ISOLATION, MOLECULAR CHARACTERIZATION AND PHYLOGENIC ANALYSIS OF *SERRATIA MARCESCES* ISOLATED FROM SOIL

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ABSTRACT

Serratia marcescens isolated from soil and identified on the basis of biochemical characteristics. This isolate was identified to produce extracellular Dnase, this isolate was subjected to molecular characterization by 16srRNA analysis. This sequenced sample was analysed for nucleotide matching from gene bank by the nucleotide blast. The strain showed 89% sequence similarity with *Serratia marcescens* Strain MTRI3 and labeled as *Serratia marcescens.*

Key Words: *Serratia marcescens*, (MTR3),16srRNA gene, soil isolate.

INTRODUCTION

Serratia marcescens(MTRI3) gram negative, non- spore forming bacterium belonging to genus *Serratia* and family Enterobacteriaceae (Holt *et al*, 1994) host strain of *Serraia marcescens* is motile with peritrichous flagella and produce a red diffusible pigment called Prodigiosin, In the soil *Serratia marcescens* might play a role in the biological cycle of metals by mineralizing organic iron and dissolving gold and copper (Eberi *et al*.,1999). Different plant associated role have been put forwarded for *Serratia marcescens*, including that of of a herbicide degradation bacterium (Selvakumar *et al*, 2008). Althought *Serratia marcescens* is conditional pathogenic bacterium that is capable of causing disease in diverse organisms, including humans (Richards *et al*.,2000), insects (Adamo,2004), and plants (Roberts *et al*., 2007), it is also a very important industrial strain which has been applied in fermentation for the production of various enzymes (Fu *et al*., 2004; Khardenavis *et al*.,2009; Ustariz *et al*., 2008). In this paper we report isolation and characterization of *Serratia marcescens* (MTR3) from soil.

MATERIAS AND METHODS

Isolation Screening and Identification

Soil samples were collected from different parts of Akola region and screened for *Serratia marcescens*. The collected soil samples were serially diluted and plated on nutrient agar plates after incubation and preserved at 4°C. The isolated colonies were identified based on Bergey's classification of determinative bacteriology.

A loopful of culture was inoculated in pre-sterilized 100ml nutrient broth. The broth flask was kept in a shaker at 120 rpm for 16-18h at 30°C. The culture broth was centrifuged at 6,000 rpm 30 min. Cell suspension was prepared using sterile distilled water and adjust to 0.2 OD using UV- Visible spectrophotometer. One percent of the above under UV-visible spectrophotometer was used as inoculums for the production of Prodigiosin. The bacterial isolate was subcutured in 100 ml of nutrient broth and incubated in a rotary shaker for 48hrs at 37°C, and then extracted Prodigiosin from production broth or medium (Slater *et a*l., 2003).

Pigment Extraction

The organism were harvested by centrifugation at 6,000 rpm for 30 minutes. The supernatant was discarded and the pellet was resuspended in acidified ethanol (4% 1M HCL in 96Ml ethanol). The mixture was vortexed and the suspension was transferred to a fresh vial and observed under UV- visible spectrophotometer at 534nm. The prodigiosin produced was quantified using known concentration of prodigiosin.

Determination of absorption spectra

Spectral analysis was made on dried pigment by dissolving in 10ml of absolute ethanol. Acidic condition for spectral analysis were obtained by adding 1ml of 1N Hydrochloric acid, to 10ml of the ethanol extract.(Williams *et al*., 1955). Spectral analysis was made on a UV-Visible Spectrometer.

DNA Extraction:

1. Bacterial Genomic DNA was isolated using the Insta Gene TM Matrix Genomic DNA isolation kit – As per the kit instruction below procedure followed.**col:**
2. An isolated bacterial colony was picked and suspend in 1ml of sterile water in a microfuge tube.
3. Centrifuge it for 1 minute at 10,000–12,000 rpm to remove the supernatant.
4. Add 200 µl of Insta Gene matrix to the pellet and incubate at 56 °C for 15 minutes.
5. Vortex at high speed for 10 seconds and place the tube in a 100 °C in heat block or boiling water bath for 8 minutes.
6. Finally, vortex the content at high speed for 10 seconds and Spin at 10,000–12,000 rpm for 2 minutes.
7. In result, 20µl of the supernatant was used per 50 µl PCR reaction.

2. PCR Protocol:

Using below 16S rRNA Universal primers gene fragment was amplified using MJ Research Peltier Thermal Cycler.

Primer Details:

Primer Name	Sequence Details	Number of Base
27F	AGAGTTTGATCMTGGCTCAG	20
1492R	TACGGYTACCTTGTTACGACTT	22

*Add 1*µL of template DNA in 20 µL of PCR reaction solution. Use 27F/1492R primers used for bacteria, and then PCR reaction performed with below conditions : Initial Denaturation 94℃ for 2 min and then 35 amplification cycles at 94℃ for 45 sec, 55℃ for 60 sec, and 72℃ for 60 sec. Final Extension at 72℃ for 10 min. DNA fragments are amplified about 1,400bp in the case of bacteria. Include a positive control (*E.coli* genomic DNA) and a negative controlin the PCR.

4. Sequencing protocol

Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Sequencing Primer Details:

Name of the Scientist	Service	Service Code No.
	Bacterial Identification – 16s rRNA Sequencing	IDSEQ01

5. Bioinformatics protocol:

1. The 16s r RNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of our sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.

2. The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar 2004). The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana 2007).Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as, Substitution model.

3. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering. (Dereeper *et al*., 2008).

RESULT AND DISCUSSION

Colony morphology

The Colonies of MTRI3 were observed on nutrient agar medium plates after 24hrs of incubation at 37^0C. Colony morphology on nutrient agar medium showed smooth, circular, entire and pigmented colonies (Fig2). Growth condition were optimized by testing growth on nutrient agar plates, Minimal media along with temperature range(25, 30, 37, 45 & 55°C) and pH (. 3, 5, 7, 11 & 12.) Maximum growth was obtained at temperature 30^0C and pH 7. The bacterial isolate elaborated the pigment at 30^0C. and the rate was reduced as the temperature increases. Williams & Hussain Quadri(1970) Reported that the no prodigiosin was observed when the temprature was sahifted to 27^0C. A complete block in prodigiosin was observed in most of the basically used media tested at 37^0C was similar to the result observed by Pryce & Terry(2000).

The production of Prodigiosin has been shown to be influenced by numerous environmental factors including media composition and pH (Weinberg.1970; Williamsons *et al*., 2005). Furthermore, the formation of Prodigiosin by *Serratia marcescens*(MTRI3) in buffered medium with a pH of 7.0 was 10 times greater than the formation in an unbuffered medium.

Effect of various oil substrate in which medium amended with peanut, sesame, coconut soya and neem oil. It was observed that maximum amount of prodigiosin resulted in the medium with peanut oil broth. Giri *et al.,(*2004) reported peanut seed powder increased the production of prodigiosin and they have concluded that the saturated form of fatty acid plays a role in enhanced cell growth and prodigisin production.

Effect of various Nitrogen source was observed in which 0.1% of Ammonium chloride as a N source supported maximum Prodigiosin production, In presence of Ammonium oxalate good pigmentation was observed, however in presence Ammonium nitrate and Ammonium citrate only little Prodigiosin was produced as compared to Ammonium oxalate (Silverman and Munoz,1973).

Effect of various incubation period was also observed It was found that 72 hrs optimized period. Prodigiosin production increases from 12to 72 hrs, maximum production was observed at 72hrs. Further increase in incubation does not show effect on Prodigiosin production. Devaraj N. *et al*.,(2009) has reported that, significant pigment production was observed on fifth and seventh day of incubation whereas the Prodigiosin completely reduced after seventh day of incubation.

Effect of various carbon source was observed , While considering the basic role of carbon source in augmenting the pigment production, the addition of maltose produced maximum pigment. Anna R Oller (2005) reported that glucose and sorbitol had a repressive effect on prodigiosin production synthesis. Chang *et al.,*(2000) has reported 3mg/ml of prodigiosin when dextrose was used in the medium.

Microscopic, Biochemical and Enzymatic analysis

The cells stained Gram negative upon gram staining. They were rod shaped and arranged singly Grimont F. (1981). Motility of the strain was observed under wet mount which indicates the presence of flagella. Various biochemical tests were performed to characterize the strain and the isolate showed negative for indole test, negative for methyl red, positive for vogesprosker and positive for citrate utilization test. These observation were in accordance with reported biochemical characters of MTRI3.

Further tests performed to study the enzymes likes gelatinase, protease and dnase which are important in virulence characters expression by *serratia marcescens*(MTRI3). When culture inoculated on to a DNA agar medium, zone of clearance was observed around the colonies when flooded with 2 NHCL which showed the presence of an extracellular Dnase enzyme. Gelatin liquefaction was observed on gelatin agar which confirms the presence of gelatin enzyme. Protease production was observed on skim milk agar plates as clear zones around the colonies around the colonies after 24hrs. Different sugar fermentation tests were carried out further to analyze the biochemical reactions in the organisms. This isolate was able to ferment lactose (Table 1). These results match with some reference papers on *Serratia marcescens.*

Molecular characterization

In spite of tentative biochemical identification of the isolate as *Serratia marcescens*(MTRI3) a detailed molecular characterization was undertaken in order to identify the position of the isolate MTRI3.Part of the 16s rRNA gene sequence of MTRI3 was amplified and analysed(Table1,2 & Fig1)

Table 1: Blast Analysis for MTRI3: Blast Report - Query name : isolate-3_contig_1- Query length : 1493

Query		Subject					Score			Identities			
Start	End	Description	AC	Length	Start	End	Bit	Raw	EV	Match	Total	Pct.(%)	Strand
1	1488	Serratia marcescens strain B3R3, complete genome	CP013046.1	5471439	4277	2791	2730	1478	0.0	1485	1488	99	Plus/Minus
1	1488	Serratia marcescens strain RSC-14, complete genome	CP012639.1	5127030	1211480	1209994	2730	1478	0.0	1485	1488	99	Plus/Minus
1	1488	Serratia sp. CC-SYL-A 16S ribosomal RNA gene, partial sequence	KC169816.1	1510	5	1491	2730	1478	0.0	1485	1488	99	Plus/Plus
1	1488	Pseudomonas fluorescens gene for 16S ribosomal RNA, partial sequence	AB091837.2	1506	20	1506	2730	1478	0.0	1485	1488	99	Plus/Plus
1	1480	Serratia sp. 4034 16S ribosomal RNA gene, partial sequence	JX566600.1	1529	29	1507	2726	1476	0.0	1479	1480	99	Plus/Plus
1	1488	Serratia marcescens strain SmUNAM836, complete genome	CP012685.1	5207023	3447863	3446377	2724	1475	0.0	1484	1488	99	Plus/Minus
1	1491	Serratia marcescens strain IHB B 7064 16S ribosomal RNA gene, partial sequence	KJ721215.1	1510	18	1507	2724	1475	0.0	1486	1491	99	Plus/Plus
1	1488	Serratia sp. FS14, complete genome	CP005927.1	5249875	573056	571570	2724	1475	0.0	1484	1488	99	Plus/Minus
1	1488	Uncultured bacterium clone YZ22 16S ribosomal RNA gene, partial sequence	KJ457339.1	1503	17	1503	2724	1475	0.0	1484	1488	99	Plus/Plus
1	1488	Bacterium A2(2013) 16S ribosomal RNA gene, partial sequence	KF114399.1	1534	17	1503	2724	1475	0.0	1484	1488	99	Plus/Plus

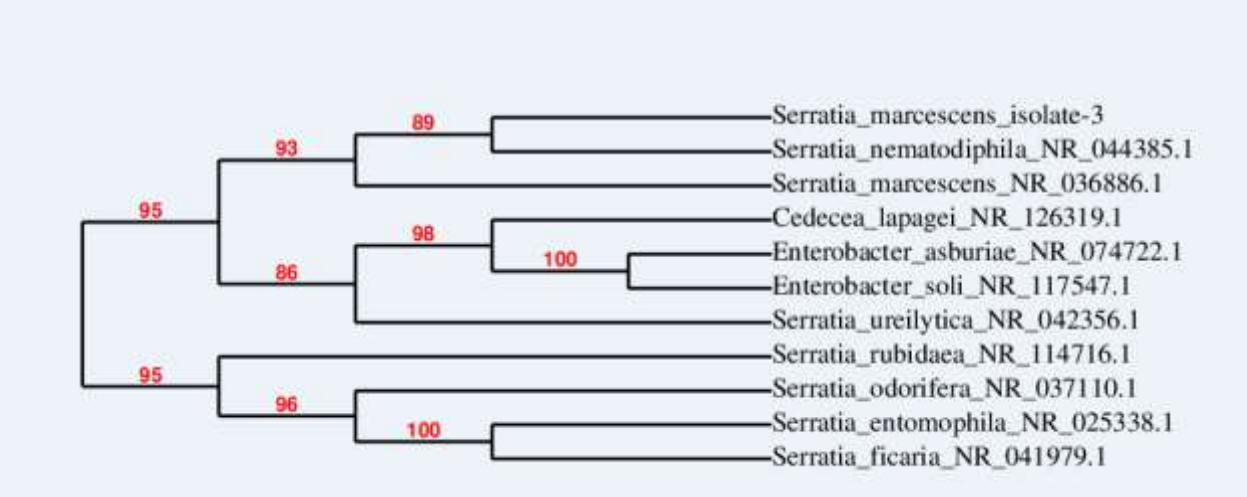

Fig 1: Phylogeny tree for MTRI3

Table 2: Phenotypic Characters of MTRI3

Sr. No.	Test	Results
1	Gram staining	Gram negative rods arranged singly
2	Pigment production	Positive
3	Motility	Positive
4	Indole	Negative
5	Methyl red	Negative
6	Voges proskeur	Positive
7	Citrate	Positive
8	Gelatin liquefaction	Positive
9	Casein hydrolysis	Positive
10	Catalse	Positive
11	Glucose fermentation	Positive
12	Lactose fermentation	Positive
13	Sucrose fermentation	Positive
14	Fructose Fermentation	Positive

Fig2: Growth of Serratia marscens on nutrient agar.

Fig3: Dnase Positive Test.

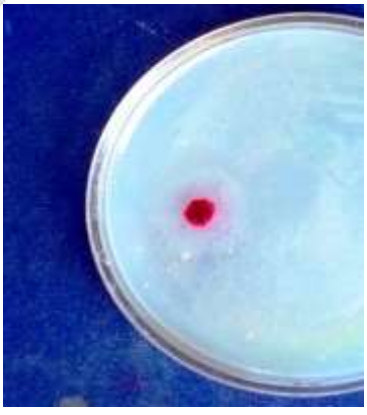

Fig4: Gelatin liquefaction Positive Test.

CONCLUSION

The bacterium isolated from soil of shri shivaji college Akola was identified by using different microscopic, biochemical, enzymatic tests as a member of Enterobacteriaceae according to bergeys manual of systematic bacteriology. The red pigment prodigiosin production confirmed it to be a *Serratia marcescens*(MTRI3). As this isolate has the potential of producing extracellular Dnase activity, molecular characterization and phylogenetic analysis was carried. Phylogenetic analysis proved the isolate to be a *Serratia marcescens* was named as *Serratia marcescens*(MTRI3).

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