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RESEARCH ARTICLE

Isolation, Molecular Characterization and *In-silico* Analysis of *Erwinia carotovora* from Rotten Vegetables

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ABSTRACT

Erwinia carotovora, a plant pathogen that causes soft-rot disease, dependent on the production and secretion of a complex arsenal of plant cell wall degrading enzymes. Biochemical characteristics of Erwinia carotovora with other bacterial species from spoiled Potatoes, carrots and tomatoes have been conducted to know biological parameters and pathogenicity relationships. Gram staining, Isolation of DNA and Northern blotting methods has been conducted in the present experimentation. Gram staining results shows purple coloured rod shaped Gram +ve bacteria. Biochemical activity has also shown good results. The DNA isolate of bacteria such as Erwinia carotovora, Aeromonas hydrophila, Pseudomonas *aeruginosa* has observed bands at 12000 bp, Staphylococcus aureus has observed bands at 12,500 bp, and Xanthomonas citri has observed bands at 11,000 bp. In these five organisms, highest molecular weight is observed in Staphylococcus aureus and lowest molecular weight observed in Xanthomonas citri. The species contains translocation and biodegradable proteins related to other bacterial species. *Erwinia carotovora* that causes soft-rot diseases in plants like Potatoes, Carrots and Tomatoes containing convergence with other species such as *Aeromonas hydrophila, Pseudomonas aeruginosa*, and shows divergence with *Xanthomonas citri and Staphylococcus aureus*.

KEYWORDS

Erwinia carotovora, Biochemical tests, Clinical isolation, Gram staining, DNA isolation, SDS PAGE

INTRODUCTION

Erwinia carotovora that causes soft-rot diseases in plants produce a variety of extracellular pectic enzymes[1,2,3,4] such as pectate lyase isozymes, cellulase, polygalacturonase, and protease to evaluate the correlation between patterns of pectic enzyme production and taxonomic classification, from representative strains. *Erwinia carotovora* subsp. *carotovora* developed soft rot following 24 hours cold storage at 0°C.

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Erwinia carotovora subsp. carotovora *Erwinia* soft root causes destructive and serious damages to many vegetable crops including potato, in the field, transit and storage periods[5]. Spread of disease occurred during cold storage of dormant hosta rhizome divisions and through vegetative propagation.

Bactericides and disinfectants tested were ineffective while used as a protectant prior to exposure to 0°C. Supernatants achieved from the polygalacturonase grown cultures of three strains of *Erwinia carotovora* subspecies *carotovora*[6,7]. The isoelectric focusing profiles of pectate lyase and polygalacturonase be present nearly identical for strains of *Erwinia*

carotovora subspecies *carotovora* showing three pectate lyase isozymes with higher isoelectric points[8]. *Erwinia carotovora* regulates expression of virulence factors and antibiotic gathering done by an*N*-3-oxohexanoyl-l-homoserine lactone independent upon quorum sensing mechanism[9,10]. The marine algae *Delisea pulchra* harvests halogenated furan ones known to antagonize 3-oxo-C6-HSL activity on *Erwinia carotovora*[11].

Biochemical characteristics be situated examined by the API 20E and API 50CHE systems[12]. Numerical analysis exhausting the Euclidean distance coefficients and clustering by the unweight average pair group method indicated that these *Erwinia carotovora* subspecies *atroseptica* strains formed a distinct cluster (Subphenon A1) that could be differentiated from other *Erwinia carotovora strains*[13]. Three non-potato strains similarly belonged to this group; two of these were from tomato and the other from Chinese cabbage.

L-asparaginase was extracted from Erwinia carotovora and purified by ammonium sulfate fractionation (60–70%), Sephadex G-100, CM cellulose, and DEAE sephadex chromatography[14]. An enzyme, oligogalacturonide *trans*-eliminase (OGTE), which degrades *O*-(4-deoxy-β-l-5-*threo*-hexopyranos-4-enyluronic acid)-(1 → 4)-d-galacturonic acid into two molecules of 4-deoxy-5-hexoseulose uronic acid, has been purified from cell-free extracts of Erwinia carotovora[15]. This enzyme also severs usual di-galacturonic acid to one molecule of d-galacturonic acid and one molecule of the deoxyketuronic acid, indicating amylase reaction. In the occurrence of Erwinia cell-free extracts and NADH, the deoxyketuronic acid is converted to 2-keto-3-deoxy-d-gluconic acid, presumably with the intermediate formation of 3-deoxy-d-*glycero*-2,5-hexodiulosonic acid[16]. A transposon that allows transcriptional fusions to a promoter less β- glucuronidase gene, a transmuted of *Erwinia carotovora* subsp. *carotovora* SCC3193 poor in extracellular protease production and soft-rot pathogenicity in plants existed isolated[17]. The DNA sequence of the cloned fragment contained two complete and one partial ORFs in *Erwinia carotovora*[18,19,20].

Erwinia primers did not show the amplification of the 119-bp fragment as seen with the pure cultures of pathogen[21]. Virulence of the plant pathogen *Erwinia carotovora* subspecies *carotovora* is dependent on the production and secretion of a complex arsenal of plant cell wall degrading enzymes. Production of these exo-enzymes is controlled by a global regulatory mechanism[22], virulence in a plant pathogen and bioluminescence in a marine bacterium, and may represent a general mechanism by which bacteria modulate gene expression in response to changing environmental conditions[23]. The virulent mutants in one of the regulatory loci, expI, show a pleiotropic defect in the growth phase-dependent transcriptional activation of exo-enzyme gene expression[24].

Evaluation of a potential drug, Neomycin is conducted previously to suppress the development of economically important plant pathogenic bacteria Erwinia Carotovora[25]. Although the extracellular levels of these enzymes are extremely low while the bacterium is grown in salts-yeast extract-glycerol (SYG) medium, the enzymatic activities are highly induced in SYG medium supplemented with celery extract.

The polygalacturonase-encoding[26] pehA gene from *Erwinia carotovora* subsp. *carotovora* strain SCC3193 and bla gene of pBR322 has been constructed using *in vitro* gene fusions. Various plant diseases that caused by pathogenic microbes cause several problems like residues in edible plant parts, resistant strains and environment pollution[27,28,29]. Crystal structures of *Erwinia carotovora* L-asparaginase complex with L-aspartate and L-glutamate were determined at 1.9 and 2.2 Å[30]. Microbes have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents. Though pharmacological industries are producing a number of new antibiotics in the last three decades, resistance to

these drugs by microorganisms has increased to great extant[31].

Screen the antibacterial activity of nine ethno veterinary plants traditionally used for the treatment of gastrointestinal complications[32]. Antimicrobial activity of the plant pathogens used to different microorganisms including laboratory standard bacterial sample[33].

MATERIALS AND METHODS

Collection of Samples

The samples have been collected during raining season from the vegetable Market place of Gantastambam, Vizianagaram. The Spoiled Potatoes, Carrots and Tomatoes have been collected and the experimentation has been conducted in the Microbiology and Biochemistry laboratory, GITAM University.

Isolation on Agar Medium

A 250 ml conical flask has been taken and all contents of Agar medium such as NaCl, Peptone, Beef extract, Agar, yeast extract are mixed to the 100 ml of distilled water.

They are heated with hot air oven for 2min, and have been sterilized in an autoclave at 121°C for 20 minutes. Petriplates has been cleaned with surgical spirit. After autoclaving the petri plates, the Agar medium has to be poured in petriplates. After pouring added microbial species and spread the inoculate with L shaped glass rod.

Gram Staining

To a normal saline, add one loop full of bacterial culture and prepare to spread smear of fresh bacteria, flooded with the primary stain "Crystal violet" for 1min. Gently wash flooded smear with mordent "Grams Iodine" for 1 min and gently wash with tap water, not longer than 2 seconds.

Decolorize with 95% ethyl alcohol, drop by drop until alcohol run almost clear showing only in blue tinge. Gently wash the slide in tap water for 2 seconds to remove the Ethyl alcohol. Counterstain with flood the smears of "Safranin" for 45min and allow to stain for 1 minute. Wash with tap water and blot dry gently between sheets of bibulous paper. Examine under 100x oil immersion lens.

DNA Isolation

Bacterial culture should be taken, centrifuge at 10000rpm for 5min and remove supernatant. Collect the bacterial Pellet. Add 250 µl of Tris EDTA, 250 µl of Ethidium Bromide and 4 µl of protease. Keep it water bath at 70 ^{0}C / 20min. Centrifuge at 5000rpm/5min and remove pellet. Collect the supernatant and add equal volumes of PCI (25:24:21). Centrifuge 5000 rpm/5min and collect supernatant (Aqueous layer). Add equal volume of chloroform and centrifuge at 5000 rpm for 5 min. Collect supernatant and add 1/10 volume of 3 molar Sodium acetate and 2M isopropanol. Gently shack the tubes for few seconds and centrifuge at 5000rpm for 5 min. discord the supernatant and take the Pellet. Wash the pellet with 70% alcohol, centrifuge again and again take the pellet. Lastly wash with 70% alcohol and dry the pellet. Add Tris EDTA to the dried pellet and stored at 4 ^{0}C.

Electrophoresis

Prepare the agarose by dissolving 1gm of agarose in 100 ml of 1x Tris Acetate Buffer. Take 1 gm agarose in 100 ml of TAE buffer, dissolve it by boiling and cool the tubes to 55^{0}C. Add 5 µl of Ethidium Bromide (EB) and pour into the gel casting tray. After pouring gel, place the comb such that the gel thickness is to be around 4 to 8 mm. Allow solidifying. The electrophoretic chamber should be filled with 1x TAE buffer before applying sample. Add the sample and the power has to be switched on at 50 or 100 v for about 1-2 hours. Observe sample migration.

Protein Interaction

Erwinia carotovora atroseptica SCRI1043 contains MiaB-like tRNA modifying enzyme from base 3036349 to 3048348 (12000 bp). Protein interaction network analysis using string has been constructed for MiaB to analyze the function of this protein with other proteins.

RESULTS

Gram Staining

The experimentation shows purple coloured, rod shape, Gram +ve bacteria and the isolated sample may be *Erwinia carotovora*. Further confirmation has been made with biochemical tests (Figure 1).

Figure 1: Gram staining of *Erwinia carotovora*

Biochemical Tests Conducted on *Erwinia Carotovora*

The TSI test on *Erwinia carotovora* has observed positive due to production of H_2S. Indole test observed as positive due to unchanged color with indole. MR test observed positive to the test culture after adding reagents and formed red color. The Voges Proskauer test observed negative result due to unchanged colour with reagents. The citrate test observed negative due to lack of reaction with citrates after culture incubation. Urease test observed positive on test culture due to increase in P^H. The Nitrate test observed positive result due to slow reaction of culture with reagents and develop red colour. Catalase test observed positive with catalase enzyme producing bubbles. The oxidase test observed negative result due to unchanged color in the medium.

Erwinia Carotovora Electrophoresis Results

The bacteria such as *Erwinia carotovora*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, has observed bands at 12000 bp, *Staphylococcus aureus* has observed bands at 12,500 bp, *Xanthomonas citri* has observed bands at 11,000 bp (Table 1 and Figure 2). In these 5 organisms highest molecular weight is *Staphylococcus aureus* has observed bands at 12,500 bp and lowest molecular weight is *Xanthomonas citri* has observed bands at 11,000 bp.

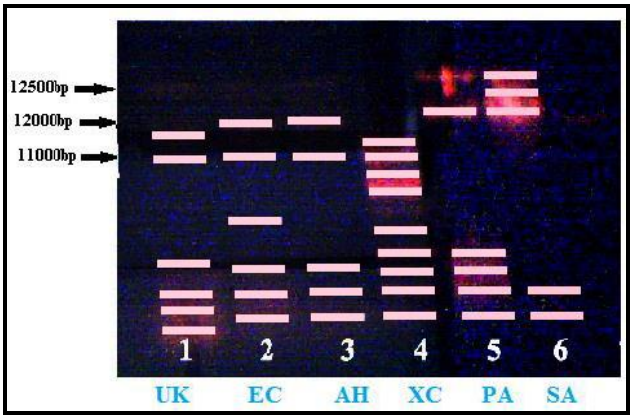

Figure 2: Molecular weight confirmation of *Erwinia carotovora*

Note: 1. Unknown; 2. *Erwinia carotovora*; 3. *Aeromonas hydrophila*; 4. *Xanthomonas citri*; 5. *Pseudomonas aeruginosa*; 6. *Staphylococcus aureus*.

Table 1: Electrophoresis result

S.No	Name of Organism	Molecular Weight
1	*Erwinia carotovora*	12,000 bp
2	*Aeromonas hydrophila*,	12,000 bp
3	*Pseudomonas aeruginosa*	12,000 bp
4	*Staphylococcus aureus*	12,500 bp
5	*Xanthomonas citri*	11,000 bp

Protein Interaction

The data provides that the proteins involve in tRNA delta(2)-isopentenylpyrophosphate transferase, Apolipoprotein N-acyltransferase, DNA mismatch repair protein, Magnesium and cobalt efflux protein CorC, Recombinase A, Translation initiation factor IF-2, Chaperone protein DnaJ, Diaminopimelate epimerase etc. Hence *Erwinia carotovora* contains sequences that is related to other bacterial species with the above mentioned characters (Figure 3).

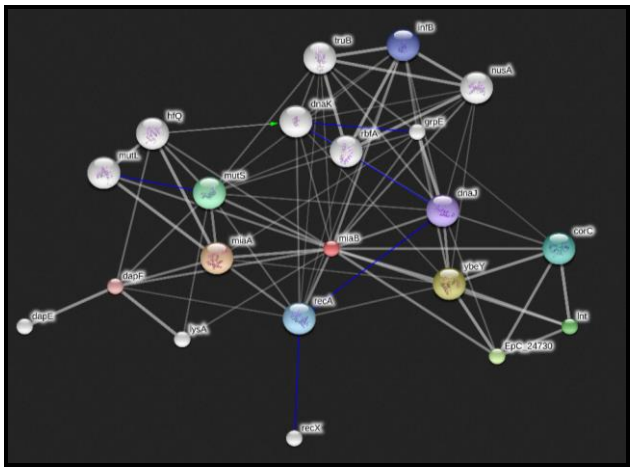

Figure 3: Protein interaction networks using string functional association interaction networs

DISCUSSION

A plant pathogenic enterobacterium, *Erwinia carotovora* is the causative agent of soft rot and blackleg potato diseases[34], causes severe losses in agriculture[35]. Various Bacterial species produce antibiotics that interfere with the normal biological functions of their competitors in order to gain competitive advantages. Various signal interference mechanisms existing in natural ecosystems may explored as a new version of antagonism for prevention of bacterial infections[36].

The synthesis of extracellular pectate lyase occurs in *Erwinia carotovora* and in nonpathogenic isolate of Pseudomonas fluorescence[37]. The pathogen and nonpathogen regulates pectate lyase synthesis, is related to pathogenicity of soft-rot bacteria. Biochemical tests to determine *Erwinia carotovora* gave the results related to Romeiro et al, 1988[38].

Hadas et al, 2001 has isolated *Erwinia carotovora* ssp. carotovora (Ecc) from commercial pepper (Capsicum annuum) seed lots and identified according to biochemical and pathogenicity tests, cellular fatty acid composition, and PCR reaction with primers based on the pel gene sequence[39]. DNA relatedness to bacterial taxonomy shows to stimulate greater awareness and use in (i) the identification of grossly atypical strains in a taxon; (ii) the recognition of new taxa; (iii) the classification of poorly studied groups of organisms; and (iv) changes in existing classifications[40]. The present work has shown closer relationship of *Erwinia carotovora* with other bacterial species based on southern blotting technique.

CONCLUSION

Erwinia carotovora is a bacterial species associated with other bacterial species with good biodegradable ability.

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