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ECOBIOLOGICAL AND BIOINFORMATIC STUDY OF SOIL BACTERIUM FROM AGRO-ECOSYSTEM

Suruchi Kumari *et al.*

BLAST

Kocuria strain

Expectation value

Alignment

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SURUCHI KUMARI, NEHA YADAV, SARITA KUMARI, PRIYANKA SAHA, SITARA JABEEN, B. S. RAIPAT[1] AND M. P. SINHA*

Department of Zoology, Ranchi University, Ranchi - 834 008

[1]Department of Zoology, St. Xavier's College, Ranchi - 834 001

E-mail: m_psinha@yahoo.com

ABSTRACT

The soil sample and earthworm midden collected from agro-ecosystem were analyzed for ecobiological and bioinformatic study. The initial bacterial population (number /g soil) in soil was $36.3 \pm 1.50 X 10^9$ which significantly increased to $51.1 \pm 1.35 X 10^9$ ($p < 0.01$) in earthworm midden (on 0 day). A sharp decline in bacterial population was observed till 42nd day of observation. Microbial population was higher (60.78 %) in midden than soil sample (42nd day). Bacterium isolated from earthworm middens from agro-ecosystem, on the basis of genomic analysis using 16s rDNA probe and PCR amplicon a band of 1500bp was identified as *Kocuria* sp HO-9042 having highest population in midden which is closely related to *Kocuria rosea* strain and *Kocuria* sp. RM1 as revealed by BLAST. In the distribution of 100 blast hits on the query sequence of 1500 bp matched the alignment scores ≥ 200. Sequence producing significant alignments by BLAST closely matched to *Kocuria* sp HO-9042, and 10 different strains of *Kocuria* spp were also found to be close to this species. Expectation value of all these strains was 0.0 which depicts that all the strains are homogenous to *Kocuria* sp HO-9042. *Kocuria* spp have been reported to possess hydrocarbon degrading capabilities and considered to the major agents for remediation of contaminated soil. The paper deals in detail the ecobiology and some aspects of bioinformatic study of bacterial species which was found in midden but absent in soil.

*Corresponding author

INTRODUCTION

Abundance, biomass structure and activity of decomposers in the soil foodweb have been used as indicators of ecosystem health since they are responsible for over 80% of total soil metabolism (Brady and Weil, 2004; Coleman *et al.,* 1992). The productivity and stability of soil as a medium for plants growth depends greatly on the balance between living and non living component. Energy stored in crop plants is recycled through decomposition by micro and macro organism in soil. Earthworm middens are stable structures characterized by higher nutrients contents, microbial biomass and activity than uningested material, thereby constituting hotspots of microbial – driven process such as nutrient release or nutrient immobilization and decomposition. Qualitative and Quantitative microbial activities are the key factor for productivity and sustainability of soil health for maintenance for crop production (Pankhurst *et al.,* 1996; Nannipieri *et al.,* 2003; Tilak *et al.,* 2005).

More than 80% of the carbon which passes through the heterotrophic component of the ecosystem is released by micro-organisms and the amount of nitrogen passing through the soil microbial population is more than twice that passes through the primary producer (Heal and Maclean, 1975). Hence studies on microbial ecology and their ecogenetics are essential for soil management.

16Sr DNA gene has been the preferred gene target for describing soil microbial diversity and for establishing phylogenetic relationship between unknown and uncultivated micro-organisms. The genome of isolated bacterium was used to describe the physiology, ecology and evolution (Zwolinski, 2007).

To assess the distribution and evolutionary conservation of two distinct prokaryotic repetitive elements consensus oligonucleotide were used in PCR amplification. Widespread distribution of these repetitive DNA elements in the genome of various micro-organisms enable rapid identification of bacterial species and strains and be useful for the analysis of prokaryotic genomes (Versalovic *et al.,* 1991).

The ‘Gene search’ finds genes based on partial or exact matches to a string of characters in specified IMG (Integrated Microbial Genome) field. Similarity sequence searches are implemented via BLAST programme. (Altschul *et al.,* 1990).

The phylogenetic profiler allows the identification of genes in a genome (organism) of interest that have homologs in one group of organisms and lack homologs in another group of organisms (Markowitz *et al*., 2006).

This work was therefore aimed at a comparative examination of microbial population in the middens of an earthworm (*Lampito mauritii*) commonly found in soil from agro-ecosystem of Ranchi, and the identification of dominant bacterium of midden by genomic analysis on the basis of which the phylogeny of the species has been traced.

MATERIALS AND METHODS

The soil sample (Table 1, edaphic factors) were collected from the agro-ecosystem of Ranchi and were brought to the laboratory in sterilized condition and immediately processed for bacteriological isolation in the laboratory.

Serial dilution plating technique (Parkinson *et al.,* 1971) was used for estimating the bacterial population in soil sample. For enumeration of bacterial population, 1g soil sample was diluted in test tube containing 9mL of sterilized distilled water and the process was repeated to get a final dilution of 10^{-7}. 1mL inoculums of the primary suspention was taken and Czapek Dox agar media was used for culture.
The colony of midden were taken for the genomic analysis. DNA was isolated from the culture of isolated bacterium. Its quality was evaluated on 1.2% agarose gel, a single band of high molecular weight DNA has been observed. Fragment of 16Sr DNA gene was amplified by PCR from the above isolated DNA. The PCR

Table 1: Characteristic of soil sample taken from Agro-ecosystem

Characteristics	Value M±SD; n=3
pH	5.9 ±0.40
Organic Carbon (mg cg^{-1}, soil)	7.45 ±1.47
Nitrogen (mg Ng^{-1}, soil)	0.61 ±0.14
Phosphorous (kg p hec^{-1}, soil)	30.3 ±2.05
Potassium (kg K hec^{-1}, soil	151.5±5.86

amplicon was purified to remove contaminants by using a QIA quick purified kit (Qiagen, Hilden, Germany), after seaken GTG (FMC) agarose gal electrophoresis (1 X Trisacetate EDTA or 1 X tris borate EDTA running buffer). Forward and reverse DNA sequencing was carried out by using BDT v 3.1 cycle sequencing kit on ABI 3730 X 1 genetic analyzer and consensus was generated by Aligner software.
The 16Sr DNA gene sequence was used to carry out BLAST with nrdatabase of NCBI gene bank database (Marchler-Bauer *et al.,* 2000, Pruitt *et al.,* 2005). Based on maximum identity score first ten sequences were selected and sequence producing significant alignments.

RESULTS AND DISCUSSION

Number of bacterial CFU isolated from soil sample was varied from 36.3±1.50 X 10^9 to 7.9±0.90 X 10^9 in cropland soil and in midden population varied from 51.1±1.350 X 10^9 to 12.7±0.750 X 10^9 (Fig. 1).

The bacterial population in soil in the beginning was 36.3 ±1.50 X 10^9 and in midden was 51.1±1.35 X 10^9.There after a sharp decline in bacterial population in soil and midden was observed. In soil bacterial population gradually decresed to 32.8±2.80 X 10^9, 24.5±2.44 X 10^9, 21.4±2.55 X 10^9,18.9±1.665 X 10^9,

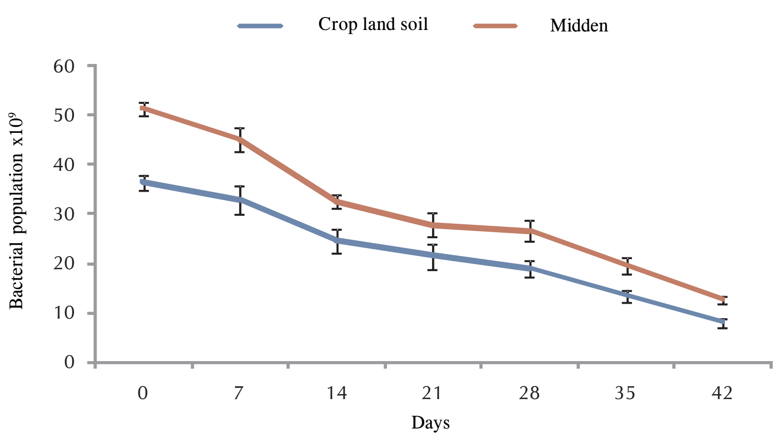

Figure 1: Bacterial population in crop land soil and earthworm midden

Table 2: Bacterial population CFU of bacteria isolated from soil (values are per g soil).

Day	Bacterial population in soil (M±SD)	Bacterial population in midden (M±SD)	% change
0	36.3±1.504 X 10^{9*}	51.1±1.350 X 10^{9*}	+40.77%
7	32.8±2.809 X 10^{9*}(-9.64)	44.9±2.417 X 10^{9*}(-12.13)	+36.89%
14	24.5±2.441 X 10^{9*}(-32.50)	32.5±1.305 X 10^{9*}(-36.39)	+33.19%
21	21.4±2.553 X 10^{9*}(-41.04)	27.8±2.451 X 10^{9*}(-45.59)	+29.90%
28	18.9±1.665 X 10^{9*}(-47.93)	26.6±2.136 X 10^{9*}(-47.94)	+40.7%
35	13.3±1.171 X 10^{9*}(-63.36)	19.6±1.662 X 10^{9*}(-61.64)	+47.36%
42	7.9±0.907 X 10^{9*}(-78.23)	12.7±0.750 X 10^{9*}(-75.14)	+60.78%

Values in parenthesis are percentage decrease(-); *= Changes produced are significant (p=0.01); n=3

13.3±1.171 X 10^9 and 7.9±0.907 X 10^9 on 7^{th},14^{th},21^{st},28^{th},$35^{th}$and 42^{nd} day respectively. On 7^{th} day of observation bacterial population in midden was 44.9±2.41 X 10^9 which was decreased upto 12.7±0.75 X 10^9. The population showed significant increase from soil to midden. The bacterial population in midden was always higher than soil. The percentage change between the bacterial population of soil and midden in the beginning was 40.77% which gradually decreased to 36.8%, 33.19% and 29.9% on 7^{th}, 14^{th}, and 21^{st} day respectively (Table 2). There after the population in midden increased by 40.7% (on 28^{th} day) 47.36% (on 35^{th} day) and 60.78% (on 42^{nd} day). Bacterial population has been reported higher in midden compared to the standaridized soils ingested by the earthworm (Daniel and Anderson, 1992). Earthworm casts have been reported to be much more microbiologically active and richer in micro-flora than their surrounding undigested soils (Daniel and Anderson, 1992).

Morphological data pertaining to different bacterial colonies on nutrient agar plates of soil and middens sample and their staining response to Gram stain are presented in Table 3 and 4.

Table 3: Colony morphology and staining response of soil sample

Shape	Margin	Elevation	Colour	Gram stain
45% Punctiform	45% Entire	75% Flat 25% Raised	75% White 25% yellow	-ve cocci
40% Irregular	40% Undulate	100%Umbonate	80% Cream 20% White	+ve bacilli
15% Circular	15% Entire	80% Flat 20% Raised	60% Yellowish 40% Brown	+ve cocci

Table 4: Colony morphology and staining response of earthworm middens sample

Shape	Margin	Elevation	Colour	Gram stain
60% Punctiform	60% Entire	80% Flat 20% Raised	80% White 20% Yellow	-ve cocci
30% Circular	30% Entire	85% Raised 15% Flat	85% Pink 15% Brown	+ve cocci
10% Irregular	10% Undulate	100% Umbonate	100% Yellow	+ve bacilli
Filamentous	Filamentous	100% Raised	100% Yellow	+ve bacilli

The developed bacterial colonies on the nutrient agar plates with respect to their shape and margin were of three types *i.e.* circular-entire, punctiform-entire and irregular undulate in both samples but in midden filamentous colony was also found. About 30% of the colonies were circular in shape and pink in colour which was used for genomic study from midden sample.

The bacterium was identified as *Kocuria* sp. HO-9042 (GenBank Accession Number: DQ531634.2) based on nucleotide homology and phylogenetic analysis.
Kocuria is a member of the Micrococcaceae family and consist of 11 species. It was previously classified into the genus of Micrococcus but was dissected from Micrococcus based on phylogenetic and chemotaxonomic analysis (Stackebrandt *et al.,* 1995).

Colony morphology was observed on nutrient agar medium after incubation at 37° for 48 hr. The colonies were pink, circular, slightly convex, opaque and approximately 2mm in diameter. The bacterium *Kocuria* sp. HO-9042 was gram positive aerobic and coccoid cells (Fig. 2).

The almost complete 16Sr DNA gene sequence (1500 bp) for strain *Kocuria* sp. HO-9042 was determined when resolved on Agarose gel (Fig. 3). Amplification of the 16S rDNA gene from the *Kocuria* sp. was done with 8F primer with 550 bp and 1492R primer with 985 bp was observed. Consensus sequence of 1401 bp DNA was generated from forward and reverse sequence data using alinger software.

Multiple alignments of the most closely related actinobacteria and calculation of levels of sequence similarity with identified bacterium *Kocuria* sp. HO-9042 were carried out by using BLAST (Marchler-Bauer *et al.,*

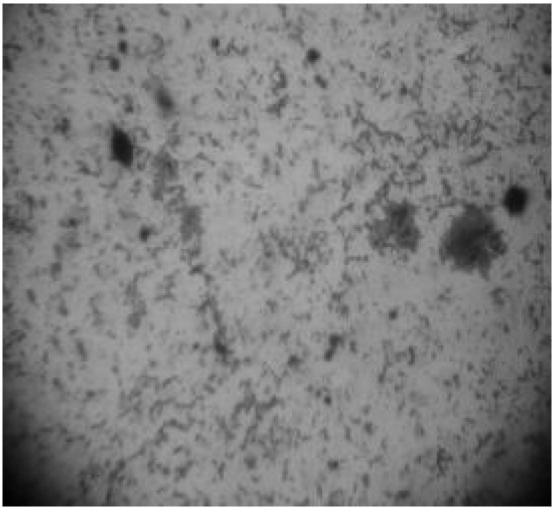

Figure 2: Gram staining of identified bacterium *Kocuria* sp HO-9042

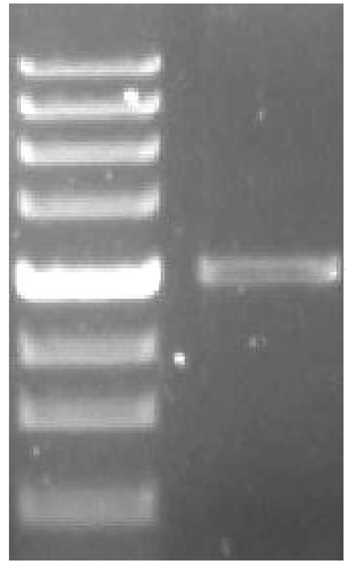

Figure 3: Gel image of 16S rDNA amplicon
Lane 1: DNA marker; Lane 2: 16S rDNA amplicon band

Table 5: Sequence producing significant alignment by BLAST

Accession	Description	M.S	T.S	Q.C	E.V	M.I
DQ531634.2	*Kocuria* sp. HO-9042	2588	2588	100%	0.0	100%
EU660350.1	*Kocuria rosea* strain CT22	2555	2555	100%	0.0	99%
AY345428.1	*Bacterium* K2-25	2553	2553	100%	0.0	99%
DQ448711.1	*Kocuria* sp. CNJ770 PL04	2510	2510	100%	0.0	99%
EF675625.1	*Kocuria* sp. RM1	2497	2497	100%	0.0	98%
AB302331.1	*Actinobacterium* C18 gene	2481	2481	99%	0.0	98%
GU217694.1	*Kocuria* sp. ljh-7	2475	2475	100%	0.0	98%
AB330815.1	*Actinobacterium* C20	2471	2471	99%	0.0	98%
DQ059617.1	*Kocuria aegyptia* strain YIM 70003	2459	2459	99%	0.0	98%
EU372971.1	*Kocuria* sp. E7	2453	2453	100%	0.0	98%

M.S. = Max score; T. S. = Total Score; Q.C = Query coverage; E.V = Expected value; MI= Max ident

2000, Pruitt *et al.,* 2005) with the nrdatabase of NCBI genbank. Triggering the extention of the 100 blast hits combined with a new heuristic for generating gapped alignments yielded a gapped BLAST programme and checked each entry in the database independently against a query sequence of amino acids. Sequence producing significant alignment by BLAST, the close matches the query sequence of *Kocuria* sp. HO-9042, 10 different strains of *Kocuria* were found (Table 5). The expectation value (E) of all these *Kocuria* sp. strain was 0.0, which showed that all sequence of different strain is homogenous to *Kocuria* sp. HO-9042.

The phenotypic features and complete sequence of 16Sr DNA revealed that *Kocuria* sp. HO-9042 strain showed 99% sequence similarity with *Kocuria rosea* strain CT22 (Flugge, 1986; Stackebrandt *et al.,* 1995) and 98% sequence similarity with *Kocuria* sp. RM1 and *Kocuria aegyptia* strain 71M 70003 (Li *et al.,* 2006). The type strain *Kocuria rosea* has been reported to cause catheter related bacterium (Altuntas *et al.,* 2004) and the majority of strains are non-pathogenic.

The RM1 strain has been reported to grow at pH 10.5 in buffered and unbuffered media and utilize 40 different carbon substractes. Isolation of actinomycetes, *Kocuria* sp. which produce high amount of xylanase from Bauxite residue and offers a new source of xylanase producing strains (Krishna *et al.,* 2008). *Kocuria* sp. HO-9042 showed 98% sequence similarity with these strain (*Kocuria*. sp. RM1). Members of this genus *Kocuria* are gram positive, aerobic, non-encapsulated, non-halophilic, non-endospore forming, with the presence of the fatty acid anteiso $C_{15:0}$ and MK-7 (H_2) and MK-8 (H_2) as the major menaquinones (Zhou *et al.,* 2008).

Gram positive bacteria can be divided into two major sub-division the phylum Actinobacteria (High G+C gram positives) and the phylum Firmicutes (low G+C gram positives; Gontang *et al.,* 2007). The DNA G+C contents of strain *Kocuria* sp. HO-9042 are 65 moL% as determined by the HPLC method (Kumagai *et al.,* 1988). The diagnostic diamino acid of the cell-wall peptidoglycan is L-lysine and contain MK-8 (H_2) and MK-9(H_2) as major menaquinones (Zhou *et al.,* 2008).

Genotypic and morphological characteristics are used to describe the species Kocuria was *Kocuria turfanensis* sp. (type strain HO-9042= CCTCCAB206107= KCTC 19307). The gene bank accession number for the 16Sr DNA gene sequence of strain HO-9042 was DQ 531634.2.

A phylogenetic tree was constructed using the neighbor-joining method of Saitou and Nei (1987) from Kimura-2 parameter values (Kimura, 1980,1983) by using MEGA4 (Tamura *et al.,* 2007). The topology of the phylogenetic tree was evaluated by using the bootstrap resampling method of Felsenstein (1985) with 500 replicates.

Pairwise similarities between the PCR amplified nucleotide sequence were used to construct distance matrices for phylogenetic analysis based on percentage of divergence between the sequence (Table 6).

Unrooted phylogenetic tree was constructed and branches corresponding to partitions reproduced is less than 50% bootstraps replicated are collapsed. The % of replicates trees in which the associated texa clustered together to the bootstrap test 500 replicates is shown next to the branches.

Table 6: Distance matrix

Sample -A	1		0.000	0.001	0.001	0.002	0.002	0.002	0.002	0.002	0.002	0.002
DQ531634.2	2	0.000		0.001	0.001	0.002	0.002	0.002	0.002	0.002	0.002	0.002
EU660350.1	3	0.001	0.001		0.000	0.002	0.002	0.002	0.002	0.002	0.002	0.002
AY345428.1	4	0.001	0.001	0.000		0.002	0.002	0.002	0.002	0.002	0.002	0.002
DQ448711.1	5	0.005	0.005	0.005	0.005		0.002	0.002	0.002	0.002	0.000	0.002
EF675625.1	6	0.006	0.006	0.006	0.006	0.008		0.002	0.001	0.002	0.002	0.001
AB302331.1	7	0.005	0.005	0.005	0.005	0.000	0.008		0.002	0.002	0.000	0.002
GU217694.1	8	0.006	0.006	0.005	0.005	0.008	0.003	0.008		0.002	0.002	0.001
DQ059617.1	9	0.007	0.007	0.007	0.007	0.010	0.004	0.010	0.004		0.002	0.002
AB330815.1	10	0.005	0.005	0.005	0.005	0.000	0.008	0.000	0.008	0.010		0.002
EU372971.1	11	0.006	0.006	0.006	0.006	0.008	0.003	0.008	0.001	0.004	0.008	

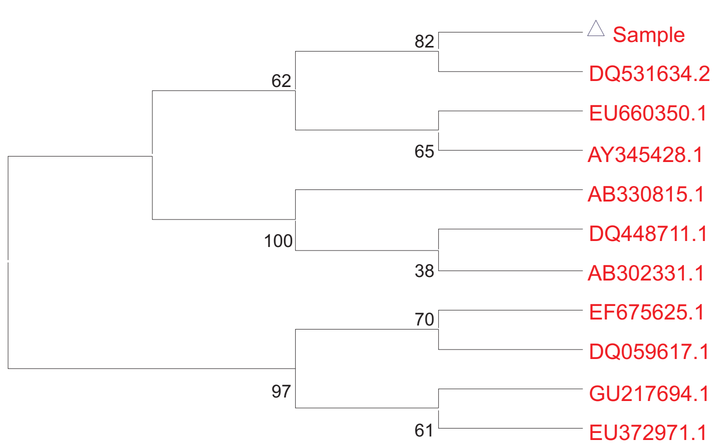

Figure 4: Phylogenetic tree showing position of Kocuria sp HO-9042 (Δsample)

Phylogenetic analysis revealed that the strains closest relative were *K. rosea* strain CT22 (EU660350.1) and Bacterium K_2-25 (Ay345428.1) showing respective 16Sr DNA gene sequence similarity higher than 99% and distantly to *K.* sp. E7 (EU372971.1). The distance from *K.* sp HO-9042 in the phylogenetic tree the descending order to EU660350.1, AY345428.1, AB330815.1, DQ448711.1, AB302331.1, EF675625.1, DQ059617.1, GU217694.1, EU372971.1 (Fig. 4). This further demonstrated that although there is slight divergence or variation among the strain but very much similar. The evolutionary distance was computed in the units of the number of base substitutions per site. Codon positions included were $1^{st}+2^{nd}+3^{rd}$ non-coding. All position containing gaps and missing data were eliminated from datasheet.

Harwati et al., (2007), first time reported degradation of compounds of Arabian light crude oil by *Kocuria rosea* and *Kocuria aegyptia.* Nazina *et al.,* (2002) isolated a strain *K. erythromyxa* from an oil field. On the basis of genomic properties (16Sr DNA similarity of 99.9%, DNA-DNA reassociation of 95%) of the type strain of *Kocuria rosea* and *K. erythromyxa* indicates that these taxa are members of the same species. According to rule 42 (union of taxa of equal rank), *K. rosea* (Stackebrandt *et al.,* 1995) has priority over *K. erythromyxa* (Rainey *et al.,* 1997; Schumann *et al.,* 1999).

Tumaikina *et al*., (2008) isolated *K. rosea* from the pondweed surface that grew on agar medium with crude oil as carbon source. *K. rosea* CMG2042 grew on all three PAHs (Polycyclic aromatic hydrocarbons) *K. flavia* grow on naphthalene, phenanthrene (Ahmed *et al.,* 2010). On plates of agar medium with or without yeast extracts colonies of both the strain had accumulated oil around them. *K. rosea* had higher growth and oil accumulating in comparision to *K. flava*. It is concluded that *K. flava* and *K. rosea* was able to utilize naphthalene as sole carbon and energy source (Ahmed *et al.,* 2010).

By the consent of nature, there are micro-organisms ubiquitously distributed in soil and aquatic environment which have hydrocarbons degrading capabilities and considerd to be the major agents for remediation of contimanated sites (Leahy and Colwell, 1990; Boonchan *et al.,* 2000; Widada *et al.,* 2002; Zhong *et al.,* 2007; Lin and Cai, 2008). Contamination of hydrocarbons, either terrestrial or aquatic, truly acts as selection pressure for these indigenous micro-organisms. Micro-organisms possess the greatest enzymatic diversity which they use to mineralize millions of organic compounds to capture the chemical energy for their growth (Dagley, 1987; Lawrence and Lynda, 1999). In this way, the identified bacterium *Kocuria* sp. HO-9042 is also very useful for degrading the hydrocarbons in agroecosystem.

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