz ultrasonic disintegrator, model JY96, Ningbo, China) (45 cycles of 5 s on and 5 s off at 280 W). The sonicate was centrifuged (10,000 rpm, 4°C, 15 min) and the supernatant was collected and used as CFEs.

Glyphosate metabolism. The assay for the pathway(s) of glyphosate degradation involved addition of 0.3 ml of CFEs to 2.7 ml of phosphate buffer (0.05 M; pH 7.0) with 0.5 g L⁻¹ glyphosate. Reaction mixtures were incubated in a water bath at 35°C for 2 h. Thereafter, incubation was stopped by adding 0.5 ml of 1 M HCl to the reaction mixtures. The decrease of the concentrations of glyphosate and accumulation of its metabolites (AMPA, glyoxylate, sarcosine, glycine and formaldehyde) were monitored using HPLC. AMPA was determined by the method of Dong et al. (2001). Sarcosine and glycine were determined by the method of Chen et al. (2005). Formaldehyde was determined by the method of Wang et al. (2012). Glyoxylate was determined by the method of Qureshi et al. (1982). The standard curve was obtained by using standard samples. Quantitation was based on peak areas.

Results

Isolation and screening of the glyphosate-degrading strain

More than 40 bacterial strains were isolated from the soil samples by the method mentioned above. The glyphosate degradation activity was confirmed by the glyphosate degradation rate. All isolates were found to possess the ability to degrade the herbicide. Among them, one of the isolates, designated as CB4, showed the highest glyphosate degradation rate, so it was

chosen for further study.

Identification of the glyphosate-degrading strain CB4

Light microscopy showed strain CB4 was gram-positive and rod shaped. In the following operations, strain CB4 was identified as *Bacillus cereus*/*Bacillus thuringiensis* A by the Biolog Microstation System after 16–24 h incubation with PROB, SIM and DIS of 99%, 0.943 and 0.85, respectively. Table 1 shows the metabolic fingerprints of strain CB4 in the Biolog GP2 Microplate. The chemicals listed in Table 1 correspond to the chemicals used by the microplate of Biolog.

The 16S rRNA gene sequences of strain CB4 were obtained (comprising 1,177 nucleotides) and submitted to GenBank (<http://www.ncbi.nlm.nih.gov>). The sequence displayed the highest similarity (99%) to that of *Bacillus cereus* (GenBank accession JF758862). A phylogenetic tree was constructed based on the 16S rRNA coding gene sequences of the isolate and the nearest relatives (Fig. 1). On the basis of the two methods, Strain CB4 was identified as *Bacillus cereus*. The gene sequence was deposited in the GenBank database under the accession number FJ611936.

Effect of pH on growth and glyphosate degradation by strain CB4

Strain CB4 was inoculated into MSM with 6 g L⁻¹ glyphosate at different pH values (pH 2.0–12.0) and incubated at 35°C for 7 days. The growth and glyphosate-degradation activity of the strain CB4 at different pH values are shown in Fig. 2a.

The test showed that the glyphosate degradation of strain CB4 was positively related with the growth. Weak acidic conditions (pH 6.0–7.0) benefited both the growth and the glyphosate biodegradation. At pH 6, the growth reached maximum, and the glyphosate degradation rate reached 94.13%. However, the degradation of glyphosate along with the growth of strain CB4 was inhibited under acidic conditions (pH 2.0–5.0) and alkaline conditions (pH 8.0–12.0).

Effect of temperature on growth and glyphosate degradation by strain CB4

Strain CB4 was incubated at different temperatures (25, 30, 35 and 40°C) in MSM containing glyphosate 6 g L⁻¹ at pH 6.0 for 7 days. As is shown in Fig. 2b, the optimal temperature for the growth of strain CB4 and glyphosate degradation was 35°C.

Table 1. Metabolic fingerprints of strain CB4 in a Biolog GP2 microplate.

Carbon source	CB4	Carbon source	CB4	Carbon source	CB4
Water	—	Dextrin	+	<i>N</i> -Acetyl-D-galactosamine	+
α -Cyclodextrin	+	Glycogen	+	<i>N</i> -Acetyl-D-glucosamine	—
L-Arabinose	—	D-Cellobiose	+	α -Methyl-D-galactoside	—
D-Arabitol	—	D-Fructose	+	β -Methyl-D-galactoside	—
D-Ribose	—	Maltotriose	+	α -Methyl-D-glucoside	—
Arbutin	—	D-Mannitol	—	γ -Hydroxy butyric acid	—
α -D-Lactose	—	D-Psicose	+	<i>p</i> -Hydroxy-phenylacetic acid	—
Lactulose	—	D-Raffinose	—	<i>N</i> -Acetyl-L-lactamide glutamic	±
Maltose	+	Xylitol	—	D-Fructose-6-phosphate	—
Palatinose	+	D-Xylose	—	α -D-Glucose-1-phosphate	—
D-Tagatose	—	D-Malic acid	—	D-Glucose-6-phosphate	—
D-Trehalose	+	L-Malic acid	+	D-L- α -Glycerol phosphate	+
Turanose	—	L-Asparagine	+	α -Hydroxy butyric acid	+
Lactamide	—	Thymidine	+	β -Hydroxy butyric acid	—
<i>m</i> -Inositol	—	Uridine	+	Pyruvic acid methyl ester	+
Amygdalin	—	L-Fucose	—	Succinic acid mono-methyl ester	—
L-Lactic acid	+	L-Serine	+	Adenosine-5'-monophosphate	±
L-Alaninamide	—	Putrescine	—	Thymidine-5'-monophosphate	+
D-Alanine	+	2,3-Butanediol	—	Uridine-5'-monophosphate	+
L-Alanine	+	Glycerol	+	β -Methyl-D-glucoside	+
Adenosine	+	D-Sorbitol	—	D-Lactic acid methyl ester	+
Inosine	+	Stachyose	—	D-Gluconic acid	—
Salicin	—	Sucrose	+	α -D-Glucose	+
Inulin	—	D-Melezitose	—	2'-Deoxy adenosine	+
D-Galactose	—	D-Melibiose	—	α -Methyl-D-mannoside	—
D-Galacturonic acid	—	L-Rhamnose	—	Glycyl-L-glutamic acid	+
D-Mannose	—	Propionic acid	—	L-Alanyl-glycine	+
Acetic acid	—	L-Glutamic acid	+	Sedoheptulosan	—
Mannan	—	Pyruvic acid	+	α -Ketovaleric acid	+
L-Pyroglutamic acid	—	Succinic acid	—	3-Methyl-D-glucose α -ketoglutaric acid	—
Succinamic acid	—	Gentiobiose	—		

“+”, utilized; “—”, not utilized; “±”, half utilized.

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences of strain CB4 and related species.

The tree was evaluated by the neighbor-joining method based on 1,000 replications. The scale bar represents 0.01 substitutions per nucleotide position, using *Paenibacillus polymyxa* (AM062684) as an outgroup.

Fig. 2. Effects of initial pH (a), incubation temperature (b), initial glyphosate concentrations (c) and inoculation amounts (d) on bacterial growth and degradation of glyphosate by strain CB4.

The error bars indicate the standard error of the mean.

Effect of glyphosate concentrations on growth and glyphosate degradation by strain CB4

The growth and the glyphosate degradation activity of strain CB4 at different glyphosate concentrations (4, 6, 8, 10 and 12 g L⁻¹) are shown in Fig. 2c.

Among different concentrations of glyphosate, 6 g L⁻¹ glyphosate exhibited the highest growth-promoting capability. With a rise in concentrations of glyphosate from 6 g L⁻¹ to 12 g L⁻¹, the growth-promoting capability decreased.

Strain CB4 could degrade glyphosate at all concentrations tested. The optimal concentration of glyphosate was 6 g L⁻¹, for 94.16% of the added glyphosate was degraded within 7 days. With a rise in concentrations of glyphosate 6–12 g L⁻¹, the degradation was inhibited. The degradation rate reached 79.6% when the concentration of glyphosate was 12 g L⁻¹.

Effect of inoculation amounts on growth and glyphosate degradation by strain CB4

The growth and the glyphosate degradation activity of strain CB4 at different inoculation amounts are shown in Fig. 2d. The culture was added to MSM containing glyphosate concentration of 6 g L⁻¹ with inoculation amounts of 1, 3, 5, 10, 20, 30 and 40% (v/v), and incubated at 35°C for 7 days. Strain CB4 exhibited significant growth at 3–10% inoculation levels, particularly at 5%. Compared to the growth of strain CB4, the degradation was less affected by the inoculation amount. The entire degradation rate stayed above 90% at different inoculation amounts. Considering both the growth of strain CB4 and glyphosate degradation, the optimal inoculation amount was selected at 5%.

Growth curve of strain CB4 and glyphosate degradation curve

In the experiments above, the optimal conditions were found as follows: initial pH 6.0, culture temperature 35°C, glyphosate concentration 6 g L⁻¹, and in-

Fig. 3. Growth curve of strain CB4 and glyphosate degradation curve in MSM containing 6 g L^{-1} glyphosate as the sole carbon source.

oculation amount 5%. Under the optimal conditions, the growth curve and the glyphosate degradation curve were obtained and are shown in Fig. 3.

The growth curve obtained during the growth of strain CB4 in MSM was similar to an "S" shape. The lag phase was from 0 to 48 h; the log phase was from 48 to 96 h; and the stationary phase began from 96 h. The degradation rate increased constantly with the increase in time. The degradation was inhibited in the initial 48 h. This indicated that the strain required an acclimation period before the rapid degradation occurred. The degradation was rapid from 48 h to 108 h and relatively stable after 108 h. Strain CB4 utilized 94.47% of glyphosate. The result indicated that the glyphosate degradation of strain CB4 was positively related with the growth. The optimal degradation time was selected at 5 days to achieve the ideal effect.

Glyphosate metabolism

The degradation through the cleavage of the C-N bond by the enzyme glyphosate oxidoreductase was assayed by measuring the decrease of glyphosate and accumulation of glyoxylate and AMPA in the reaction mixture. The degradation through the cleavage of

the C-P bond by C-P lyase was assayed by measuring the decrease in glyphosate and accumulation of sarcosine, glycine and formaldehyde concentrations. In the experiments, AMPA and glyoxylate were abundant in cell-free extracts of strain CB4 (Table 2), thus indicating the presence of glyphosate oxidoreductase capable of cleaving the C-N bond of glyphosate. Meanwhile, sarcosine, glycine and formaldehyde were found to be the breakdown products in cell-free extracts of strain CB4 incubated on glyphosate (Table 2), indicating that the initial cleavage of glyphosate was at the C-P bond. Therefore, this pointed out glyphosate breakdown in strain CB4 was achieved by a C-P lyase activity and a glyphosate oxidoreductase activity.

Discussion

Strain CB4, capable of degrading glyphosate, was isolated from the polluted soil in a herbicide plant through selective enrichment, screening and purification. According to sequence analysis of 16S rRNA and the Biolog method, the strain was identified as *B. cereus*. It was reported previously that *B. cereus* could degrade 2-aminoethylphosphonate (La Nauze and Rosenberga, 1968), chlorpyrifos (Lakshmi et al., 2009), pentachlorophenol (Singh et al., 2009), benzene (Dou et al., 2010), etc. To our knowledge, this is the first report on degradation of glyphosate, the broad-spectrum herbicide, by *B. cereus*.

Further tests were carried out to confirm the growth of strain CB4 in MSM and glyphosate biodegradation activity. The optimal pH was found to be pH 6.0. Pipke et al. (1987) reported that the degradation of glyphosate by an *Arthrobacter* sp. was also pH dependent, showing an optimum at pH 6.0–7.0, and it indicated a preferential uptake of the glyphosate dianion (Wauchope, 1976). This was an important feature of an organism to be employed for bioremediation of variable environments.

The optimal temperature for the growth of strain CB4 was 35°C , and the degradation activity reached the

Table 2. Intracellular concentrations of glyphosate metabolites in cell-free extracts of *Bacillus cereus* CB4.

Metabolites	Concentration (mg L^{-1})	Metabolites	Concentration (mg L^{-1})
Glyphosate	37.1 ± 0.03	Sarcosine	4.2 ± 0.03
AMPA	25.94 ± 0.05	Glycine	9.75 ± 0.04
Glyoxylate	13.0 ± 0.02	Formaldehyde	10.79 ± 0.01

" $\pm$ " indicates the standard error of the mean.

Fig. 4. The two potential glyphosate degradation pathways.

maximum at the same time. It was possible that some key enzyme(s) responsible for glyphosate degradation had their optimum enzymatic activity at 35°C.

The optimal concentration of glyphosate was 6 g L⁻¹. However, the degradation rate reached 79.6% when the concentration of glyphosate was 12 g L⁻¹. There were some reports regarding bacterial degradation of glyphosate by *Pseudomonas* sp. strain PG2982 (Kent-Moor et al., 1983), *Arthrobacter* sp. strain GLP-1 (Pipke and Amrhein, 1988) and *E. coli* (Huynh et al., 1988), etc. However, to our knowledge, *B. cereus* is the first reported strain that can degrade glyphosate concentration up to 12 g L⁻¹, which is the maximum solubility of glyphosate in water at room temperature.

The inoculation amounts affected the growth of strain CB4. This might be caused by the toxicity of glyphosate to the bacterial cells. But it had a weaker effect on the glyphosate degradation, which stayed above 90% at different inoculum amounts.

In the experiments above, the optimal conditions were found as follows: initial pH 6.0, incubation temperature 35°C, glyphosate concentration 6 g L⁻¹, inoculation amount 5% and incubation time 5 days. Under the optimal conditions, strain CB4 utilized 94.47% of glyphosate.

Analysis of metabolites in cell-free extracts gave us reasons to propose the glyphosate catabolism pathways. Figure 4 summarizes the available knowledge about glyphosate degradation pathways (Rojano-Delgado et al., 2012; Sviridov et al., 2011). Glyphosate degradation of glyoxylate and AMPA by glyphosate oxidoreductase was reported in some bacteria, such as *Flavobacterium* sp. strain GD1 (Balthazor and Hallas, 1986), *Arobacterium radiobacter* (McAuliffe et al., 1990), *Arthrobacter atrocyaneus* ATCC 13752 (Pipke and Amrhein, 1988), etc. The C-P lyase activity could also degrade glyphosate to sarcosine, which eventually formed formaldehyde and glycine in a reaction

catalyzed by sarcosine oxidase. Examples of microbes identified to have C-P lyases were *Pseudomonas pseudomallei* 22 (Penaloza-Vazquez et al., 1995), *Pseudomonas* sp. GS (Albrecht et al., 1991), *Arthrobacter* sp. strain GLP-1 (Pipke et al., 1987), *Rhizobium meliloti* 1021 (Liu et al., 1991), *Streptomyces* StC (Obojska et al., 1999), etc. In this report, we revealed the glyphosate catabolism pathways by *B. cereus*, which could degrade glyphosate via both AMPA and sarcosine pathways. *Pseudomonas* sp. strain LBr (Jacob et al., 1988) and *Ochrobactrum anthropi* GPK 3 (Sviridov et al., 2011) were reported capable of degrading glyphosate via two concurrent pathways. The two bacteria utilized glyphosate as a phosphorus source and their C-P lyases were inhibited by AMPA (Sviridov et al., 2011). The cause of the phenomena might be an enzyme which was inducible under phosphate starvation. However, *B. cereus* CB4 could utilize glyphosate as the sole source of carbon and its C-P lyase could degrade glyphosate in phosphate-rich environments. This may indicate that the sensitivity of the C-P lyase was different from the C-P lyases in *Pseudomonas* sp. strain LBr and *Ochrobactrum anthropi* GPK 3, although the pathways of glyphosate catabolism were somewhat hypothetical as none of its enzymes had been isolated or characterized.

This is the first report about *B. cereus* isolated with the capacity to utilize herbicide glyphosate and degrade glyphosate up to 12 g L⁻¹. This strain has a remarkable potential for application in bioremediation and wastewater treatment. Based on the above research, we have started a cooperative effect with the herbicide plant. Further research lies in the reassessment of strain CB4 in the activated sludge process and the exploration of the potential to increase wastewater treatment capacity.

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