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PRODUCTION OF INDOLE ACETIC ACID BY 5-FLURO-TRYPTOPHAN RESISTANT MUTANT OF *RHIZOBIUM* SP. ISOLATED FROM ROOT NODULE OF PULSE LEGUME *PHASEOLUS MUNGO* L.

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ABSTRACT

A symbiotic strain was isolated from the root nodules of the legume pulse *Phaseolus mungo* L and designated as PM 25. The strain was identified as *Rhizobium* sp. on the basis of the physiobiochemical characteristics and 16S rDNA sequence based homology. The Minimum Inhibitory Concentration of the analogue 5-fluro-tryptophan was 3.0 mM. The analogue (5FT) resistant mutants were screened from wild PM 25 strain after mutagenesis with N-methyl-N´-nitro-N-nitrosoguanidine (NTG) as a mutagenic agent. The analogue resistant mutants were isolated from 5.0 mM and 10.0 mM 5-fluro-tryptophan supplemented Bergersen's medium. These mutants produced tryptophan and IAA in Bergersen's medium without supplemented with tryptophan. The wild type PM 25 was unable to produce tryptophan and IAA in tryptophan non-supplemented medium but capable of producing IAA in tryptophan supplemented media. So, this *Rhizobium* sp. is able to follow tryptophan dependent pathway and able to convert tryptophan to IAA.

KEY WORDS: **Indole Acetic Acid (IAA), 5-fluro-tryptophan, *Rhizobium, Phaseolus mungo***

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INTRODUCTION

The legume family (Fabaceae) is the third largest (after Orchidaceae and Asteraceae) family of angiosperms [1] with approximately 730 genera and over 19400 species worldwide.[2] Rhizobia are Gram negative bacteria of the family Rhizobiaceae and forming root nodule by symbiotic association in the plants belonging to the family Leguminosae (Fabaceae). Currently, this Rhizobiaceae of Alpha-proteobacteria group includes 92 species within 13 genera.[3] A successful symbiosis is the result of a complex series of interactions between the host and the symbiont.[4] The symbionts are assumed to contribute a part of each of the hormones to the nodule.[5] It is reported that rhizobia could produce IAA in culture.[6] Besides nitrogen fixation, the root nodules of leguminous plants contain appreciable amount of different phytohormones, which play an important role in genesis, and development of root nodules.[7] Most of the explored herbs are pulses, and are studied in relation to nitrogen fixation by the root nodules. The plant *Phaseolus mungo* is an herbaceous legume pulse. However seeds of the legume plant were not only the important sources of protein and phosphoric acid but played a significant role in Indian diet. The plant produced plenty of root nodules. Among which mature nodules were small, pink in color, mostly oval to circular in shape. Type of IAA biosynthesis pathway vary with different bacteria.[8] Rhizobia were able to follow tryptophan dependent pathway and was converted tryptophan to IAA. [9] IAA production by *Rhizobium* sp. in culture was reported by several workers.[10] The IAA content in the nodule and supply to the host was taken as the second line of symbiosis in the root nodule–*Rhizobium* association.[11] The present study describes the isolation of symbiont from *Phaseolus mungo* and its identification by physio-biochemical characters and 16 S rDNA sequence based homology. Attempts were also made to isolate tryptophan analogue resistant mutants and to study their ability to produce tryptophan and IAA. This investigation is assumed to make a better understanding on the IAA biosynthesis pathway of *Rhizobium* sp.

MATERIALS AND METHODS

The plant, microorganism, medium and growth conditions

The leguminous pulse herb *Phaseolus mungo* (L.) was selected for the study. The strain was isolated following the method of Ghosh et al. [12]. The medium for bacterial growth was yeast extract mineral medium with 1% mannitol (YEM)[13] and 0.1% $CaCl_2$, H_2O rather than NaCl and $CaCO_3$ at pH 7.0 and supplemented with different isomers of tryptophan (L-, DL- and D-tryptophan). The bacteria were incubated in 30 ml medium in 100 ml conical flasks in three replicates at 30±2^oC on a rotary shaker for 24 h. The growth of the bacteria was measured turbidimetrically by Spectrophotometer at 540 nm. A number of physiological and biochemical tests were performed separately in YEM medium. The mutation experiments were performed in Bergersen's synthetic medium.

Identification, 16s rDNA sequencing and phylogenetic affiliation of the symbiont

The isolated symbiont was identified as a species of *Rhizobium* (strain PM 25) by 16S rDNA sequence homology. Genomic DNA was extracted following the method of Johnson.[14] Amplification, purification of the amplicons and sequencing of 16S rDNA was carried out.[15] A continuous stretch of 1401 nucleotide long gene sequences of 16S rRNA gene was used to search for similar sequences from RDP database release 10.1 (http://rdp.cme.msu.edu/) and NCBI site (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using various online tools (CLASSIFIER, SEQMATCH). After confirmation of generic affiliation, sequences from type strains of different species were retrieved from GenBank. All these sequences were aligned by CLUSTAL_X programme[16] and edited manually. A phylogenetic tree showing

relationship between *Rhizobium* sp. strain PM 25 and other reference strains were constructed by neighbour-joining (NJ) method with Jukes and Cantor correction using TREECON software for Windows as described by Saha and Chakrabarti.[15]

Estimation of IAA and tryptophan

The culture medium after growth was centrifuged and the cell-free supernatant was used for IAA [17] and tryptophan [18] extractions. The extracted IAA was estimated Spectrophotometrically [19] using a standard curve prepared from authentic IAA (Sigma chemical Co. USA) and tryptophan was estimated following Hassan. [20]

Standardization of time for NTG (N-methyl-N´-nitro-N-nitrosoguanidine) treatment

For mutagenic treatment of the selected isolate, M/20 Tris meleate buffer (pH 6.0) was used with N-methyl-N´-nitro-N-nitrosoguanidine. [21] Before mutagenesis the killing effect of NTG and survival of the cells to NTG (500µg/ml) exposed for different time was determined. Bacterial cells were grown overnight on agar slants of Bergersen's synthetic medium. Cells were harvested and washed twice with M/20 Tris maleate (TM) buffer (pH 6.0). The cells ($5x10^7$ cfu/ml) were treated with 500 µg NTG/ml for 60 min at 37^{o}C. The treated cells were washed two times with TM buffer at pH 6.0. Cell suspension (0.1 ml) was taken out 0 to 60 min at 10 min interval from the reaction mixture and was grown in medium (YEM) at 30±2^{o}C for 48 h and the colonies appeared on each plate were counted. The time required for 50% survival (Lethal dose 50, LD 50) was taken as the standard time for exposure to NTG.

Determination of minimum inhibitory concentration (MIC) of 5-fluro-tryptophan (Analogue)

The sensitivity of the bacterium (*Rhizobium* sp.) to 5-fluro-tryptophan (5-FT) an analogue of tryptophan was tested. The stock solution of analogue was sterilized by filtration and added at various concentrations to the sterilized Bergersen's agar medium. Freshly grown cells of the bacteria were streaked on the plates containing different concentration of the analogue and incubated at 30±2^{o}C for 7 days and the colonies appeared along the streak was checked to find out the MIC[22] .

Isolation of analogue resistant mutants

Freshly grown cells of the bacterium were treated with NTG solution (500 µg/ml) for 30 min at 37^{o}C. Cells were washed twice with TM buffer (pH 6.0) and were grown on 20 ml of Bergersen's synthetic medium for overnight at 30±2^{o}C on a rotary shaker. The cells were then spread directly on the surface of the agar medium containing the analogue 5-FT to higher concentration which completely inhibited the growth (MIC) of the wild strain. Plates were incubated at 30±2^{o}C for 7 days for appearance of colonies resistant to the treated analogue. A hallow of satellite colonies around the larger colony of the mutant 5-FT resistant colonies and non hallow 5-FT resistant colonies were picked up and checked further for their resistance to the analogue. Finally tryptophan and IAA production ability of the picked up analogue resistant strains along with the wild strain were checked. IAA and tryptophan were estimated by the following methods mentioned earlier.Statistical analyses were also made [23].

RESULTS AND DISCUSSION

The leguminous pulse *Phaseolus mungo* produced plenty of root nodules. The symbiont isolated from pink in color, mostly oval to circular in shape, mature healthy root nodules, was identified to be a species of *Rhizobium* and was supposed to be *R*. *phaseoli* as the host is *P*. *mungo*. Re-nodulation experiments following Koch's postulate confirmed the host. As compared with fast growing *Rhizobium* sp. [24] the working isolate PM25 was positive for catalase and indole production, PHB granule formation, VP test, nitrate reduction and 2% NaCl tolerance and was negative for amylase and protease activities. It was capable of producing acid but not gas from mannitol, galactose, maltose, xylose, myo-inositol, raffinose, fructose, sucrose, and glucose (data

not shown). Molecular phylogenetic analysis based on near complete 16S rRNA gene sequence suggested the taxonomic affiliation of the strain to the genus *Rhizobium*. In the NJ tree (Fig. 1) the strain was placed within *Rhizobium tropici* - *Rhizobium multihospitium* cluster. Based on biochemical and phylogenetic evidences the isolate PM 25 was identified as *Rhizobium* sp. Production of tryptophan in substantial amount was restricted due to complicated and branched pathway with stringent regulatory mechanism. Several techniques were employed in bacteria to obtain mutant with relaxed regulatory control wherein a metabolite in question accumulated in large quantities [25]. A good number of reports were available where the regulatory mechanisms have been overcome by isolation of analogue resistant mutants for the overproduction of tryptophan [26, 27]. The *Rhizobium* sp. (strain PM 25) could tolerate the tryptophan analogue i.e. 5-FT upto the concentration 2.0 mM (Table 1). So the MIC of the analogue for the strain was 3.0 mM (Table 1). With a view to produce tryptophan by the *Rhizobium* sp. (PM 25), attempts were made to isolate the mutants resistant to the tryptophan analogue i.e. 5-FT. Eight such 5FT resistant rhizobial strains have been isolated and tested for their ability for the production of tryptophan (Table 2).

For obtaining analogue (5-FT) resistant mutant prior to mutagenic treatment the sensitivity of the *Rhizobium* sp. to this analogue was determined. Many colonies appeared on 5.0 mM than 10.0 mM 5-FT supplemented agar medium. However, all such resistant mutants may not be regulation defective; the resistant may develop by some other reasons. The colonies appeared on 5.0 mM and 10.0 mM concentrations of 5-FT were picked up as analogue resistant mutants. The mutant strains were designated as analogue resistant mutants viz. $5FT^R$ 5A, $5FT^R$ 5B, $5FT^R$ 5C and $5FT^R$ 5D isolated from 5.0 mM 5FT supplemented medium. The mutant strains were designated as analogue resistant mutants viz. $5FT^R$ 10A, $5FT^R$ 10B, $5FT^R$ 10C and $5FT^R$ 10D isolated from 10.0 mM 5FT supplemented medium (Table 2). In order to study tryptophan and IAA productivity of the said 5-FT resistant mutants, they were cultivated in Bergersen's liquid medium without supplementing any tryptophan as precursor of IAA and analogue (Table 2). It was observed from the Table -2 that among the eight tryptophan producing 5-FT resistant mutants those were produced hallow satellite colonies are capable to produced tryptophan, which utilized for the production of IAA in culture, but those mutant strains were not produced the hallow satellite colonies which were unable to produced tryptophan or IAA in culture. The production of IAA was greatly varied with the amount of tryptophan produced by the mutants. This result strongly support that the tryptophan was the precursor of IAA production as already established earlier[28] and IAA biosynthesis of *Rhizobium* followed tryptophan dependent pathway. Production and metabolism of IAA in the root nodules and roots of the legume pulse *Phaseolus mungo* are already established [29]. The production of IAA in culture by the *Rhizobium* sp. isolated from the root nodules of the plant is also studied[12]. So from the above research work it can be concluded that the *Rhizobium* sp. might produce a significant amount of IAA symbiotically from the overproduction of tryptophan after undergoing change in their tryptophan biosynthesis pathway during different stress conditions.

Table 1
Determination of minimum inhibitory concentration (MIC) of Analogue (5-fluro-tryptophan)

Conc. of Analogue (mM)	Growth
0.0	++++
0.1	++++
0.5	+++
1.0	++
2.0	+
3.0	-
4.0	-
5.0	-
10.0	-

Table 2
Tryptophan and IAA production by the analogue resistant mutant

.**Bacterial strains**		**Growth OD at 540nm**	**Tryptophan production (µg/ml)**	**IAA production (µg/ml)**
Rhizobium strain (Wild)	PM 25 (*Rhizobium* sp.)	0.36	ND	ND
Analogue resistant mutant isolated from 5.0 mM 5FT supplemented medium	$5FT^R$ 5A	0.23±0.003	28.0±0.006	26.0±0.556
	$5FT^R$ 5B	0.24±0.006	ND	ND
	$5FT^R$ 5C	0.22±0.003	17.0±0.333	22.0±0.889
	$5FT^R$ 5D	0.21±0.009	ND	ND
Analogue resistant mutant isolated from 10.0 mM 5FT supplemented medium	$5FT^R$ 10A	0.28±0.009	43.0±0.333	37.0±0.668
	$5FT^R$ 10B	0.21±0.007	25.0±0.881	18.0±0.578
	$5FT^R$ 10C	0.29±0.006	ND	ND
	$5FT^R$ 10D	0.27±0.012	ND	ND

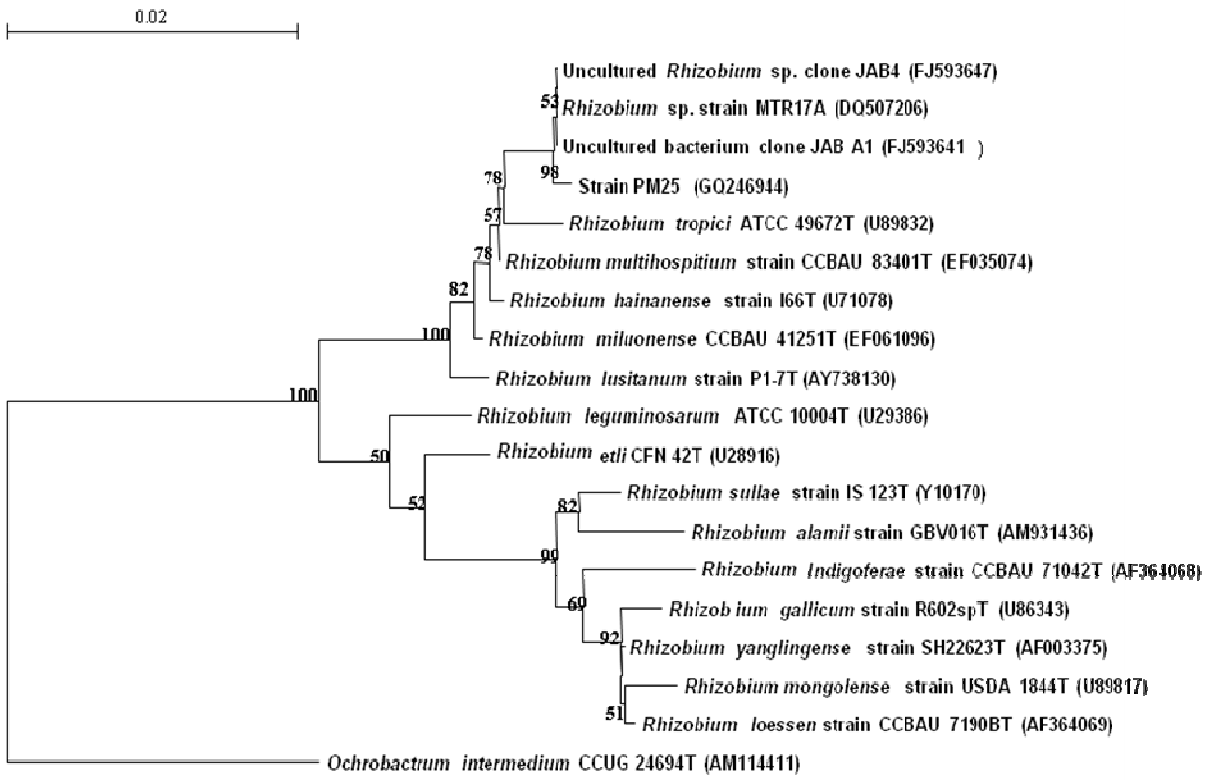

Figure 1

Phylogenetic tree based on neighbour-joining method, showing the relationships between PM 25 and other related taxa with some uncultured bacterium clones. Bootstrap values of 100 replications are shown at the branch. The tree was generated using TREECON and Jukes & Cantor correction. Sequence from Ochrobactrum intermedium CCUG 24694T (AM114411) strain was taken as an out group. Bar 0.1 base substitutions per site.

CONCLUSION

The *Rhizobium* sp. could generally produce IAA in culture when the media are supplemented with tryptophan. With a view to produce IAA in culture medium without supplementation of tryptophan, attempts were made to check the ability of IAA production by the tryptophan producing 5-FT resistant rhizobial strain. It was observed that some 5-FT resistant mutants were capable to produce IAA in culture without supplementation of tryptophan in growth medium. The production of IAA was greatly varied with the amount of tryptophan produced by the mutants. This result once again strongly support that the tryptophan was the precursor of IAA production. The work done here represents a new idea about tryptophan producing 5-FT analogue resistant rhizobial strain having the capacity of producing IAA from the excess tryptophan, as produced by the strain and opens a new line of research.

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Conflict of Interest

The authors declare that they have no conflict of interests.

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