Desulfovibrio marinisediminis sp. nov., a novel sulfate-reducing bacterium isolated from coastal marine sediment via enrichment with Casamino acids

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To obtain amino acid-utilizing sulfate reducers, enrichment culture was carried out with a medium containing Casamino acids and sulfate and inoculated with coastal marine sediment from the eutrophic Tokyo Bay, Japan. A sulfate reducer, designated strain C/L2^T, was isolated from the sulfide-producing enrichment culture after further enrichment with lactate and sulfate by means of the agar shake dilution method. Cells of strain C/L2^T were vibrio-shaped, Gram-negative, motile rods (0.7–1.0 μm wide and 1.0–3.5 μm long) with single polar flagella. The optimum temperature for its growth was 37 °C, the optimum pH was around 7.5 and the optimum NaCl concentration was 20–25 g l^{-1}. Hydrogen, formate, lactate, pyruvate, fumarate, malate, succinate, ethanol, propanol, glycerol, glycine, alanine, serine, aspartate, Casamino acids, peptone and yeast extract were used as electron donors. Sulfate, sulfite and thiosulfate each served as an electron acceptor, but elemental sulfur, nitrate, fumarate, acrylate and 2,4,6-tribromophenol did not. Disproportionation of thiosulfate was not observed. Desulfoviridin, *c*-type cytochromes and catalase were present. The major respiratory quinone was MK-6(H_2). The G+C content of the genomic DNA was 46.2 mol%. Comparisons based on 16S rRNA gene sequences and on dissimilatory sulfite reductase gene sequences clearly showed that strain C/L2^T belonged to the genus *Desulfovibrio*: its closest relatives were the uncharacterized *Desulfovibrio* sp. strain TBP-1 (16S rRNA gene sequence similarity of 99.4 %) and *Desulfovibrio acrylicus* DSM 10141^T (16S rRNA gene sequence similarity of 98.7 %). The level of DNA–DNA hybridization with *Desulfovibrio acrylicus* DSM 10141^T was 10.3 %. On the basis of the data from this study and the physiological and phylogenetic differences that exist between the isolate and *Desulfovibrio acrylicus*, strain C/L2^T represents a novel species of the genus *Desulfovibrio*, for which the name *Desulfovibrio marinisediminis* sp. nov. is proposed. The type strain is C/L2^T (=NMRC 101113^T=JCM 14577^T=DSM 17456^T).

Sulfate-reducing bacteria (SRB) play a major role in the mineralization of organic matter in coastal marine sediments (Jørgensen, 1982). In such environments, SRB decompose short-chain fatty acids, especially acetate, which are produced by fermentative bacteria (Sørensen *et al.*, 1981; Winfrey & Ward, 1983; Christensen, 1984; Parkes *et al.*, 1989; Fukui *et al.*, 1997). Although SRB are considered to play only a limited role in the oxidation of amino acids (Burdige, 1989, 1991; Hansen & Blackburn, 1995; Parkes *et al.*, 1989), some SRB are able to use several single amino acids: examples include species of the genera *Desulfovibrio* (Baena *et al.*, 1998; Stams *et al.*, 1985; van der Maarel *et al.*, 1996), *Desulfobacterium* (Rees *et al.*, 1998), *Desulfobulbus* (Sass *et al.*, 2002), *Desulfomusa* (Finster *et al.*, 2001) and *Desulfotomaculum* (Stams & Hansen, 1986). Hansen *et al.* (1993) showed that the rate of amino acid turnover was greatly inhibited by the addition of molybdate (a specific

Abbreviations: DSR, dissimilatory sulfite reductase; SRB, sulfate-reducing bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *dsrAB* gene sequences of strain C/L2^T are AB353727 and AB218445, respectively.

A phase-contrast microphotograph of cells of strain C/L2^T and a phylogenetic tree based on *dsrAB* gene sequences are available as supplementary material with the online version of this paper.

inhibitor of sulfate reduction) in experiments involving the incubation of fish food-amended sediment collected from below a marine fish farm. We also reported that the rate of sulfate reduction was more stimulated by the addition of Casamino acids than by the addition of lactate and that glutamate was accumulated in the presence of molybdate in the incubation of sediment slurry collected in the highly eutrophic area of Tokyo Bay, Japan, where algal blooms were often observed in warm seasons (Takii, 2003). These findings suggest the active participation of SRB in amino acid oxidation in sediments rich in organic matter. To isolate amino acid-utilizing SRB from sediment, enrichment culture was carried out with Casamino acids and sulfate, using surface sediment from Tokyo Bay as the inoculum. The sulfate-reducing enrichment culture obtained contained various morphologically different types of bacteria. After further enrichment with lactate and sulfate, a sulfate reducer, designated strain C/L2^T, was isolated. Here, we describe this strain as representing a novel species of the genus *Desulfovibrio.*

Strain C/L2^T originated from anoxic black sediment collected (using an Ekman dredge) from a dredged site (140° 00′ 2″ E 35° 37′ 19″ N) in the inner part of Tokyo Bay. The details of the site were described in a previous paper (Takii *et al.*, 2002).

The basal medium used for the enrichment, isolation and maintenance of sulfate-reducing strains was a marine, bicarbonate-buffered, sulfide-reduced medium, described by Widdel & Bak (1992). The medium contained the following (l^{-1}): 4.0 g Na_2SO_4, 0.2 g KH_2PO_4, 0.25 g NH_4Cl, 20.0 g NaCl, 3.0 g $MgCl_2.6H_2O$, 0.5 g KCl and 0.15 g $CaCl_2.2H_2O$. The following were also added (l^{-1}) to the autoclaved medium, after cooling under a stream of N_2 gas: 1 ml SL10 trace element solution, 1 ml selenite/tungstate solution, 1 ml vitamin mixture solution, 1 ml vitamin B_{12} solution, 30 ml $NaHCO_3$ solution and 7.5 ml $Na_2S.9H_2O$ solution (Widdel & Bak, 1992). The completed medium was distributed into sterile tubes or bottles (with screw caps fitted with butyl rubber discs): the vessels were filled to exclude air, unless indicated otherwise. Enrichment culture was carried out with screw-capped bottles filled with the basal medium supplemented with Casamino acids (3.5 g l^{-1}) and inoculated with a fresh sediment sample, as described previously (Takii *et al.*, 2007). After several transfers of sulfide-positive cultures, the culture was subjected to the agar shake dilution method (Widdel & Bak, 1992). Despite the performance of a repeated isolation procedure, the culture obtained contained two types of morphologically distinct cells. The culture was then successively enriched with basal medium supplemented with lactate (20 mM) as the sole substrate; the sulfate-reducing enrichment culture was subjected to the most-probable-number technique with the same medium. The highest dilution growing tubes were used to inoculate an agar shake culture. Purity was investigated by means of phase-contrast microscopy (Optiphot; Nikon), using cultures with various substrates. Strain C/L2^T was isolated and maintained in a small screw-capped tube filled with basal medium supplemented with lactate.

The cell morphology was examined using phase-contrast microscopy. Flagella were observed under a transmission electron microscope (JEM-1010; JEOL) using exponentially growing cells without staining. Gram staining was performed using the Hucker method (Doetsch, 1981) and the KOH test was performed according to the method of Gregersen (1978).

Duplicate culture tubes were used in all experiments. Growth at various temperatures, pH values and NaCl concentrations were studied using small screw-capped tubes filled with basal medium supplemented with sodium lactate (20 mM). The pH was adjusted with 2 M HCl or 1 M Na_2CO_3. In the NaCl tolerance tests, various amounts of NaCl (0–50 g l^{-1}) were added to basal medium or NaCl-free medium with reduced $MgCl_2.6H_2O$ (0.4 g l^{-1}). Utilization of electron donors was tested in small screw-capped tubes filled with basal medium, except in the case of H_2, and was evaluated by monitoring the growth (i.e. the optical density of the culture) for longer than 1 month and by the production of hydrogen sulfide, detected by cupric reagent (Widdel & Bak, 1992). To test for H_2 utilization, the gas phase was filled with H_2/CO_2 (80 : 20, v/v) in small tubes closed with butyl rubber stoppers and screw caps. Fermentation was tested with basal medium modified such that it lacked any electron acceptors and contained dithionite (final concentration, 40 mg l^{-1}) instead of sodium sulfide. Thiosulfate (20 mM), sulfite (5 mM), elemental sulfur (1 %), nitrate (10 mM), acrylate [20 mM with yeast extract (1 g l^{-1})] and 2,4,6-tribromophenol [100 and 200 μM with yeast extract (1 g l^{-1})] were tested as sole electron acceptors in the modified basal medium containing lactate (20 mM) as electron donor. Formate was used as an electron donor with acetate (2 mM) and yeast extract (0.5 g l^{-1}) to test for the utilization of fumarate (20 mM) as an electron acceptor. To test for disproportionation, modified basal medium (containing 20 mM thiosulfate and 2 mM acetate) was used.

The optical densities of liquid cultures were measured by inserting the screw-capped culture tubes directly into a photometer (Tokyo Photoelectric). For the analysis of end products, a 40 ml sample of the modified basal medium in 70 ml serum vials was used. The gas phase was N_2. Fatty acids were analysed by means of HPLC (Purdy *et al.*, 1997) and GC (Takii *et al.*, 2007). Hydrogen in the gas phase was analysed by using GC as described previously (Takii *et al.*, 2007). Desulfoviridin was tested as described by Postgate (1959). Cytochromes were identified by redox-spectroscopy (dithionite reduced minus ferricyanide oxidized) of cell-free extracts at 540–570 nm using a UV-3000 spectrophotometer (Shimadzu). Catalase was detected by pouring a 3 % H_2O_2 solution onto a cell pellet in a centrifugation tube. Respiratory quinones were extracted with chloroform/methanol (2 : 1, v/v), purified with a Sep-Pak Plus column (Waters) and analysed by using a reversed-phase

HPLC system (Beckman System Gold with a Hewlett Packard Zorbox ODS column) as described by Shintani *et al.* (2000).

The DNA G+C content was measured at the Techno Suruga Co. Ltd (Shizuoka, Japan), using HPLC as described by Katayama-Fujimura *et al.* (1984).

16S rRNA gene fragments were amplified by means of a PCR using a MicroSeq 500 16S rDNA gene Bacterial Identification PCR kit (Applied Biosystems) and sequenced with a MicroSeq 500 16S rDNA gene Bacterial Identification sequencing kit on a gene analyser system (ABI PRISM 3100; Applied Biosystems) at the Techno Suruga Co., Ltd. The dissimilatory sulfite reductase (DSR) gene of strain C/L2^T was amplified with primers DSR1F and DSR4R (Wagner *et al.*, 1998). The DSR amplification products were directly sequenced using primers DSR1F and DSR4R and the internal primer DSR1Fl (Dhillon *et al.*, 2003) on a DNA sequencer (ABI 377; Applied Biosystems). The 16S rRNA gene sequences obtained and the deduced amino acid sequences of the α- and β-subunits of the DSR gene were aligned and compared with reference sequences by using the ARB program package (Ludwig *et al.*, 2004) and CLUSTAL W, version 1.6 (Thompson *et al.*, 1994), respectively. The phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) with the ARB program package or the MEGA2 program (Kumar *et al.*, 2001). Bootstrap analysis based on 1000 replicates was performed (Felsenstein, 1985).

DNA–DNA hybridization was performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). DNA was isolated from bacterial cells by using chromatography on hydroxyapatite according to the procedure of Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) with the modifications reported by Huß *et al.* (1983), using a Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostatted 6×6 multicell changer and a temperature controller with an *in situ* temperature probe (Varian).

The sediment sample collected from the inner part of Tokyo Bay (140° 00′ 2″ E 35° 37′ 19″ N) was inoculated into basal medium supplemented with Casamino acids (3.5 g l^{-1}) as a carbon and energy source. A stable sulfate-reducing enrichment culture was successfully established. After further enrichment with lactate, strain C/L2^T was isolated by repeated agar shake culture.

The purity of the strain was confirmed by the morphological homogeneity of cells grown on various substrates under a phase-contrast microscope and by the identical partial sequences of the 16S rRNA gene obtained from several colonies in the agar shake culture.

The cells of strain C/L2^T were vibrio-shaped (0.7–1.0 μm wide and 1.0–3.5 μm long) and were motile, by means of single polar flagella, in the exponential growth phase (see Supplementary Fig. S1 available in IJSEM Online). Most cells rapidly changed to short rods and cocci (less than 1 μm wide), showing no motility after the exponential phase when cultivated with lactate, pyruvate or Casamino acids. However, this morphological change was delayed when cells were cultivated with fumarate or succinate. No sporulation was observed. Cells stained Gram-negative and behaved like Gram-negative cells in the KOH lysis test.

The optimum growth temperature for strain C/L2^T on lactate and sulfate was 37 °C. No growth was observed at 10 or 42 °C. The strain grew between pH 6.3 and 8.6, with an optimum around pH 7.5. The NaCl concentration for growth was 0.5–35 g l^{-1}, the optimal concentration being 20–25 g l^{-1}.

The isolate showed anaerobic growth by sulfate reduction using various organic substrates as electron donor, i.e. lactate, pyruvate, fumarate, malate, succinate, ethanol, propanol, glycerol (each at 20 mM), glycine, alanine, serine, aspartate (each at 10 mM), Casamino acids (3.5 g l^{-1}), peptone (5 g l^{-1}) or yeast extract (1 g l^{-1}). When Casamino acids were added as a substrate (3.5 g l^{-1}), only four amino acids (serine, alanine, threonine and glycine) had been almost completely utilized after 5 days incubation (Takii *et al.*, 2007). Vitamins were not required for growth on lactate and sulfate, because the cultures could be transferred (1 % inoculation size) to the vitamin-free medium at least three times. However, the addition of yeast extract (1 g l^{-1}) increased cell yield as much as twofold. Growth on hydrogen or formate required the presence of yeast extract (0.5 g l^{-1}) in addition to acetate as a carbon source. Lactate (19.9 mM) was oxidized in the presence of sulfate to acetate (18.6 mM), CO_2 and H_2S. Pyruvate, fumarate, Casamino acids, peptone and yeast extract were fermented in the absence of sulfate but not lactate. The fermentation products from pyruvate (10.7 mM) were acetate (8.2 mM), succinate (0.9 mM), H_2 and CO_2. Fumarate (10.4 mM) was converted to acetate (7.7 mM), succinate (4.8 mM), H_2 and CO_2 in the sulfate-free medium. Fermentative growth on pyruvate and fumarate was weak. A Stickland reaction was not observed with alanine–glycine or alanine–proline. Disproportionation of thiosulfate was not observed. Sulfate, thiosulfate and sulfite served as electron acceptors in the presence of lactate as the energy and carbon source, but this was not the case with elemental sulfur, nitrate, fumarate, acrylate or 2,4,6-tribromophenol.

Cytochromes of the *c* type were present in the soluble and membrane fractions of strain C/L2^T, as revealed by the absorption maximum at 550 nm in the redox-differential spectrum. Desulfoviridin was present. Catalase was produced. The major respiratory quinone was MK-6(H_2). The G+C content of the genomic DNA was 46.2 mol%.

Phylogenetic analysis of an almost full-length 16S rRNA gene sequence (1533 bp) from strain C/L2^T showed that the strain fell within the genus *Desulfovibrio*, family *Desulfovibrionaceae*, order *Desulfovibriales*, in the *Deltaproteobacteria* (Fig. 1). Its closest relative was the

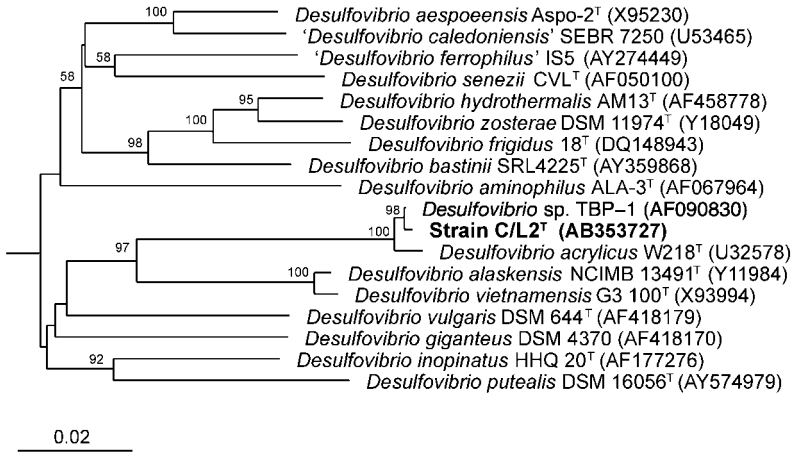

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, indicating the position of strain C/L2^T among related members of the class *Deltaproteobacteria*. Bootstrap percentages (based on 1000 replications) are shown at nodes where >50 %. *Desulfomicrobium apsheronum* (GenBank no. U64865) was used as an outgroup. Bar, 2 % sequence divergence.

uncharacterized *Desulfovibrio* sp. strain TBP-1 (sequence similarity of 99.4 %; Boyle *et al.*, 1999) and the closest species with a validly published name was *Desulfovibrio acrylicus* DSM 10141^T (98.7 %; van der Maarel *et al.*, 1996). These two were coherently clustered with strain C/L2^T. Analysis of the approximately 600-amino-acid sequence of the gene for DSR (an enzyme essential to sulfate reduction) also revealed that strain C/L2^T was robustly associated with the genus *Desulfovibrio* (see Supplementary Fig. S2 available in IJSEM Online).

Strain C/L2^T was similar to members of the genus *Desulfovibrio* (belonging to the *Deltaproteobacteria*) in the following physiological and chemotaxonomic respects: H_2, lactate, pyruvate, ethanol, fumarate and malate were each used as an electron donor in the presence of sulfate; growth in the absence of an electron acceptor was possible from the fermentation of pyruvate or fumarate; desulfoviridin was present; and MK-6(H_2) was the major menaquinone (Widdel & Bak, 1992). The phenotypic similarities were also supported by results of sequence comparisons based on 16S rRNA and *dsrAB* genes.

Table 1 shows the characteristics that serve to differentiate between strain C/L2^T and closely related species. Strain C/L2^T differs from *Desulfovibrio acrylicus* in being unable to utilize acrylate as an electron acceptor; *Desulfovibrio acrylicus* was so named because of its unique ability to decompose acrylate. Unlike *Desulfovibrio acrylicus*, strain C/L2^T possessed catalase and was able to utilize sulfite as an electron acceptor and to oxidize aspartate. Strain C/L2^T was unable to oxidize cysteine, unlike *Desulfovibrio acrylicus*, which could use this substrate. In spite of the high level of 16S rRNA gene sequence similarity (98.7 %) between strain C/L2^T and *Desulfovibrio acrylicus* DSM 10141^T, DNA–DNA hybridization revealed only 10.3 % relatedness. As the recommended threshold value for DNA–DNA relatedness for the definition of species is 70 % (Wayne *et al.*, 1987), this result confirmed that the two strains are not related at species level. The high level of 16S rRNA gene sequence similarity (99.4 %) between strain

Table 1. Characteristics used for distinguishing strain C/L2^T (*Desulfovibrio marinisediminis* sp. nov.) from related species of the genus *Desulfovibrio*

Taxa: 1, strain C/L2^T (data from this study); 2, *Desulfovibrio* species TBP-1 (Boyle *et al.*,1999); 3, *Desulfovibrio acrylicus* (van der Maarel *et al.*, 1996). All taxa listed reduce sulfate and thiosulfate to sulfide, but do not reduce ritrate. All use hydrogen, formate, fumarate, lactate and pyruvate as electron donors, but not acetate, propionate or butyrate as electron donors. All taxa have desulfoviridin. +, Used or present; –, not used or absent; ND, not described.

Characteristic	1	2	3
Cell morphology	Vibrioid; later coccoid	Vibrioid; later spherical	Vibrioid to rod-shaped
Temperature range for growth (°C)	15–40	ND	ND
Optimum temperature for growth (°C)	37	ND	30–37
pH range for growth	6.3–8.6	ND	ND
Optimum pH for growth	7.5	ND	7.4
Range of NaCl for growth (g l^{-1})	0.5–35	<0–75	6–40
Optimum NaCl for growth (g l^{-1})	20–25	37.5	18
DNA G+C content (mol%)	46.2	ND	45.1
Catalase	+	ND	−
Electron acceptors			
Sulfite (5 mM)	+	+	−
Elemental sulfur	−	+	−
Acrylate (10 mM)	−	−	+
2,4,6-Tribromophenol (100–200 μM)	−	+	ND
Substrates utilized			
Succinate	+	−	+
Propanol	+	ND	+
Glycerol	+	ND	+
Cysteine	−	ND	+
Aspartate	+	ND	−

C/L2^T and the uncharacterized strain TBP-1 indicated that they are closely related to each other phylogenetically. However, there were clear differences between strains C/L2^T and TBP-1 in the usage of electron acceptors and in the tolerance of salt: TBP-1 was able to reduce elemental sulfur and 2,4,6-tribromophenol, unlike the novel isolate, and strain TBP-1 was able to grow with NaCl at concentrations up to 75 g l^{-1} (optimally at 37.5 g l^{-1}) whereas strain C/L2^T did not show significant growth at NaCl concentrations above 35 g l^{-1}. Unfortunately, strain TBP-1 is not available at the moment, so further phenotypic comparisons and DNA–DNA hybridization experiments are not possible.

Although strain C/L2^T showed only a limited ability to utilize amino acids, the metabolism of amino acids was extensively enhanced in co-culture of the strain with *Dethiosulfatibacter aminovorans*, an amino acid-fermenting bacterium isolated from the same enrichment culture (Takii *et al.*, 2007). This stimulation was due to the scavenging of H_2 by strain C/L2^T (confirmed by the absence of H_2 in the co-culture). Recently, Haouari *et al.* (2006) isolated *Desulfovibrio* species closely related (>99 % 16S rRNA gene sequence similarity) to strain TBP-1 from Tunisian marine sediments, by using peptone as substrate. Like strain C/L2^T, these strains might also contribute to amino acid decomposition as hydrogen scavengers in marine environments.

On the basis of its phylogenetic and phenotypic properties, it is proposed that strain C/L2^T represents a novel species in the genus *Desulfovibrio*, for which the name *Desulfovibrio marinisediminis* sp. nov. is proposed.

Description of *Desulfovibrio marinisediminis* sp. nov.

Desulfovibrio marinisediminis (ma.ri.ni.sed′i.mi′nis. L. adj. *marinus* of the sea, marine; L. gen. n. *sediminis* of a sediment; N.L. gen. n. *marinisediminis* of a marine sediment).

Vibrioid cells (0.7–1.0 μm wide and 1.0–3.5 μm long) rapidly change to a coccoid form after the exponential phase of growth. Gram-negative. Cells are motile by means of single polar flagella. Mesophilic. Optimum growth occurs at 37 °C and at a pH around 7.5. Grows with NaCl at 0.5–35 g l^{-1} (optimally at 20–25 g l^{-1}). Strictly anaerobic. Able to use sulfate, sulfite and thiosulfate as electron acceptors, with the production of sulfide, but unable to use elemental sulfur, nitrate, fumarate, acrylate or 2,4,6-tribromophenol. Does not show disproportionation of thiosulfate. Hydrogen, formate, lactate, pyruvate, fumarate, malate, succinate, ethanol, propanol, glycerol, glycine, alanine, serine, aspartate, Casamino acids, peptone and yeast extract are used as electron donors. Pyruvate, fumarate, Casamino acids, peptone and yeast extract can be utilized fermentatively. Acetate, propionate, butyrate, benzoate, butanol, valine, leucine, isoleucine, proline, methionine, phenylalanine, tryptophan, threonine, cysteine, asparagine, glutamine, glutamate, histidine, lysine, arginine, gelatin and sugars (glucose, mannose, fructose, maltose and sucrose) are not used as sole substrates for growth. Desulfoviridin, *c*-type cytochromes and catalase are present. The G+C content of the genomic DNA of the type strain is 46.2 mol%.

The type strain, C/L2^T (=NMRC 101113^T=JCM 14577^T=DSM 17456^T), was isolated from a Casamino acid-containing enrichment culture inoculated with anoxic sediment collected from a highly eutrophic area of Tokyo Bay, Japan.

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