Cloning, expression and characterization of a novel GH5 exo/endoglucanase of Thermobifida halotolerans YIM 90462\textsuperscript{T} by genome mining

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The 1389-bp \textit{thc}el5A gene, which encodes a family 5 of glycoside hydrolases (GH5), was screened from the draft genome of \textit{Thermobifida halotolerans} YIM 90462\textsuperscript{T}. ThCel5A was most similar (77% identity) to a GH5 endoglucanase from \textit{Thermobifida fusca} YX, followed by cellulases from \textit{Nocardiopsis dassonvillei} subsp. \textit{dassonvillei} DSM 43111, \textit{Nocardiopsis alba} ATCC BAA-2165, and \textit{Kribbella flavida} DSM 17836. The deduced amino acid sequence of ThCel5A, which consisted of 462 amino acid residues, encompassed a family 2 cellulose-binding module and a GH5 catalytic domain. Notably, ThCel5A hydrolysed soluble as well as insoluble cellulose substrates. The enzymatic hydrolysis assay showed that the activity of recombinant ThCel5A was optimized at pH 8.0 and 50°C. Moreover, it retained hydrolytic activity in the presence of various metal ions and >90% activity within the range of pH 8.0–9.0 after 30 min at 50°C. These results suggested that this enzyme has considerable potential in industrial applications.

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\textbf{[Key words:} GH5 exo/endoglucanase; Genome; \textit{Thermobifida halotolerans} YIM 90462\textsuperscript{T}; Industrial applications]\n
The study of cellulases has attracted attention for more than 50 years, mainly because of the increased interest in developing processes to effectively treat and utilize cellulosic wastes as inexpensive carbon sources\textsuperscript{(1,2)}. However, the hydrolysis of cellulose is a complex and expensive process due to its recalcitrance structure\textsuperscript{(3)}. Generally, three cellulases are involved in the breakdown of cellulose, and they are all glycoside hydrolases that break $\beta$-1,4-linkages in cellulose with different specificities. Endoglucanase (EC 3.2.1.4) cleaves the internal bonds of cellulose, exposing the individual cellulose polysaccharide chains, while exoglucanase (EC 3.2.1.91) attacks the ends of crystalline cellulose, releasing cellobiose from the reducing or non-reducing ends of the cellulose chain; finally, $\beta$-glucosidase (EC 3.2.1.21) hydrolyses cellobiose and cello-oligosaccharide to glucose, which can be converted to bio-products, e.g., bioethanol\textsuperscript{(4)}. Therefore, the complete hydrolysis of cellulose into monosaccharides requires an array of cellulases that act synergistically to break the various chemical linkages\textsuperscript{(5)}. Thus, the discovery and development of multifunctional cellulases is very useful in reducing the cost of efficiently converting cellulose to glucose\textsuperscript{(6)}.

Based on sequence homology and structure similarity, the cellulases were grouped into the family of glycoside hydrolases (GH) along with other enzymes. According to the glycoside hydrolase (GH) classification in the CAZy database, glycoside hydrolases are classified into more than 100 families\textsuperscript{(6,7)}. Among them, the 5 glycosyl hydrolase family is the most widely conserved family of cellulases and contains members in all three domains of evolutionary descent, including more than 2000 archaea, prokaryotes and eukaryotes\textsuperscript{(8,9)}. Nevertheless, all GH5 enzymes share a common $\left(\beta/\alpha\right)_{\text{b}}$-barrel fold and cleave glycosidic bonds via a retaining mechanism that involves general acid-base hydrolysis, in which two strictly conserved carboxylates (usually two glutamates) located at the C-terminus of $\beta$-strands 4 and 7 catalyse the reaction\textsuperscript{(9)}. To date, more than 30 structures of GH5 have been available in the protein databank (PDB), but significant variations in the surface loops might account for the different substrate specificities\textsuperscript{(10)}. Thus, the GH5 family encompasses many cellulolytic enzymes, such as endoglucanases, cellobiohydrolases, mannanases, xylanases and xylanoglucanases (Carbohydrate Active Enzymes database, http://www.cazy.org/GH5.html), which suggests that it has wide application value in the bioconversion industries\textsuperscript{(9)}.

\textit{Thermobifida} species display outstanding (hemi)cellulolytic decomposition capabilities, of which \textit{Thermobifida fusca} YX has become a model organism for the study of cellulose-degrading bacteria because of its ability to utilize various plant cell wall polymers and secrete multiple cellulases: three endoglucanases, two exoglucanases, and an endo/exoglucanase (11,12). \textit{Thermobifida halotolerans} YIM 90462\textsuperscript{T}, which is an aerobic, thermophilic and salt-tolerant actinomycetal strain, was first isolated by our group from a salt mine sample (Yunnan, Southwest China). Three glycoside hydrolases (ThCel9A and ThXynA) of \textit{T. halotolerans} YIM 90462\textsuperscript{T} had been identified in earlier studies, and all of them were highly stable and alkali-resistant\textsuperscript{(13–15)}. To determine whether this strain encodes the GH5 cellulase, we first scanned the draft genome of \textit{T. halotolerans} YIM 90462\textsuperscript{T} and then cloned and heterologously expressed the putative GH5 cellulase in Escherichia coli.
The evaluation of the biochemical characterization of the recombinants enzyme indicated the identified cellulase is a good candidate for biotechnological applications that involve cellulose degradation.

**Materials and Methods**

**Bacterial growth** Thermobifida halotolerans YIM 90462(T—KCTC 19123—DSM 44531) was cultivated at 45°C in Luria–Bertani medium. E. coli Top10 (Invitrogen, China) and BL21(DE3) (Novagen, Germany) strains were grown at 37°C in Luria–Bertani broth or agar supplemented with the appropriate antibiotic (13).

**Bioinformatic analyses** The draft genome of strain T. halotolerans YIM 90462 was sequenced and annotated by the Beijing Genomics Institute-Shenzhen (BGI-Shenzhen). A similarity analysis of the predicted gene of GHS cellulase was carried out using the BLAST (P) program (http://www.ncbi.nlm.nih.gov). The deduced amino acid sequence was analysed with the EXPASY tools (http://expasy.org/). The signal peptide in the deduced amino acid sequence was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/). The Pfam site (http://www.sanger.ac.uk/Software/Pfam/) was used to determine the domain structure. The nucleotide sequence of the GHS cellulase gene (thcel5A) was deposited into the GenBank database under accession number JQ043264.

**Gene cloning** Genomic DNA was prepared as described previously (13). The coding sequence, including the predicted signal peptide, was amplified by PCR using two primers: thcel5AF (CGGCACATATGGTCCCCGCGGAGCTGG, containing an NdeI site) and thcel5AR (ATATCCAGTCCCCACAGCCTGG, containing an Xhol site). The PCR mixture and procedure were carried out as described in the specification for Top10 (Toyobo, Japan). The PCR fragments were inspected on 1% agarose gels and purified with an Omega DNA gel extraction kit (Biotek, USA). The vector pET28a (Novagen) and the PCR product were digested with NdeI and Xhol and then treated with T4 DNA ligase (Fermentas, USA). The recombinant plasmid was transformed into E. coli BL21(DE3) and E. coli Top10. After confirming the correct construct by nested PCR and sequencing, E. coli BL21(DE3) were transformed to express the positive isolated constructs.

**Gene expression and protein purification** The transformant was grown overnight at 37°C in LB medium supplemented with 30 μg/ml kanamycin. Three hundred millilitres of terrific broth containing kanamycin were inoculated with 3 ml of E. coli Top10(DE3) culture and incubated overnight to an OD600 of 0.6. IPTG (isopropyl-β-D-thiogalactopyranoside) was then added to a final concentration of 1 mM. After incubation on a rotary shaker (200 rpm) for approximately 24 h at 37°C, the culture supernatant was collected by centrifugation at 12,000 × g for 10 min at 4°C. The expressed His6-tagged proteins in the supernatant were purified using chelated Ni-nitrilotriacetic acid chromatography (Ni/NTA; Merck, Germany) as described previously (13). The eluted proteins were concentrated by ultracentrifugation using a Centricon centrifugal device with a 10-kDa membrane (Millipore, USA). The protein concentration was determined with the Bradford method.

**Protein electrophoresis and activity assay** The molecular weight of the target protein was assessed with standard sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). Proteins fixed in the gels were visualized with Coomassie brilliant blue or Congo red (16). The activity of recombinant cellulase was detected in a reaction mixture that contained 50 mM Tris–HCl buffer (pH 8.0), 1% sodium carboxymethyl cellulose (CMC, low viscosity; Sigma, USA) as substrate and 30 μg/ml of enzyme protein. The reactions were performed at 50°C for 30 min. The reaction was stopped by 3,5-dinitrosalicylic (DNS), and the treated mixture was boiled for 5 min. After cooling, the absorbance of the solution was measured at 540 nm, and the concentration of reduced sugar was estimated using a glucose standard curve as reference.

**Determination of pH and temperature optima** The optimum pH value of the purified enzyme for the hydrolysis of 1% CMC was determined at 50°C for 30 min from pH 3.0 to 10.5 using appropriate buffers: McIlvaine buffer (0.2 M Na2HPO4/0.1 M citric acid; pH 3.0 to 7.5), 50 mM Tris–HCl buffer (pH 7.5 to 9.0) and 50 mM glycine–NaOH buffer (pH 9.0 to 10.5). The temperature optima of the purified cellulase was determined by incubating a mixture of the purified enzyme and 1% CMC at various temperatures ranging from 20°C to 90°C at intervals of 1°C in 50 mM Tris–HCl buffer and pH 8.0 for 30 min. The reactions were stopped by adding DNS solution. All of the above experiments were carried out in triplicate, and the maximum activity was defined as 100%. The results are reported as the mean ± standard deviation.

**Effects of different metal ions and other reagents** The effects of various metal ions on the activity of purified enzyme were determined by adding metal ions to a final concentration of 5 mM. The effect of other reagents on the purified cellulase activity was determined in the presence of 1 mM DTT, 1 mM EDTA, 1 mM PMSF or 1% SDS. The reaction mixtures containing various additives were incubated for a final concentration of 5 mM. The effect of other reagents on the purified cellulase activity was determined in the presence of 1 mM DTT, 1 mM EDTA, 1 mM PMSF or 1% SDS. The reaction mixtures containing various additives were incubated with 1% CMC under standard assay conditions, and the cellulase activity was

![FIG. 1.](image-url) (a) Phylogenetic tree of Th Cel5A and its close homologue cellulosomes constructed using their amino acid sequences. Bootstrap values (n = 1000 replicates) are percentages in calculation. Accession numbers are given behind each species name. (b) The conserved domains of protein sequences of the ThCel5A of Thermobifida halotolerans YIM90462. Glycosyl hydrolase family 5 domains are indicated by the red box, and the predicted active sites are marked with purple lollipops. The green box indicates the carbohydrate-binding module (CBM2).
assessed with the DNS method. The inhibition or activation of the activity was presented as the activity relative to that of the control, which was measured without the addition of metal ions or other reagents.

Substrate specificity and kinetic parameters The substrate specificity of the purified enzyme was determined by performing the assay with different substrates: Avicel (Sigma), CMC (Sigma), barley glucan (Sigma), laminarin (Sigma), filter paper (GE Healthcare, USA), chitin (Sigma), lichenan (Megazyme, UK), beechwood xylan (Sigma), birchwood xylan (Sigma), and oat spelt xylan (Sigma). The concentration of all substrates was 1%, and the enzyme activity was measured by incubating it with the substrate in 50 mM Tris-HCl buffer (pH 8.0) at 50°C for 30 min. The amount of reducing sugar released was determined with the DNS method, and the activity against CMC was defined as 100%. The $K_m$ and $V_{\text{max}}$ values for the purified enzyme were determined using CMC as the substrate, with concentrations ranging from 2 mg/ml to 18 mg/ml in 50 mM Tris-HCl buffer (pH 8.0) at 50°C. The reaction rate versus the substrate concentration was plotted according to the Lineweaver–Burk method (17).

RESULTS

Gene cloning and analysis From the draft genome annotation for *T. halotolerans* YIM 90462T, one 1389-bp predicted gene encoded a GH5 cellulase. The open reading frames (ORF) (designated as ThCel5A) encoded a protein of 462 amino acids with a predicted 40-residue signal peptide and a predicted molecular mass of 49.6 kDa. As shown in Fig. 1a, the BLAST result indicated that ThCel5A amino acid sequence was most similar (77% identity) to that of a GH5 cellulase from *Thermobiida fusca* YX (YP_288962.1) (18), followed by cellulases from *Nocardiopsis dassonvillei* subsp. *dassonvillei* DSM 43111 (YP_003679306.1), *N. alba* ATCC BAA-2165 (YP_006643032.1), and *Kribbella flavida* DSM 17836 (YP_003379608.1) with identities of 49%, 49% and 48%, respectively. Protein domain analysis showed that ThCel5A harboured a family 2 carbohydrate-binding module (CBM) and the catalytic domain of glycoside hydrolase family 5 (GH5; residues 172–430) (Fig. 1b). Like all other GH5 cellulases, two conserved glutamates (E294 and E386) located in the active site catalysed the reaction (19).

Protein expression and purification To evaluate whether the cellulose activity of ThCel5A was similar to that of other GH5 enzymes, this protein, including the signal peptide, was heterogeneously expressed in *E. coli* BL21(DE3) and purified from the culture supernatants with the Ni/NTA resin method. The purified ThCel5A produced a single protein band on SDS-PAGE with an apparent molecular weight of approximately 50 kDa, which was consistent

<table>
<thead>
<tr>
<th>Bacteria species</th>
<th>MW (kD)</th>
<th>Temperature optima (°C)</th>
<th>pH optima</th>
<th>$K_m$ (mg/ml)</th>
<th>$V_{\text{max}}$ (μmol/min/mg)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Thermobifida halotolerans</em> YIM 90462T</td>
<td>49.6</td>
<td>50</td>
<td>8.0</td>
<td>34.2</td>
<td>1240.7</td>
<td>This study</td>
</tr>
<tr>
<td><em>Thermobifida fusca</em> YX</td>
<td>46</td>
<td>80</td>
<td>5.5</td>
<td>5.1</td>
<td>48.7</td>
<td>18</td>
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<tr>
<td><em>Bacillus amyloliquefaciens</em> DL-3</td>
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<td>50</td>
<td>7.0</td>
<td>ND</td>
<td>ND</td>
<td>1</td>
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<tr>
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<td>75</td>
<td>6.5</td>
<td>1.39</td>
<td>ND</td>
<td>4</td>
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<tr>
<td><em>Paenibacillus xylanilyticus</em> K5-03</td>
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<td>40</td>
<td>6.0</td>
<td>ND</td>
<td>ND</td>
<td>24</td>
</tr>
<tr>
<td><em>Bacillus</em> sp. strain KSM-N252</td>
<td>51.2</td>
<td>55</td>
<td>10.0</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td><em>Xanthomonas</em> sp. EC102</td>
<td>52</td>
<td>70</td>
<td>5.5</td>
<td>25.6</td>
<td>1.44</td>
<td>32</td>
</tr>
<tr>
<td><em>Cytophaga hutchinsonii</em></td>
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<td>45</td>
<td>5.0</td>
<td>ND</td>
<td>ND</td>
<td>33</td>
</tr>
</tbody>
</table>

ND, no data.
with the theoretical value of 49.6 kDa (Fig. 2, lane 4). Obviously, the molecular weight of ThCel5A falls in line with previous studies on cellulases (Table 1). The ability of the purified protein to hydrolyse CMC incorporated into the gel was examined, and the zymogram analysis showed that the recombinant enzyme could hydrolyse CMC, as indicated by a clear zone in the gel after destaining with 1 M NaCl (Fig. 2, lane 5). These results indicated that the expression and purification procedures for the enzyme were effective and that ThCel5A exhibited CMCase activity even after renaturation.

**Effect of pH and temperature on enzyme activity** The effect of the pH on the CMCase activity of the purified cellulase was examined at various pH values ranging from pH 3.0 to pH 10.5, as shown in Fig. 3b. The cellulase activity toward CMC was maximized at 8.0, while 98% and 95% of the maximum activity were observed at pH 8.5 and pH 9.0, respectively, suggesting that the enzyme is an alkaline cellulase. Although the purified ThCel5A was active only between pH 7.0 and 10.5, it could maintain greater than 50% of its maximum activity from pH 7.5 to 9.5 after alkaline treatment for 30 min. However, its activity decreased sharply when the pH value approached 10.0. The effect of the temperature on the hydrolysis rate was also evaluated (Fig. 3a) between 20°C and 90°C at pH 8.0 for 30 min ThCel5A activity increased rapidly from 20°C to 50°C, reached its peak at 50°C, and its relative activity began to decline thereafter; therefore, the optimal reaction temperature of recombinant ThCel5A was 50°C. The enzymatic activity of recombinant ThCel5A remained stable when incubated at temperatures of 40°C and 60°C for 30 min because it retained more than 50% of its maximum activity in this range. These findings indicated that ThCel5A is a mesophilic cellulase.

**Effects of different chemicals on cellulase activity** The effects of metal ions on the activity of ThCel5A were measured in the presence of metal ions at a concentration of 5 mM (Fig. 4a). As shown in Fig. 4, the addition of 5 mM Ba²⁺, Ca²⁺ and Mn²⁺ could
enhance the CMCase activity by 27%–38%, and the addition of 5 mM Ag⁺ and Cd²⁺ inhibited the CMCase activity by approximately 70% and 40%, respectively, while most other metal ions did not significantly affect the activity. Notably, the heavy metal ion Pb²⁺ exerted a minimally negative effect. The relative activities of the purified cellulase in the presence of 1 mM DTT, PMSF, EDTA and 1% SDS are shown in Fig. 4b. EDTA and SDS inhibited the CMCase activity by 37% and 78%, respectively, whereas DTT boosted the enzymatic activity to 132%. However, PMSF did not markedly influence ThCel5A activity. The characterization of ThCel5A in the presence of these four chemicals indicated that ThCel5A is a metal-ion-dependent enzyme that includes thiol groups but lacks serine in its active site.

Substrate specificity and kinetic parameters of ThCel5A activity Various polysaccharide substrates were used to determine the optimal substrate for ThCel5A (Fig. 5). Among the tested substrates, ThCel5A exhibited broad substrate specificity with high preferences towards lichenan that contained β-1,3-1,4-linkages. However, its activity on β-1,3-1,4-linked barley glucan was not as high as that on β-1,4-linked CMC and filter paper. Though the enzyme exhibited significant activity toward Avicel, significant activity against other substrates that did not include a β-1,4-linked glucose unit was not observed. Taken together, these data indicate that ThCel5A is not only active towards amorphous cellulose but also towards crystalline cellulose, such as Avicel and filter paper, indicating its endo-mode and exo-mode activity against cellulose. The kinetic constants for CMC were obtained from Lineweaver-Burk plots, and the $K_m$ and $V_m$ values of recombinant ThCel5A were 34.2 mg/ml, 1240.7 μmol/min/mg, respectively. With CMC as substrate, the $K_m$ and $V_m$ values are higher than ThCel5A of $T$. fusca XY (18) (Table 1), which indicated ThCel5A has lower affinity but higher catalytic efficiency than ThCel5A.

DISCUSSION

One of the major challenges to the economically viable production of transportation fuel from inedible plant matter is the high cost of cellulase mixtures that are commonly employed in biomass-to-biofuel conversion processes (20). A prerequisite to overcome this obstacle is the availability of an increasing number of enzymes that hydrolyse cellulose, hemicellulose and other polysaccharides into fermentable sugars at conditions suitable for industrial use (21). Fortunately, the number of microbial genomes that have been published in public databases has explosively grown, which presents unprecedented opportunities for probing enzyme genes (22). Here, we detected a GH5 cellulase gene from the draft genome of $T$. halotolerans YIM 90462T that contained 1389 base pairs. When the putative cellulase was heterogeneously expressed in $E$. coli, we found that the recombinant enzyme (ThCel5A) exhibited high activity on CMC and filter paper (Fig. 5). At this point, ThCel5A was superior to the most similar GH5 cellulase from $T$. fusca XY (ThCel5A), which showed low activity towards filter paper (23). Noticeably, our enzyme preferentially hydrolysed lichenan, which was distinct from a GH5 cellulase from $T$. fusca XY (TfCel5B) (11). However, a GH5 recombinant endoglucanase from yaks rumen uncultured microorganism could also degrade lichenan, CMC, and Avicel (6). Thus, these findings may be promising because the difficulty in degrading cellulolic materials is primarily attributed to the crystalline structure of the cellulose substrate (11).

Over the years, a large number of bacteria and fungal strains have been reported to produce GH5 cellulase (24), but such studies of halophilic actinomycetes are limited. Compared to other groups of extremophilic microorganisms, such as thermophiles and alkaliphiles, halophiles have not often been utilized in biotechnological processes, even though researchers recently predicted that halophiles may be used to break down biomass material and form biofuel products (25). The ThCel5A we cloned from $T$. halotolerans YIM 90462T was tolerant to most ions, especially to Pb²⁺ and Ag⁺, which are well-known to be toxic to enzymes by oxidizing their sulphydryl groups (26). In contrast, Ca²⁺ and Mn²⁺ stimulated the activity of ThCel5A, which was most likely due to the ability of Ca²⁺ to stabilize an enzyme conformation with a higher affinity for the substrate, while Mn²⁺ hyper-stabilized the catalytic core (26,27). However, Endo et al. found that the activity of Egl-252, an alkaline GH5 endoglucanase from an alkaliphilic Bacillus isolate, was more stable in the absence of CaCl₂ (28). Although the mechanisms involved in the effects of many ions on the recombinant enzyme are unclear, understanding the effect of metal ions on enzyme activity is important because many industrial applications require their addition at various stages of the process (16,29).

In most cases, the enzymatic hydrolysis of cellulose requires pre-treatment. Thus, the stability of the cellulolytic enzymes employed at the optimal conditions is essential for efficient hydrolysis (30). Therefore, the thermal and pH stabilities are important parameters of enzymes. As expected, the optimal temperature of ThCel5A (50 °C) is in parallel with that of another cellulase from halotolerans YIM 90462T (ThCel5A) (13), which suggests that ThCel5A is also stable at a moderately high temperature. Thus, ThCel5A might be a more competitive cellulase for industrial applications at a moderately high temperature. The optimum pH of a variety of GH5 cellulases ranged from 4.0 to 10.0, such as an endoglucanase Ce15A from Paenibacillus xylanilyticus KJ-03 (pH 6.0) (24), Xcel5A from Xanthomonas sp. EC102 (pH 5.5) (31), ChCel5A from Cytophaga hutchinsonii (pH 5.0) (32), and an alkaline endoglucanase Egl from Bacillus isolate(pH 10.0) (Table 1) (28). Interestingly, the ThCel5A from T. halotolerans YIM 90462T examined in this study retained more than 90% of its maximum activity between pH 8.0 and 9.0. This property was concordant with an endoglucanase Cel6A from halotolerans YIM 90462T, which also retained high activity after incubation at alkaline conditions (33). Thus, this enzyme could serve as an effective additive in laundry detergents in detergent formulations to remove soils from cotton fabrics without damaging said fibers (28).

In conclusion, we identified a GH5 exo/endoglucanase from the draft genome of $T$. halotolerans YIM 90462T for the first time. This cellulase hydrolysed both soluble and insoluble cellulose substrates. Additionally, the enzyme possessed a number of other
useful traits, including alkaline tolerance, metal ion resistance and moderately high temperature stability. These features make the enzyme ideally suited for industrial applications, including the production of bioethanol, textile industry applications, and detergent industry applications.

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