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Mutations Associated with Pyrazinamide Resistance in *pncA* of *Mycobacterium tuberculosis* Complex Organisms

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A gene (*pncA*) with mutations associated with pyrazinamide resistance in *Mycobacterium tuberculosis* complex members was characterized in 67 pyrazinamide-resistant and 51 pyrazinamide-susceptible isolates recovered from diverse geographic localities and anatomic sites and typed by IS*6110* profiling. All pyrazinamide-susceptible organisms had identical *pncA* alleles. In striking contrast, 72% of the 67 resistant organisms had *pncA* mutations that altered the primary amino acid sequence of pyrazinamidase. A total of 17 previously undescribed mutations were found, including upstream mutations, missense changes, nucleotide insertions and deletions, and termination mutations. The mutations were arrayed along virtually the entire length of the gene. These data are further evidence that most drug resistance in *M. tuberculosis* is due to simple mutations occurring in chromosomally encoded genes rather than to acquisition of resistance genes by horizontal transfer events.

Pyrazinamide (PZA) is a nicotinamide analog that has been used for almost 50 years as a first-line drug to treat tuberculosis (13, 32). PZA is bactericidal to semidormant mycobacteria (7) and reduces total treatment time (6, 15). Although the exact biochemical basis of PZA activity in vivo is not known, under acidic conditions it is thought to be a prodrug of pyrazinoic acid, a compound with antimycobacterial activity (24).

The finding that PZA-resistant strains lose amidase (pyrazinamidase and/or nicotinamidase) activity and the hypothesis that amidase is required to convert PZA to pyrazinoic acid intracellularly (13) led to the recent cloning and characterization of the *Mycobacterium tuberculosis* gene (*pncA*) encoding pyrazinamidase (23). DNA sequencing showed that four of four PZA-resistant clinical isolates had *pncA* mutations thought to confer resistance. Three of these alterations were missense substitutions (affecting amino acids 63, 138, and 145), and the fourth change was a deletion of nucleotide 162 that led to production of a nonfunctional truncated polypeptide. Moreover, all six *Mycobacterium bovis* isolates studied had the same single C→G point mutation at nucleotide 169, resulting in an H57D substitution. This amino acid substitution was thought to be the molecular basis for PZA resistance in *M. bovis*, a species naturally resistant to this antimicrobial agent.

In an effort to further understand the molecular basis of PZA resistance in *M. tuberculosis*, we analyzed the DNA sequence of *pncA* in a diverse sample of PZA-resistant and -susceptible clinical isolates. All susceptible isolates had the identical wild-type *pncA* sequence. In striking contrast, the analysis identified 17 previously undescribed mutations that alter the primary amino acid sequence or result in other PncA changes and are confined to PZA-resistant organisms (23). These data confirm and extend previous findings (23) and are further evidence that most antibiotic resistance in *M. tuberculosis* is due to point mutations occurring in chromosomally encoded genes.

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MATERIALS AND METHODS

Bacterial strains. Sixty-seven PZA-resistant and 51 PZA-susceptible *M. tuberculosis* isolates recovered from diverse geographic localities and anatomic sites were studied. Virtually all strains were characterized for their IS*6110* subtypes by an internationally standardized protocol (28) after growth to confluence on Lowenstein-Jensen medium. The collection included organisms representing the range of IS*6110* fingerprint diversity found in the species (25). In addition, the isolates included members of three genetic categories to which all *M. tuberculosis* isolates can be assigned based on sequence polymorphisms in *katG* and *gyrA* (27). The sample also included isolates assigned to the so-called W family of IS*6110* types (1). This group of genetically related organisms has caused widespread disease in New York City and has recently been disseminated to other localities in the United States and Europe (1). The sample also included PZA-resistant and -susceptible strains assigned to the same IS*6110* subtype. Ten isolates of *M. bovis* recovered from animals (cattle and deer) and humans were also analyzed. One isolate each of *Mycobacterium microti* and *Mycobacterium africanum* was studied to represent the other two *M. tuberculosis* complex species. Mycobacterial culturing and DNA isolation were conducted in biosafety level 3 laboratories.

Automated DNA sequencing of the *pncA* locus. A 630-bp region that includes the entire open reading frame of *pncA* and 82 bp of upstream putative regulatory sequence was analyzed in all 67 PZA-resistant and 13 of 51 PZA-susceptible isolates. This region was sequenced by first amplifying a 673-bp region with the following oligonucleotide primers: 5′-GCTGGTCATGTTCGCGATCG-3′ (forward) and 5′-CAGGAGCTGCAAACCAACTCG-3′ (reverse). A 488-bp region (nucleotides 30 to 518) of *pncA* was analyzed for 38 of the 51 PZA-susceptible organisms. This region was sequenced by first amplifying a 558-bp segment of the entire open reading frame of *pncA* with the following oligonucleotide primers: 5′-ATGCGGCGTTGATCATCG-3′ (forward) and 5′-CAGGAGCTGCAAACCAACTCG-3′ (reverse). A GeneAmp System 9600 thermocycler (Perkin-Elmer Corp., Foster City, Calif.) was used for target amplification with the following parameters: an annealing step at 60°C for 21 s, extension at 72°C for 15 s, and a denaturation step at 94°C for 21 s. Each reaction was preceded by an initial denaturation step at 94°C for 60 s and terminated with a final extension step at 72°C for 5 min. DNA sequencing reactions were performed with the *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, Calif.) by using the forward and reverse primers described above. An internal primer (5′-ATTCCTCGTCGTGGCCACC-3′) was used to sequence the 3′ end of the *pncA* open reading frame for the 67 PZA-resistant isolates. Sequence data generated with an ABI 377 automated instrument were assembled and edited electronically with the EDITSEQ, ALIGN, and MEGALIGN

TABLE 1. *pncA* nucleotide and amino acid changes in PZA-resistant *M. tuberculosis*

IS*6110* subtype(s)[a]	No. of isolates	Change(s) in:	
		Nucleotide sequence	Amino acid
001	1	A→G at 139	T47A
	2	C→G at 169	H57D
	1	G→T at 203	W68L
	1	T insertion at 397	
	1	T→C at 416	V139A
	1	A→G at −11	
BP	1	A→G at −11	
AB	1	G→C at 362	R121P
ED2	1	C insertion at 403	
DP	2	A→G at 410	H137R
H	7	ΔG at 70	
	1	T→C at 353	W119R
M	1	C→G at 123	Y41STOP
N	2	T→C at 280; T→C at 281	F94P
N2 and N6	4	AGGTCGATG insertion at 388	
P and P3	8	T→C at 254	L85P
W and W family[b]	10	A→G at 139	T47A
W12	1	T→C at 56	T76P
W18	1	A→C at 226	P54T
W22	1	C→A at 160	L19P

[a] Arbitrary designations assigned by the Tuberculosis Laboratory, Public Health Research Institute, New York, N.Y.

[b] Includes isolates typed as W (n = 2), W1 (n = 2), and W8, W6, W31, W13, W25, and W17.

programs (DNASTAR, Madison, Wis.) and compared with a published sequence (23) for *pncA* (GenBank accession number U59967).

PZA susceptibility testing. Isolates were tested for PZA susceptibility with either the BACTEC radiorespiratory method (8, 22) or the conventional proportion method with a PZA concentration of 25 μg/ml in 7H10 agar (pH 5.5) (3).

RESULTS

***pncA* sequences in PZA-susceptible organisms.** All 51 PZA-susceptible isolates studied had identical wild-type *pncA* sequences.

***pncA* mutations in PZA-resistant *M. tuberculosis*.** Compared to a reference *pncA* sequence (23), all 130 *M. tuberculosis* complex isolates analyzed had a C→T transversion at nucleotide 69. This synonymous change is an error in the deposited sequence and will not be discussed further.

Among the 67 PZA-resistant *M. tuberculosis* isolates studied, 19 (28%) organisms lacked nucleotide sequence changes in *pncA*. Thirty-one organisms, representing 21 distinct IS*6110* subtypes, had mutations that resulted in single amino acid substitutions in PncA (Table 1). Four strains had a 9-bp insertion beginning at nucleotide 388. Seven strains with the same IS*6110* subtype had a deletion of a guanine residue at nucleotide 70. This mutation resulted in production of a truncated polypeptide due to the creation of a stop codon downstream from the deletion. Two organisms had single base pair insertions, at nucleotide 397 in one and at nucleotide 403 in the other, and one isolate had a mutation that created a stop codon. Two organisms had an upstream mutation at nucleotide −11, resulting in an A-to-G change (Fig. 1).

A total of four distinct missense mutations were identified among PZA-resistant isolates assigned to the IS*6110* W family (1) (Fig. 2). One organism typed as W22 had a CTG→CCG (L→P) change in codon 19. An isolate with the W12 type had an ACT→CCT mutation resulting in a T76P substitution, and a W18 isolate had a CGG→ACG (P54T) change. All other PZA-resistant W family members had identical ACC→GCC nucleotide substitutions, which produced a T47A amino acid replacement (Fig. 2).

Mutations in PZA-resistant isolates assigned to other IS*6110* types. To test the idea that other PZA-resistant isolates which were grouped based on their IS*6110* profiles had the same *pncA* mutation, we examined six isolates assigned to IS*6110* type P and two isolates with related type P3. These organisms are multidrug resistant and have caused tuberculosis cases in New York City (1). All eight isolates had identical L85P amino acid substitutions caused by a CTG→CCG mutation. Similarly, two PZA-resistant IS*6110* type N isolates had the same F94P replacement caused by a dinucleotide change (TTC→CCC). Interestingly, four isolates considered variants of this IS*6110* type (N2) lacked the F94P substitution but had a 9-bp insertion between nucleotides 378 and 379. This inser-

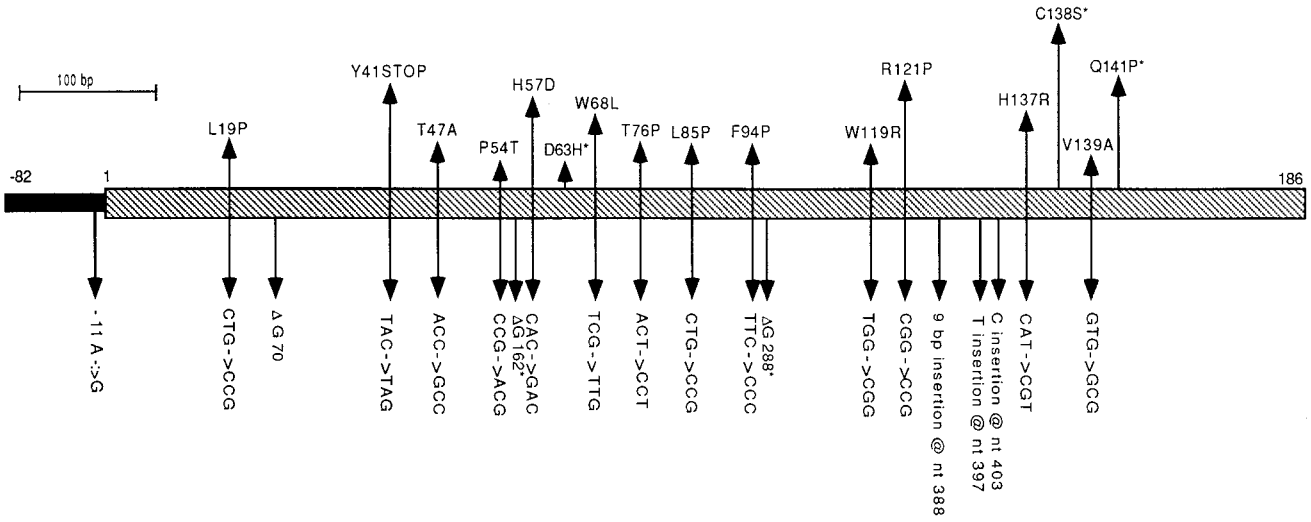

FIG. 1. Representation of mutations associated with PZA resistance in the 558-bp *pncA* gene of *M. tuberculosis* complex members. The nucleotide change is shown below the schematic, and the corresponding structural alteration is displayed above. Asterisks denote changes previously described in reference 23. nt, nucleotide.

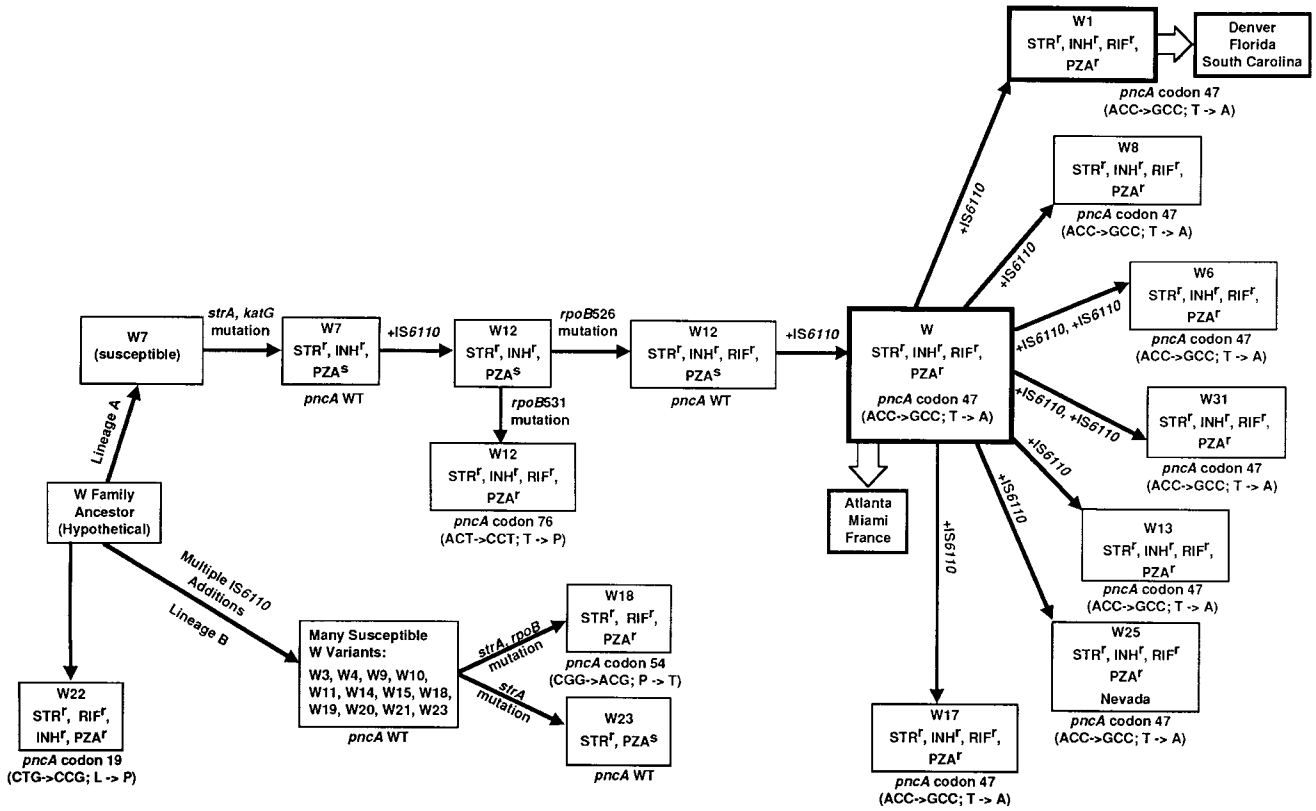

FIG. 2. Schematic of the distribution of *pncA* alleles among isolates assigned to the W clone family of *M. tuberculosis*. The figure is modified from a similar one published recently (1). The schematic shows a plausible pathway to explain the molecular evolution and distribution of W clone family members; it also illustrates that PZA resistance arose multiple times, independently, among members of the family. Importantly, all organisms hypothesized to be derivatives of the W type (W1, W8, W6, etc.) have identical codon 47 alterations (ACC→GCC; T→A), a result strongly consistent with the hypothesis that these organisms are related by recent descent.

tion appears to have occurred due to slipped-strand mispairing (14). Two isolates with different IS*6110* subtypes had a transversion from A to G at nucleotide −11 upstream of the *pncA* start codon. This mutation would potentially alter the promoter strength and result in a *pncA* down regulation. Finally, two isolates classified as IS*6110* type DP had an H137R alteration caused by a CAT→CGT substitution.

Sequences of PZA-susceptible and -resistant organisms with the same IS*6110* type. PZA-susceptible and -resistant organisms were available for two IS*6110* types, W12 and H. For both types, PZA-resistant organisms had mutations that would alter the primary amino sequence of PncA. In contrast, the PZA-susceptible isolates contained the wild-type *pncA* allele.

***pncA* in other *M. tuberculosis* complex members.** Scorpio and Zhang (23) identified a substitution (C→G) in nucleotide 169 of *pncA* that occurred only among the six *M. bovis* isolates they studied. We analyzed the *pncA* sequences of 10 *M. bovis* isolates, including strains with one to eight IS*6110* copies. Eight strains had the C→G substitution at position 169, but two strains lacked this change. The isolates of *M. africanum* and *M. microti* analyzed had identical wild-type *pncA* sequences.

DISCUSSION

Common theme of simple nucleotide changes resulting in single amino acid replacements. Most resistant organisms with *pncA* changes had nucleotide substitutions resulting in single amino acid replacements. This discovery extends the common observations that have emerged from the study of antimicrobial agent resistance in *M. tuberculosis* (18, 27a). For example, rifampin resistance in 96% of these organisms is attributable to substitutions occurring in a 27-amino-acid region of the beta subunit of RNA polymerase encoded by *rpoB* (11, 18). Similarly, many isoniazid-resistant organisms have single amino acid changes in the enzyme catalase-peroxidase, encoded by *katG* (18, 20). Our data also confirm and extend earlier findings reported by Scorpio and Zhang (23), who identified three clinical isolates with PZA resistance attributable to individual amino acid replacements at positions 63, 138, and 141.

It is noteworthy that approximately 40% of all distinct amino acid substitutions found in this and an earlier study (23) involved replacement with a proline residue (Fig. 1). These changes would be expected to significantly alter protein structure if located in alpha-helical areas, thereby enhancing the likelihood that enzyme function would be detrimentally affected. Although it is a formal possibility that the preponderance of these proline substitutions occurred based merely on codon utilization, this explanation seems unlikely because no unusually increased frequency of recovery of proline substitutions was noted among KatG mutants in isoniazid-resistant *M. tuberculosis* strains (20).

Identification of an apparent slipped-strand mispairing event in a PZA-resistant organism. Four resistant isolates with the same or closely similar IS*6110* types each had a 9-bp insertion that apparently arose due to a slipped-strand mispairing event. Apparent slipped-strand mispairing events have also

been identified relatively rarely in the *rpoB* gene in rifampin-resistant isolates (11) and in the *katG* gene in isoniazid-resistant bacteria (20). Slipped-strand mispairing processes are a major mechanism for mediating DNA sequence evolution (14) and bacterial virulence factor variation (17, 19). As noted above, this mechanism also provides a convenient strategy for altering the structures of proteins participating in antimicrobial agent interaction and thereby inducing drug resistance.

Occurrence of nucleotide deletions. Our data and those reported elsewhere (23) show that single nucleotide changes resulting in frameshift mutations can be associated with PZA resistance. The occurrence of one of these deletions at nucleotide 70 strongly suggests that the affected organism can maintain viability in the complete absence of pyrazinamidase activity. Although we did not measure enzyme function, Scorpio and Zhang (23), using the semiquantitative method described by Wayne (29), found a lack of pyrazinamidase production by a resistant clinical isolate with a deletion of guanine 162.

Large numbers of distinct mutations associated with resistance. Our analysis found that a large number of distinct *pncA* mutations are associated with PZA resistance and that these changes are distributed throughout virtually the entire length of the gene. These data imply that enzymatic activity can be altered by a wide variety of mechanisms. In the absence of a three-dimensional crystal structure, it is impossible to confidently predict how all of the structural changes identified would be expected to result in altered enzyme function. Several studies have documented a nonrandom relationship between distinct structural changes in drug targets and the level of antimicrobial agent resistance (2, 4, 16, 21, 31). Hence, it will clearly be important to perform analogous studies with the battery of strains containing the precisely defined mutant alleles identified in this study.

Utility of automated DNA sequencing to differentiate between PZA-susceptible and -resistant organisms. The identification of a broad spectrum of *pncA* mutations associated with resistance, coupled with their wide distribution in the *pncA* gene and the lack of nucleotide changes in susceptible organisms, means that the automated DNA sequencing strategy used in this study is ideal for differentiating between PZA-susceptible and -resistant *M. tuberculosis* strains. This strategy is rapid and unambiguous, does not employ radioactivity, and can be used to analyze large numbers of isolates, all of which make the method suitable to a clinical microbiology or other diagnostic laboratory. Moreover, because our analysis found that distinct PZA-resistant clones were characterized by the same mutation, the resulting data can be used to infer epidemiologic and phylogenetic relationships. In addition, we have shown elsewhere that this strategy can readily be used to predict the *M. tuberculosis* antimicrobial agent susceptibility phenotype with growth obtained in early BACTEC cultures (10, 21a, 25).

Lack of synonymous substitutions. The identification of only a single synonymous mutation in *pncA* in the 130 strains characterized adds to data (12) documenting the remarkable paucity of silent nucleotide changes among members of the *M. tuberculosis* complex recovered from global sources. As noted elsewhere (12), the most parsimonious explanation for this observation is that the organisms shared a recent ancestor, perhaps less than 20,000 years ago.

***M. bovis* and other *M. tuberculosis* complex members.** It has long been speculated (30) that *M. tuberculosis* sensu stricto arose from *M. bovis*. If this idea is correct, it implies that the ancestral condition is the occurrence of Asp57 rather than the His57 identified among the other three named species of the *M. tuberculosis* complex. Moreover, it implies that defective pyrazinamidase activity and PZA resistance were the ancestral conditions in the complex. *M. tuberculosis* sensu stricto arose well before the introduction of PZA into widespread clinical use in humans, which means that PZA use was not a factor bearing on the origin of the amino acid 57 polymorphism. Unlike Scorpio and Zhang (23), we identified two *M. bovis* isolates that had His57 rather than the anticipated Asp57. Further analysis of these two organisms for molecular markers reported to differentiate among members of the *M. tuberculosis* complex (5, 26) indicated that isolates of *M. bovis* with PncA His57 exist. Hence, differentiation of *M. bovis* from *M. tuberculosis* sensu stricto based on the polymorphism in *pncA* codon 57 is not 100% specific, as suggested by the earlier analysis (23).

The existence of *M. bovis* with His57 rather than Asp57 also suggests an alternative evolutionary hypothesis in which it is not necessary to postulate that the ancestral condition was an organism defective in pyrazinamidase function. In the alternative hypothesis, *M. bovis* with His57, or an unidentified related organism with His57, was ancestral. This organism was then transmitted to humans, developed other traits associated with *M. tuberculosis* sensu stricto, and became abundant globally. From the His57 *M. bovis* progenitor there arose the Asp57 lineage that has defective pyrazinamidase activity.

Lack of *pncA* changes in PZA-resistant organisms. Our analysis found that 28% of PZA-resistant organisms lacked changes in the entire 558-bp open reading frame and in an 85-bp upstream region of *pncA*. Notwithstanding the technical problems associated with PZA susceptibility testing and relatively poor interlaboratory concordance (9), the failure to document a *pncA* mutation in all resistant organisms implies the existence of at least one additional gene participating in PZA resistance. Additional studies are under way to identify the molecular basis for PZA resistance in organisms lacking *pncA* mutations.

Strains of the W family of organisms. The W family of *M. tuberculosis* organisms was originally identified based on IS*6110* profiling (1). This group of organisms gained national prominence because several of the members are resistant to all first-line antituberculosis agents and several second-line drugs (1). Recently, with data obtained from IS*6110* profiling, automated DNA sequencing of several genes with drug resistance-conferring mutations, and other strategies, a scenario to explain the molecular evolution of these organisms was advanced (1). Central components of the evolutionary pathway were the existence of two major lineages and an IS*6110* type W clone that spawned a series of derivative subclones, all marked by identical complex arrays of resistance-conferring mutations. All *pncA* data generated in the present study strongly support the evolutionary pathway advanced earlier (1). Importantly, the occurrence of identical *pncA* mutations (ACC→GCC; T47A) clearly demonstrates that PZA resistance probably arose once in a W progenitor that had already acquired resistance to rifampin, isoniazid, and streptomycin (Fig. 2).

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