***Mycobacterium frederiksbergense* sp. nov., a novel polycyclic aromatic hydrocarbon-degrading *Mycobacterium* species**

Pia Willumsen,¹ Ulrich Karlson,¹ Erko Stackebrandt²
and Reiner M. Kroppenstedt²

¹ Department of Microbial Ecology & Biotechnology, National Environmental Research Institute, Roskilde, Denmark

² Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany

Author for correspondence: Reiner M. Kroppenstedt. Tel: +49 531 2616 227. Fax: +49 531 2616 418. e-mail: kdt@DSMZ.de

A polycyclic aromatic hydrocarbon-degrading bacterium isolated from coal tar-contaminated soil in Denmark was characterized by a polyphasic approach. Phylogenetically and chemotaxonomically, it was related to members of the genus *Mycobacterium*. The isolate contains chemotaxonomic markers that are diagnostic for the genus *Mycobacterium*; i.e. the *meso* isomer of 2,6-diaminopimelic acid, arabinose and galactose as diagnostic whole-cell sugars, MK-9(H₂) as the principal isoprenoid quinone, a mycolic acid pattern of α -mycolates, ketomycolates and wax-ester mycolates, unbranched saturated and unsaturated fatty acids plus a small amount of tuberculostearic acid and a significant amount of a C_{18:0} secondary alcohol. Based on the unique combination of chemical markers among mycobacteria, it is proposed that the isolate should be assigned to a new species, *Mycobacterium frederiksbergense* sp. nov. This novel species is phylogenetically closely related to *Mycobacterium diernhoferi*, *Mycobacterium neoaurum* and *Mycobacterium hodleri*. The type strain of *M. frederiksbergense* is strain FAn9^T (= DSM 44346^T = NRRL B-24126^T).

Keywords: *Mycobacterium frederiksbergense* sp. nov., polycyclic aromatic hydrocarbon degradation, polyphasic taxonomy

INTRODUCTION

The suborder *Corynebacterineae* of the order *Actinomycetales*, class *Actinobacteria*, constitutes a phylogenetically coherent group that includes the genera *Corynebacterium*, *Dietzia*, *Rhodococcus*, *Nocardia*, *Skermania*, *Gordonia*, *Tsukamurella*, *Williamsia* and *Mycobacterium* (Stackebrandt *et al.*, 1997; Kämpfer *et al.*, 1999). These genera can be differentiated easily from other bacteria by a combination of chemical markers, i.e. *meso*-diaminopimelic acid in the cell wall and the heteropolysaccharide arabinogalactan connecting the mycolic acids (α -branched β -hydroxylated long-chain fatty acids) with the cell wall. Individual genera of the *Corynebacterineae* can be differentiated

from each other by their lipids, e.g. the chain length and type of mycolic acids, the type of quinone and qualitative and quantitative differences in their fatty acid patterns (Goodfellow, 1989; Kroppenstedt, 1985; Lechevalier, 1994).

This taxon harbours many degraders of xenobiotica: members of *Rhodococcus* are able to degrade hydrocarbons, chlorophenols, polychlorinated biphenyls and sulfonated azo dyes (Bell *et al.*, 1998), mycobacteria degrade diverse polycyclic aromatic hydrocarbons (PAH) and polychlorophenols (Kleespies *et al.*, 1996; Häggblom *et al.*, 1994; Willumsen & Karlson, 1997), while *Gordonia* metabolize alkanes (Kummer *et al.*, 1999). Most members of the suborder *Corynebacterineae* are usually considered slow growers but, as they are excellent survivors of unfavourable conditions, they can compete successfully with fast-growing strains, e.g. pseudomonads and related taxa, which are also known to metabolize xenobiotica (Bell *et al.*, 1998; Bock *et al.*, 1996; Lang, 1995). The present study is a polyphasic characterization of a novel scotochromogenic mycobacterium.

Abbreviations: FAME, fatty acid methyl ester; MACP, mycolic acid cleavage product; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PAH, polycyclic aromatic hydrocarbon.

The EMBL accession number of the 16S rDNA sequence of *Mycobacterium frederiksbergense* FAn9^T is AJ276274.

METHODS

Isolation. Strain FAn9^T was isolated from coal tar-contaminated soil on the site of a former gas works at Frederiksberg, Denmark. Bacteria were extracted from soil and isolated by direct plating as described previously (Willumsen & Karlson, 1997). PAH-coated agar plates (Kiyohara *et al.*, 1982) were inoculated in the dark at 15 °C for 15–18 d. Strain FAn9^T was distinguished from other soil bacteria as colonies surrounded by a clear zone in the fluoranthene layer on PAH-coated agar. The strain was purified further by repeated transfer to Trypticase soy agar and PAH-coated agar plates.

Characterization and biochemical tests. Micromorphological properties were determined by phase-contrast microscopy. Gram staining, acid–alcohol-fastness, colony morphology, the ability to grow at various temperatures, pigment production and photoreactivity were determined after 2 weeks of growth in Middlebrook medium or on Trypticase soy broth (TSB) agar at 37 °C using the proposed minimal standards for the genus *Mycobacterium* (Lefford, 1980; Vincent Lévy-Frébault & Portals, 1992). The catalase test was performed as described by Kubica & Pool (1960). Nitrate reductase and Tween 80 hydrolysis were detected as described by Bönike (1961). The protocols used to determine growth on xylose, trehalose, mannitol and sorbitol as sole sources of carbon were those of Silcox *et al.* (1981). Additional carbon source utilization and quantitative enzyme tests were performed in standard microtitration plates (F-form, Greiner) as described by Kämpfer *et al.* (1990) with the following modifications: carbon source utilization test medium was yeast nitrogen base (Difco) supplemented with yeast extract (0.02 g l⁻¹), K₂HPO₄ (1.74 g l⁻¹) and KH₂PO₄ (0.36 g l⁻¹); enzyme test medium contained 0.05 M Tris/HCl buffer, 0.05% (w/v) Casamino acids (Difco) and 0.05% (w/v) yeast extract (Difco). The panels were inoculated with a standardized bacterial suspension of 0.5–1.0 McFarlane units in 0.9% (w/v) NaCl and then incubated for 3 d at 28 °C. After 50 µl of a filter-sterilized indicator solution (4 mM MTT; 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) and phenazine methosulfate (Sigma) had been added to the auxanographic test and control wells, incubation was continued for 24 h in the dark. The formation of the deep-blue-coloured formazane was taken as an indication of carbon source utilization (Kirchhoff *et al.*, 1992). Reading of the auxanographic test results was carried out photometrically by using a Multiscan MCC340 MKII photometer (Flow Laboratories). The catabolism of a carbon source was considered positive if the A₅₄₀ of the sample was greater than 0.129 (control). The results of the enzyme tests were evaluated visually. The type strains of the closely related species *Mycobacterium diernhoferi*, *Mycobacterium neoaurum* and *Mycobacterium hodleri* were tested under identical conditions.

The capacity of strain FAn9^T to degrade different PAHs in liquid batch experiments was determined by measuring the amount of ¹⁴CO₂ produced during mineralization of ¹⁴C-labelled phenanthrene, fluoranthene, pyrene, fluorene, anthracene and benzo(a)pyrene, as described previously (Willumsen *et al.*, 1998).

Determination of chemotaxonomic properties. For fatty acid analyses and HPLC mycolic acid analyses, strain FAn9^T and the related species were cultivated on Middlebrook 7H10 agar, enriched with Middlebrook OADC (DSMZ medium no. 645; DSMZ, 1998) at 37 °C for 1 week. In order to obtain sufficient cell material for analysis of the other

chemical markers, FAn9^T was grown in GPHF liquid medium (DSMZ medium no. 553) on a rotary shaker (90 r.p.m.) at 28 °C for 1 week. The cells were collected by centrifugation, washed once with water and lyophilized.

Analyses of cell wall amino acids and sugars. Amino acids and sugars of whole-cell hydrolysates were analysed by TLC as described previously (Staneck & Roberts, 1974).

Extraction and analysis of isoprenoid quinones. Isoprenoid quinones were extracted from 100 mg freeze-dried cells by using the small-scale integrated procedure described by Minnikin *et al.* (1984). The dried menaquinones were dissolved in 200 µl 2-propanol and filtered and the extract was used for HPLC analysis without any further purification. A 5 µl aliquot of the extract was separated by HPLC using an RP-18 column (250 × 4 mm) packed with Lichrospher 100 (particle size, 5 µm). The column temperature was kept at 40 °C. Acetonitrile/2-propanol (65:35, v/v) was used as the mobile phase. The flow rate was 1 ml min⁻¹ (Kroppenstedt, 1985; Kroppenstedt *et al.*, 1981).

Analysis of fatty acids. The fatty acid methyl esters (FAMES) were obtained from 40 mg wet weight of cells by saponification, methylation and extraction, as described previously (Schröder *et al.*, 1997). The FAME mixtures were separated by GC (model 5898A; Hewlett Packard) controlled by MS software (Microbial ID). Peaks were integrated automatically and fatty acid identities and percentages were determined using the Microbial Identification standard software package (Sasser, 1990).

Analysis of mycolic acids by TLC. Mycolic acids were analysed following the method of Minnikin *et al.* (1980). For differentiation between α'-α-methoxy- and ketomycolates, the method of Luquin *et al.* (1991) was used.

Analysis of mycolic acids by HPLC. For mycolic acid analyses by HPLC, samples containing about 40 mg wet cells of the four strains were harvested from Petri dishes and saponified by KOH. The free mycolic acids were extracted and transferred to their bromophenacyl esters as described previously by Butler *et al.* (1992) and Miller (1997). Low and high molecular mass internal standards (Ribi ImmunoChem Research) were added to the samples. The mycolic acid bromophenacyl ester mixtures were separated by HPLC (Series 1050; Hewlett Packard) fitted with a C18 Ultrasphere XL cartridge column (Beckman) at 35 °C. The HPLC was operated by the SHERLOCK System software (MIDI Inc).

Analyses of mycolic acid cleavage products (MACPs). For preparation of the mycolic acid methyl esters, the procedure described for fatty acids was used. Ten microlitres of 0.2 M methanolic trimethylsulfonium hydroxide was added to the extract to enhance the pyrolysis of the mycolic acid esters in the hot injector block of the GLC. For analyses of the MACPs, the GC conditions described previously by Müller *et al.* (1998) were used.

16S rDNA sequence determination. Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA and purification of PCR products were carried out using procedures described previously (Rainey *et al.*, 1996). Purified PCR products were sequenced using a Taq DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) as directed in the manufacturer's protocol. Sequence reactions were electrophoresed using an Applied Biosystems 373A DNA sequencer. The 16S rDNA sequence was aligned manually with published sequences from representatives of the actinomycete sublines of descent included in the DSMZ database of sequences. Distance analysis was done according to De Soete (1983).

RESULTS AND DISCUSSION

Cells of strain FAn^{9T} were Gram-positive, non-spore forming, acid-alcohol-fast and non-motile. They were rod-shaped (length about 2 µm, diameter 1 µm). The scotochromogenic, smooth colonies were cadmium yellow. In liquid media, the cells clumped together, probably as a result of their hydrophobic cell wall surface. On Middlebrook and TSB media, growth appeared after 5–7 d at 30 °C. The strain produced a catalase and was positive for nitrate reductase but negative for urease. It was able to hydrolyse Tween 80 but did not grow on MacConkey agar. Acid was produced from D-xylose, trehalose and mannitol but not from sorbitol.

In mineral salt medium, strain FAn^{9T} was able to mineralize phenanthrene, fluoranthene and pyrene aerobically. In addition, these substrates were used as sole sources of carbon and energy (data not shown). No loss of degradation ability was observed after subcultivating the strains on rich media like TSB agar or Middlebrook for an extended period, which suggests that the degradation of phenanthrene, fluoranthene and pyrene is not plasmid-mediated. No growth or mineralization could be observed for anthracene, fluorene or benzo(a)pyrene.

The almost complete 16S rDNA sequence of strain FAn^{9T}, consisting of 1474 nucleotides, was aligned to the DSMZ database of type strains of *Mycobacterium* species. *M. neoaurum* ATCC 25795^T, *M. diernhoferi* ATCC 19340^T and *M. hodleri* DSM 44183^T were the closest neighbours, respectively exhibiting 98.7, 98.0 and 97.7% similarity. Similar and even higher values were found to separate the type strains of fast-growing mycobacteria species, such as the pairs *Mycobacterium aichiense* ATCC 27820^T and *Mycobacterium parafortuitum* DSM 43528^T, *M. diernhoferi* ATCC 19340^T and *M. neoaurum* ATCC 25795^T or *M. parafortuitum* DSM 43528^T and *Mycobacterium gilvum* ATCC 43909^T. A dendrogram of relatedness, generated by distance analysis of Kimura's two-parameter correction of similarity values to compensate for multiple nucleotide exchange (Felsenstein, 1993), is shown in Fig. 1. *M. diernhoferi*, *M. neoaurum* and *M. hodleri* were the closest neighbours, forming a separate subclade. This subclade was recovered by other treeing algorithms (maximum-likelihood and maximum-parsimony; trees not shown).

The results of the chemotaxonomic data match those expected for mycobacteria. Whole-cell hydrolysates of strain FAn^{9T} contained the cell wall diamino acid meso-diaminopimelic acid. The cell-wall sugars were arabinose and galactose. The combination of these chemical markers grouped this isolate into the cell wall type IV actinomycetes (Lechevalier & Lechevalier, 1970). Our analysis of the mycolic acids by one-dimensional TLC revealed a multispot pattern, which is diagnostic for members of the genus *Mycobacterium*. Based on the position of the α-mycolates on TLC, we can assume that the mycolic acid chain lengths are in

Fig. 1. Phylogenetic dendrogram obtained by distance matrix analysis showing the position of strain FAn^{9T} (= DSM 44346^T) among phylogenetic neighbouring members of the rapidly growing mycobacteria. Bootstrap values above 80% are indicated at branch-points. Bar, 1 nucleotide substitution per 100 positions.

the 70–90 carbon atoms range. Two quinones, MK-8(H₂) (30%) and MK-9(H₂) (70%), could be detected by HPLC. A differentiation within the genus *Mycobacterium* was obtained by the separation of the mycolic acids by TLC in two directions (Minnikin *et al.*, 1980). The analyses revealed four spots, which could be identified as α-mycolates, ketomycolates and ω-carboxymycolates plus alcohols (wax-ester mycolates). This pattern is distributed widely among mycobacteria and can be found in about 40% of all mycobacteria, including the closely related species *M. neoaurum*, *M. hodleri* and *M. diernhoferi* (Hinrikson & Pfyffer, 1994; Luquin *et al.*, 1991; Vincent Lévy-Frébault & Portaels, 1992). Pyrolysis of the mycolic acid methyl esters revealed mainly a saturated FAME with a chain length of 22 carbon atoms. This differentiates FAn^{9T} from *M. hodleri* and *M. diernhoferi*, which release a C₂₄ FAME in addition. Mycolic acid HPLC elution profiles are well suited to the separation of mycobacteria to the species level (Butler *et al.*, 1996). Comparison of the mycolic acid HPLC elution profile of FAn^{9T} with our mycolic acid database and data from the literature showed that this mycolic acid pattern was unique among the mycobacteria (Butler *et al.*, 1991, 1992, 1996). It showed two clusters of peaks. One cluster was composed of a homologous series of wax-ester mycolates and the other contained α-mycolates plus ketomycolates. α-Mycolates and ketomycolates co-elute and cannot be separated under standard HPLC conditions. As expected, FAn^{9T} was separated very well by this method from its closely related neighbours *M. neoaurum*, *M. hodleri* and *M. diernhoferi* (Fig. 2). The fatty acid pattern from whole-cell hydrolysates was composed of unbranched saturated and unsaturated fatty acids. Only small amounts of tuberculostearic acid were synthesized by this strain. Two secondary alcohols (C18:0-2OH, C20:0-2OH) could be detected

Fig. 2. Mycolic acid HPLC elution profiles of FAn9^T (= DSM 44346^T) and the closely related species *M. diernhoferi* DSM 43524^T, *M. neoaurum* DSM 44074^T and *M. hodleri* DSM 44183^T. LIS, Low-molecular-mass internal standard; HIS, high-molecular-mass internal standard.

Table 1. Composition of FAMES derived from whole-cell hydrolysates of strain FAn9^T and related mycobacteria

Values are percentages of total fatty acids. Secondary alcohols that are released from wax esters are included. Examples of abbreviations: 16:0, hexadecanoic acid (palmitic acid); 18:1 *cis*-9, *cis*-9-octadecenoic acid (oleic acid); 18:0 10-methyl, 10-methyloctadecanoic acid (tuberculostearic acid); 20:0 alcohol, 2-eicosanol.

Fatty acid	FAn9 ^T	<i>M. neoaurum</i> DSM 44074 ^T	<i>M. hodleri</i> DSM 44183 ^T	<i>M. diernhoferi</i> DSM 43524 ^T
14:0	5.45	5.72	5.74	6.23
15:0	0.66	—	1.05	—
16:1 <i>cis</i> -7	6.30	3.48	1.49	2.17
16:1 <i>cis</i> -9	—	5.23	2.42	0.77
16:1 <i>cis</i> -10	3.41	—	6.07	5.65
16:0	31.44	28.31	25.17	25.27
16:0 10-methyl	—	—	0.76	—
18:2 <i>cis</i> -9,10	—	—	0.63	—
18:1 <i>cis</i> -9	34.83	30.46	20.27	20.57
18:0	2.27	1.96	1.24	3.98
18:0 10-methyl	1.39	5.57	18.46	11.15
20:0	1.36	—	—	—
18:0 alcohol	10.78	15.07	13.93	18.00
20:0 alcohol	1.71	1.74	2.77	4.57

Fig. 3. Dendrogram based on fatty acid profiles showing the relationships between strain FAn9^T and some phylogenetically related *Mycobacterium* species. *C.*, *Corynebacterium*.

in addition (Table 1). These were released from wax esters during preparation of the FAMES. This GLC elution profile differentiates FAn9^T quite well from the phylogenetically related species (Fig. 3).

The results of the physiological tests obtained from microtitre plates by means of the MTT reduction test revealed that FAn9^T was able to utilize only eight of the 26 carbon sources tested (Table 2).

Differentiation of strain FAn9^T from other fast-growing mycobacteria

The results of the physiological, chemotaxonomic and phylogenetic analyses indicate clearly that strain FAn9^T represents a new species of the genus *Myco-*
bacterium. The phylogenetic position of this organism is within the cluster defined by *M. diernhoferi*, *M.*

neoaurum and *M. hodleri*, the latter being another PAH-degrading mycobacterium. All four strains synthesize α -mycolates, ketomycolates and wax-ester mycolates, but can be differentiated into two groups by their mycolic acid pyrolysis ester. Strain FAn9^T and *M. neoaurum* released a C22:0 FAME whereas, in *M. diernhoferi* and *M. hodleri*, C24:0 was found in addition. A clear separation between FAn9^T and its neighbours and all the other mycobacteria was obtained by the unique mycolic acid bromophenacyl ester HPLC profile of FAn9^T, which was different from those of *M. neoaurum*, *M. diernhoferi* and *M. hodleri* (Fig. 2). Strain FAn9^T can be differentiated by conventional tests from *M. hodleri*, which is negative for nitrate reduction and does not utilize trehalose but can grow on sorbitol, from *M. diernhoferi*, which is non-chromogenic, does not hydrolyse Tween 80, lacks catalase activity and is unable to utilize trehalose, and from *M. neoaurum*, which lacks catalase activity and shows variable reactions for Tween 80 hydrolysis, nitrate reaction and xylose utilization. Table 2 lists other discriminative tests that are useful for the differentiation of FAn9^T from its phylogenetically related neighbours.

Description of *Mycobacterium frederiksborgense* sp. nov.

Mycobacterium frederiksborgense (fre.de.riks.ber.gen'se. N.L. gen. n. *frederiksborgense* of Frederiksborg, Denmark, referring to the place of isolation).

Gram-positive, acid-fast, non-spore-forming, non-motile short rods, sometimes coccid without branching. Smooth, scotochromogenic colonies, cadmium yellow in colour, appear after 5 d on TSB agar and Middlebrook agar. Grows at 15–37 °C. Shows a positive reaction for catalase and nitrate reduction but

Table 2. Physiological properties of strain FAn9^T and closely related species

Results are based on the type strains tested. Data for *M. hodleri* were taken from Kleespies *et al.* (1996). All strains gave positive results for utilization of gluconate, *i*-inositol, putrescine, *p*-nitrophenyl β -D-xyloside and mannitol and were negative for benzoate, 4-aminobenzoate, 4-hydroxybenzoate, citrate, D-sucrose, D-turanose and 2-deoxythymidine 5'-*p*-nitrophenyl phosphate. Characteristics other than utilization of carbon compounds are scored as: +, more than 85% of strains are positive; –, fewer than 15% of strains are positive; v, variable. Assimilation of auxanographic substrates was detected photometrically by means of reduction of the redox dye MTT (Kirchhoff *et al.*, 1992) and is scored as: +, $A_{540}(\text{test}) - A_{540}(\text{control}) > 0.129$; –, $A_{540}(\text{test}) - A_{540}(\text{control}) < 0.129$.

Characteristic	FAn9 ^T	<i>M. diernhoferi</i>	<i>M. neoaurum</i>	<i>M. hodleri</i>
Formation of pigment*	S	N	S	S
Growth at 42 °C	—	—	v	—
Tween 80 hydrolysis	+	—	v	+
Nitrate reduction	+	+	v	—
Catalase activity	+	—	—	+
Growth on:				
Xylose	+	+	v	+
Trehalose	+	—	+	—
Sorbitol	—	—	—	+
Utilization of:				
<i>N</i> -Acetyl D-glucosamine	+	—	—	+
D-Glucosaminic acid	+	—	+	—
L-Rhamnose	+	—	—	+
D-Ribose	—	—	—	+
D-Arabitol	—	—	—	+
2-Hydroxyvalerate	+	+	+	—
2-Oxoglutarate	—	+	+	+
Succinate	+	—	—	+
L-Alanine	—	+	—	+
L-Aspartate	—	—	—	+
L-Leucine	—	+	—	+
L-Proline	—	+	—	+
L-Valine	—	—	—	+
Acetamide	—	—	+	—
Phenylacetic acid	—	—	+	—
Quinate	—	—	—	+
<p>-Nitrophenyl phosphorylcholine</p>	—	+	+	—

* Data from Kleespies *et al.* (1996). s, Scotochromogenic; n, non-chromogenic.

negative for urease. Tween 80 is hydrolysed but no growth occurs on MacConkey agar. D-Xylose, trehalose and mannitol are used as sole sources of carbon but glucitol (sorbitol) is not. Able to utilize *N*-acetyl D-glucosamine, gluconate, D-glucosaminic acid, *i*-inositol, putrescine, L-rhamnose, succinate and 2-hydroxyvalerate, whereas acetamide, phenylacetic acid, L-alanine, D-arabitol, L-aspartate, benzoate, 4-aminobenzoate, 4-hydroxybenzoate, citrate, glutamate, 2-oxoglutarate, L-leucine, L-proline, putrescine, quinate, D-ribose, sucrose, D-turanose and L-valine are not used. *p*-Nitrophenyl β -D-xyloside is hydrolysed but *p*-nitrophenyl phosphorylcholine and 2-deoxythymidine 5'-*p*-nitrophenyl phosphate show no change in colour. Mineralizes the PAHs phenanthrene, fluoranthene and pyrene. The fatty acid pattern from whole-cell methanolysates is composed of tetradecanoic acid (5%), palmitoleic acid (6%), *cis*-11-hexadecenoic acid (3%), palmitic acid (31%), oleic

acid (35%), stearic acid (2%), tuberculostearic acid (1%) and eicosanoic acid (1%). Significant amounts of the alcohols 2-octadecanol (11%) and eicosanol (2%) are also present. TLC of mycolic acid methanolysates reveals α -mycolates, ketomycolates and ω -carboxymycolates plus 2-eicosanol (wax-ester mycolates). The mycolic acid HPLC elution profile is unique and can be used for differentiation from the closely related species *M. diernhoferi*, *M. neoaurum*, *M. hodleri* and from all other mycobacteria.

Isolated from the site of a former gas works at Frederiksberg, Denmark. Strain FAn9^T (= DSM 44346^T = NRRL B-24126^T) is the type strain.

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