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Pseudomonas plecoglossicida as a novel organism for the bioremediation of cypermethrin

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Abstract

The ever increasing use of pesticides has increased the propensity to identify microorganisms for bioremediation. This research study has been carried out for physical, chemical and microbial characterization from animal waste with special reference to cow dung for its potential use in bioremediation of hazardous compounds like pesticides. Physico-chemical data shows favorable conditions for the growth and proliferation of microbial consortium. The microbial consortium assessed from the animal waste (cow dung) mainly comprised of *Pseudomonas sp., Actinomycetes sp.,Cellulomonas sp., Escherichia coli, Flavobacterium sp., Serratia sp., Nocardiasp., Sarcinasp., Salmonellasp., Staphyloccocus aureus, Alcaligens sp., Bacillus sp., and Fungi*. In order to isolate and identify the potential microorganism the microbial consortium were exposed to increasing concentrations of pesticide cypermethrin viz., 10mg/L, 25mg/L, 50mg/L and 100/mg/L using scale up process technique. The potential organism resistant to higher concentration was identified by 16s rDNA technique. The organism was identified as *Pseudomonas plecoglossicida* with NCBI BLAST homology and is a novel organism for bioremediation of hazardous compounds.

Keywords: Bioremediation, Cypermethrin, *Pseudomonas plecoglossicida*, BLAST.

Introduction

Environmental pollution caused by the release of a wide range of compounds as a consequence of industrialization has now become a major problem. Thousands of hazardous waste sites have been generated worldwide resulting from the accumulation of xenobiotic in soil and water over the years. Many chemicals are used in agriculture including fertilizers, pesticides (soil and seed treatment), plant growth regulators, disinfectant, veterinary drugs etc., are directly or indirectly affecting the environment. Increasing pesticide use in recent years has led to public concern about the social and environmental impacts of pesticide residues. At present India is the largest producer of pesticides in Asia, the Indian pesticide industry with 82000 MT of production for 2005-06 is ranked second in Asia (behind China) and ranks twelfth in the world for the use of pesticides with an annual production of 90,000 tons. A vast majority of Indian population (56.7 percent) is engaged in agriculture and is, therefore, exposed to the pesticides used in agriculture. In the present research study, the microbial consortium from the animal waste (cow dung) has been assessed and the potential microorganism for the bioremediation of hazardous waste compounds with special reference to cypermethrin has been assessed using scale-up process technique at increasing concentrations of cypermethrin and by sequencing microorganism and developing phylogenetic tree. Using the NCBI BLAST, a homology was established for the microorganism and it has been reported as a novel organism for potential use in bioremediation of pesticides.

Materials and Methods

Materials

All chemicals, reagents and solvents used were of HPLC grade purchased from Ranbaxy India ltd. Media used for the inoculation techniques were from Hi-media (India). Before the experiment all the glasswares were cleaned by chromic acid and subsequently washed

thoroughly and rinsed with double glass distilled water and dried at 110^{0}C for about 6 hours.

Sample collection for enrichment study

Cow dung sample was collected and used as source of biomass. The sample was immediately processed on the same day and the physico-chemical parameters were studied as per Standard Methods for the Examination of Water and Waste water, 17th edition, APHA (1989). The microbial enrichment technique was started immediately after the collection of the sample.

Enrichment and cultivation of mixed bacterial culture from cow dung

Cow dung was used as the source of biomass for initial inoculation in the media. One gram of cow dung was inoculated in 9ml of sterile physiological saline (0.85%) in sterile 30ml saline tube and mixed thoroughly for two to three minutes. All the debris (unbroken seeds undigested food particle etc.) was allowed to settle down for about one hour before inoculating in to fresh sterile 100ml nutrient broth medium (Hi-media) in 250 ml Erlenmeyer flask. The flask was incubated at 28^{0}C on rotary shaker at 150rpm for 24 hrs. After 24 hrs of growth, dense turbidity was observed. The microbial characteristic of the first inoculated broth was studied.

Enrichment of cypermethrin degrading monoculture

Above enriched microbial culture was used for further enrichment process. 1ml of enriched culture was added in to 100ml of fresh and sterile FTW medium (Herman & Frankenberger, 1999). FTW medium is comprised of (in g L^{-1}): K_2HPO_4, 0.225; KH_2PO_4, 0.225; $(NH_4)_2SO_4$, 0.225; $MgSO_4 \cdot 7H_2O$, 0.05; $CaCO_3$, 0.005; and $FeCl_2 \cdot 4H_2O$, 0.005 blended with 1 mL of trace elements solution (Focht, 1994). The Focht trace element solution contained (in mg L^{-1}): $MnSO_4 \cdot H_2O$, 169; $ZnSO_4 \cdot 7H_2O$, 288; $CuSO_4 \cdot 5H_2O$, 250; $NiSO_4 \cdot 6H_2O$, 26; $CoSO_4$, 28; and $Na_2MoO_4 \cdot 2H_2O$, 24. The pH of minimal medium was adjusted at 7.2 with the help of 0.1N HCL and 0.1N NaOH and autoclaved at 15 psi for 20 mins. Trace element solution was autoclaved separately and added to the minimal medium just before use.

Pesticide spiking

250ml Erlenmeyer flasks were used for spiking the pesticide. The flasks were first autoclaved and dried completely at 110^{0}C for 6hrs before the pesticide was added. 100µl of acetone pesticide containing was aseptically added to each sterilized and dried flask in laminar flow hood allowing the acetone to evaporate overnight. Thereafter, 100 mL of the fresh culture medium was added to the spiked flask. 1ml of the above enriched broth was use to inoculate the culture medium.

Isolation of genomic DNA for 16S rDNA technique

The genomic DNA was isolated using Bacterial Genomic DNA Isolation Kit (RKT09-Chromous biotech Pvt. Ltd). The kit consists of Bacterial gDNA Suspension Buffer (5X), bacterial gDNA Lysis Buffer (1X), Buffer: 5ml concentrate. Elution Buffer, Spin Columns: 25 nos., with collection tubes. RNase A (DNase free, 10mg/ml). All the reagents were stored -20^{0}C. Working range buffers were prepared prior to use.

Amplification of 16S rDNA

16s rDNA analysis was performed by genomic DNA isolation followed by direct PCR using 16s rDNA specific primers. Specific primers (Forward primer 5 to 24 and Reverse Primer---1482 to 1503), were designed for the consensus region of 16s rDNA of the bacteria and used for amplification of full sequence of the eubacteria. The PCR product size was 1.5kb (Fig 2). PCR mixtures 100µl, 16s reverse primers and 16s forward primers 100ng, MgCl2 1.5 mM final conc. dNTPs (2.5 mM each) 4 µl, Taq DNA polymerase 1 µl, Double glass distilled water to make up the volume to 100 µl (All PCR reagents were of Chromous make). Genomic DNA isolated was used as a template DNA. The PCR amplification was performed using ABI 2720 model programmed as follows: Initial denaturation for 5mins at 94^{0}C, denaturation for 30 secs at 94^{0}C, annealing at 55^{0}C for 30 sec and extension at 72^{0}C for 2 mins, final extension 72^{0}C for 15 mins and number of cycles 35.

Sequence analysis

The ~1.5kb rDNA fragment was amplified using high –fidelity PCR polymerase. The PCR product was sequenced bi-directionally using the forward, reverse and internal primer. The sequencing was carried out in ABI 3730xls model. The sequence data was aligned and analyzed to identify the bacterium and its closest neighbors. Different softwares like Weighbor Tree, Jukes-Cantor Correction, Bootstrap were used for the analysis.

Biochemical characteristics

The biochemical characteristics were examined with Himedia kit for biochemical characterization. Catalase activity of the bacterial isolate was tested with 3% H_2O_2, and oxidase activity was tested with 1% tetramethyl-p-phenylendiamine reagent paper disc.

Results and Discussion

The use of synthetic pesticides has become an indispensable tool in agriculture for the control, prevention and mitigation of pests. The data available indicates the 2-3% of pesticide is actually used and rest persists in soil and water causing environmental pollution leading toxicity to the biodata. The remediation of pesticides residue from soil and water is of prime importance to decontaminate the environment. The bioremediation using the microbial action is the recent technique for environmental clean-up. Therefore, the identification of microbial consortium and potential organism present in the microbial consortium will be beneficial for the bioconversion of pesticides. In the present research study, the animal waste (cow dung) has been characterized for the physico-chemical parameters. The data obtained from the physico-chemical is presented in Table 1. The microbial consortium mainly comprised of bacteria, fungi and actinomycetes. The physicochemical parameter assessed clearly indicates that the cow dung slurry provides favourable conditions for the growth and proliferation of microorganisms (Fulekar 2005). The various parameters such as pH, temperature, COD, BOD etc. were studied. The COD of the cow dung was found to be very high suggesting a high persistence of organic compounds. BOD was also around 9 mg/L suggesting that there are many organisms which are thriving in the extreme conditions. The microorganisms present have been found to have potential for the bioremediation of the hazardous waste compounds. The bacterial isolate that survived higher cypermethrin concentration viz CYP was tested for its growth on *Pseudomonas agar* and nutrient agar. The pigmentation was enhanced in the Pseudomonas agar as compared to nutrient agar. The colonies formed were large, circular with an entire margin and emanated a fluorescent pigment. The colony characteristic of the CYP is detailed in Table 2.

Total viable count of the bacteria was performed (Microbiological methods by C.H.Collins et.al), and was found that the number of viable organisms were very high which indicates that many different types of bacteria are able to grow on the available nutrient in the cow dung and they can be studied by using this type of enrichment technique. Apart from bacteria, *Yeast*, *Molds*, *Fungi* and *Actinomycetes* were also detected. *Escherichia coli* was also found in remarkable number. The various microorganisms among the population consisted of *Pseudomonas sp., Streptoccocus sp., Alcaligens sp., Bacillus sp., Sarcina sp., Sarratia sp,.Cellulomonas., Actinomycetes sp. and Nocardia sp.*, were also found to be present. *Bacillus stearothermophillus*, which can survive the ruminant temperature of cattles, were also detected. Among the Fungi the principle organisms that were studied includes *Penicillium sp*., *Aspergillu sps.*, *Rhizopus sp*., and *Mucor sp*. The biochemical assays of the isolate are given in Table 4. The CYP isolate was found to be lysine decarboxylase, ornithine decarboxylase and citrate positive. Whereas Indole, Methyl red, Voge's Prokaur negative. The CYP isolate was also tested for its carbohydrate utilization and was found to be a lactose nonfermenter, characteristic of Pseudomonas, and glucose, Malonate, xylose positive. It was found to be oxidase positive which is also a characteristic of the *Pseudomonas sp.*

In order to identify the organism by 16S rDNA technique, the genomic DNA of the organism was first isolated (Fig 1) and subjected to direct PCR using 16s rDNA specific primers (Fig 2). The amplicon of 1.5kb was then used for DNA sequencing and in order to identify the closely

matching species, the sequence so obtained was analyzed using appropriate Database and Similarity Matrix programs. Phylogenetic Tree Builder uses sequences aligned with System Software aligner. A distance matrix was generated using the Jukes-Cantor corrected distance model. The phylogenetic tree was created using Weighbor with an alphabet size 4 and length size 1000 (Bruno et. al 2000 & Wiley et al 1991). Jukes and Cantor created a formula that calculates the distance taking into account more than just the individual differences (Jukes-Cantor 1969). DNA sequences were compared with already submitted sequences in nucleotide databases which are available at NCBI website using BLAST technique for the sequence alignment and similarity search (Fulekar 2008). Homologous segment pairs identified by BLAST were pre-aligned, allowing faster multiple alignments with CLUSTAL W. The table of sorted phylogenetic distances computed by CLUSTAL W simplifies the reading of the results compared to direct reading of a BLAST file. On the basis of the phylogenetic tree generated, the microbe was found to match closely with *Pseudomonas plecoglossicida* (NCBI accession no. FJ587217). The next closest homologue was found to be Pseudomonas sp. PNP6-2 (NCBI accession no. EU851059). Information about other close homologue for the microbe can be found from the alignment view Table 5. *Pseudomonas plecoglossicida* as confirmed with cultural characteristics and biochemical test is a non-fluorescent, Gram-negative, rod-shaped, motile bacterium. *P. plecoglossicida* has been placed in the *Pseudomonas putida* group. So far there is no report of *P. plecoglossicida* used in the bioremediation of pesticides. The pesticide degrading property of this microbe is being studied which can provide beneficial future technique for the decontamination of the hazardous waste by bioremediation.

Acknowledgement

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Table 1: Physico-chemical parameters of cow dung

Physico-Chemical Parameters of Cow dung		
Sr. No.	**Parameter**	**Results**
1.	pH	7.3
2.	Temperature	28^0C
3.	Alkalinity/100gm	1.12meq
4.	Dissolved oxygen	10mg
5.	% Organic carbon	0.37
6.	Phosphorus	0.79mg/L
7.	Kjeldahl Nitrogen	8.7
8.	Sulphate	27mg/L
9.	Calcium (mg/100g)	8.7
10.	Sodium (mg/100g)	93.245

11.	Potassium (mg/100g)	162.50
12.	Magnesium (mg/100g)	145.95
13.	COD	230mg/L
14.	BOD	9mg/L

Table 2: Colony characteristics chart

Colony Characteristics Chart		
1.	Size	2mm
2.	Shape	Circular
3.	Pigmentation/Color	Non-fluorescent
4.	Margin	Entire
5.	Elevation	Slightly raised
6.	Opacity	Translucent
7.	Consistency	Butyrous
8.	Gram's Nature	Gram Negative Coccobacilli
9.	Motility	Motile

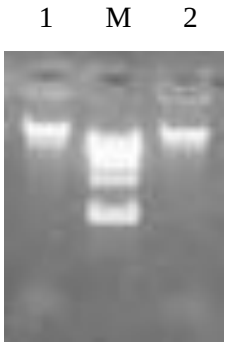
Table 3: Total microbial characteristics of cow dung

1.	Total Viable count/g	$6.5x10^{10}$
2.	Total coliform count/g	$1.89x10^{9}$
3.	Total Yeast count and mold count/g	$7.2x10^{4}$
4.	*Pseudomonas* count/g	$5.9x10^{4}$
5.	*Actinomycetes* count/g	$8.3x10^{5}$
6.	*Esherichia coli* count/g	$2.36x10^{4}$
7.	Anaerobic bacterial count	<30
8.	Thermophilic bacterial count	$7.9x10^{2}$
9.	Anaerobic spore count	Nil
10.	Thermophilic spore count	Nil
11.	Anaerobic thermophilic spore count	Nil
12.	*Salmonella*/25g	Absent
13.	*Staphylococcus aureus* count/25g	Absent
14.	*Shigella sp.*	Absent
15.	Fecal *Streptococcus sp*	Present
16.	*Flavobacterium sp*	Absent
17.	*Alcaligens sp.*	Present
18.	*Bacillus sp.*	Present
19.	*B.stearothermophilus and B.cereus*	Present
20.	*Cellulomonas sp.*	Present
21.	*Streptococcus sp.*	Present
22.	*Sarcina sp.*	Present
23.	*Serratia sp.*	Present
24.	*Nocardia sp.*	Present
25.	*Mucor sp.*	Present
26.	*Rhizopus Stolonifer*	Present
27.	*Aspergillus sp.*	Present
28.	*Penicillium sp.*	Present

Table 4: Biochemical characteristics of the CYP isolate

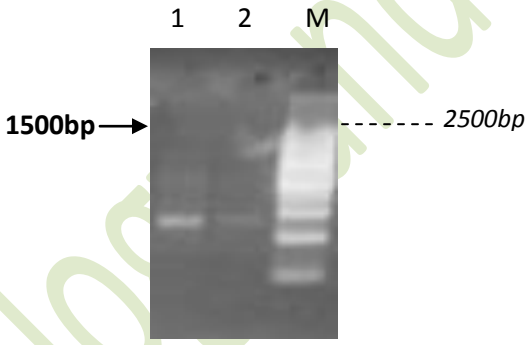
1.	ONPG	-ve
2.	Lysine decarboxylase	+ve
3.	Ornithine decarboxylase	+ve
4.	Urease	-ve
5.	Phe Deamination	-ve
6.	Nitrate reduction	-ve
7.	H_2S production	-ve
8.	Citrate utilization	+ve
9.	Voge's Prokaur	-ve
10.	Methyle red	-ve
11.	Indole	-ve
12.	Malonate	+ve
13.	Esculin	-ve
14.	Arabinose	-ve
15.	Xylose	+ve
16.	Adonitol	-ve
17.	Rhamnose	-ve
18.	Cellobiose	-ve
19.	Melibiose	-ve
20.	Saccharose	-ve
21.	Raffinose	-ve
22.	Trehalose	-ve
23.	Glucose	+ve
24.	Lactose	-ve
25.	Oxidase	+ve

Fig 1: Extraction of genomic DNA from bacterial sample



Lane description:
M: 1kb DNA Ladder (Chromous Cat. No. LAD03)
Lane 1 & 2. Extracted genomic DNA

Fig 2: PCR amplification of 16s rDNA fragment from bacterial sample



Lane description:
M: 500bp DNA Ladder (Chromous Cat. No. LAD02)
Lane 1 & 2. PCR amplified product from extracted DNA

Table 5: The aligned sequence data for 1444 bp is as follows:

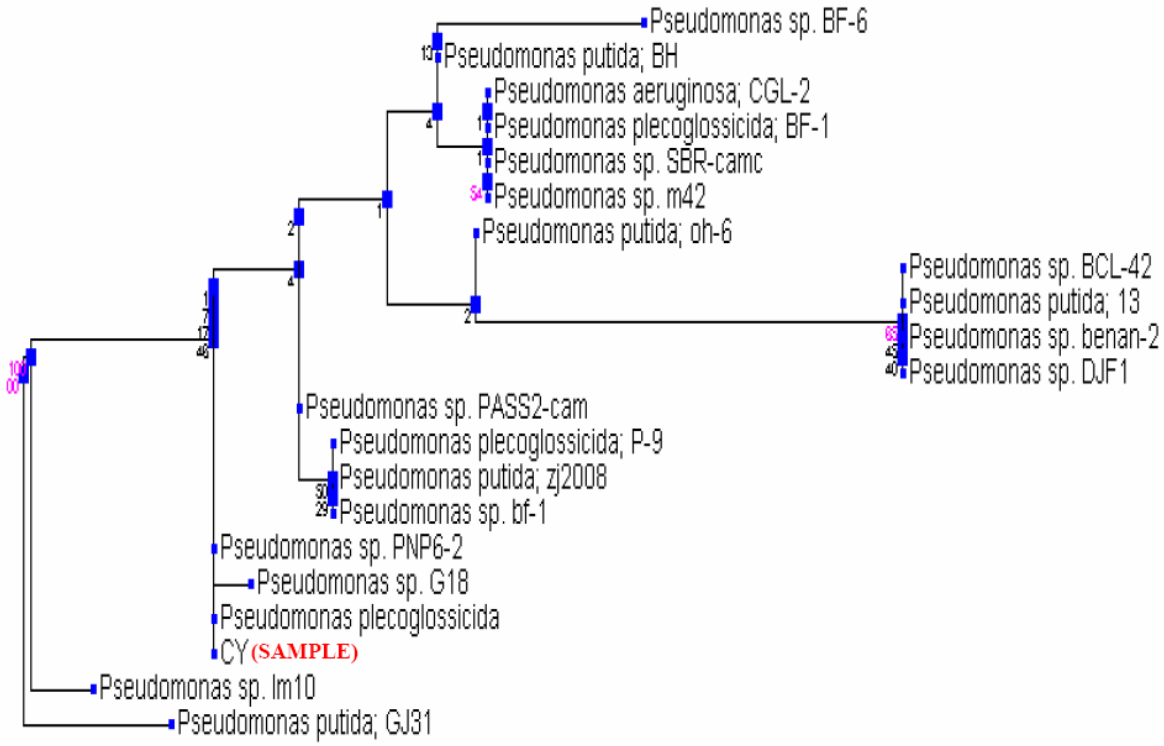
AGCCTGCGGCGGCCTACACATGCAAGTCGAGCGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGT
GAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTAC
GGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAA
TGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGA
CTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAA
GAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGCTAATACCTTGCTGTTTTGACGTTAC
CGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTA
CTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATC
CAAAACTGGCGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAA
GGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAG
GATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGAATCCTTGAGATTTTAGTGGCGCAG
CTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATGCAGAGAACTTTCC
AGAGATGGATTGGTGCCTTCGGGAACTCTGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTT
GGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTG
CCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGC
TACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCTCACAAAACCGATCGTAGTCCGGATCG
CAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCC
GGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTCAGGAGG
ACGGTACCACGGTGGATTCATACTGG

ALIGNMENT VIEW and DISTANCE MATRIX TABLE:

(With CY sequence taken as reference sequence)

S_ab score	Organism Name	NCBI Accession I
0.992	Pseudomonas putida; GJ31;	AY332610
0.990	Pseudomonas putida; BH;	D85996
0.990	Pseudomonas sp. DJF1;	DQ885956
0.990	Pseudomonas sp. BCL-42;	EF028692
0.994	Pseudomonas aeruginosa; CGL-2;	EU147006
0.991	Pseudomonas sp. lm10;	EU240462
0.994	Pseudomonas sp. BF-6;	EU289802
0.995	Pseudomonas sp. m42;	EU375660
0.990	Pseudomonas putida; 13;	EU438845
0.994	Pseudomonas sp. G18;	EU442267
0.991	Pseudomonas sp. PNP6-2;	EU851059
1.000	Pseudomonas putida; oh-6;	EU862319
0.989	Pseudomonas sp. benan-2;	EU871646
0.999	Pseudomonas putida; zj2008;	FJ004920
0.995	Pseudomonas sp. SBR-camc;	EU446283
0.989	Pseudomonas sp. PASS2-cam;	EU446288
0.993	Pseudomonas sp. bf-1;	EU857432
0.996	Pseudomonas plecoglossicida; P-9;	FJ493170
0.994	Pseudomonas plecoglossicida; BF-1;	FJ592171
0.996	Pseudomonas plecoglossicida;	FJ587217

PHYLOGENETIC TREE:



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