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Isolation and characterization of a chlorpyrifos-degrading bacterium from agricultural soil and its growth response

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A soil bacterium capable of utilizing chlorpyrifos as sole carbon source was isolated by selective enrichment on mineral medium containing chlorpyrifos. The bacterial isolate, designated MS09, was identified and characterized as a strain of *Providencia stuartii* based on biochemical characteristics and 16S rRNA sequence analysis. Growth studies showed that *P. stuartii* strain MS09 utilized chlorpyrifos to grow in Luria-Bertani broth containing different concentrations of chlorpyrifos at 50 -700 mg/L. However, the optimum concentration that supported bacterial growth over 24 h was found to be 50 - 200 mg/L chlorpyrifos. When compared with the control, a significant increase in bacterial growth was noted at a low concentration of chlorpyrifos (50 mg/L), whereas at higher concentrations (300 - 700 mg/L) an increased lag phase was observed, without inhibiting growth of the pesticide- utilizing bacterium. A literature survey revealed that no data is available regarding the role of *P. stuartii* on pesticide biodegradation.

Key words: Pesticide, chlorpyrifos, *Providencia sp.* (MTCC No 8099), biodegradation.

INTRODUCTION

The application of pesticides for pest control in rice-based cropping systems is general practice in India. Insecticides are the dominant group of pesticides used in most rice-growing countries like India. Since pesticides are very toxic by design, they have the potential to adversely impact on ecosystem health. Organophosphorous (OP) insecticides such as parathion, methamidophos and chlorpyrifos are a group of highly toxic agricultural chemicals widely used in plant protection. As these pesticides cause extensive damage to non-target organisms, studies regarding their degradation have received considerable attention from soil microbiologists. Several reports suggest that contamination of soil by these pesticides as a result of their bulk handling at the farm yard or following application in the field or accidental release may lead to occasional contamination of a wide range of water and terrestrial ecosystems (Singh et al., 2004). Extensive use of chlorpyrifos contaminates air, ground water, rivers and lakes. The contamination has been found up to about 24 km away from the point of use, Chlorpyrifos-oxon and 3,5,6-trichloro-2-pyridinol (TCP) are the two potent transformation products of chlorpyrifos, have been found in groundwater. Such contamination can led to the insecticides and their transformation products are being transported long distances (Bhagobaty et al., 2007).If pesticides are not degraded or detoxified rapidly enough, the risk of their off-site migration may pose a health risk to humans. Increasing awareness of the potential adverse effects of pesticides has resulted in greater public pressure to ass -ess, monitor and minimize off-site impacts. Soil bacteria that utilize several pesticides have been isolated from the soil. They include a metamitron-degrading *Rhodococcus sp*. (Parekh et al., 1994), as well as chlorpyrifos-degrad-ing *Flavobacterium sp*. (ATCC 27551), *Pseudomonas di-minuta* strain (Gm) and *Pseudomaonas putida* (Sethuna-than and Yoshida, 1973; Rani and Kumari, 1994). Pestici-des in soil and water can be degraded by biotic and abio- tic pathways; however, biodegradation by microorganism

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is the primary mechanism of pesticide breakdown and detoxification in many soils. Thus, microbes may have a major effect on the persistence of most pesticides in soil.

Chlorpyrifos [O,O–diethyl O–(3,5,6- trichloro-2- pyridyl) phosphorothioate] is used worldwide as an agricultural insecticide. Chlorpyrifos (cpf) is characterized by P-O-C linkages as in other organophosphorous pesticides such as parathion and diazinon. Several attempts have been made to isolate chlorpyrifos-degrading bacteria from agricultural soil, albeit not successful (Mallick. et al., 1999; Racke et al., 1990). However, chlorpyrifos has been shown to be degraded co-metabolically in liquid media by bacteria (Horne et al., 2002; Mallick et al., 1999) and various organophosphorous-degrading genes have been isolated from different microorganisms from different geographical regions, some of which have been shown to hydrolyze chlorpyrifos (Horne et al., 2002; Mulbry et al., 1986; Richins et al., 1997; Serdar et al., 1982). Enhanced degradation of chlorpyrifos by *Enterobacter* strain B-14 was reported by Singh et al. (2004). Yang et al. (2005) isolated *Alkaligenes faecalis* DSP3, which is capable of degrading chlorpyrifos and 3, 5, 6-trichloro-2- pyridinol (TCP). Six chlorpyrifos-degrading bacteria were isolated using chlorpyrifos as the sole carbon source by an enrichment procedure (Yang et al., 2006). The main objective of the present study involved the isolation and identification of chlorpyrifos-utilizing bacteria from agricultural soil using an enrichment culture technique, and to assess the growth response of one of the isolates in liquid medium containing different concentrations of chlorpyrifos.

MATERIALS AND METHODS

Pesticide used

Commercial-grade insecticide chlorpyrifos (20% E.C.) was obtained from the Regional Agricultural Research Station, Tirupati A.P, India. It was used throughout the experimental studies, because it may more closely resemble the active compound that microorganisms are likely to be exposed to in the soil environment.

Media

Mineral salts medium (MSM) enriched with chlorpyrifos was used for isolation and characterization of chlorpyrifos-degrading bacteria. The carbon source in MSM was replaced with chlorpyrifos. The MSM has the following composition in (g/L): KH_2PO_4, 4.8; K_2HPO_4, 1.2; NH_4NO_3, 1.0; $MgSO_4.7H_2O$. 0.2; Ca $(NO_3)_2.4H_2O$, 0.04; and Fe $(SO_4)_3$, 0.001 with pH 7.0 (Rasul et al., 1988). Luria-Bertani (LB) broth (Sambrook et al., 1989) and mineral agar were used according to manufacturer's instructions (HI-MEDIA).

Isolation and taxonomic characterization of chlorpyrifos-degrading bacteria

Soil bacteria capable of degrading chlorpyrifos were isolated from agricultural soil of the Chittoor District, Andhra Pradesh, India, which had an almost 10-year history of chlorpyrifos use in pest control activities. Air-dried and sieved (<2 mm) soil samples (10 g) from farm lands (rice cultivated soil) were collected and suspended in 250-ml Erlenmeyer flasks containing 50 ml of mineral salts med-ium (Rasul et al., 1988) supplemented with chlorpyrifos (50 mg/L). The flasks were incubated on a rotary shaker at 250 rpm for 7 days at 30^0 C. At periodic intervals, a loopfull of bacterial growth from the flasks were streaked onto mineral agar supplemented with chlorpyrifos (50 mg/L) and the plates were incubated at 37^0 C for 24 h. The individual bacterial colonies that grew on the medium were subcultured onto mineral agar containing chlorpyrifos of the same concentration until pure cultures were obtained. Bacterial isolates grown on chlorpyrifos-containing agar were subjected to morphological, cultural and biochemical studies. The 16S rRNA gene of one of the bacterial strains, namely MTCC No. 8099 that displayed maximum pesticide utilization potential, was partially sequenced. The nucleotide sequences were used for BLAST analysis against the NCBI data base to obtain related sequences of related organisms. These sequences were aligned using CLUSTALW and a phylogenetic tree was constructed using the PHYLIP analysis programme. Sequencing of the 16S rRNA was carried out by Institute of Microbial Technology, Chandigarh, India.

Viable cell count determinations

Aliquots (2.5 ml) of 24-h old bacterial cultures grown in mineral salts medium were inoculated into 100-ml Erlenmeyer flasks containing 25 ml of Luria- Bertani broth supplemented with various concentrations (50 - 700 mg/L) of chlorpyrifos to test their ability to degrade the supplemental substrate (pesticide). A control was maintained with equal volume of broth containing bacterial culture, but without pesticide. Bacterial growth was followed by viable cell counts immediately after inoculation at 0 h and 2, 4, 6 and 24 h of incubation. A bacterial inoculum (1 ml) was drawn at regular intervals from the test and control cultures and serial dilutions were performed using 9 ml of sterile saline (0.85% NaCl; pH 7). Appropriate dilutions were plated in triplicate on nutrient agar and the plates incubated at 37^0 C for 24 h. The bacterial colonies were counted (CFU/ml) with a colony counter, as described by Collins and Lynes (1985).

RESULTS

Identification and taxonomic characterization of chlorpyrifos-degrading bacteria

Four morphologically distinguishable bacterial colonies were observed on mineral salts agar enriched with chlorpyrifos pesticide. Morphological, cultural and biochemical studies were carried out and are listed in the Table 1. The four isolates were identified as *P. stuartii, Serratia marcescens, Klebsiella oxytoca* and *Bacillus subtilis*, respectively, according to Bergey's Manual of Systematic Bacteriology (Vol I and II; Palleroni, 1984). One of the four bacterial isolates that grew most rapidly and luxuriously and displayed the highest chlorpyrifos-hydrolyzing capability (Figure 1) was selected, and further characterized by partial sequencing of the 16S rRNA gene.

On the basis of morphological and biochemical characteristics (Table 1), the bacterial isolate, designated MS09, was identified as a member of the genus *Providencia*. This was further confirmed by partial sequencing of the 16S rRNA gene (733 bp) and BLAST analysis, the results of which showed more than 99% similarity with three

Table 1. Biochemical characteristics of chlorpyrifos-degrading strains isolated from soil of rice fields of the Chittoor District, AP, India.

Test	I	II	III	IV
Gram's reaction	-	-	-	+
Cell shape	Small rods	Short rods	Short rods	Rodshaped
Spores	-	-	-	+
Motility	+	+	-	+
Indole test	+	-	+	-
Methyl red	+	-	-	-
Voges-Proskauer test	-	-	+	+
Catalase	+	+	+	+
Citrate utilization	+	+	+	+
Oxidase	-	-	-	-
Urease	-	-	+	-
Nitrite reduction	+	+	+	+
Casein and starch hydrolysis	-	-	-	+
Gelatin and Tween-60 hydrolysis	-	+	-	+
Esculin hydrolysis	-	+	+	+
Growth on MacConkey agar	+ NLF*	+	+ LF**	ND
Ornithine decarboxylase	-	+	-	-
Phosphatase	+	+	+	+
Acid from glucose, sucrose	+	+	+	+
Gas from glucose	-	+	+	-
Growth (temperature15^{0}C–42^{0}C)	+	+	+	+
Growth (NaCl 2-5%)	+	+	+	+
Growth (pH 8-10.5)	+	+	+	-
Utilization of glucose and adonitol as carbon source	+	+	+	+(glucose) - (adonitol)
Utilization of L-glutmic acid, L-phenylalanine, L-valine, L-histidine as nitrogen source	+	+	+	ND

Note: Values represented in the table are means of three replicates. I = *Providencia stuartii* MS09. II = *Serratia marcescens*, III = *Klebsiella oxytoca*, IV = *Bacillus subtilis*. Non Lactose Fermentor; ** Lactose Fermentor. ND=Not detected

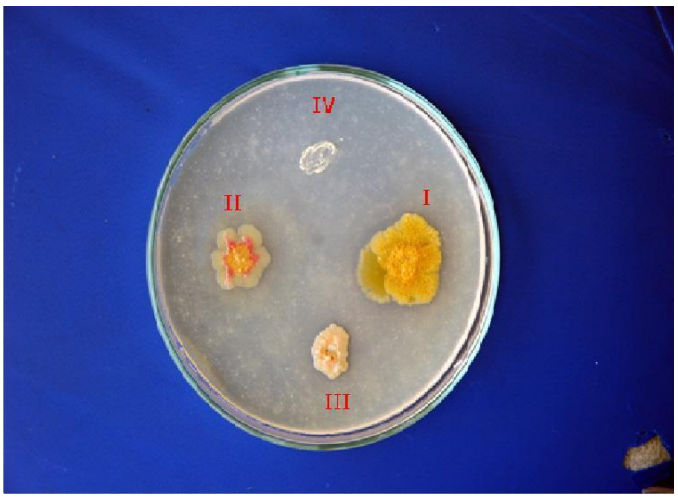

Figure 1. Screening of potential chlorpyrifos-degrading bacteria on mineral salts agar. **I =** *Providencia stuartii* MS09 **II** = *Serratia marcescens*, **III** = *Klebsiella oxytoca*, **IV** = *Bacillus subtilis*.

species of *Providencia.* These three species show similarity in biochemical characteristics and as a result, it was difficult to assign isolate MS09 to a particular species (Figure 2 and Table 2). Against this background and based on additional tests, it was decided to name the isolate as *P. stuartii* strain MS09. To our knowledge, this is the first report of a *Providencia* sp. that is capable of degrading chlorpyrifos pesticide.

Growth kinetics of *Providencia stuartii* strain MS09

Growth curve experiments were performed with different doses of chlorpyrifos in order to determine the optimum concentration of chlorpyrifos that stimulates the growth of the *P. stuartii* strain MS09*.* A Similar strategy has been reported previously by (Mishra et al., 2002; Karpouzas and Walker, 2000; Lee et al., 1998; Smith 1995; Haugland et al., 1990) while performing pesticide degradation using isolated strains of microorganisms.

Table 2. Accession numbers as used in the phylogenetic tree together with the identity of the bacterial strains.

Accession number	Identity of bacterial strain
AY870456.1	*Providencia sp. UT DM314*
AM040495.1	*Providencia vermicola*
AQ685265.1	*Musmusculus*
AM040492.1	*Providencia rettgeri*
AB616460.1	*Providencia sp. WW2*
AY803748.1	*Fomitiporia mediterranea*
AY374116.1	*Rainbow trout intestinal bacterium D22*
DQ885263.1	*Providencia rettgeri*
AY994812.1	*Providencia alcalifaciens*
DQ436917.1	*Enterobacteriaceae bacterium MB6-1*
AM040491.1	*Providencia stuartii*
AM040489.1	*Providencia rustigianii*
AM040490.1	*Providencia heimbachae*
DQ885281.1	*Hepatitis B virus*
DQ885254.1	*Schizosaccharomyces pombe*
AJ301554.1	*Arabdopsis thaliana*
AB272367.1	*Providencia heimbachae*

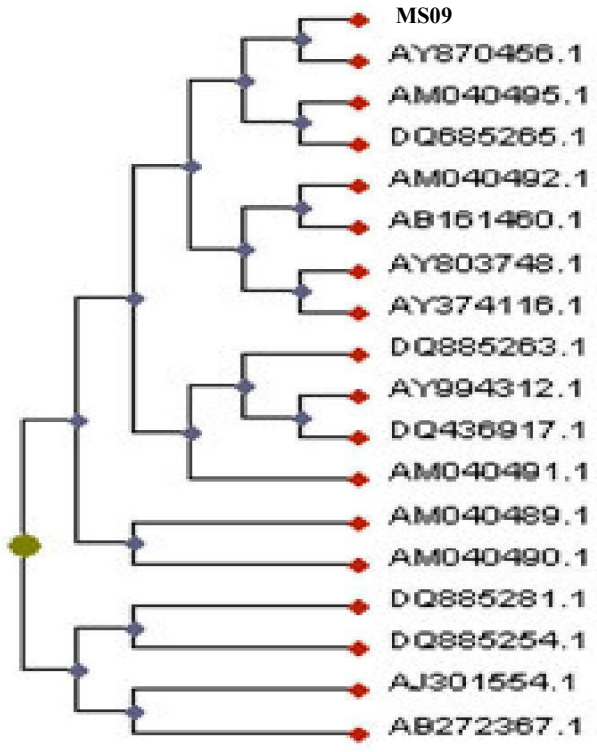

Figure 2. Phylogenetic relationship of *Providencia sp*. based on partial 16S rRNA sequence.

A phase of adaptation of *P. stuartii* strain MS09 conti-nued up to 5 h and the total viable count at the initial per-iod was 12×10^7 CFU/ml (Figure 3) . After incubation for 6 h, the total viable count was 92.5×10^7 CFU/ml with. The bacterium had a generation time of 77.2 min and specific growth rate ( ) of 0.017/h. (Mansi and Bryce, 2002). With increasing incubation time the bacterial growth also improved (Figure 3) For instance, after incubation for 24 h the total viable count increased significantly to 217×10^7 CFU/ml. This indicated that the bacterial culture, after remaining in lag phase (phase of adjustment to initiate accelerated growth) for 5 h, entered into the log phase. The total viable count of 31.5×10^7 CFU/ml at 4 h had increased to 92.5×10^7 CFU/ml at 6 h, and to 217×10^7 CFU/ml at 24 h.

When compared with the control (without chlorpyrifos), the growth pattern of *P. stuartii* strain MS09 in the med-ium containing 50 - 200 mg/L chlorpyrifos was similar up to 6 h of incubation, whereas at 24 h a significant incre-ase in total viable count was observed compared to the control (Figure 3) . The generation time was calculated to be 137 min at 50 mg/L and 165 min at 200 mg/L of chlo-rpyrifos, respectively (Mansi and Bryce, 2002). The res-ults also indicated that growth of the bacterial isolate in the presence of chlorpyrifos was stimulated in the con-centration range of 50-200 mg/L during 24 h of incubation (Figure 3). The total viable count at 24 h was 147×10^7, 24×10^7, 15×10^7, 9×10^7 and 7×10^7 with 300, 400, 500, 600 and 700 mg/L of chlorpyrifos, respectively (Figure 3). Thus, a marked reduction in the bacterial count during 24 h of incubation was noted at higher concentrations (400 - 700 mg/L) of chlorpyrifos, suggesting that bacterial enzy-mes involved in chlorpyrifos hydrolysis were suppressed at these higher concentrations and the growth rate thus decreased. However, the growth of bacteria in MSM with 400 - 700 mg/L of chlorpyrifos recovered by the seventh day and bacterial counts reached values similar to those

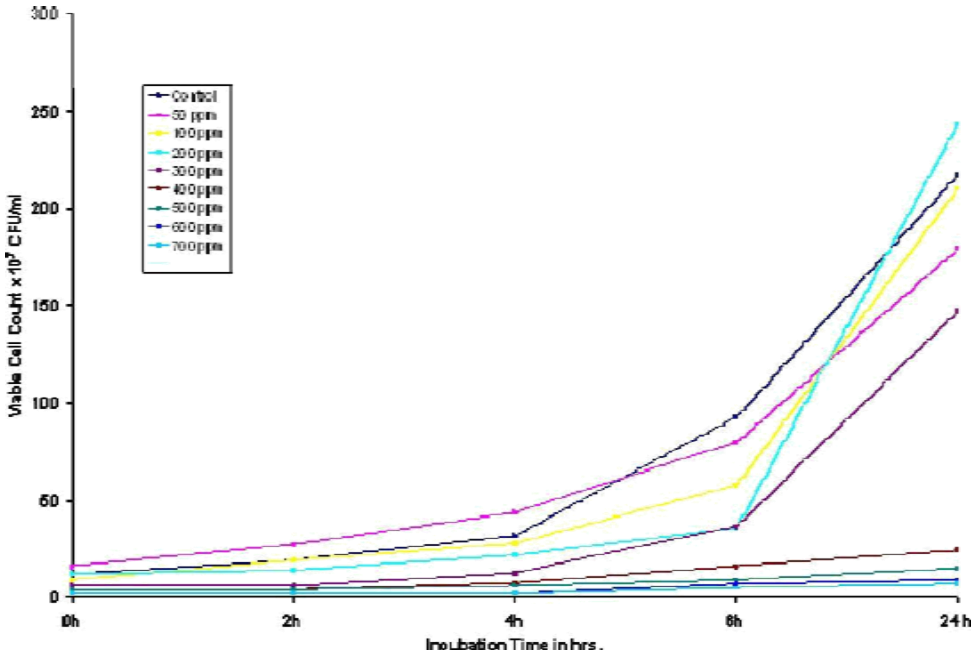

Figure 3. Growth of *Providencia stuartii* strain MS09 in Luria-Bertani medium containing different concentrations of chlorpyrifos

of bacteria grown in the growth medium for 24 h with lower concentrations of chlorpyrifos (50 - 200 mg/L). These observations indicated that the rate of substrate utilization decreased, which prolonged the lag phase in the presence of higher concentrations of chlorpyrifos. As growth kinetic studies providing an evidence of mineralization potential of organism, therefore such studies were reported by several other researchers (Maria et al., 2002; Karpouzas and Walker, 2000; Lee et al., 1998; Smith et al., 1995) while performing pesticide degradation using isolated strains of microorganisms.

DISCUSSION

In the present study, four different bacterial isolates were isolated from agricultural soil. Among them, three isolates were shown to belong to the family *Enterobacteriaceae* and one isolate belonged to the genus *Bacillus*. The isolate selected for further characterization in this study, namely isolate MS09, showed the greatest similarity to members of the order *Enteriobacteriales* and, in particular, to the *Providencia* genus. The results obtained in this study were in agreement with earlier reports that indicated the involvement of different species of *Enterobacteriaecae* in the degradation of organophosphorous insecticides like chlorpyrifos (Singh et al., 2004), phosphonate (Lee et al., 1992) and glyphosate (Dick and Quinn, 1995). Singh et al. (2004) reported that *Enterobacter* strain B-14 used chlorpyrifos as a source of carbon and phosphorous. Sethunathan and Yoshida (1973) isolated a *Flavobacterium* sp. that could use parathion as source of phosphorous but not diazinon as carbon source. Although utilization of chlorpyrifos by several soil bacteria has been reported (Singh, 2004; Sethunathan, 1973; Racke et al., 1990), this is the first report indicating that *P. stuartii* is capable of utilizing the organophosphorous pesticide chlorpyrifos as a source of carbon.

The main focus of the present study was to isolate potential chlorpyrifos-degrading bacteria and to study their growth response in the presence of different concentrations of the pesticide. Among the four bacterial isolates, *P. stuartii* strain MS09 showed better chlorpyrifos-hydrolyzing capability, as was evidenced from the broader hydrolysis zone observed on mineral salts agar (Figure 1), compared to the other three isolates. Growth experiments conducted had shown that *P. stuartii* strain MS09 is able to grow in the presence of high concentrations of chlorpyrifos and utilized it as carbon source. Similar observations were reported regarding utilization of chlorpyrifos as carbon source by bacteria isolated using an enrichment procedure (Yang et al., 2006). Some organophosphorous insecticides such as diazinon, chlorpyrifos, ethion, parathion, fonofos, malathion and gusathion are susceptible to microbial hydrolysis and serve as carbon sources for the growth of pure and mixed cultures of *Flavobacterium sp*., *Pseudomonas* sp. and *Arthrobacter* sp. (Digrak et al., 1995; Ghisalba et al., 1987).

After incubation for 24 h at 37^{o}C, high viable counts were recorded at 200 mg/L, whereas at high concentrations of chlorpyrifos (400 - 700 mg/L) the count was very low compared to the control. No inhibition of growth was observed at high concentrations (400 – 700 mg/L),

indicating that the isolated bacterium can tolerate up to 700 mg/L of chlorpyrifos. A possible explanation may be that microorganisms need an adaptation period to pro-duce the necessary degradative enzymes. This may account for the prolonged lag phase at high concentra-tions of added chlorpyrifos (Jilani and Khan, 2004). Degradation of different pesticides at high concentrations by microorganisms has been reported previously (Kar-pouzas and Walker, 2000; Struthers et al., 1998) . *Entero-bacter* sp. was able to degrade chlorpyrifos at concen-trations as high as 250 mg/L in less than two days (Singh et al., 2004) . In the presence of high concentrations of insecticides, the bacterium was greatly stressed and its growth was slowed as a consequence. During the adap-tation period, the bacteria changed from a rod shape to a coccus form with an increase in insecticide concentration (Jilani and Khan, 2004) . However, this change was tem-porary and after seven days the strain recovered to its original rod shape. The soil used for the present study had been exposed to chlorpyrifos for about ten years. In the present study, the isolated *P. stuartii* strain MS09 can tolerate up to 700 mg/L of chlorpyrifos. Hence, the tole-rance of the organism might be due to previous exposure or due to its ability to hydrolyze the supplemental subs-trate. Successful removal of pesticides by the addition of bacteria had been reported earlier for many compounds, including coumaphos (Mulbry et al., 1996), ethoprop (Karpouzas and Walker, 2000) and atrazine (Struthers et al., 1998).

Conclusion

The present study reports the identification of a bacte-rium, *P. stuartii* strain MS09, which is capable of utilizing chlorpyrifos as a source of carbon. Utilization of xeno-biotic compounds by soil microorganisms is a crucial phenomenon by which these compounds are removed from the environment, thus preventing environmental pollution. Results from the present study suggest that the isolated *P. stuartii* MS09 is able to grow in medium in the presence of added pesticide (50 - 700 mg/L) and may therefore be used for bioremediation of pesticide-conta-minated soil.

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