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16s rDNA Based Identification of Bacteria in the Organophosphates Treated Agricultural Soil

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Abstract: This work was undertaken with the aim to isolate and identify the bacteria from agricultural soil, untreated and treated with four organophosphates (Malathion, Chlorpyrifos, Phorate and Dimethoate) and find the influence of these organophosphates on them. Conventional methods of bacterial identification have major drawbacks. Therefore species level identification was carried out based on 16S rDNA sequence and GenBank database. The 16S rDNA gene is highly conserved within a species and among species of the same genus, and hence, used as the new technique for identification of bacteria to the species level. Fragment of 16S rDNA gene of the DNA of isolated bacteria was amplified by PCR . Forward and reverse DNA sequencing reaction of purified PCR amplicon was carried out with 16sF and 16sR primers. BLAST was performed and phylogenetic tree constructed using the sequences. The evolutionary distances were computed using the Kimura 2-parameter method and expressed in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4. 1359nt, 1458nt and 1309nt contig region of sample A, B and E was homologous with sequence with Genbank accession no. FJ158842.1, DQ178226.1 and HQ190844.1 which were *Sphingomonas sp.SZL-1, Pseudomonas mendocina strain PC19* and *Brevundimonas sp.XJ-412* respectively. *Sphingomonas* was affected the most followed by *Pseudomonas* by all the four organophosphates considered for study and was absent in most of the treated soil plates upto 21st day whereas *Brevundimonas* was least affected, present in almost all the treated plates as compared with controls. The three of the above characterized bacteria is found to play a significant role in bioremediation and soil fertility maintenance. Our results emphasizes on less use of organophosphates in agroecosystem to reduce biotic stress on normal soil microfauna, that would ultimately be helpful in better crop yield, thereby maintaining natural ecological balance.

Keywords: Bacterial identification, 16S rDNA, database, organophosphates, phylogenetic tree, *Brevundimonas*, contig region.

1. Introduction

Identification of bacteria is traditionally performed by isolation of the organisms and study of their phenotypic characteristics, including Gram staining, morphology, culture requirements and biochemical reactions. However, these methods of bacterial identification have major drawback. First, they cannot be used for noncultivable organisms. Second, we are occasionally faced with organisms exhibiting biochemical characteristics that do not fit into patterns of any known genus and species. Third, identification of slow growing organisms would be extremely slow and difficult (Woo et al., 2003)

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Since the discovery of PCR and DNA sequencing, comparison of the gene sequences of bacterial species showed that the 16S rDNA gene is highly conserved within a species and among species of the same genus, and hence, can be used as the new technique for identification of bacteria to the species level(Oleson and Woese., 1993)

The primary role of agriculture is to produce a reliable supply of wholesome food to feed the burgeoning world population, safely and without adverse effects on the environment. The intensified agriculture in developing countries has therefore, dictated the increasing use of agrochemicals to meet growing food demands.(Altaf et al.).Bacteria are considered to be very significant for soil fertility. Role of microorganisms in bioremediation is important for detoxification of organophosphates. The biodegradation of organophosphorus insecticides by microorganisms in soil has been widely reported. (Racke et al.,1998),(Sharmila et al., 1989), (Digrak et al.,1994), (Digrak et al., 1995). Bacterial strains of the genus Sphingomonas are often isolated from contaminated soil for their ability to use polycyclic aromatic hydrocarbons(PAH) as the sole source of carbon and energy. The direct detection of Sphingomonas strains in contaminated soil, either indigenous or inoculated is, as, of interest for bioremediation purposes (Leys et al., 2004). The isolate of the microbial cultures from soil exposed to dimethoate, an organophosphorus insecticide.that showed maximum degradation of dimethoate and was identified as Brevundimonas MCM B-427 (Deshpande et al., 2004). The use of phosphate solubilizing bacteria as inoculants simultaneously increases phosphorous uptake by the plant and crop yield strains from the genera Pseudomonas are among the most powerful phosphate solubilizers (Rodriguz and Fraga, R. 1999).

Pesticides are intended to protect crops but they may affect non target organisms and contaminate soil environment resulting in alterations of the equilibrium of soil processes for shorter or longer periods. Pesticides might affect micro-organisms by reducing their number, biochemical activity, diversity and changing the microbial community structure (Zun et al., 2008). Organophosphorus pesticides such as Malathion, Dimethoate, Chlorpyrifos and Phorate have been widely use in the field of agriculture for insect pest control.

2. Materials And Methods

Soil samples were collected as per the method of (Dutta et al., 2010). Field soil sample was collected in polythene bags from the surface layer (0-15) of the agricultural land of ICAR, Research Complex, Eastern Region, Palandu, Ranchi. Bacteria were isolated from this soil sample, serially diluted and plated on Nutrient Agar, incubated at 37˚C for 24 hrs. After incubation pure colonies streaked out and subcultured on Nutrient Agar.The presence/absence of different bacterial colonies were evaluated. The soil samples were treated with the following organophosphates at recommended dosages: Malathion, Dimethoate, Chlorpyrifos and Phorate . The presence/absence of microorganisms were determined using the dilution method (Ajaj et al., 2005). Dilution of 10^{-7} were prepared and plated on the nutrient medium(Onet, 2008).

DNA was isolated from the pure culture(marked as A) as per the protocol of (Van et al., 1997), which included mechanical lysis of cells, phenol and chloroform extrations, a potassium acetate precipitation. Its quality was evaluated on 1.2% Agarose gel (Peixoto et al., 2002) .The 16S rDNA gene was amplified as per the method of (Li et al, 2004) using universal primer and PCR product for the isolate was sequenced in both directions. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 16sF and 16sR primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer and chromatogram plot using Chromas Lite 2.0 version Applied Biosystems and Amersham MegaBace automated sequencers.. The 16S rDNA gene sequence was analysed using BLAST with nrdatabase of NCBI GenBank database to find closely related bacterial 16S rDNA sequences. Based on maximum

identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed according to Neighbour-joining method(Saitou, and Nei, 1987) using MEGA 4.0 Molecular Evolutionary Genetics Analysis software version 4 (Tamura.et al., 2007) for the studying evolutionary relationship. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein., 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1299 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.0. Same procedure was applied for colonies marked B and E. A similar protocol was followed by (Nishio et al, 2005) to isolate and identify Sphingomonas sp.

3. Results and Discussion

On quality evaluation of all isolated DNA, a single band of high molecular weight DNA was observed on 1.2% Agarose Gel . A single discrete PCR amplicon band of 1500bp of 16S rDNA was observed when resolved on Agarose Gel (Kimura, 1980) as in (fig. 1).Similar results were obtained by (Orengo et al),on amplification of 16S rDNA using universal primer an amplicon band of 1.5kb was obtained on electrophoresis. All positions containing gaps and missing data were eliminated from the dataset. These were a total of 1359, 1458 and 1309 positions in the final dataset (consensus sequence) of sample A, B, and E respectively (fig. 2, 3, 4). out BLAST with the nrdatabase of NCBI GenBank database. These were homologous to sequences with GenBank Accession No:-FJ158842.1, DQ178226.1 and HQ190844.1respectively.

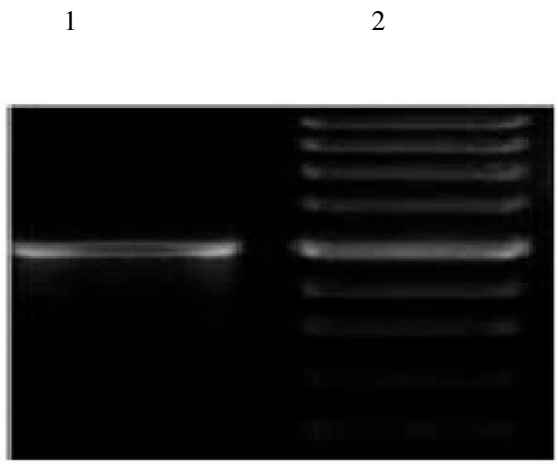


Figure 1: Lane 1: 16S rDNA amplicon
Lane 2: DNA marker

Based on nucleotide homology and phylogenetic analysis (Gurtner et al., 2001) it was found to be Sphingomonas sp. SZL-1, Pseudomonas mendocina strain PC19 and Brevundimonas sp.XJ-421 respectively.

Table 1: Identification of soil bacterial strains based on 16S rDNA sequence and GenBank database.

Isolate	Morphological details				% Similarity	GenBank accession number	Length of consensus sequence	Identified as
	Color	Shape	Margin	Elevation				
A	Yellow	Circular	Entire	Convex	100	FJ158842.1	1359bp	Sphingomonas sp. SZL-1
B		Circular	Entire	Umbonate	99	DQ178226.1	1458bp	Pseudomonas mendocina strain PC19
E	Orange	Circular	Entire	Convex	100	HQ190844.1	1309bp	Brevundimonas sp. XJ-412

Previous work by (Li et al, 2004) for isolation and identification of bacterial isolates from the space laboratory identified Sphingomonas and Brevundimonas with the following morphological features: yellow, circular and smooth colonies that were similar to the results of the present study as in table 1 and creamy white, circular, smooth colonies respectively.

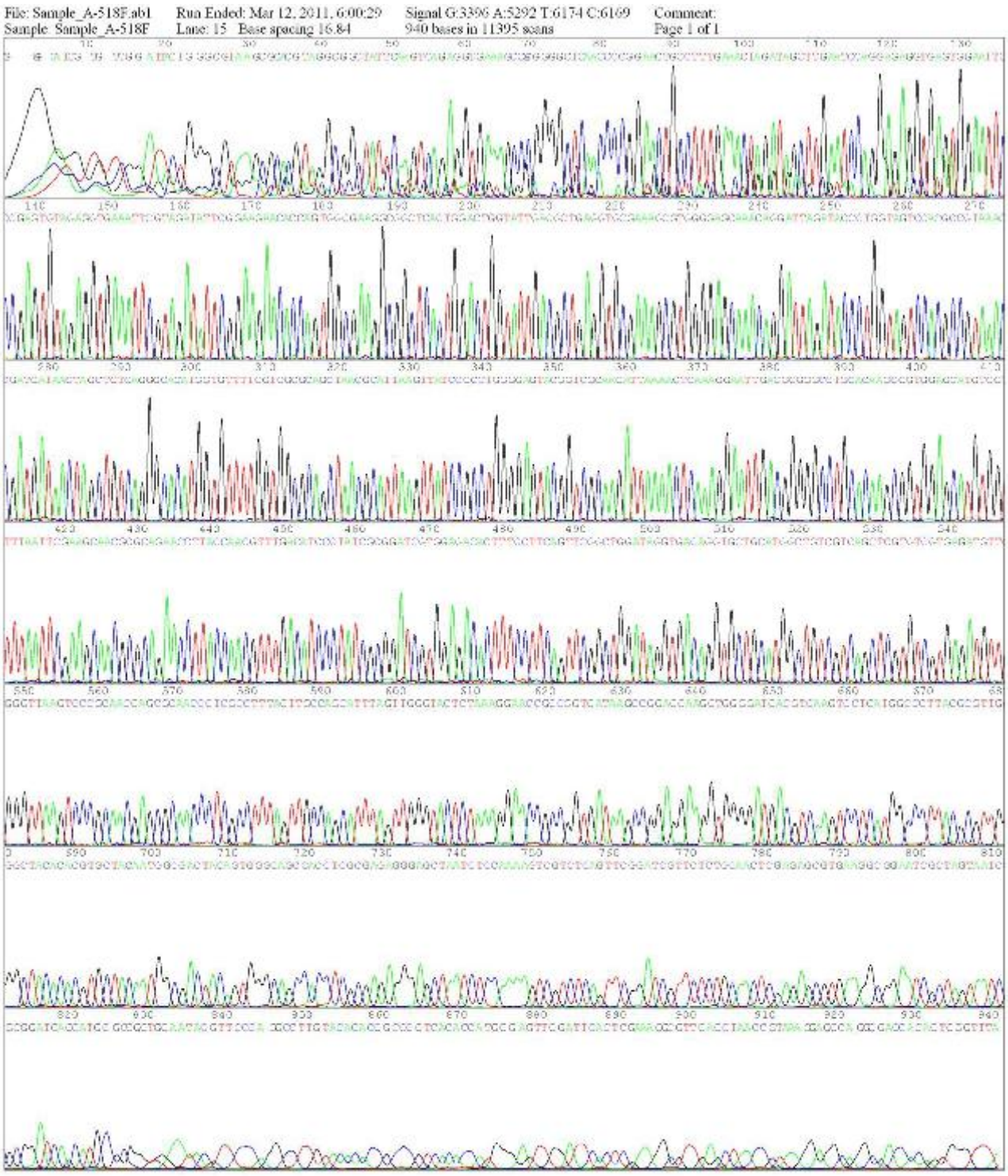


Figure 2: Sample A- 518F (*Sphingomonas sp. SZL-1*) Chromatogram of 940 bases in 31995 scans / base spacing 16.84 using Chromas Lite 2.0 version Applied Biosystems and Amersham MegaBace automated sequencers.

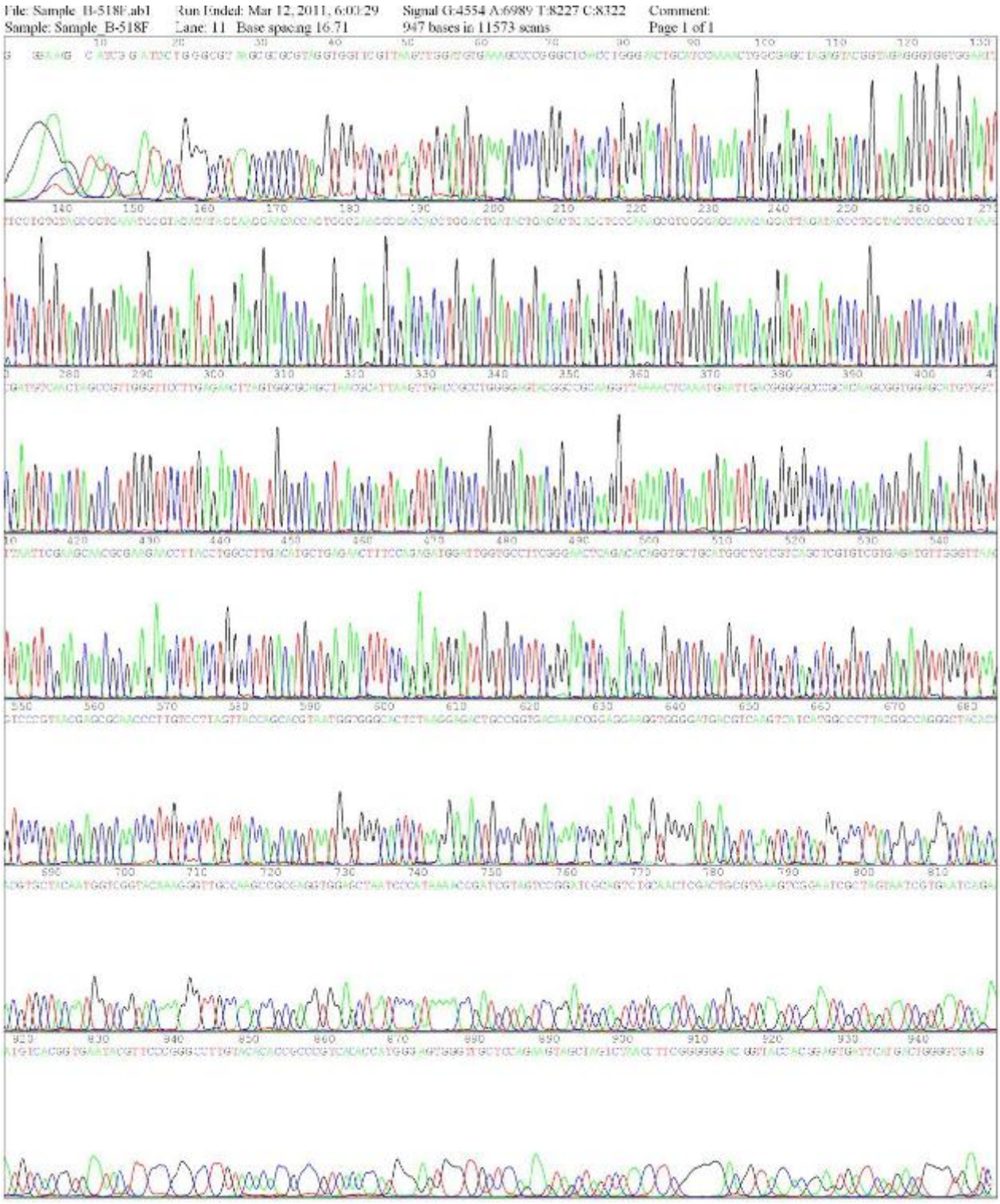


Figure 3: Sample B -518F (*Pseudomonas mendocina strain PC19*) chromatogram of 947 bases in 11573 scans / base spacing 16.71 using Chromas Lite 2.0 version Applied Biosystems and Amersham MegaBace automated sequencers.

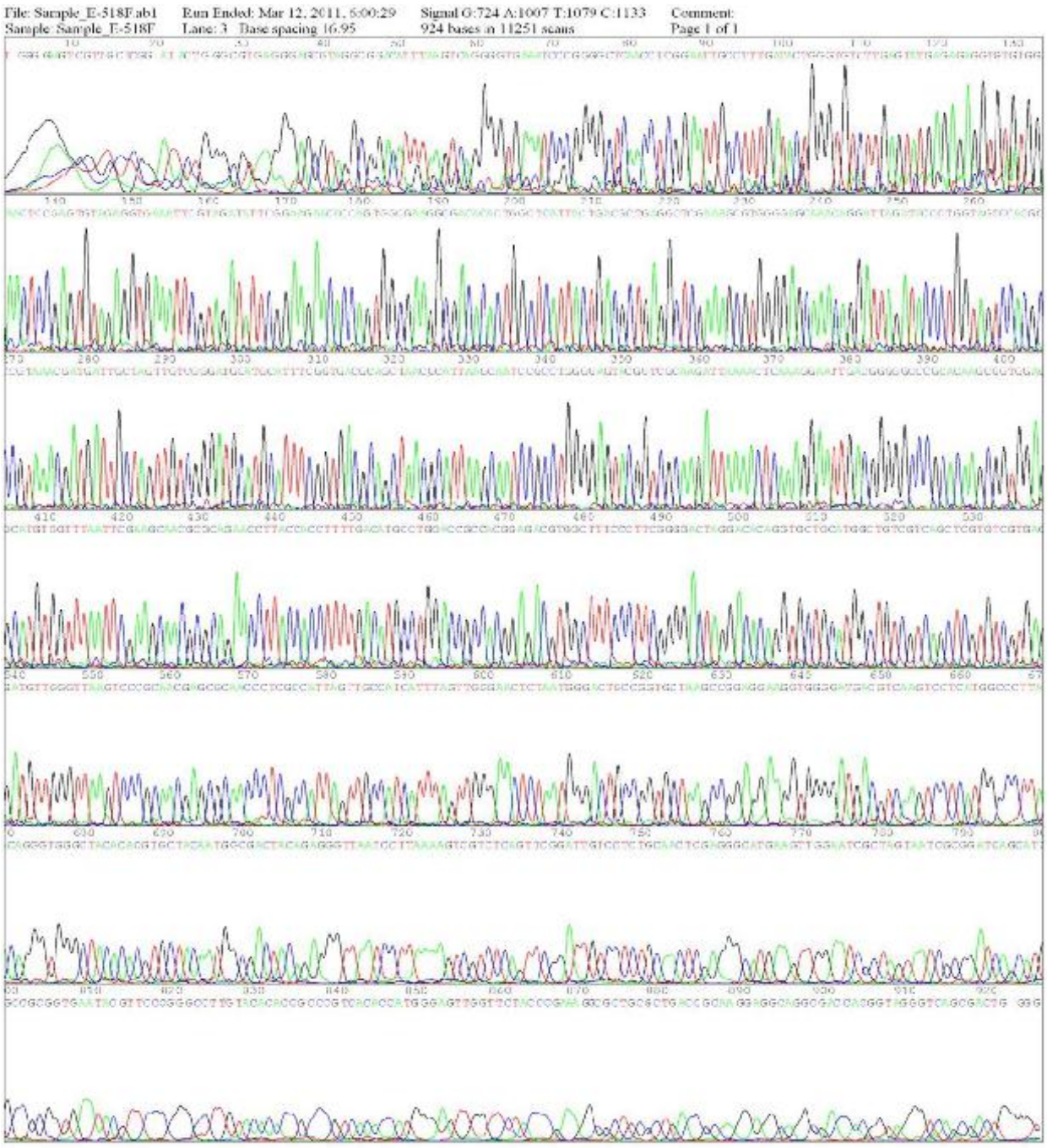
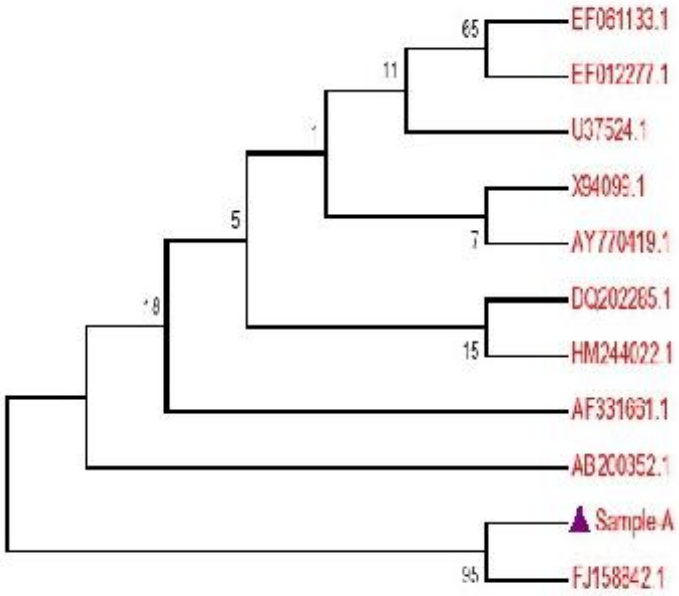
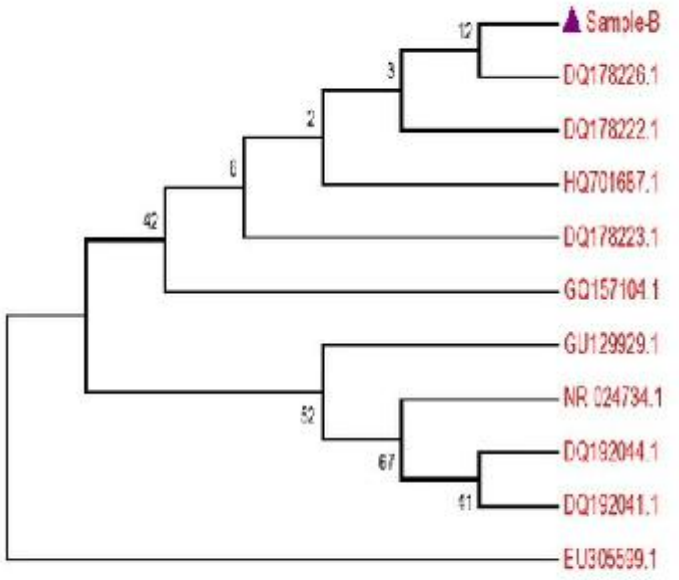


Figure 4: Sample E- 518F (*Brevundimonas sp. XJ-412)* chromatogram of 924 bases in 11251 scans / base spacing 16.95 using Chromas Lite 2.0 version Applied Biosystems and Amersham MegaBace automated sequencers.

Phylogenetic Tree:





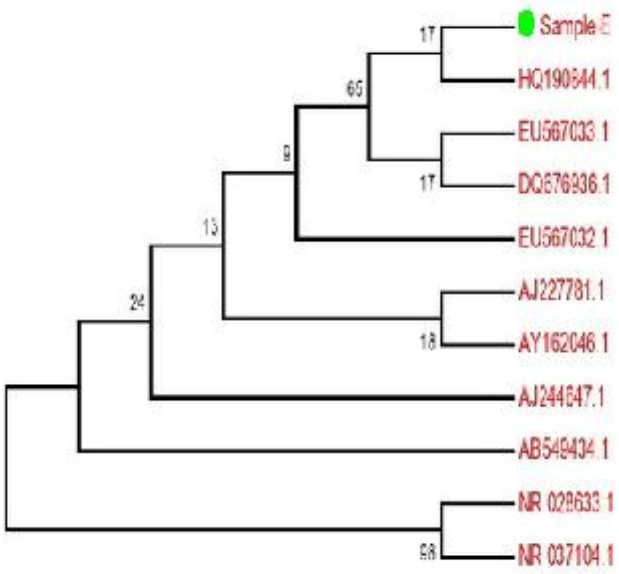


Figure 5: Evolutionary relationships of 11 taxa for 3 different Sample A, Sample B, Sample C using Mega 4.0 version Phylogenetic Analysis Tool.

A phylogenetic tree or evolutionary tree is a branching diagram or tree showing the inferred evolutionary relationships among various biological species or other entities based upon similarities and differences in their physical and/or genetic characteristics. The taxa joined together in the tree are implied to have descended from a common ancestor. Unrooted trees illustrate the relatedness of the leaf nodes without making assumptions about ancestry at all. In the case of unrooted trees, branching relationships between taxa are specified by the way they are connected to each other, but the position of the common ancestor is not (Maher, 2002) Fig 5 shows the phylogenetic tree of sample A, B and E with the ten most similar sequences.

Soil from treated sample showed significant changes in bacterial population at different post – application intervals over pre – treatment counts. There was absence of the target bacterial colonies at post application days 0, 7, 14 and 21 as can be observed in table no. 2. These microorganisms showed capability to recover rapidly in the treated soil sample. Initially at 0 day, all bacterial colonies were present. Sphingomonas was completely absent in Chlorpyrifos and Dimethoate plates after 7 to 28 days and could not recover itself. It has been observed that Chlorpyrifos has significant effect on soil microorganisms (Gilani et al.,2010) which may be due to its longer half life of 34-36 days(Singh et al.,2002) and longer persistence in soil for about 2 months (Shahida et al., 2004).

Table 2: Occurrence of Sphingomonas sp. SZL-1(A), Pseudomonas mendocina strain PC19(B), and Brevundimonas sp. XJ-412(E) in various organophosphates treated soil sample.

	Days after treatment	0 days			7 days			14 days			21 days			28 days		
S.No.	Organophosphates	A	B	E	A	B	E	A	B	E	A	B	E	A	B	E
1	Chlorpyrifos	+	+	+	-	-	-	-	+	-	-	+	-	-	+	+
2	Phorate	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+
3	Dimethoate	+	+	+	-	-	-	-	+	+	-	+	+	-	+	+
4	Malathion	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+

Among environmental resistant strains Pseudomonas species have exhibited high potential for dimethoate (Nazarian et al., 2005) which can also be concluded from table above.

4. Conclusion

Based on nucleotide homology and phylogenetic analysis, the three different bacteria, isolated from organophosphate treated plate were characterized as *Sphingomonas sp. SZL-1, Pseudomonas mendocina strain PC19* and *Brevundimonas sp.XJ-421* respectively. As compared to others, *Brevundimonas* was least affected by the pesticide, and was present in all the treated plates except for first week.

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