Molecular characterization and Phylogenetic analysis of *Serratia sp*-YAJS- An extracellular Dnase producer isolated from rhizosphere soil

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ABSTRACT

Soil isolate obtained in our laboratory was identified as *Serratia* sp., based on microscopic and biochemical characteristics. This isolate was identified to produce extracellular Dnase when tested on Dnase test agar. As very few strains are known to produce extracellular Dnase, this isolate was subjected to molecular characterization by 16srRNA analysis. The sequenced sample was analyzed for nucleotide matching from gene bank by the nucleotide blast. The strain showed 95% sequence similarity with Serratia *marcescens strain SB08* and was labeled as *Serratia sps YAJS.*

Key words: *Serratia*, 16srRNA gene, soil isolate, *Serratia sp* YAJS.

INTRODUCTION

*Serratia marcescens*is gram-negative non-spore-forming bacterium belonging to the genus *Serratia* and family *Enterobacteriaceae* (Holt *et al,* 1994) host strains of *Serratia* are motile with peritrichous flagella and produce a red diffusible pigment called prodigiosin. In the soil *Serratiamarcescens* might play a role in the biological cycle of metals by mineralizing organic iron and dissolving gold and copper (Eberl *et al,* 1999) Different plant associated roles have been put forward for *Serratia marcescens*, including that of a herbicide degradation bacterium (Silva *et al*, 2007); a plant growth promoting *rhizobacterium* (Selvakumar *et al, 2008*). Although *S.marcescens* is a conditional pathogenic bacterium that is capable of causing disease in diverse organisms, including humans (Richards *et al*, 2000) corals (Patterson *et al*, 2002), 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 sps YAJS* obtained from soil samples.

MATERIALS AND METHODS

Isolation of bacterium

Different rhizosphere soil samples were collected from college campus for screening purpose. Serial dilutions of soil samples were performed and plated on Nutrient agar medium for isolating the bacteria.

Morphological, biochemical and enzymatic characterization

Morphology was observed by gram staining and hanging drop method (Gopal Reddy *et al,* 2005). Biochemical characterization was done with different tests such as indole, methyl red, vogesproskeaur and citrate. The carbohydrate fermentation tests such as, fructose, glucose, by lactose and sucrose were carried out to check for acid and gas production. Various enzymatic tests such as gelatinase, protease and Dnase were performed to find out potential of the organism. Antibiotic sensitivity test for different antibiotics was done to check for the sensitivity of organism by disc diffusion method.

Molecular characterization by 16s rRNA:

Genomic DNA isolation

Bacterial colony from the culture plate was used directly to isolate Genomic DNA by kit devised by Indigenèse Biotechnologies. Bacterial cell pellet was dissolved in buffer and centrifuged at 10,000rpm for 2 min. After centrifugation, clear lysate was collected in new eppendorf and mixed with 50 µl of DBM.

This mixture was centrifuged at 10,000 rpm for 4min at room temperature to obtain pellet. The pellet was washed with 200µl of wash buffer. The sample was centrifuged at 10,000 rpm for 4min at room temperature to remove the traces of wash Buffer completely. 20 µl of Elution Buffer was added to the pellet, mixed and incubated at room temperature for 2-3 min. The supernatant with the DNA was collected after centrifugation at 12,000 rpm for 4min at room temperature and stored at -20^{o} C. The products were analyzed on 1% agarose gel electrophoresis.

Polymerase chain reaction

The polymerase chain reaction was carried out by following a method of Sambrook *etal* 2001. Primers used for the amplification of the 16S region in Eubacteria are:

Forward primer Bac8F: 5' AGA GTT TGA TCC TGG CTC AG3'
Reverse primer 1392 R: 5' GGT TAC CTT GTT ACG ACT T 3'

The 50 µl reaction mixture was transferred into 0.5ml microfuge amplification tube containing the mixture in following order: 10x amplification buffer (5 µl), 20 mM solution of four dNTP's ,pH8 (1 µl), 20 µM forward primer (2 µl) 20 µM reverse primer (2 µl) , Taq DNA polymerase (2 µl), nuclease free water (33 µl), DNA sample (5 µl).The reaction mixture was centrifuged at 4,000 rpm for 5 min then placed in the thermocycler .The nucleic acids amplified by setting denaturation at 94^0C for 1 min,annealing at 55^0C for 30s and extension at 72 ^{0}C for 1 min in the thermocycler and number of denaturation cycles repeated to 18 cycles.After PCR ,15 µl of amplified DNA product was run in an agarose gel electrophoresis by preparing 1.5% agarose gel and bands were viewed under the UV transilluminator.

Each PCR was set in triplicate for 18 cycles, the bands were checkedon agarose gel electrophoresis, pooled for purification by ethanol precipitation, and then the purified samples were sequenced. DNA sequencing sample was processed using ABI 3730Xl (96 capillary) electrophoresis instrument .The sequenced sample was analyzed for nucleotide matching from gene bank by the nucleotide blast, where the sequence was pasted in the FASTA format and analyzed.

Phylogenetic analysis

Nucleotide sequence was compared to those in the Gene Bank database with Basic Local Alignment Search Tool (BLAST) algorithms to identify known closely related sequences.

Phylogenetic analysis was performed after including the consensus sequence in an alignment of small ribosomal subunit sequences collected from the international nucleotide sequence library GenBank. Phylogenetic and molecular evolutionary analyses were conducted using the genetic distance-based neighbor-joining algorithms (http://www.ncbi.nlm.nih.gov).

RESULTS AND DISCUSSION

Colony morphology:

The colonies of isolate were observed on nutrient agar medium plates after 24 hrs of incubation at 37^oC. Colony morphology on nutrient agar showed smooth, circular entire and pigmented colonies (Fig 1). Growth conditions were optimized by testing growth on Nutrient agar, luria-bertin (L.B) agar, Minimal media along with range of temperature (15 27 32, 37 &40^0C) & pH (5, 6, 7, 8, & 9) required for maximum growth. Maximum growth was obtained at 32^oc with pH 7 in Nutrient broth. Red colour diffusible pigment prodigiosin production was observed after 24hr of incubation and the colour of pigment enhanced when subjected to lower temperatures (William and Quadri, 1980)

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 observations were in accordance with the reported biochemical characters of *Serratia*.

Later tests were performed to study the enzymes like gelatinase, protease & dnase which are important in virulence character expression by *Serratia*. Gelatin liquefaction was observed on gelatin agar which confirms the presence of gelatinase enzyme. When 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.Intrestingly this isolate was identified to produce big zone of clearance on Dnase agar test medium.Protease production was observed on skim milk agar plate as clear zones around the colonies after 24 hr incubation.

Different sugar fermentation tests were carried out further to analyze the biochemical reactions in the organism. This isolate was able to ferment sucrose, glucose and fructose while it could not ferment lactose (Table 1). These results match with the reported literature on *Serratia*.

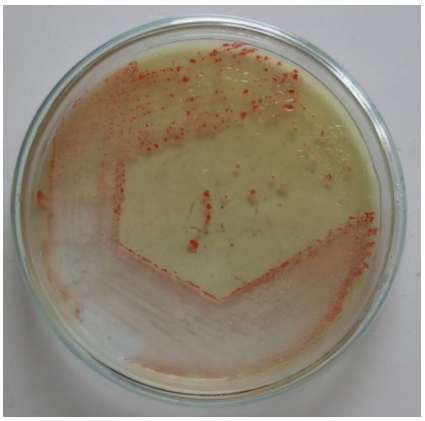
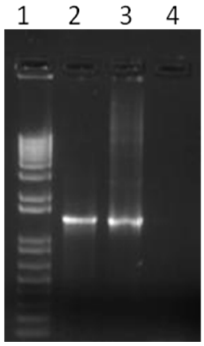


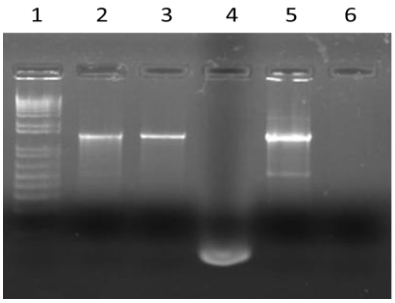
Fig. 1: Growth of *Serratia isolate* on nutrient agar medium.



1. 1Kb ladder
2. Sample pcr
3. Control pcr
4. Negative control

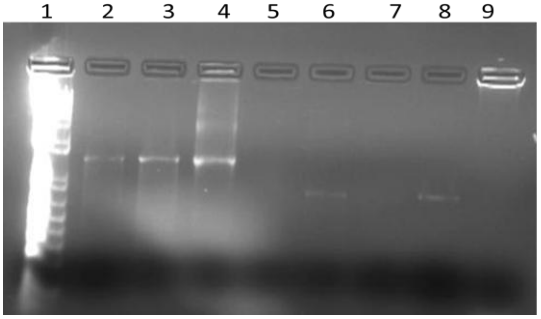
Fig. 2: Gel picture of the PCR products run on an agarose gel. The amplified band size is 1600 bp

Fig 3 :Dnase activity of the isolate, On DNA agar medium. (*activity enhanced after 48 hours)



1. 1Kb ladder
2. Positive control
3. Sample with Genomic DNA as a template
4. Sample with Bacterial lysate as a template
5. Ethanol precipitated sample
6. Negative control with out template

Fig. 4: Observation for DNA degradation with different factors



Lane # 1: 1Kb ladder
Lane # 2 :Positive control for 16s fragment
3 :PCR product with 1mM calcium chloride
4: Ethanol precipitated PCR product after two days
5: Negative control
6: GFP PCR with GFP template and Bacterial Genomic DNA
7: GFP PCR with GFP template and Bacterial lysate
8: GFP PCR with GFP template
9: Negative control.

Fig. 5: Observation of DNA degradation by amplifying GFP

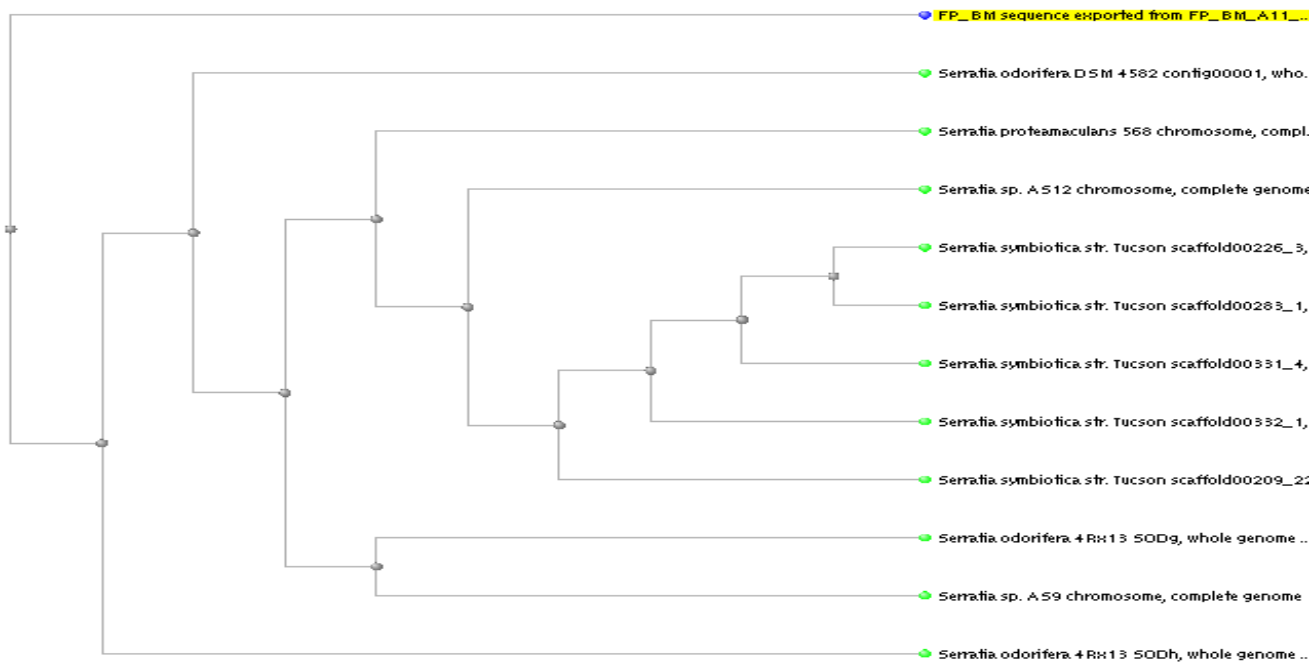


Figure 6: Analysis of the gene sequence of Serratia strain YAJS (FP_BM), Neighbor joining method.

Table 1. Phenotypic characters of Serratia isolate

Test	Result
Gram staining	Gram negative rods arranged singly
Pigment production	positive
Motility	Positive
Indole	Negative
Methyl red	Negative
Vogesproskeur	Positive
Citrate	Positive
Gelatin liquefaction	Positive
Dnase activity	Positive
Casein hydrolysis	Positive
Catalase	Positive
Glucose fermentation	Positive
Lactose fermentation	Positive
Sucrose fermentation	Positive
Fructose fermentation	Positive

The antibiotic sensitivity of the isolate was tested on nutrient agar plates by placing the antibiotics like streptomycin, chloramphenicol, trimethoprim, tetracycline and penicillin discs (Himedia). The isolate was observed more to be sensitive to streptomycin, chloramphenicol, trimethoprim, and tetracycline and resistant to pencillin. Experiments were also carried out to study whether the given isolate produces any secondary metabolites, as reports were available on carbapenam production by *Serratia.* When culture filtrates were tested on *Staphylococcus* for anti microbial activity by well method, culture filtrate showed zone of inhibition around the wells. This result stands as evidence for the presence of secondary metabolite and could be carbapenam as reported by Demain *et al,* (1995).

Molecular characterization:

In spite of tentative biochemical identification of the isolate as *Serratia*, a detailed molecular characterization was undertaken in order to identify the position of the isolate among various *Serratia* species reported so far.

Nucleic acid based diagnostic systems are known to be more rapid, sensitive and precise in identification of an organism. Thegenotyping or sequenceanalysis of highly conserved regions of the bacterial genome, such as the small subunit rRNA gene now provide a universal method of estimating the evolutionary relationships among all organisms. The genomic DNA of the isolate was extracted and amplified with primers.

Forward primer Bac8F: 5' AGA GTT TGA TCC TGG CTC AG3'

Reverse primer 1392 R: 5' GGT TAC CTT GTT ACG ACT T 3'

The amplified DNA was run on 1% agarose gel and the genome size was found to be 1600 bp.(Figure 2).DNA sequencing was processed using ABI 3730Xl (96 capillary) electrophoresis and analyzed for nucleotide matching from gene bank by the nucleotide blast, where the sequence was pasted in the FASTA format. The genomic sequences in FASTA format are as follows

1. AGTGGCGGGCGGCTTACACATGCAAGTCGAGCGGTAGCACAGGGGAGCTTGCTCCCTGGGTGACGAGCGGCGGAC
GGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGC
AGGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAATGG
CTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTAC
GGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCG
GGTTGTAAAGCACTTTCAGCGAGGAGGAAGGTGGTGAACTTAATACGCTCATCAATTGACGTTACTCGCACAAGAAGCA
CCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACAGATGGTGCAAGCGTTAATCGGAATTTACTGCGCGTACAGCGCAC
GCAGGCGGTTTGTTAAGTCAGATGTGAAATCTCCCGGGCTCAGCCTGGGCAACTGCATTTGAAACTGTCAAGCTAGAGTC
TCGTAGAGGGGCGCTACATTTCCAGGTGCAGCGATGAATGCGTAGTAGATCTGGAGGAATACACGGTGGCGAAGGCAG
GCTCCCTGCACGGACTGACGCCCAAGGTGCTAATCTTGTGCAGCAAGCCGCATTCGATACCTGGTATTCTCACGCTGTTAT
ACCAAGGTCTCAGTCTGGGA.

Intresting observations were made during the PCR amplification. When Bacterial lysate as a template used for the PCR, its product was obtained however it was degraded after two days.When PCR with the genomic DNA as a template was performed, amplified DNA was stable only when it was purified by ethanol precipitation. This DNA was also stable when 1mM calcium chloride was added to the PCR product immediately after the completion of PCR (fig. 4).

Further w hen the bacterial lysate and the Genomic DNA (of the sample) was added to the GFP PCR reaction using GFP primers and template, GFP product was present only in the reaction in which genomic DNA of the bacterial sample. The reaction in which the bacterial lysate was present did not give any GFP PCR product. The positive control of the GFP PCR (without bacterial lysate and genomic DNA) gave the amplified product of 750 bp (fig 5). These observations indicate the action of Dnase in the bacterial lysate. PCR amplified product was observed when the genomic DNA was either purified further with ethanol precipitation or by adding 1M calcium chloride which were stabilizing DNA from Dnase action.

Phylogenetic analysis

Part of the 16s rDNA gene sequence of isolate was amplified and analysed (GenBank accession No.JQ 217430).Figure 6 showed a neighbour joining phylogenetic tree based on the alignment of the nearly complete 16S rDNA gene sequence of isolate with 16SrDNA sequences of the 10 described *Serratia* type strains available in GenBank and EMBLdatabases**.** BLAST analysis revealed 95 % sequence similarity with Serratiamarcescens strain.The strain was identified and named as *Serratiasps YAJS* under gene bank accession no JQ 217430.

CONCLUSION

The bacterium isolated from rhizosphere soils of our college was identified by using different microscopic, biochemical, enzymatic tests as a member of Enterobacteriaceae according to bergeys manual of systemic bacteriology. The red pigment prodigiosin production confirmed it to be a member of genus *Serratia*. 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 to be *Serratiamarcences* and was named as *SerratiamarcencesYAJS*. The sequences were submitted to gen bank under accession number_no. JQ 217430for further study.

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