

Full Length Research Paper

Study of Multi-metal Resistance in Bacteria Highly Resistant to Mercury Isolated from Effluent of Dental Clinics

Parisa Keramati¹, Mehran Hoodaji¹ and Arezoo Tahmourespour^{2*}¹Soil Science Department, Khorasgan (Isfahan) branch, Islamic Azad University, Isfahan, Iran²Basic Medical Sciences Department, Khorasgan (Isfahan) branch, Islamic Azad University, Isfahan, Iran.

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Heavy metals pollution represents an important environmental problem. One of these metals is mercury. The aim of this research was isolating bacteria highly resistant to mercury from dental clinic effluent to investigate their growth potential in the presence of other heavy metals, such as Zn, Cu, Ni, Ag, Cd and Pb. Three dental wastewater samples were selected and their mercury concentrations, pH, EC, BOD₅ and COD were determined. The mercury-resistant bacteria were found to belong to the genera of *Pseudomonas*, *Proteus*, *Citrobacter*, *Bacillus*, *Corynebacterium* and *Staphylococcus*. In examining multi-metal resistances, the pattern of hexa-R was seen in the case of *Citrobacter* and *Pseudomonas* genera. The highest tolerated concentration of heavy metals was 25.6 mM which is related to Ag and Cu and tolerated by *Citrobacter* isolate. It is also indicated that *Citrobacter* was the most resistant isolates to Cd with significant difference ($P < 0.05$). Results of this study demonstrate the occurrence of different groups of bacteria, capable of high tolerance to mercury with a potential to tolerate a variety of other toxic heavy metals suggest that, resistance to many types of toxicants may be present in the same organism; therefore, such organisms have high potential for biotechnology purposes.

Key words: Bacteria, dental effluent, heavy metal, mercury, multi-metal resistance.

INTRODUCTION

Heavy-metals are widely used in chemistry and engineering. There is increasing interest in the biotechnology of extraction of rare metals from primary and secondary raw materials (Korenevskii et al., 1997). On the other hand, heavy metals are very toxic elements, which comprise the essential part of anthropogenic pollutants (Avtsyn et al., 1991). The presence of toxic heavy metal contaminants in aqueous streams, arising from the discharge of untreated metal containing effluents into water bodies, is one of the most important environmental issues (Rehman et al., 2008). The discharge of heavy metals into the environment as a result of agricultural, industrial and military operations, and the effects of this pollution on ecosystems and human health have been of concern for some years (Essa et al., 2002). These heavy metals include

cadmium, chromium, cobalt, manganese, mercury, nickel, silver, and zinc. Metal-polluted industrial effluents discharged into sewage treatment plants could lead to high metal concentrations in the activated sludge. Microbial populations in metal-polluted environments contain microorganisms that have adapted to the toxic concentrations of heavy metals and become "metal resistant" (Leung and Chua, 2001).

Interest in processes involving heavy metal uptake by micro-organisms has increased considerably in recent years, in particular because of the biotechnological potential of micro-organisms in metal removal and/or recovery (Lopez-Errasquin and Vazquez, 2003). Mercury is one of the most toxic heavy metals in the environment, commonly found in the global environment including lithosphere, hydrosphere, atmosphere and biosphere and its toxic nature has been known for centuries (Miretzky and Fernandez Cirelli, 2009). Mercury and its allied compounds are extremely toxic in the environment, because of its many unique properties, the use and dispersion of mercuric compounds have increased vastly, resulting in extensive heavy metal pollution (Renzoni

*Corresponding author. E-mail: atahmoures@khuisf.ac.ir, arezootahmourespour@gmail.com. Tel: 0983116278314. Fax: 00983115354045.

et al., 1998).

Mercury is recognized as a potent and widely distributed toxicant in the global environment having ability to accumulate at various levels of food chain (Jan et al., 2009). Mercury poisoning has become a problem of current interest as a result of environmental pollution on a global scale (Fitzgerald and Lamborg, 2005; Parta and Sharma, 2008). Mercury pollution is a ubiquitous problem in the present day world, as mercury concentration are rising continuously as a result of increased industrial, medicinal and domestic use of mercury (Jan et al., 2009). Exposure to mercury can come from a number of different roots, such as through dental amalgam fillings. Dental amalgam is the most commonly used dental restorative material used for dental fillings and currently, they are composed of about 50% mercury (Davis, 2003). American Dental Association (2005) in a mass balance assessment of the annual amount of amalgam discharged in dental wastewater reported that, about 29.7 tons of mercury in the form of amalgam was discharged into dental units by dental offices.

Therefore, dental clinic wastewater contains mercury and mercury resistant bacteria. Conventional techniques for removing dissolved toxic metals include chemical precipitation and sludge separation, chemical oxidation or reduction, ion exchange, reverse osmosis, filtration, adsorption using activated charcoal, electrochemical treatment and evaporative recovery may not be effective, especially when the metal concentration is lower than 100 mgL^{-1} . Also, these techniques can be expensive, they may not always be feasible and their metal-binding properties are non-specific. So, the use of microorganisms in the removal of metals from contaminated wastewater, mining, and industrial wastes is generally considered promising. These are the reasons why alternative processing methods, such as those using microbial biomass, are now being considered more seriously (Lopez-Errasquin and Vazquez, 2003; Green-Ruiz, 2006). Bioremediation techniques that involve the use of microorganisms to remove environmental contaminants have gained an increasing interest in the last few years (Gupta and Ali, 2003). Staphylococcus, Bacillus, Pseudomonas, Citrobacteria, Klebsiella, and Rhodococcus are organisms commonly used in bioremediation mechanisms (Adeniji, 2004). There are three classes of bioremediation: 1) Biotransformation, 2) Biodegradation and 3) Mineralization. These three classifications can occur either *in situ* (at the site of contamination) or *ex situ* (contaminant taken out of the site of contamination and treated elsewhere) (Leung, 2004). Metal-resistant microorganisms may be useful as indicators of potential toxicity to other forms of life (Doelman et al., 1994). Bacterial resistance to mercury in the environment is one of the numerous examples of the genetic and physiological adaptability of microbial communities exposed to contaminants (Ramond et al., 2009). The present study was aimed to isolate mercury

resistant bacteria from dental clinic wastewater and investigate their potential for growth in the presence of 6 different heavy metals such as Nickel, Lead, Copper, Zinc, Cadmium and Silver; because these heavy metals are present in many environments and so, the remediation of these metals is one of great ecological interests.

MATERIALS AND METHODS

Wastewater sampling and culture condition

Three dental clinic effluents were selected from training section (sample 1), restorative section (sample 2) and an existing unit waterline (sample 3) of the dental faculty of Islamic Azad University Khorasgan branch (Esfahan) during November and September 2009. The samples were transferred to a laboratory in sterile-dark Pyrex flasks at 4°C and processed within 18 to 24 h. The properties such as temperature, pH, EC, COD, BOD_5 were determined. Also, mercury contents were measured by cold vapor atomic absorption spectrophotometer (Buck Scientific). The samples were inoculated on PHG Agar medium [peptone (4 g/L), yeast extract (1 g/L), glucose (2 g/L), NaCl (5 g/L), Agar (15 g/L)] , (supplemented with 0.05 mM (10 ppm) HgCl_2) and incubated at 35°C for 48 h.

MIC determination

Mercury-resistant bacteria (MRB) from wastewater samples were isolated by growth on PHG Agar and PHG Broth amended by 10 ppm mercuric chloride (Merck, Germany). For determining MIC (Minimum Inhibitory Concentration) and adaptation, the PHG agar plates supplemented with different concentration of HgCl_2 were inoculated aseptically with a culture of isolated MRBs (15 isolates) in exponential growth phase and incubated for 48 h (MIC) and 7 to 10 days (adaptation). 6 isolates out of these 15 MRBs were selected and hereafter designated as bacteria highly resistant to mercury (BHRM). Gram staining and biochemical testes of BHRMs were done for probable identification.

Multi-metal resistance pattern of BHRM

To determine multi-metal resistance, PHG-II agar plates supplemented with heavy metals were used. Resistant colonies to mercury (BHRMs) were inoculated in radial streaks on PHG agar media supplemented with each heavy metal such as $\text{Pb}(\text{NO}_3)_2$, $\text{Ag}(\text{NO}_3)$, ZnCl_2 , $\text{Ni}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ and $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$. Each metal concentration in PHG agar medium was 0.4, 0.8, 1.6, 3.2, 6.4, 12.8 and 25.6 mM. For each step, the plates were incubated at 35°C for 48 h and growth was investigated. Then, the grown colonies transferred to a media with higher concentration of the same metal.

Statistical analysis

Observations were made and all the experiments run in triplicate. The experimental groups were compared and significant differences evaluated by using the variance analysis (ANOVA) and Duncan by SPSS software (ver.18). In order to draw charts, Excel software was used. Statistical significance was assumed at a P value of 0.05.

RESULT

The chemical properties of three effluent samples are

Table 1. The analysis of selected samples.

Sample	Analysis	Mercury content (mg.L ⁻¹)	pH	EC (dS.m ⁻¹)	COD (mg.L ⁻¹)	BOD ₅ (mg.L ⁻¹)
1 (Training section effluent)		3.55	7.53	0.32	157	32
2 (Restorative section effluent)		47.8	7.65	0.3	272	55
3 (Existing unit waterline effluent)		0.12	7.26	0.29	134	25

Table 2. The MIC and adaptation of MRBs to different concentrations of HgCl₂.

Strain Hg Concentration (mM)	<i>Citrobacter</i> (<i>intermedius</i>)	<i>Pseudomonas</i> (<i>mallei</i>)	<i>Proteus</i> (<i>mirabilis</i>)	<i>Staphylococcus</i> (<i>aureus</i>)	<i>Bacillus</i> (<i>megaterium</i>)	<i>Corynebacterium</i> (<i>xerosis</i>)
0.2	+	+	+	+	+	+
0.4	+	+	+	+(MIC)	+	+
0.8	+	+	+	+a	+	+(MIC)
1.6	+	+(MIC)	+(MIC)	+a	+	+ ^a
3.2	+(MIC)	+ ^a	+ ^a	+a	+(MIC)	+ ^a
6.4	+ ^a	+ ^a	+ ^a	+a	+ ^a	+ ^a
12.8	+ ^a	-	+ ^a	+ ^a	+ ^a	-
25.6	+ ^a	-	-	-	+ ^a	-
51.2	-	-	-	-	-	-

+: growth (24-48h); -: no growth; a : growth after 7 to 10 days (adaptation).

Table 3. Multi-metal resistance pattern of BHRMs.

Strain	Type of multiple resistance	Zn	Cd	Cu	Ni	Pb	Ag	g
<i>Citrobacter</i>	Hexa- R	+	+	+	+	+	+	+
<i>Pseudomonas</i>	Hexa- R	+	+	+	+	+	+	
<i>Proteus</i>	penta - R	+	-	+	+	+	+	
<i>Bacillus</i>	penta_R	+	-	+	+	+	+	
<i>Corynebacterium</i>	penta - R	+	-	+	+	+	+	
<i>Staphylococcus</i>	Tri - R	-	-	+	-	+	+	

+: growth on 3.2 mM; -: no growth or growth on < 3.2 mM.

shown in Table 1. It is clear that sample 2 (restorative section) was the most polluted samples with significant difference ($P < 0.05$). 15 mercury resistant bacteria (MRB) were isolated by growth on PHG Agar supplemented with 10 ppm mercuric chloride. The MIC and adaptation of MRBs to different concentrations of HgCl₂ were investigated and shown in Table 2. 6 isolates out of these 15 MRBs were highly resistant to mercury and designated as BHRM. The maximum MIC and adaptation were 3.2 and 25.6 mM/L, respectively which

are related to such bacteria as *Citrobacter* and *Bacillus*. The minimum MIC was 0.4 mM/L that is of *Staphylococcus*. In examining multi- metal resistances, the pattern of hexa-R were seen in the case of *Citrobacter* and *Pseudomonas* genera. The *Proteus*, *Bacillus* and *Corynebacterium* isolates could not resist in high concentration (3.2 mM/L) of Cd and their multi-metal resistance pattern was penta -R (Table3).

The results of minimum inhibitory concentration of 6 different heavy metals is shown in Figure 1. In all cases,

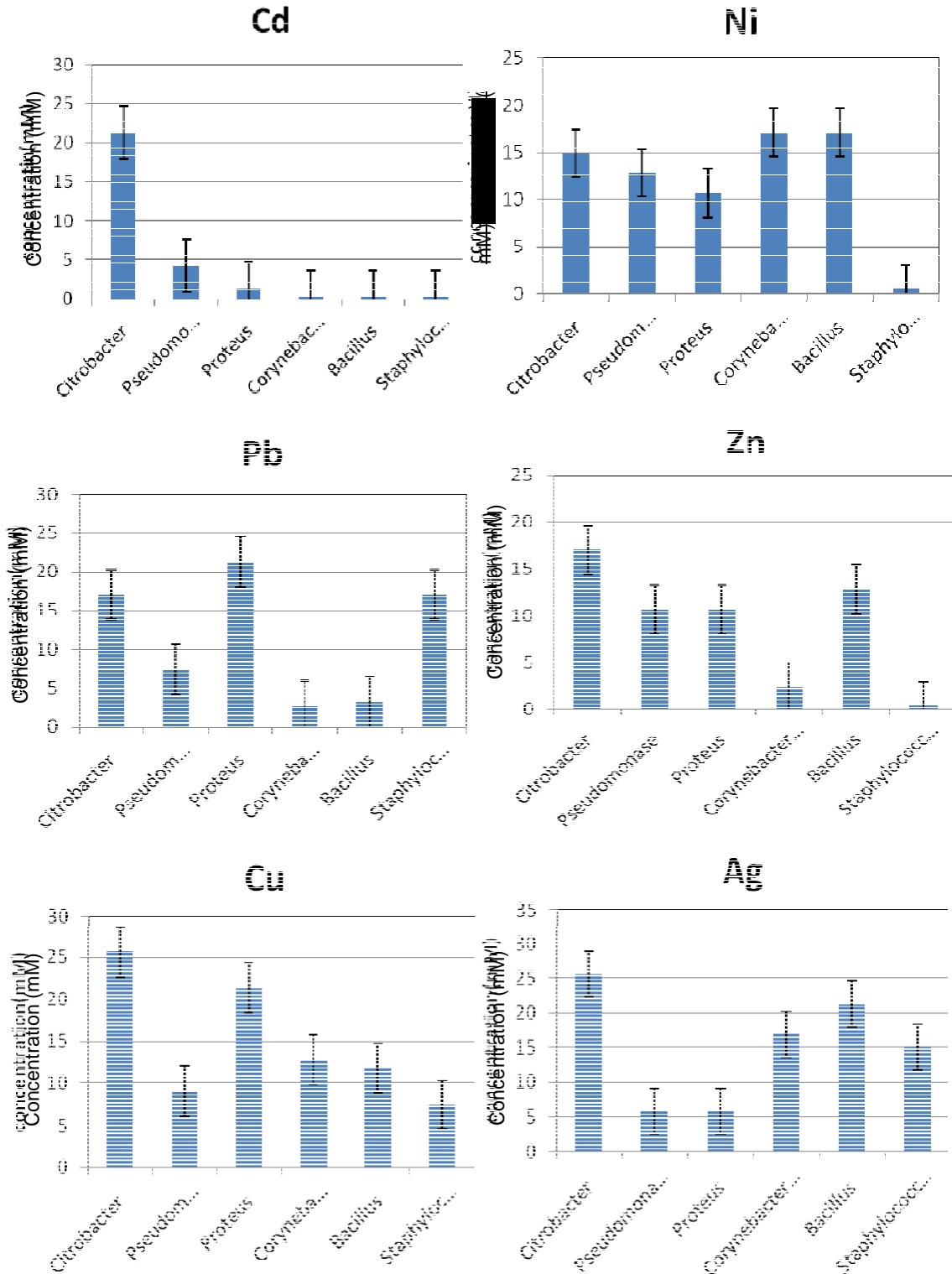


Figure 1. The MIC comparison of BHRMs to different heavy metals.

strains were able to grow in the proximity of BHRM on PHG broth medium containing 6.4 and 25.6 mM Hg within 7 to 10 days incubation. Minimum inhibitory concentration of other heavy metals was determined at

different concentrations of each tested heavy metal within 48 h incubation. The results indicated that, Citrobacter was the most resistant isolates to Cd with significant difference ($P < 0.05$). The other isolates had not

significant differences in Cd tolerance. *Staphylococcus* isolate was the most sensitive strain to Ni with significant difference ($P < 0.05$).

DISCUSSION

The mercury-resistant bacteria (MRB) were isolated and identified from three dental clinic effluents and their mercury MIC and adaptability were determined. At least, 6 isolates of highly resistant bacteria to mercury (BHRM) were selected for investigation of their multi-metal resistance. These isolates included both Gram-positive (e.g. *Bacillus*) and Gram-negative (e.g. *Citrobacter*) bacteria. The resistance of these strains to 6 different heavy metals such as copper, lead, zinc, cadmium, silver and nickel were determined with an initial metal concentration of 0.2 mM. Such organisms are important in containing the basic biological processes in contaminated habits. The Hg-resistant bacteria can reduce Hg^{2+} to elemental Hg^0 that volatilizes out of the system because of its high vapor presence (Summers and Silver, 1978). Further, some bacteria can detoxify Hg compounds by the action of organomercural and by mercuric reductase, that reduces Hg^{2+} to volatile Hg (Nakamura and Silver, 1994). Several authors have reported the presence of metallothionein-like proteins in bacterial system that exhibit resistance to several heavy metals like zinc, cadmium, nickel, cobalt and mercury (Robinson et al., 2001; Silver and Ji, 1994). Many bacterial species have been shown to develop resistance to mercury and other heavy metals (Jaysankar and Ramaiah, 2007; Fitzgerald and Lamborg, 2005; Singh et al., 2008). As for microorganisms, there are at least three mechanisms of mercury bioremediation: enzyme reduction to Hg^0 , volatilization and formation of insoluble HgS (Essa et al., 2002).

Recently, microbial bioremediation has appeared as an alternative technique to such traditional chemical treatments (Brierly, 1990). Microorganisms like bacteria, fungi, algae and actinomycetes have effectively sequestered heavy metals (Wong and So, 1993). These have been used to remove metals from polluted industrial and domestic effluents on a large scale. Microbiological detoxification of polluted water is economical, safe and sustainable (Eccles, 1995). It is well recognized that microorganisms have a high affinity for metals and can accumulate both heavy and toxic metals by a variety of mechanisms (Rehman et al., 2008). In this research, in comparison of multi-metal resistant pattern cleared that, significant differences ($P < 0.05$) were observed between some bacterial strains. For example in lead resistance pattern, a significant difference was observed between *Proteus* and *Corynebacterium*, *Bacillus* and *Pseudomonas* but there is no significant difference ($P > 0.05$) between *Proteus*, *Citrobacter* and *Staphylococcus*. In these cases, *Proteus* and *Corynebacterium* were the

most and the least resistant strains respectively. In nickel-resistant pattern, a significant difference was observed between *Staphylococcus* (the least resistant strain) and all others. *Bacillus* and *Corynebacterium* had maximum resistance to nickel. In cadmium-resistant pattern, a significant difference was observed between all of these strains. *Citrobacter* had maximum resistance and each of the gram-positive bacteria had no resistance. In zinc-resistant pattern, a significant difference was observed between *Staphylococcus* with other strains except *Corynebacterium*. *Citrobacter* had maximum resistance to zinc and *Staphylococcus* had minimum resistance.

In copper-resistant pattern, a significant difference was observed between *Citrobacter* with other strains except *Proteus*. *Citrobacter* and *Staphylococcus* showed maximum and minimum resistance respectively. In silver-resistant pattern, a significant difference was observed between *Pseudomonas* and other strains except *Proteus*. *Citrobacter* had maximum resistance to silver and *Proteus* had minimum resistance. In whole, the *Citrobacter* strain, exhibited the highest tolerance to all tested metals and *staphylococcus* strain exhibited the lowest tolerance to metals. In previous researches, bacteria like *Escherichia coli*, *Klebsiella*, *Alcaligenes*, *Bacillus*, *Micrococcus*, *Pseudomonas* (Pahan et al., 1990) and *Acinetobacter* (Petrova et al., 2002) were recognized as mercury resistant bacteria. It was also reported that, clinical isolates of *Staphylococcus aureus* is resistant to mercury (Jan et al., 2009). Holtz et al. (2005) in a study of mercury resistant bacteria found that, Hg-resistance was almost exclusively restricted to *Pseudomonas frederiksbergensis* and *Pseudomonas migulae* groups. In another research on the assimilation and detoxification of selenium and mercury, *Pseudomonas fluorescens* was a mercury resistant bacteria (Belzile et al., 2006).

In this study, *Proteus* sp., *Citrobacter* sp. and *Corynebacterium* sp. which were not mentioned in previous researches or have not been considered thoughtfully were isolated as mercury-resistant bacteria. The results showed that, mercury colored concentration which the isolates were able to grow in, was much higher than the previous ones. For example, in a study of Jaysankar et al. (2003), bacteria highly resistant to mercury that were isolated from seawater and sediment samples were capable of growth at 50 ppm of mercury. Also, Ready et al. (2007) investigated the effect of amalgams exposure on mercury resistant bacteria, the mercury chloride was used at concentrations ranging from 0.125 to 512 μ M and bacteria with a $HgCl_2$ MIC of 325 μ M were regarded as Hg resistant. One of the reasons for such high resistance among the isolated bacteria in this study could be related to the way of isolating mercury-resistant bacteria and it is of great import. In most of the past researches, the isolating of mercury-resistant bacteria were done by direct culturing

in a solid medium containing mercury and a lower degree of resistance was observed (Karbasizadeh et al., 2003; Wagner-Dobler et al., 2003). Mercury-resistant bacteria in this study were isolated by initial enriching with presentation of 10 ppm mercury chloride. Initial enriching adapts bacteria to mercury stress. So, the isolated bacteria would be able to tolerate in even higher concentrations.

Additionally, the bacteria in each stage were incubated after culturing in liquid medium about a week and then transferred to a medium with a higher mercury concentration resulting in bacterial adaptation. The pathways of Hg modification are not yet understood. The BHRM investigations during this study have many unique characteristics. We find that these dental effluent isolates capable of high resistance to Hg were able to detoxify many other heavy metals, and this is useful for bioremediation of heavy metals. Mercury levels prescribed by Bureau of Indian standards and WHO after a survey in 2003 are 0.001 mg/L for drinking water and 0.01 mg/L for industrial effluents (Jan et al., 2009); and the lowest effluent mercury concentrations for many waste streams in US. EPA (1997) ranging from 0.5 to 50 µg/L, which mercury content measured in these three wastewater samples are more than standards, therefore the high tolerance of isolated bacteria to mercury in this study could be related to the existence of bacteria in dental effluent containing mercury and their adaptation during passing time.

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