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Molecular Characterization of Psychrotrophic Serratia marcescens TS1 Isolated from Apple Garden at Badran Kashmir

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Abstract: Summary A psychrotrophic bacterial strain was isolated from the apple garden around Badran Magam Kashmir using deoxyribonulease toluidine blue cephalothin agar. The psychrotrophic organism was characterized by all biochemical tests and showed similarity with *Serratia marcescens*. The genomic level confirmation done with 16s rDNA the sequence showed 98% sequence similarity with *Serratia marcescens* and thus strain named as *Serrtia marcescens* TS1 and was deposoited in gene bank.

Key words: Psychrotrophic organism, *Serratia marcescens* TS1, 16s rDNA genomic analysis, apple garden isolate

INTRODUCTION

Serratia marcescens is a gram-negative bacteria belonging to the genus *Serratia* and family enterobacteriaceae [9]. It is on the whole smaller than the average coliform bacterium. Similarly there is great variation in their ability to produce a non-diffusible red pigment, prodigiosin or 2-methyl-3amyl-6-methoxy prodigiosin [24] that is formed in presence of oxygen and a suitable temperature. Most strains of *Serratia marcescens* are motile with peritrichious flagella, which are usually best seen in culture grown at temperature below 37^0C. Strains producing the non-diffusible red pigment prodigiosin seem to be toxic to protozoa [12]. In the soil *Serratia marcescens* might play a role in the biological cycle of metals by mineralizing organic iron and dissolving gold and copper [3]. A mineralization role has also been attributed to cold tolerant *Serratia* associated and can grow at low temperature [15]. A yellow diffusible pigment, 2-hydroxy-5-carboxymethyl muconic acid semialdehyde, is produced from the Meta cleavage of 3, 4-dihydroxyphenylacetic acid (3, 4 DHP) by the enzyme 3, 4-DHP 2, 3-dioxygenase [23].

MATERIALS AND METHODS

Soil sample: Soil samples were collected from the cold place of dense apple garden around at Badran Magam in Kashmir and processed for the islation of psychrotrophic bacteria.

Isolation of the Bacterium: The organisms were isolated [6] by using the Deoxyribonuclease Toluidine Blue-cephalothin agar which contains: Deoxyribonucleic acid 41g/l, Sodium chloride 1g/l, Toludine blue 0.20g/l, Agar 15g/l, having p^H 7.3 and 5ml of 100mg of sterile cephalothin/ml. All ingredients were prepared and sterilized then poured on sterilized plates. The soil samples were serially diluted and spread over the medium and incubated at 20^0C for 24 hours.

Identification of the Bacterium: The morophology and biochemical characterization of isolated strain TS1 was performed [11].

1.Microscopic morphology: Microscopic morphology was observed by gram staining, flagella staining and by hanging drop method.

2. Biochemical test: Biochemical tests carried out such as indole, methyl red, voges proskeaur, citrate, triple sugar iron, carbohydrate fermentation viz xylose, arabinose, lactose, rhamnose, fructose, sucrose, sorbitol, and glucose, to find out the biochemical characters of strain TS1

3. Enzyme Hydrolysis test: Various enzymatic hydrolysis tests such as deoxyribonuclease test, Gelatinase test, Casein hydrolysis test and Lipid hydrolysis test were carried to find out organism

Molecular confirmation of most promising strain TS1 by 16s rDNA:

Extraction of bacterial DNA: The DNA was extracted from the bacterial isolate [8] by taking a single colony of bacterial strain TS1 was inoculated into the 50ml of

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Luria Bertani broth and incubated at 20^0C on rotatory shaker for overnight. Then 1.5 ml of culture was transferred into eppendroff tubes and spin at 8000rpm for 10 minutes. The supernatant were discarded and drained on tissue paper. The pellet was resuspended into 400ml of TE buffer, to this 32ml of lysosome was added and incubated at 37^0C for 30 minutes. After incubation 100ml of 0.5mM EDTA was added followed by 60ml of 10% SDS and 1.5ml of proteinase K (50ml/1ml) respectively then incubated at 50^0C for 60 minutes. After incubation tubes were brought at room temperature and 250ml of Phenol: Chloroform: Isoamylalchol in the ratio of 25:24:1 were added and centrifuged at 10000rpm for 10 minutes. The aqueous phase was transferred to another eppendroff tubes and RNAase was added at final concentration of 50ml/ml and mixture was incubated at 60^0C for 1 hour. After incubation DNA was precipitated with ice-cold ethanol and precipitated DNA was collected by centrifugation at 5000rpm for 10 minutes. Finally the pellet was washed with 70% ethanol and completely air dried and resuspended in l00µl of TE buffer at p^H 8.0.

Polymerase Chain Reaction: The polymerase chain reaction was carried out by following a method of Sambrook and Russel (2007). DNA sample of strain TS1, Primer 16s rDNA Forward primer 5' TAG GGA AGA TAA TGA CGG 3' Reverse primer 5' CCT CTA TCC TCT TTC CAA CC3', 10X amplification buffer contains 500mM KCl**,** 100mM Tris-HCl (p^H 8.3)**,** 15mM $MgCl_2$**,** DNTP solution (20mM) having p^H 8.0**,** Taq DNA polymerase**,** Thermal cycler programmed with desired amplification protocol**,** Ethidium bromide 0.5m g/ml, 1500bp DNA marker. The 50ml reaction mixture was transferred into 0.5ml microfuge amplification tube containing mixture in following order: 10X amplification buffer 5ml, 20mM solution of four dNTP p^H 8.0 1ml, 20mM forward primer 2ml, 20mM reverse primer 2ml, Taq DNA polymerase 2ml, Nuclease free water 33ml, DNA sample 5ml, total volume 50ml. The reaction mixture was gently centrifuged at 4000rpm for 5 minutes inorder to settle down the mixture which might had got layered on the walls of microfuge amplification Tube. The reaction mixture was placed in the thermal cycler fitted with heated lid. The nucleic acids were amplified by setting denaturation at 94^0C 1 minute, Annealing at 55^0C for 30 seconds and extension at 72^0C for 1 minute in the thermal cycler and number of cycles repeated 30 cycles**.** After polymerase chain reaction 15ml of amplified DNA product was run in an agrose gel electrophoreses by preparing 1.5% agarose gel and DNA bands were viewed under the UV transilluminator.

Automated Sequencing: The sequencing of the genomic DNA amplicon coding for strain TS1 was carried out at scientific synergy company Canal Bank Road Chennai, India using an instrument ABI3130, Amersham Biosciences United kingdom [21].

Purification: 1µl of PCR reaction mixture was aliquated into a 0.6ml microfuge tube and diluted to a total volume of 20µl with distilled water. Then 20µl of 4M-ammonium acetate was added and mixed well. To this 40µl of isoproponal was added and mixed well. It was left at room temperature for 10 minutes and after that the tubes were centrifuged at 12,000g for 10 minutes to obtain the DNA precipitate. The supernatant was removed, discarded and the pellets washed with 70% ethanol to obtain pure DNA. Then pellet was dried under vacuum and resuspended in 20µl TE buffer.

Materials for Cycle Sequencing: Prism Tm ready reaction dideoxy terminator premix, I: ABI mix 1.5µM dd ATP viz 94.7µM dd TTP, 0.42µM dd GTP, 43.3µM dd CTP, 168.43mM Tris p^H 9.0, II: 4.2mM $(NH_4)SO_4$, III: 0.42U/µl Ampli Taq DNA polymerase

Cycle sequencing of PCR products: The following reagents were mixed in 0.6ml microfuge tube: 5µl of DNA template, 1µl of primer used for PCR, 4.4µl sterile water. To this 9.5µl of ABI prism ready mixture ddNTP primer was added. The reaction mixture was placed in a thermocycler and following conditions were employed, Denaturation at 94^0C for 1 minute, Annealing at 55^0C for 30 second, Extension at 72^0C for 1 minute, Total reaction cycle – 30 and samples were kept at 4^0C.

Materials for Cycle Sequencing Products: Phenol: water: Chloroform (17:18:15), 2M sodium acetate (p^H 4.5), 100% and 70% ethanol, Sequencing gel (6%), 10X TBE buffer contains; 890mM Tris Borate, 890mM Boric acid, 20mM EDTA (p^H 8.3), 12ml of 40% urea/acrylamide (W/V), (19:1) Acryl amide/Bisacrylamide, 20ml distilled water, 1g mixed bed ion exchange resin, TEMED, 10% W/V ammonium per sulphate.

Extraction of Cycle Sequencing Products: 80µl of sterile water was added to PCR DNA sample, to this 100µl of phenol: water: chloroform (68:18:14) mixture was added and vortexes well, then sample was centrifuged at 12000g for 1 minute. The supernatant was transferred to a clean eppendroff tube and 15µl of 2M sodium acetate, 300µl of 100% ethanol were added and mixed well. The mixture was centrifuged at 12000g for 15 minutes at room temperature. The supernatant was removed and pellet washed with 70% ethanol then dried under vacuum and resuspended in 20µl of TE buffer.

Sequencing Sample Preparation: 4µl of deionizer foramide: 50mM EDTA having p^H 8.0 was added to sequencing product tube and mixed then centrifuged briefly. Before loading the sample was heated at 90^0C for 3 minutes and transferred immediately to ice. Then sample was loaded on to automated DNA sequence (ABI3130) fitted with 6% polyacrylamide gel and was run, then result were analyzed for determining the phylogenetic relationship by submitting to gene bank and accession number were obtained.

Phlyogenetic Tree Analysis: Nucleotide sequence was compared to those in the Gene Bank database with Basic Local Alignment Search Tool (BLAST) algorithms to identify known closely releated sequences by Chromo Software. The tree was generated by the neighbour joining [19] algorithm implemented in phydit the assemblage of 16s rDNA gene sequences in each library was analyzed by rarefaction analysis using Ecosim [7] to assess the extent to which the diversity of microbial communities was represented by the library at the class and species level. The number of species in each clone library was determined [22] comparing closely related sequence using b12 seq (http:// www.ncbi. nlm. nih. gov/blast/b12seq/wblast2.cgi). The 16s rDNA sequences exhibiting a percentage of similarity of 97% or lower [4] were considered for species authentication.

RESULTS AND DISCUSSION

Colony Morphology of Soil Isolates: After 48 hours of incubation in deoxyribonuclease toluidine blue cephalothin agar medium $21.1x10^4$ CFU were isolated from the soil collected from the apple garden around at Badran Magam in Kashmir. All the isolates showed visible growth at 20^0C. The purified colony morphology appears as red colour pigmented convex and relatively opaque centre effuse, colorless, almost transparent periphery and irregular crenate edge surrounded by a clear zone around the colonies were observed.

Microscopic Appearance of Strain TS1: The microscopic examination showed gram-negative rods in gram staining and in flagella staining it showed a pertrichious flagella while under wet mount it showed active motility.

Biochemical Tests: The various biochemical tests were performed and found that isolated strain TS1 belonged to genus *Serratia marcescens* are shown in Table 1.

Carbohydrate Fermentation Test: The strain TS1 has shown both fermentation and non-fermentation raction are described in Table 2. Thus confirmed biochemically that it belongs to *Serratia marcescens.*

Enzyme Tests: The organisms *Serratia marcescens* have utilized the protein gelain and led the liquefaction of the medium that was a positive result (Figure 1). In case of deoxyribonuclease test the organism have produced deoxyribonuclease an extracellular enzyme by showing the zone around the colonies thus indicated positive result (Figure 2). The strain TS1 has utilized the casein and there was a zone of hydrolysis around the colonies (Figure 3). The strain has utilized the lipid in the medium and there was zone around the colonies (Figure 4).

Fig. 1: Gelatin had hydrolyzed by *Serratia marcescens* TS1 by showing liquefaction in the nutrient gelatin medium.

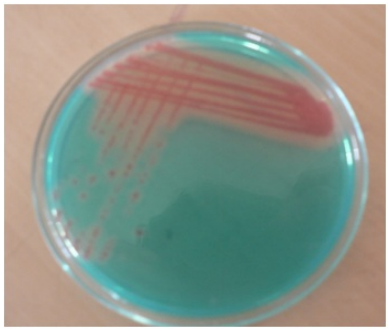

Fig. 2: Deoxyribonuclease activity of *Serratia marcescens* TS1 by sowing a clear zone around the colonies.

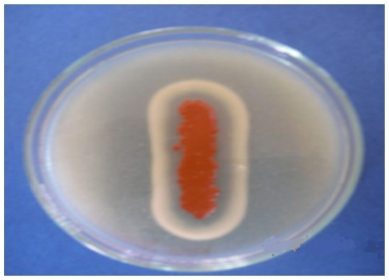

Fig. 3: Casein hydrolysis by *Serratia marcescens* TS1 by showing zone formation around colonies

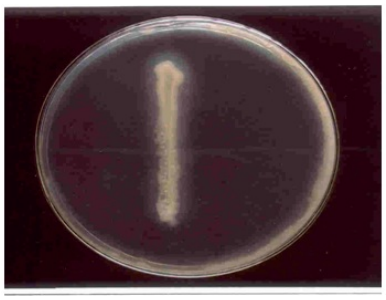

Fig. 4: Lipolytic activity of *Serratia marcescens* TSI by showing a clear zone around the colonies.

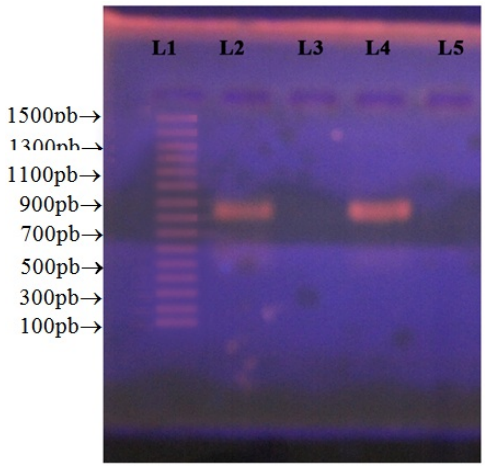

Fig. 5: PCR Product; DNA of *Serratia marcescens* TSI L1-Marker (1500bp), L2, L4, DNA Samples (920bp)

Molecular Characterization of Strain: The genomic DNA isolated from strain TSI when visualized under U.V transilluminator. The sharp orange bands were found in agarose gel electrophoresis. The genomic DNA was amplified with 16s rDNA primer, Forward primer: 5' TAG GGA AGA TAA TGA CGG3' Reverse primer: 5' CCT CTA TCC TCT TTC CAA CC 3'.The amplified PCR product when run in agarose gel electrophoresis, strain TS1 was found having a molecular weight approximately of 920bp when compared with the DNA marker (Figure 5). The strain TSI was identified and named as *Serratia marcescens* TS1 under Gene Bank ACC.No-GU046543. The genomic seguence obtained are

CATGCAAGTCGAGCGGTAGCACAGGGGAGCTT
GCTCCCTGGGTGACGAGCGGCGGACGGGTGAG
TAATGTCTGGGAAACTGCCTGATGGAGGGGGA
TAACTACTGGAAACGGTAGCTAATACCGCATA
ACGTCGCAAGACCAAAGAGGGGGACCTTCGGG
CCTCTTGCCATCAGATGTGCCCAGATGGGATTA
GCTAGTAGGTGGGGTAATGGCTCACCTAGGCG
ACGATCCCTAGCTGGTCTGAGAGGATGACCAG
CCACACTGGAACTGAGACACGGTCCAGACTCC
TACGGGAGGCAGCAGTGGGGAATATTGCACAA
TGGGCGCAAGCCTGATGCAGCCATGCCGCGTG
TGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTT
CAGCGAGGAGGAAGGTGGTGAACTTAATACGT
TCATCAATTGACGTTACTCGCAGAAGAAGCACC
GGCTAACTCCGTGCCAGCAGCCGCGGTAATAC
GGAGGGTGCAAGCGTTAATCGGAATTACTGGG
CGTAAAGCGCACGCAGGCGGTTTGTTAA

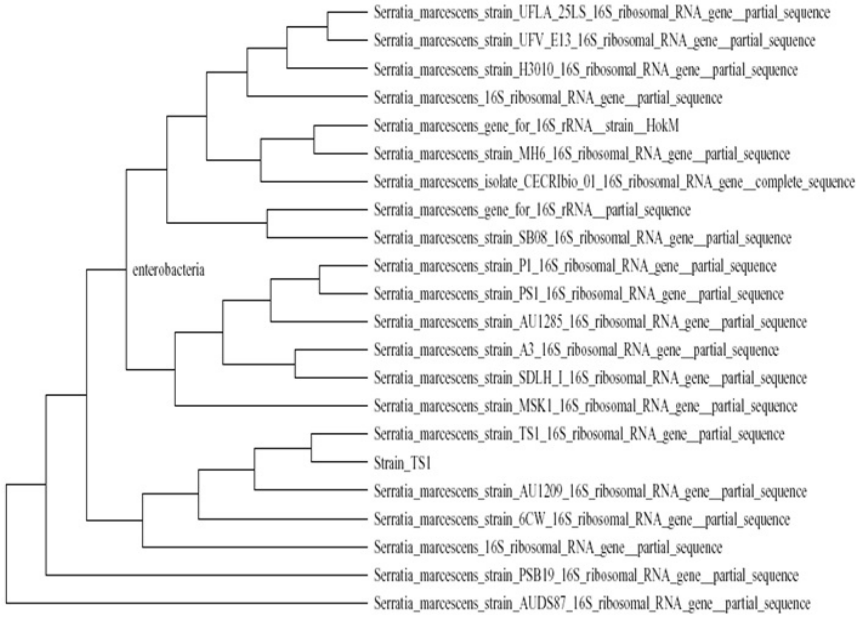

Fig. 6: Phylogenetic tree analysis of strain TSI was drawn by multiple sequence alignment with neighbor joining method.

Table 1: Biochemical identification profile of Serratia marcescens TS1

S.NO	Biochemical test	Result
1	Indole	N
2	Methyl Red	N
3	Voges Proskauer	P
4	Citrate	P
5	Triple sugar iron	A/K G-ive
6	Hydrogen sulphide production	N

N-negative, P-positive, A/K - Acid/Alkaline
G-v gas negativ

Table 2: Carbohydrate fermentation profile of Serratia marcescens TS1

S.NO	Carbohydrate	Result
1	Glucose	F
2	Sucrose	F
3	Sorbitol	F
4	Fructose	F
5	Xylose	NF
6	Rhaminose	NF
7	Lactose	NF
8	Arabinose	NF

F-fermentation, NF- no fermentation

GTCAGATGTGAAATCCCCGGGCTCAACCTGGG
GAACTGCATTTGAAACTGGCAAGCTAGAGTCTC
GTAGAGGGGGGTAGAATTCCAGGTGTAGCGGG
TGAAATGCGTAGAGATCTGGAGGAATACCGGT
GGCGAAGGCGGCCCCCTGGACGAAGACTGACG
CTCAGTGCGAAAGCGTGGGGAGCAAAGCAGGA
TTAGATACCCTGGTAGTCCACGCTGTAAACGAT
GTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGC
TTCCGGAGCTAACGCGTAATCGACCGCCTGGG
GGAGTACGGCCGCAAGGTTAAAACTCAATGAA
TTGACTGGGGCCCGCACAAGGCGGTGGGAGCA
TGTGGTTTAATCGATGCAACGC.

The 16s rDNA sequences of the amplified products revealed that the strains TS1 had unique sequences which matched with *Serratia marcescens* present by phylogenetic tree (Figure 6).

Discussion: A strain producing the non-diffusible red pigment prodigiosin seems to be toxic to protozoa [12] and this may an ecological advantage in soil and water. However it seems that pigmented bacteria are more often isolated from unpolluted water (from springs or wells) than from the polluted water (river water down stream from cities). Pigmented species and biotypes of *Serratia* often exhibit pink or red colonies on nutrient agar. Use of low phosphate agar without glucose such as peptone glycerol agar is best in order to demonstrate pigmentation [24]. The increase in temperature leads decrease in prodigiosin production but increase in growth while the decrease in temperature leads more prodigiosin production in presence of oxygen and decrease in growth. The selected strain was named as strain TSI. The colonies were subcultured to get a purified colony [18]. When the strain TS1 observed under microscope the colonies appeared gram-negative rod shaped bacteria pink colour [10]. The bacterial strain TS1 appeared dark black rods an outer coat that bears dark stained flagella seen peritrichious tiney weavey threads of greater length than cells. The flagella helps in motility, swarming motility is a type of population migration behavior characteristic of some bacterial species on solid medium [5]. Extracellular nuclease is produced by bacteria are usually reported as deoxyribonuclease and ribonuclease [6]. The use of the agar plate technique for determining the activity of microorganisms on nucleic acid demonstrated the *Serratia marcescens* could degrade both DNA and RNA the present study reveled that *Serratia marcescens* strain TS1 have degraded the DNA by producing an enzyme deoxyribonuclease there was a zone around the colonies and colour change from blue to red. The genomic DNA was isolated from the strain TSI [10]. The orange colour sharp bands were observed. The orange colour bands in agrose gel were due the intercalating of ethidium bromide is a fluorescent dye. The need for rapid and reliable identification of this organism has become more important in the past few years. Although conventional biochemical, phenotypical and genotypical methods [14] for identifying *Serratia* strains are reliable. They require several days to complete but the PCR technology has the advantage of not only higher sensitivity and specificity but also a reduction in time and workload. The amplified product of genomic DNA was obtained with 16s rDNA by forward primer 5' TAG GGA AGA TAA TGA CGG 3' and reverse primer 5' CCT CTA TCC TCT TTC CAA CC 3'. The molecular weight was about 960bp in the strain TSI [27,25,26]. The whole cyele was repeated for 30 times and amplified product was obtained [20]. Nucleic acid based diagnostic systems including polymerase chain reaction methods as well as the application of DNA and RNA probes are well known sensitive techniques for more rapid detection and specific identification of an organism there fore hold promise for sensitive and specific detection within much shorter time [13]. The genotyping is easiest technique as reproducible as ribotyping and with almost the same ability to discriminate different strains [16]. The DNA was used as template to amplify by polymerase chain reaction by designed two primers that is forward and reverse primer with reaction mixtures at appropriate conditions [17]. The PCR product was run in agrose gel electrophroses showed 960bp for strain TSI. The DNA sequence of the strain TSI having Gene Bank ACC.NO.GUO46543 when compared to those in Gene bank data bases with BLAST were found 98% similarites with *Serratia marcescens*. This strain named as *Serratia marcescens* TS1 (Gene bank ACC. No. GU046543). The pholygenetic tree showed resemblence with *Serratia marceseens*.

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