

Effect of triclosan on *Salmonella typhimurium* at different growth stages and in biofilms

Mina Tabak¹, Keren Scher¹, Efrat Hartog¹, Ute Romling², Karl R. Matthews³, Michael L. Chikindas³ & Sima Yaron¹

¹Department of Biotechnology and Food Engineering, Technion, Haifa, Israel; ²Microbiology and Tumorbiology Center, Karolinska Institutet, Stockholm, Sweden; and ³Department of Food Science, Cook College, Rutgers, The State University of New Jersey, New Brunswick, NJ, USA

Correspondence: Yaron Sima, Department of Biotechnology and Food Engineering, Technion-Israel Institute of Technology, Haifa 32000, Israel. Tel.: +972 0 4 8292940; fax: +972 0 4 8293399; e-mail: simay@tx.technion.ac.il

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Abstract

Triclosan is a potent biocide that is included in a diverse range of products. This research was aimed to investigate the susceptibility of planktonic and biofilm-associated *Salmonella enterica* serovar Typhimurium to triclosan, and to identify potential mechanisms of adaptation. The effect of triclosan was studied on planktonic *Salmonella* (log and stationary phases), on biofilm-associated cells, on bacteria derived from disrupted biofilms and on a biofilm-deficient mutant. An eight-log reduction of exponentially growing cells was observed with 1000 µg mL⁻¹ triclosan within 10 min, a 3.6-log reduction in stationary cells and a 6.3-log reduction in stationary cells of a biofilm-deficient mutant ($P < 0.05$). Biofilm-associated cells were tolerant (1-log reduction). However, biofilm-derived cells showed sensitivity to triclosan similar to stationary-phase cells. Triclosan induced the transcription of *fabI* and *micF*. Within biofilms, triclosan also up-regulated the transcription of *acrAB*, encoding for an efflux pump, *marA*, and the cellulose-synthesis-coding genes *bcsA* and *bcsE*. Thus, *Salmonella* within biofilms could experience reduced influx, increased efflux and enhanced exopolysaccharides production. Our results demonstrated that the tolerance of *Salmonella* towards triclosan in the biofilm was attributed to low diffusion through the extracellular matrix, while changes of gene expression might provide further resistance to triclosan and to other antimicrobials.

Introduction

Triclosan is a bisphenol antimicrobial agent that has a broad range of activity (Russell, 2004). Triclosan is bacteriostatic at concentrations ranging between 0.025 and 100 µg mL⁻¹, and bactericidal at higher levels (Suller & Russell, 1999, 2000; Escalada *et al.*, 2005). It is used as a preservative, antiseptic and disinfectant in a diverse range of products. Owing to its extensive use and stability, triclosan and its derivatives can now be found in wastewater, deposited in environmental sediments and concentrated in aquatic biota (Adolfsson-Erici *et al.*, 2002).

The inhibitory activity of triclosan results from blocking lipid synthesis through specific inhibition of the NADH-dependent enoyl-acyl carrier protein reductase FabI (McMurry *et al.*, 1998b; Levy *et al.*, 1999; Heath & Rock, 2000). At higher concentrations, triclosan is likely to damage the bacterial membrane (Villalain *et al.*, 2001). Gram-negative bacteria use multiple mechanisms to develop

resistance to triclosan, including mutations in the enoyl reductase, alteration of the cell envelope, active efflux and expression of triclosan-degradative enzymes (Schweizer, 2001a; Yazdankhah *et al.*, 2006). The main physiological change resulting from adaptation to triclosan, as described so far in *Escherichia coli* and *Salmonella*, is the overexpression of efflux pumps, particularly the AcrAB efflux pump (Levy, 2002; Braoudaki & Hilton, 2004, 2005). As active AcrAB was also associated with increased resistance to many other structurally unrelated antimicrobials (McMurry *et al.*, 1998a; Randall & Woodward, 2002; Randall *et al.*, 2004), there might be a link between triclosan usage and antibiotic resistance (McMurry *et al.*, 1998a; Levy, 2000, 2002).

Most data on the antimicrobial activity of triclosan were collected from studies using planktonic microorganisms rather than biofilm-associated microorganisms. Plastics incorporated with triclosan are now heavily marketed for use in the food processing industry, but very little is known about the effect of triclosan-containing plastics on the

microbial loads of the foods, and none of these studies examined the effect of triclosan on biofilms (Yazdankhah *et al.*, 2006). A number of research projects studied the antimicrobial activity of triclosan in environments that may contain biofilms, like on the floor (Moretro *et al.*, 2006), in sink drains (McBain *et al.*, 2003b) or within mouthrinse formulations (Arweiler *et al.*, 2002). In these experiments, triclosan prevented the formation of biofilms, but its effect, if any, on viability in existing biofilm was equivocal or not studied.

In the present research, we studied the effect of triclosan on the viability of the foodborne pathogen *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) within the biofilm in comparison with planktonic cells in different growth phases, and identified mechanisms of adaptive tolerance to the antimicrobial.

Materials and methods

Bacterial strains

Salmonella typhimurium ATCC 14028-1 s NaI^r (wt) and its mutants MAE52 and MAE190 were described previously (Zogaj *et al.*, 2001). While the wt forms biofilms at 28 °C, MAE52 also forms biofilms at 37 °C. The MAE190 mutant does not form a biofilm due to deletion of the genes encoding for the production of cellulose and curli (Romling *et al.*, 2000; Gerstel & Romling, 2001).

Biofilm formation

Overnight cultures of MAE52 were diluted (1:30) in fresh Luria–Bertani (LB) broth without NaCl and incubated in 24-well micro-plates (1.5 mL) for 24 h at 37 °C with gentle shaking (130 r.p.m.). Under these conditions, MAE52 forms a biofilm at the air–liquid surface. Biofilms were gently removed from the surface of the broth with sterile tweezers and washed with 10 mL saline. Enumeration of cells in the biofilm was conducted after disruption of the biofilm with glass beads as was described previously. As was shown before, each biofilm contained *c.* 10⁸ CFU (Scher *et al.*, 2005).

Preparation of cells in different phases of growth

The following cultures were used: MAE52 within biofilms (biofilm-associated cells), MAE52 originating from disrupted biofilms (biofilm-derived cells) and planktonic bacteria at the log phase of growth and at the stationary phase (wt, MAE52 and MAE190).

To prepare the MAE52 biofilm-associated cells and biofilm-derived cells, biofilms were formed as described above, and used in their entirety (biofilm-associated cells) or

agitated with glass beads for 1 min in saline, followed by centrifugation (32 g, 5 min) to remove unbroken small particles of the biofilm matrix, and a second centrifugation step (4500 g, 15 min) to pellet the cells (biofilm-derived cells).

MAE52 planktonic cells at the stationary phase were prepared by collecting the broth under the biofilm after 24 h of incubation at 37 °C. The stationary cultures of wt and MAE190 strains (which do not form biofilms at 37 °C) were prepared by incubation under the same conditions as MAE52.

Log-phase cultures were obtained by growing all strains for 3.5 h to OD_{600 nm} 0.4–0.5. All planktonic cells were then collected by centrifugation (4500 g, 15 min) and resuspended in saline to a final concentration of *c.* 10⁸ CFU mL⁻¹.

Determination of minimal inhibitory concentration (MIC) and minimal bacteriocidal concentration (MBC) for planktonic cells

A stock solution of 5000 µg mL⁻¹ triclosan (Irgasan, Fluka BioChemika) was prepared in 50% ethanol (McBain *et al.*, 2003a; Aiello *et al.*, 2004). Dilutions of predetermined concentration were made either in medium or in saline before each experiment.

Determination of the MIC was conducted in broth according to the recommendation of the National Committee for Clinical Laboratory Standards (NCCLS, 2003) using triclosan concentrations of 0.06–2 µg mL⁻¹ and a final inoculum of 10⁸ CFU mL⁻¹. The final concentration of ethanol did not exceed 0.02% and had no growth-inhibitory properties.

MBC, i.e. the concentration required to obtain at least a 3-log reduction (Sherris, 1990), was determined for 10⁸ CFU mL⁻¹ in saline, LB or Mueller–Hinton broth (MHB) supplemented with 2–1000 µg mL⁻¹ triclosan. Following incubation for 24 h at 37 °C, cultures were harvested (4500 g for 15 min), diluted 10-fold in saline and plated on LB agar. Colonies were counted after 24 h of incubation at 37 °C.

Effect of triclosan on viability

To determine the effect of triclosan on the viability of biofilm-associated cells, each prewashed biofilm was placed in 2 mL triclosan solution (1000 µg mL⁻¹) for 5, 10, 30 and 60 min at room temperature. Then, the biofilms were washed twice with 10 mL saline, disrupted with glass beads as previously described (Scher *et al.*, 2005) and plated onto LB agar.

Triclosan (final concentration of 1000 µg mL⁻¹) was also added to all the planktonic cells described above. Aliquots were taken at 5, 10, 30 and 60 min, collected by centrifugation, washed and resuspended in saline, diluted 10-fold and

plated. Controls were treated with saline or with saline and ethanol. All experiments were conducted three to five times in duplicate.

RNA isolation, cDNA synthesis and qRT PCR

Stationary MAE52 cells and biofilms were treated with 500 µg mL⁻¹ triclosan for 10 and 30 min as described above. Controls were biofilms and stationary cells treated with 5% ethanol. Treated cells were washed with 10 mL saline, and following disruption of the biofilms with glass beads, all samples were harvested by centrifugation, resuspended in 2 mL saline and were added to 4 mL of RNAprotect Bacteria Reagent (QIAGEN, Valencia, CA). Total RNA was extracted with the RNeasy Mini Kit (QIAGEN). The DNA remaining in the sample was removed with RNase-Free DNase (QIAGEN). cDNA was synthesized from the RNA template (250 ng) using the Reverse-iT 1st-strand synthesis Kit (ABgene) and was diluted 1:5 in water to obtain the working solution.

Real-time PCR for the quantification of *micF*, *acrA*, *marA*, *fabI*, *bcsA*, *bcsE* and *agfB* mRNAs was performed using 4 µL working solution of cDNA, 100 to 250 nM concentrations of each primer (Table 1) and 10 µL AbsoluteTM QPCR SYBR Green Mix (ABgene) in 20 µL total volume. A three-step protocol was used in Rotor-Gene 3000 (Corbett Research): (i) denaturation (15 min at 95 °C); (ii) an amplification and extension program repeated 40 times (1 s at 95 °C, 15 s at appropriate annealing temperature and 20 s at 72 °C); and (iii) a melting curve program of heating from 72 to 99 °C, at a heating rate of 1 °C per 5 s.

The concentrations of the experimental transcripts were calculated from the linear regression of a standard curve and standardized by referring the 16S rRNA gene calculated

concentrations. For each culture, the concentration of the sample that was treated without triclosan was set as 1, and the other samples were calculated proportionally. Analysis was performed twice in triplicates, and the average numbers are shown. No amplification product was obtained when the total RNA was used as a template in PCR reactions using the same primers.

Data analysis

Data were analyzed with MICROSOFT EXCEL version 7, and statistically processed using the One-Way ANOVA method, followed by the Tukey–Kramer test in case of ANOVA showing significance. *P* values < 0.05 were regarded as significant.

Results

Susceptibility to triclosan depends on the growth phase

The MIC for wt, MAE52 and MAE190 in the log phase was 0.5 µg mL⁻¹. For the same three strains, the MBC was 500 µg mL⁻¹, which is 1000-fold higher than the MIC, and was not dependent on the media (saline, LB or MHB).

We compared the capability of triclosan (1000 µg mL⁻¹ i.e. 2 × MBC) to kill log-phase cells, stationary cells, biofilm-associated cells and biofilm-derived cells (Fig. 1). Exposure of the log-phase cells (all three strains) to 1000 µg mL⁻¹ triclosan resulted in at least 8-log reduction of viable cell counts. A significant difference (*P* < 0.05) was observed between the mutant incapable of biofilm formation (6.3-log reduction) and the other two strains (3.6-log reduction) at the stationary phase (Fig. 1). Only a 1-log reduction was observed with regard to the CFU of the cells within the biofilm. However, the biofilm-derived cells, which were treated with triclosan after disruption of the biofilm, had susceptibility to triclosan similar to the stationary cells (3.9-log reduction, Fig. 1). In all cultures, the majority of the cell death was achieved within the first 10 min.

Triclosan up-regulated transcription of specific genes in the biofilm

Quantification of the transcription of *micF*, *acrA*, *marA*, *fabI*, *bcsA*, *bcsE* and *agfB* in biofilm and stationary MAE52 cells before- and after-exposure to a subinhibitory concentration (500 µg mL⁻¹) of triclosan was achieved by qRT PCR. 16S rRNA gene was used to standardize the results as its amount was similar in biofilm and stationary cells and did not change after exposure to triclosan (Fig. 2).

Transcription of *fabI* in stationary cells was fourfold higher than in biofilm-associated cells (*P* < 0.05) (data not shown). Triclosan up-regulated transcription of *fabI* in

Table 1. Oligonucleotides used in this study

Gene	Sequence
<i>micF</i> F/R	GCTATCATCATTAACCTTTATTATTACCGT/ GGGTAAACAGACATTCAGAAGTGA
<i>acrA</i> F/R	CGGTGAATTTACAGGCGTTA/ AAACCTCGAGTGCCGATTT
<i>marA</i> F/R	AAAAGTGTCTGAGCGTTCAGGATATTCCA/ GCGTCCGCCAGATAGAGAATGGGCTCGTT
<i>fabI</i> F/R	CAGCATTGACGCTATGTTT/ TTAACATAATCGCCGTCCAG,
<i>bcsA</i> F/R	AGTTGTAGCCCACCTCATT/ GGAAGGGCAGAAAGTGAATC
<i>bcsE</i> F/R	ATACTCTTTGCCCCACGATT/ TATGCAGGCTTTCTGACGTT
<i>agfB</i> F/R	TGATCTGGCTCGTTCAGAGT/ CTATTATCCGTGCCGACTTG
*16S rRNA gene F/R	CGGGGAGGAAGGTGTTGTG/ GAGCCCGGGATTTCACATC

*Primers of 16S rRNA gene according to Fey et al., (2004).

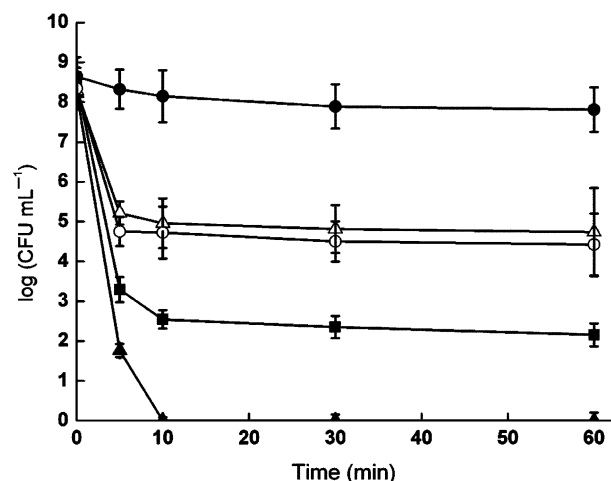


Fig. 1. Survival of *Salmonella typhimurium* wt, MAE52 and MAE190 (biofilm-deficient mutant) upon a 60-min exposure to triclosan ($1000 \mu\text{g mL}^{-1}$) in saline. Log-phase cells of all three strains (▲), stationary cells, wt and MAE52 (△), stationary cells MAE190 (■), biofilm-associated cells MAE52 (●) and biofilm-derived MAE52 (○). Zero values indicate that alive cells were not detected at the minimum level of sensitivity ($< 30 \text{ CFU mL}^{-1}$). The bars represent the SE of the mean.

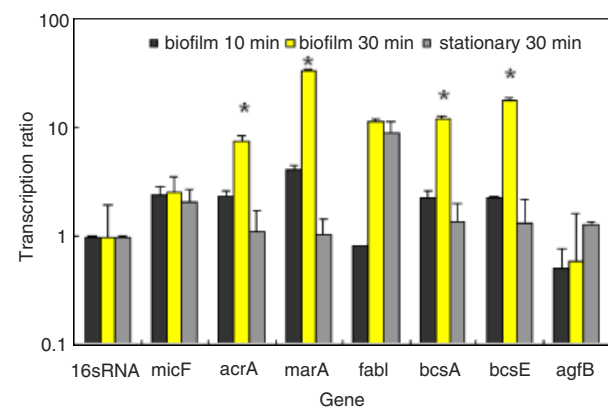


Fig. 2. Induction of 16S rRNA gene, *micF*, *acrA*, *marA*, *fabI*, *bcsA*, *bcsE* and *agfB* in biofilm and stationary *Salmonella typhimurium* MAE52 by $500 \mu\text{g mL}^{-1}$ triclosan. Numbers of all genes (except 16S rRNA gene) represent the ratio of normalized transcription after incubation with triclosan to non treated cells as determined by qRT-PCR. Transcription of 16S rRNA gene was normalized by total RNA taken to the assay. Error bars represent the SE of the mean. Asterisks represent statistical differences between stationary and biofilm-associated cells after a 30-min exposure ($P < 0.05$).

biofilm-associated cells and stationary cells by 10-fold after 30 min (Fig. 2). Triclosan also induced *micF* transcription in both cultures, but just by twofold. Transcription of *marA* and *acrA* was 17-fold lower in biofilm-associated cells ($P < 0.05$) (data not shown). Triclosan did not affect significantly the transcription of *marA* and *acrA* in the stationary cells, but induced the transcription of these genes in the biofilm-associated cells ($P < 0.05$; Fig. 2).

We showed that cells within biofilms were protected from the effects of exposure to triclosan. To elucidate whether triclosan up-regulates *bcsA*, *bcsE* and *agfB*, genes involved in biofilm formation, we compared their transcription levels before and after exposure to triclosan. Increased transcription of *bcsA* and *bcsE* was observed after exposure of biofilm-associated cells to triclosan, while the transcription in stationary cells was not affected. Induction was noticeably higher after 30 min ($P < 0.05$; Fig. 2). A slight but not statistically significant reduction in the *agfB* transcription was observed only in the triclosan-exposed biofilms (Fig. 2).

Discussion

This is the first study that compared the effect of triclosan on the viability of planktonic and biofilm-associated cells, and showed a significant difference between the susceptibility of log-phase *Salmonella* cells, stationary cells and cells within biofilms. Triclosan was efficient in controlling exponentially growing cells, and the MIC ($0.5 \mu\text{g mL}^{-1}$) was in the scale reported for other clinical and environmental bacteria (Randall *et al.*, 2001, 2004; Aiello *et al.*, 2004; Braoudaki & Hilton, 2005). Stationary and biofilm cells, the most widely represented states of microorganisms living in the environment, survived exposure to $1000 \mu\text{g mL}^{-1}$ triclosan ($2000 \times$ MIC), a concentration that was lethal for log-phase cells. Our observation that cells in a biofilm were resistant to triclosan supports the study of Junker & Hay (2004). It can also explain why plastic containing $1500 \mu\text{g mL}^{-1}$ triclosan did not effectively reduce bacterial populations on meat surfaces, whereas the same bacteria were sensitive to triclosan in plate assays (Cutter, 1999). The considerable difference between susceptibility of log-phase and stationary cells was also observed in *E. coli* (Escalada *et al.*, 2005), but was unlike the previous observations in *Enterococcus hirae* and *Staphylococcus aureus* (Suller & Russell, 2000; Escalada *et al.*, 2005), supporting the hypothesis that the inhibitory activity of triclosan against *E. coli* and *Salmonella* may result from multiple mechanisms and not from just targeted action against the bacterial cytoplasmic membrane (McMurry *et al.*, 1998b; Levy *et al.*, 1999; Heath & Rock, 2000).

The triclosan sensitivity of the biofilm-derived cells was identical to that of stationary cells, suggesting that the matrix has a significant role in the resistance of biofilm-associated cells probably via reduction of diffusion. The low activity of triclosan attributed to low diffusion was also reported for biofilm-associated oral *Streptococcus mutans* (Steinberg *et al.*, 2006). Furthermore, curli or cellulose, the main components of the biofilm matrix, might also have a protective effect on planktonic *Salmonella* via diffusion into the cells from the broth, as the mutant MAE190, which has deletions in the genes coding for curli and cellulose synthesis, was considerably more sensitive to triclosan

compared with the other strains. This was not seen when we treated the same *Salmonella* mutant with chlorine (Scher *et al.*, 2005).

The lower triclosan susceptibility of stationary and biofilm-associated cells compared with log-phase cells suggests that the cells probably adapted to triclosan by an alteration of gene expression or by changes in the permeability of triclosan through the envelope, and not by mutations. The effect of subinhibitory concentrations of triclosan on expression of genes has not been studied before. Based on previous reports about triclosan activity and about strains that developed resistance to triclosan (McMurry *et al.*, 1998a; Heath *et al.*, 1999; Levy *et al.*, 1999; Braoudaki & Hilton, 2004, 2005), we chose to focus on specific genes that might be activated by triclosan during the adaptation process. We hypothesized that as FabI is the triclosan target, *Salmonella* cells would increase the FabI synthesis after exposure to triclosan, in order to eliminate the inhibition of fatty acid synthesis. Indeed, *fabI* transcription was induced in response to triclosan by stationary and biofilm-associated cells. This is an observation that, to the best of our knowledge, has not been described previously.

The transcription of *marA* and *acrA* was investigated, as mutants with decreased susceptibility to triclosan showed higher expression of broad-spectrum efflux pumps like AcrAB (McMurry *et al.*, 1998a; Schweizer, 1998, 2001b; Braoudaki & Hilton, 2005). Other potential changes in the outer membrane that might affect the permeability like alteration in LPS were not observed during exposure of *Salmonella* to triclosan (Braoudaki & Hilton, 2005). We found that triclosan increased the transcription of *acrAB* and its activator *marA*, adding triclosan to the antimicrobial agents that induce *marA* like tetracycline (Seoane & Levy, 1995). However, this induction was just in biofilms. The *marA* gene encodes for MarA, a global regulator that controls the expression of more than 60 genes, many of which have a role in bacterial stress response including *acrAB* (Barbosa & Levy, 2000). If we assume that a higher transcription of the genes *acrAB* and *marA* is directly correlated with a higher expression of these proteins, and given that the overexpression of MarA, AcrAB and *micF* has also been linked to the increased resistance to many different antibiotics, organic solvents and disinfectants (Alekhshun & Levy, 1997, 1999; Yaron *et al.*, 2003), we propose that within the biofilm, there is a relationship between the use of triclosan and antibiotic resistance.

Thus, triclosan and antibiotics not only share multidrug efflux systems as a common mechanism of resistance, but triclosan also induces expression of these efflux pumps. However, the up-regulation of *acrAB* and *marA* by triclosan in the biofilm will probably mediate resistance only to low concentrations of antibiotics, because it was shown that expression of AcrAB only protected *E. coli* in biofilms

against low concentrations of ciprofloxacin (Maira-Litran *et al.*, 2000).

The extracellular matrix produced by *Salmonella* in the biofilm is composed of proteinaceous components and exopolysaccharides. The main exopolysaccharide is cellulose, which is produced and secreted by enzymes encoded in the *bcsABZC* and *bcsEFG* operons (Zogaj *et al.*, 2001; Solano *et al.*, 2002). In *Salmonella*, the *bcs* genes are constitutively transcribed (Zogaj *et al.*, 2001). Enhanced cellulose biosynthesis via transcriptional up-regulation of the structural genes *bcsA* and *bcsE* by triclosan in biofilm-associated cells might contribute to extended biofilm formation. It should be noted, however, that triclosan did not induce the *bcs* genes in planktonic cells, but only in cells that were already within the biofilm matrix. Induction of biofilm formation by aminoglycosides antibiotics has been observed in *Pseudomonas aeruginosa* and *E. coli* (Hoffman *et al.*, 2005), but to the best of our knowledge has not been observed for biocides.

We demonstrated that the concentrations used in consumer products (usually ranging from 600 to 20 000 $\mu\text{g mL}^{-1}$) (Suller & Russell, 1999) should be effective for the prevention of growth, but might not be effective in killing *Salmonella*, particularly not within biofilms. This underscores the need for a revision of the issue of the antimicrobial efficacy of triclosan against microorganisms in biofilms when triclosan is incorporated into products such as soaps, dishwashing liquids, food storage containers and other kitchen utensils and medical devices. Two mechanisms contributed to the enhanced resistance of the biofilm-associated cells: the presence of the exopolysaccharide matrix that probably reduced diffusion, and specific changes in transcription of genes. These changes exhibit a strong potential to enhance the resistance of biofilm-associated cells to low concentrations of other antimicrobials, an issue that should be further investigated in the future.

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