16S Ribotyping and classification of endophytes of finger millet (*Eleusine coracana* L. Gaertn.) variety VL149

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Jr. Scientist Cum Asst. Professor

CERTIFICATE

This is to certify that the thesis titled **"16S Ribotyping And Classification Of Endophytes Of Finger Millet (*Eleusine coracana* L. Gaertn.) Variety VL149"** submitted in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE IN BIOTECHNOLOGY** of the **COLLEGE OF BIOTECHNOLOGY** of Post-Graduate Studies, Birsa Agricultural University, Ranchi, Jharkhand is a record of bonafide research carried out by **Ms NIDHI KUNAL** under my supervision and guidance. No part of thesis has been submitted for any other degree or diploma.

It is further certified that help or information received during the course of this investigation and preparation of the thesis has been duly acknowledged.

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Date:.....

Place: Ranchi ***NIDHI KUNAL***

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ABBREVIATIONS

~	Approximately
µl	micro litre
µM	micromolar
A	Adenine
bp	base pairs
BLAST	Basic Local Alignment Search Tool
Cacl2	Calcium chloride
°C	degree Celsius
DNA	Deoxyribo Nucleic Acid
dNTPs	Deoxyribo Nucleotide Tri Phosphate
FAO	Food and Agriculture Organization
F&R	Forward and Reverse
Hrs	hours
ICRISAT	International Crops Research Institute for the Semi-Arid Tropics
IPTG	Isopropyl thiogalactoside
ISR	Institute of Seismological Research
ITS	Internal Transcribed Spacer
LB	Luria Bertani
Lbs	pounds
ml	mililitre
mins	minutes
mg/ml	milligram per milliliter
mg	milligram
Mm	milimolar
M	molar
NCBI	National Centre for Biotechnology Information
no.	number
ng/µl	nanogram per microliter
nm	nanometer
ori	origin of replication
OD	Optical Density

PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
pmole/μl	picomoles per microliter
RT	room temperature
rpm	revolutions per minute
RNase	Ribonuclease
rDNA	ribosomal Deoxyribo nucleic acid
S	sydberg's unit
s	seconds
Taq	*Thermus aquaticus*
T	thymin
TE	tris EDTA
u	units
u/μl	units per microliter
U	uridine
X-gal	5-bromo 4-chloro 3-indolyl-β-D-galacto pyranoside

CHAPTER 1

INTRODUCTION

Finger millet [*Eleusine corocana* (L) Gaertn.] is a staple cereal, widely grown in the semi-arid areas of Eastern and Southern Africa as well as South Asia (Kiran and Thakur *et al*., 2012). The crop is believed to have been first cultivated in Uganda about 5000 years ago. Today, finger millet is an important minor millet in the tropics grown in more than 25 countries of Africa and Asia and accounts for 12% of the global millet area (ICRISAT, 2008).

The major finger millet producers are Uganda, India, Nepal, China, Kenya, and Ethiopia. The annual production is estimated at 841, 000 metric tons on 460, 000 hectares of land, making it the second most important cereal after maize (Wanyera, 2007). The world total production of millet grains at last count was 762712 metric tons and the top producer was India with an annual production of 334500 tons (43.85%) (FAO 2012). Currently, finger millet has been recognized as a highly nutritious food for the weak and immuno-compromised people in Uganda (Takan *et al*., 2012). The grains are a rich source of protein (5.6 to 12.7 %), proportionately higher in brown seeded than in white seeded genotypes. The grain contains high levels of essential amino acids with 44.7% lysine, threonine, and valine above the 33.9% of FAO reference content of similar essential amino acids in rice, wheat and sorghum and maize (Singh and Raghuvanshi, 2012). Besides, finger millet grain has sulphur containing amino acids that are crucial for human health, and rich source of minerals like, calcium, iron, zinc, and manganese (Bhatt *et al*., 2003; Singh and Srivastava, 2006).

Eleusine species occupy diverse habitats, ranging from open, dry places to forests under-covers. Finger millet is extensively grown in the semi-arid regions of Africa and India (Werth *et al*., 1994; Das *et al.,* 2007).

The crop is cultivated in diverse eco-geographical areas where it displays high variability in vegetative, floral, and seed morphology. Hilu and de Wet (1976) and Upadhyaya *et al*. (2007) identified three eco- geographical races: African highland race cultivated in the

East African highlands, a lowland race grown in the lowlands of Africa and south India and an Indian race with its centre of diversity in northeast India. Natural selection also has played a significant role in finger millet evolution (Hilu and de Wet, 1995).

Extensive amounts of finger millet germplasm exist (Bennetzen *et al*., 2003) with ICRISAT holding 5,000 accessions, University of Agricultural Sciences in Bangalore (4,500) accessions, National Dry land Farming Research Station of Kenya (1,500), Gene bank of Kenya (1,000), Plant Genetic Resource Centre in Ethiopia (1,000) and University of Georgia in US (1, 500) and Uganda (5,000). These germplasm provide plant breeders with the necessary genetic material, to develop farmer-desired varieties besides being natural conservation facility for desirable genes due to genetic variability (Fakrudin *et al*., 2004: Das *et al*., 2007).

Finger millet and its wild relatives belong to the genus *Eleusine* (*Poaceae*, subfamily *Chloridoideae*). The genus includes annual and perennial species that are native to East Africa (Phillips, 1972; Dida, 1998; Andersson and De Vicente, 2010) which are *E. corocana, E. indica, E. intermedia, E. jaegeri, E. floccifloia, E. kigezienesis, E. multiflora* and *E. semisterilis* and one New World species i.e., *E. tristachya* native to Argentina and Uruguay (Andersson and De Vicente, 2010). The primary centre of diversity for the genus *Eleusine* is East Africa where 8 of the approximately 10 species occur. India is considered to be a secondary centre of diversity. The genus includes diploids and tetraploids with basic chromosomes number of 8, 9, and10. This has been extended by widespread introduction of the crop (*E. corocana*) throughout the tropics and the common weed often associated with cultivation *E. indica* (Dida *et al*., 2007; Liu and Peterson *et al.,* 2011). A lot of diversity has been reported in the genus *E. corocana, E. africana, E. indica* and *E. kigezienesis* (Dida, 2007). A study of the genome has reported a close relationship between *E. corocana, E. africana, E. indica* and *E. kigezienesis* with allotetraploid attributes (Liu *et al*., 2011).

ENDOPHYTES

An endophyte is an endosymbiont, often a bacterium or fungus, that lives within a plant for at least part of its life cycle without causing apparent disease. Endophytes are

ubiquitous and have been found in all species of plants studied to date; however, most of the endophyte/plant relationships are not well understood. Endophytes are also known to occur within lichens and algae. Endophytes may benefit host plants by preventing pathogenic or parasitic organisms from colonizing them. Extensive colonization of the plant tissue by endophytes creates a "barrier effect", where the local endophytes outcompete and prevent pathogenic organisms from taking hold. Endophytes may also produce chemicals that inhibit the growth of competitors, including pathogenic organisms.

Endophytes are also known to increase expression of defense-related genes in plants, making plants more resistant to many potential pathogens. Bacterial endophytes are a class of endosymbiotic microorganisms widespread among plants that colonize intercellular and intracellular spaces of all plant compartments. They colonize plant apoplast, the intercellular spaces of the cell walls and xylem vessels of plant roots, stems and leaves, and they are also found in tissues or flowers (Compant *et al*., 2011), fruits (de Melo Pereira *et al*., 2012) and seeds (Trognitz *et al*., 2014). Population densities of endophyte bacteria are extremely variable in different plants and tissues and have shown to vary from hundreds to reaching as high as 9×10^9 of bacteria per gram of plant tissue (Jacobs et al., 1985; Misaghi, Donndelinger, 1990; Chi *et al*., 2005). Plant and endophytic bacteria association includes vast diversity of bacterial taxa and plant hosts.

The tight association between host-plant and endophytes is mediated through the action of compounds produced by both, the microorganisms and the host cells (Reinhold-Hurek, Hurek, 2011; Brader *et al*., 2014). The endophytes aid nutrient availability and uptake, enhance stress tolerance, and provide disease resistance (Ryan *et al*., 2008; Hamilton *et al*., 2012). The growth promoting capability of endophytes is due to the production of plant growth hormones, alterations of endogenous plant hormone production or activities that increases accessibility of nutrients, such as nitrogen and phosphorus (Glick, 2012). Production of a wide range of compounds such as antibiotics or chitinase by endophytes inhibits growth of plant pathogens and thus protects the hosts (Raaijmakers, Mazzola, 2012; Christina *et al*., 2013; Brader *et al*., 2014; Wang *et al*., 2014). A latent disease defense mechanism is also stimulated by endophytes, this is called induced systemic

resistance (ISR), that confers an enhanced level of protection to the host from a broad spectrum of pathogens (Pieterse *et al*., 2014). Due to these properties endophytes can be used in the form of bioinoculants to promote plant growth and health (Mei, Flinn, 2010).

The characterization of endophytic isolates has generally been done after plant surface disinfection. Lodewyckx *et al.* (2002) characterized the main methods used for the bacteria isolation and 81 bacterial species that form endophytic associations with plants. One of the early reviews by Hallman *et al.* (1997) presented a list of bacterial endophytes isolated from various plant parts of different agricultural crops. The list of endophytes and plants harbouring them has been supplemented by later studies (Rosenblueth, Martinez-Romero, 2006; Bacon, Hinton, 2007; Ryan *et al.*, 2008).

RIBOTYPING

Ribotyping is a molecular technique for bacterial identification that uses information from rRNA-based phylogenetic analyses. It is a rapid and specific method widely used in clinical diagnostics and analysis of microbial communities in food, water, and beverages. All bacteria have ribosomal genes, but the exact sequence is unique to each species, serving as a genetic fingerprint. Therefore sequencing the particular 16S gene and comparing it to a database yields identification of the particular species.

The present study concentrates on 14 endophytes (13 bacteria and 1 fungus), which had been isolated and characterized on the basis of colony features and biochemical reactions earlier in the laboratory (Rani, 2016). The objective was to amplify the 16S rDNA from the bacterial endophytes followed by sequencing for taxonomic classification in combination with the colony and biochemical characteristics. The lone fungal endophyte attempted to be identified on the basis of 18S rDNA sequencing, using the ITS region of the major rRNA transcript.

CHAPTER 2

REVIEW OF LITERATURE

2.1 IDENTIFICATION OF ENDOPHYTES

The term endophyte (Gr. endon, within; phyton, plant) was first coined by De Bary (De Bary, 1866). Vogl in 1898 reported the presence of endophytes, he revealed a mycelium residing in the grass seed *Lolium temulentum* which is now defined as a class of endosymbiotic microorganisms, that are able to colonize and healthfully coexist with plant tissues (Kloepper and Beauchamp, 1992). Hallmann *et al* (1997) defined endophytes as those bacteria that can be isolated from surface-disinfested plant tissue or extracted from within the plant, and that do not visibly harm the plant. Endophytic bacteria had been thought to be weakly virulent plant pathogens but were recently discovered to have several beneficial effects on host plants, such as plant growth promotion and increased resistance against plant pathogens and parasites. In general, endophytic bacteria originated from the epiphytic bacterial communities of the *rhizosphere* and *phylloplane*, as well as from endophyte-infested seeds or planting materials. Endophytic bacteria may gain entrance to plants through natural openings or wounds. They also actively penetrate plant tissues using hydrolytic enzymes like cellulases and pectinases. In general, endophytic bacteria occur at lower population densities than pathogens, and at least some of them do not induce a hypersensitive response in the plant, indicating that they are not recognized by the plant as pathogens. Evolutionarily, endophytes appear to be intermediate between saprophytic bacteria and plant pathogens, but it can only be speculated as to whether they are saprophytes evolving toward pathogens, or are more highly evolved than plant pathogens and conserve protective shelter and nutrient supplies by not killing their host. Overall, the endophytic microfloral community is of dynamic structure and influenced by biotic and abiotic factors, with the plant itself constituting one of the major influencing factors. Since endophytic bacteria rely on the nutritional supply offered by the plant, any parameter affecting the nutritional status of the plant could consequently affect the endophytic community

Grobbelaar *et al* (1987) identified 41 isolates of the cyanobionts in the coralloid roots of 31 cycad species, which included all the species indigenous to the Republic of South Africa. One isolate (from *Encephalartos hildebrandtil)* appeared to be a species

of *Calothrix*. All the other isolates were identified as species of *Nostoc*. Twenty eight isolates were assigned to *N. commune,* 5 to *N. punctiforme,* and 2 each to *N. ellipsosporum, N. paludosum* and *N. sphaericum* while 1 isolate was identified as *N. muscorum*. In the case of 2 cycad species, isolates were made from more than one individual. Of the 10 isolates from 7 individuals of *Encephalartos transvenosus* 8 were *N. commune* and 2 *N. paludosum*. One of the 2 isolates from *Encephalartos manikensis* was *N. ellipsosporum* and the other *N. punctiforme.* One isolate from each cycad species reduced acetylene to ethylene. One isolate from each of 12 cycad species were able to fix nitrogen. Although the cyanobacteria in all the isolates tested for acetylene reduction were contaminated with other bacteria (which were not identified), it was demonstrated that they did not contribute to the nitrogenase activity of the cyanobiont cultures.

Araújo *et al* (1999) isolated actinomycetes from surface sterilized leaves and roots of maize. A total of 53 isolates were obtained, 31 of them from leaves and 22 from roots. The genus *Microbispora* was the most frequently found followed by the genera *Streptomyces* and *Streptosporangium*. From the isolated *actinomycetes*, 43.4% showed antimicrobial activity against one or more tested bacteria and yeast.

Tanaka *et al* (1999) examined diversity of endophytic microbes in plants, and devised a method for isolation. They isolated at least one kind of microbe from all the plants examined. The microbes isolated included 162 fungi and 166 bacteria from 57 plants in Hokkaido Japan. Also 86 fungi and 56 bacteria were obtained from 20 plants in Java, Indonesia. The isolates showed interesting bioactivities. Nine filamentous fungi were selected and taxonomic study was carried out on the basis of 18S ribotyping. The experiment showed that endophytes from various plants have great diversity and useful characteristics for biotechnological applications.

Filamentous fungi include both sexual (*Epichlo ̈e*) and asexual (*Neotyphodium*) species. As a group they are genetically diverse and form both antagonistic and mutualistic associations with temperate grasses. Moon et al (1999) reported the development of a microsatellite-based PCR system for fingerprinting this group of fungi with template isolated from either culture or infected plant material. M13mp19 partial genomic libraries were constructed for size-fractionated genomic DNA from two endophyte strains. These libraries were screened with a mixture of DIG-labeled dinucleotide and trinucleotide repeat probes. Positive clones were sequenced, and 9 unique microsatellite loci were identified. An additional microsatellite was identified in the 3’ untranscribed region of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG

CoA) reductase gene from *N. lolii* Lp19. Primers were designed for each locus and a panel of endophytes, from different taxonomic groupings was screened to determine the degree of polymorphism. On the basis of these results a multiplex assay was developed for strain identification with fluorescently labeled primers for 5 of these loci. Using this system the size of the products amplified was precisely determined by automated analysis, and an allele profile for each strain was readily generated. The assay was shown to resolve endophyte groupings to the level of known isozyme phenotype groupings. In a blind test the assay was used successfully to identify a set of endophytes in *planta*. A reference database of allele sizes was established for the panel of endophytes examined, and was expanded as new strains were analyzed.

Caruso *et al* (2000) investigated the fungal and *actinomycete* populations of internal tissues of woody branches, shoots and leaves of different plants belonging to the genus *Taxus*. Twenty two plants of *Taxus baccata* and 1 of *Taxus brevifolia* were sampled in different habitats located in central-northern Italy. Also 150 fungal and 71 *actinomycete* strains were isolated, some of them from woody tissues, others from herbaceous ones. Both microbial populations were composed of representatives of different genera and the presence of *actinomycetes* inside living tissues of plant above ground organs was reported for the first time. Fungi and *actinomycetes* were screened for taxane production. Different liquid media were used for strain growth and the culture extracts were assayed by a competitive inhibition enzyme immunoassay (CIEIA) using a commercial kit employing a polyclonal antibody. The production of taxanes was detected in 15 out of 150 fungal strains and in 10 out of 71 *actinomycetes,* isolated both from woody and herbaceous tissues. In both populations, the production of taxanes was quantitatively low and only in few cases reached 50-100 ng per litre. The capability to produce taxanes was not related to the geographical origin of isolation but with a particular tree from which the fungus was isolated.

Guo *et al* (2000) carried out a survey of the endophytic fungi in fronds of *Livistona chinensis* in Hong Kong. The endophyte assemblages identified using morphological characters consisted of 16 named species and 19 ‘ morphospecies ’, the latter group based on cultural morphology and growth rates. Arrangement of taxa into morphospecies did not reflect species phylogeny, and therefore identification of selected morphospecies was further based on rDNA sequence analysis. The 5n8S gene and flanking internal transcribed spacers (ITS1 and ITS2) regions of ribosomal DNA (rDNA) from 19 representative morphospecies were amplified by the PCR and

sequenced. Phylogenetic analysis based on 5n8S gene sequences showed that these morphospecies were filamentous *Ascomycota*, belonging in the *Loculoascomycetes* and *Pyrenomycetes*. Identification was conducted by means of sequence comparison and phylogenetic analysis of both the ITS and 5n8S regions. Results showed that one belonged to the genus *Diaporthe* and its anamorph *Phomopsis* of the *Valsaceae*. one was inferred to be *Mycosphaerella* and its anamorph *Cladosporium* of the *Mycosphaerellaceae*. 7 morphospecies were placed in the genus *Xylaria* of the Xylariaceae. 3 were close to the Clypeosphaeriaceae. 2 were closely related to the *Pleosporaceae* within the *Dothideales*. The other 5 morphospecies probably belonged in the *Xylariales*.

Tan *et al* (2001) studies showed that like other microorganisms invading plant tissues, endophytes produce extracellular hydrolyases as a resistance mechanism to overcome attack by the host against pathogenic invasion and get nutrition from the host. Enzymes such as pectinases, esterases, cellulases and lipases, 60 proteinase, α-1,4-glucan lyase and phosphatases had been documented with different endophytes. Enzymatic activities closely related to the host-specificity of the endophytes were demonstrated. The actions of these enzymes lead to the possibility that the 'genetic recombination' of the endophyte with the host might occur in evolutionary time. This could be the reason why some endophytes can produce some phytochemicals originally characteristic of the host. The extended significance of the productivity of endophytes for some important phytochemicals such as Taxol lies in that it provides an alternative strategy for easing the impact of the growing population on plants, which are needed for the preservation of biodiversity and the ecosystem. As a poorly investigated store of microorganisms 'hidden' within the host plants, endophytes obviously are a rich and reliable source of bioactive and chemically novel compounds with huge medicinal and agricultural potential.

Suryanarayanan *et al* (2001) isolated mitosporic fungi and several sterile forms as endophytes from the leaf tissues and aerial roots of *Ficus benghalensis (Moraceae).* Although similar number of endophyte species was present in lamina and petiole, the endophytic fungi more densely colonized the petiole. The species composition and the colonization frequency of the endophytes were more for the aerial roots entering the soil when compared with those growing in the air since the roots recruited some endophytes from the soil. The endophyte assemblages of the leaf and aerial root and of the aerial

root growing in the air and soil showed little overlap suggesting that the nature of the host tissue as well as the environment determine the endophyte composition of a host.

Zinniel *et al*.(2002) determined the prevalence, properties, persistence, and types of endophytic bacteria in agronomic and native plants. In the study, 853 different endophytic colonizing bacterial strains were isolated from four agronomic crop species and 27 prairie plant species. Six of the most promising colonizing strains were identified taxonomically as species of *Cellulomonas*, *Clavibacter*, *Curtobacterium*, and *Microbacterium* by fatty acid, carbon source utilization, and 16S rRNA gene sequence analyses.

Taechowisan *et al* (2002) isolated endophytic *actinomycetes* from surface-sterilized tissues of 36 plant species (*Rhinacanthus communis, Amaranthus gracilis, Brassica juncea, Brassica oleracea, Cyperus difformis, Cyperus iria, Ocimum tenuiflorum, Coffea Arabica, Coriandrum sativum, Zingiber cassumunar, Zingiber officinale*, etc.) using humic acid–vitamin (HV) agar as a selection medium. Of the 330 isolates recovered, 212 were from roots, 97 from leaves and 21 isolates from stems with a prevalence of 3.9, 1.7 and 0.3%, respectively. Identification of endophytic *actinomycetes* was based on their morphology and the amino acid composition of the whole-cell extract. Most isolates were classified as *Streptomyces sp.*; with the remainder belonging to *Microbispora sp*., *Nocardia sp*. and *Micromonospora sp.*. Four isolates were unclassified and 23 were lost during subculture. The most prevalent group of isolates were the *Streptomyces sp.* occurring in 6.4% of the tissue samples of *Zingiber officinale*. Investigation of the plant by scanning electron microscopy revealed that 7.5% of the root and 5% of the leaf samples contained endophytes. Three of the *Streptomyces sp.* isolates strongly inhibited *Colletotrichum musae*, 5 were very active against *Fusarium oxysporum* and 2 strongly inhibited growth of both the fungi.

Arnold *et al.*(2003) presented the evidence that fungal endophytes associated with a woody angiosperm reduce leaf damage and loss due to a major pathogen. Firstly extensive field surveys in lowland Panama were used to characterize the diversity, spatial structure, and host affinity of natural endophyte infections in the economically important rainforest tree, *Theobroma cacao* (Malvaceae). Inoculation of leaf tissues by endophytes frequently isolated from naturally infected, asymptomatic hosts demonstrated significant reduction in damage by foliar pathogen (*Phytophthora* sp.). Together with the ecological context afforded by field surveys and *in vitro* experiments,

the antipathogen protection demonstrated the capacity of diverse, horizontally transmitted, and ubiquitous endophytes to play an important role in host plant defense.

Studies by Coombs *et al.* (2003) was the first report of the isolation and identification of endophytic actinobacteria from healthy wheat plants. The work was part of a larger study that characterized the actinobacterial endoflora of cereal plants and assessed their role in pathogen defense and growth regulation of their hosts.

Promputtha *et al* (2005) identified the sporulating endophytic fungi isolated from *Magnolia liliifera* to genus or species level using morphological characters. Non-sporulating isolates were generally termed as 'mycelia sterilia' and were grouped as 'morphospecies' based on similar cultural characters. Mycelia sterilia were grouped to 31 morphospecies and were further identified based on ribosomal DNA (rDNA) sequence analysis. The 5.8S gene, ITS1 and ITS2 regions of rDNA from all morphospecies were amplified and sequenced. Phylogenetic analysis indicated that MS88 were related to the genus *Massarina (Lophiostomataceae*), MS9, MS11 and MS47 to *Xylaria (Xylariaceae)*, MS19 to *Glomerella (Phyllachoraceae),* MS25 to *Hypoxylon (Xylariaceae),* MS27 to *Bionectria (Bionectriaceae)* and the remaining 24 morphospecies were related to *Phomopsis* and *Diaporthe (*anamorphic *Phomopsis).*

Rosenblueth *et al* (2006) through her studies on endophytic bacterial diversity revealed a large richness of species. Some endophytes were seed borne, but others had mechanisms to colonize the plants that were being studied. Bacterial mutants unable to produce secreted proteins were impaired in the colonization process. Molecular analysis showed that plant defense responses limit bacterial populations inside plants. Some human pathogens, such as *Salmonella* spp., had been found as endophytes, and these bacteria were not removed by disinfection procedures that eliminate superficially occurring bacteria.

Firmicutes are a phylum of bacteria, most of which have Gram-positive cell structure. *Megasphaera*, *Pectinatus*, *Selenomona* and *Zymophilus* have a porous pseudo-outer membrane that causes them to stain Gram-negative. Scientists once classified the *Firmicutes* to include all Gram-positive bacteria, but have recently defined them to be of a core group of related forms called the low-G+C group, in contrast to the *Actinobacteria.* They have round cells, called cocci (singular coccus), or rod-like forms (bacillus). Many *Firmicutes* produce endospores, which are resistant to desiccation and can survive extreme conditions. They are found in various environments, and the group includes some notable pathogens. Those in one family,

the *heliobacteria,* produce energy through photosynthesis. *Firmicutes* play an important role in beer, wine, and cider spoilage. The group is typically divided into the *Clostridia,* which are anaerobic, the *Bacilli*, which are obligate or facultative aerobes, and the *Mollicutes*, which are parasites.

The *"Proteobacteria"* are a major phylum of Gram-negative bacteria. They include a wide variety of pathogens, such as *Escherichia*, *Salmonella*, *Vibrio*, *Helicobacter*, *Yersinia*, and many other notable genera. Others are free-living (nonparasitic), and include many of the bacteria responsible for nitrogen fixation. Carl Woese established this grouping in 1987, calling it informally the "purple bacteria and their relatives". Because of the great diversity of forms found in this group, the *"Proteobacteria"* are named after Proteus, a Greek god of the sea capable of assuming many different shapes and is not named after the genus *Proteus*. The group is defined primarily in terms of ribosomal RNA (rRNA) sequences. The *Proteobacteria* are divided into six classes with validly published names, referred to by the Greek letters alpha through epsilon and the *Acidithiobacillia* and *Oligoflexia*. These were previously regarded as subclasses of the phylum, but they are now treated as classes. These classes are monophyletic. The genus *Acidithiobacillus*, part of the Gammaproteobacteria until it was transferred to class *Acidithiobacillia* in 2013, is paraphyletic to *Betaproteobacteria* according to multigenome alignment studies. The *Alphaproteobacteria* grow at very low levels of nutrients and have unusual morphology such as stalks and buds. They include agriculturally important bacteria capable of inducing nitrogen fixation in symbiosis with plants. The type order is the *Caulobacterales,* comprising stalk-forming bacteria such as *Caulobacter*. The *Betaproteobacteria* are highly metabolically diverse and contain chemolithoautotrophs, photoautotrophs, and generalist heterotrophs. The type order is the *Burkholderiales*, comprising an enormous range of metabolic diversity, including opportunistic pathogens. The *Gammaproteobacteria* are the largest class in terms of species with validly published names. The type order is the *Pseudomonadales*, which include the genera *Pseudomonas* and the nitrogen-fixing *Azotobacter*. The *Deltaproteobacteria* include bacteria that are predators on other bacteria and are important contributors to the anaerobic side of the sulfur cycle. The type order is the *Myxococcales*, which includes organisms with self- organising abilities such as *Myxococcus* spp. The *Epsilonproteobacteria* are often slender, Gram-negative rods that are helical or curved. The type order is the *Campylobacterales*, which includes important food pathogens such as *Campylobacter* spp. The *Oligoflexia* are filamentous

aerobes. The type order is the *Oligoflexales*, which contains the genus *Oligoflexus*. The *Acidithiobacillia* contain only sulfur-oxidising autotrophs. The type order is the *Acidithiobacillales*, which includes economically important organisms used in the mining industry such as *Acidithiobacillus* spp.

Proteobacterial classes with validly published names include some prominent genera: e.g.:

- *Alphaproteobacteria: Brucella, Rhizobium, Agrobacterium, Caulobacter, Rickettsia, Wolbachia, etc.*
- *Betaproteobacteria: Bordetella, Ralstonia, Neisseria, Nitrosomonas, etc.*
- *Gammaproteobacteria: Escherichia, Shigella, Salmonella, Yersinia, Buchnera, Haemophilus, Vibrio, Pseudomonas, etc.*
- *Deltaproteobacteria: Desulfovibrio, Geobacter, Bdellovibrio, etc.*
- *Epsilonproteobacteria: Helicobacter, Campylobacter, Wolinella, etc.*
- *Oligoflexia: Oligoflexus.*
- *Acidithiobacillia: Acidithiobacillus thiooxidans, [Thermithiobacillus tepidarius]*.

Ulrich *et al.* (2007) studied the composition of endophytic bacteria colonizing the aerial parts of poplar using a multiphasic approach. The terminal restriction fragment length polymorphism analysis (RFLP) of 16S rRNA genes demonstrated the impact of different hybrid poplar clones on the endophytic community structure. Detailed analysis of endophytic bacteria using cultivation methods in combination with cloning of 16S rRNA genes amplified from plant tissue revealed a high phylogenetic diversity of endophytic bacteria with a total of 53 taxa at the genus level that included *Proteobacteria, Actinobacteria, Firmicutes* and *Bacteroidetes*. The community structure displayed clear differences in terms of the presence and relative proportions of bacterial taxa between the four poplar clones studied. The results showed that the genetic background of the hybrid poplar clones corresponded well with the endophytic community structure. Out of the 513 isolates and 209 clones identified, *Actinobacteria*, in particular the family *Microbacteriaceae*, made up the largest fraction of the isolates, whereas α and β proteobacteria dominated the clone library. The most abundant genera among the isolates were Pseudomonas and *Curtobacterium,* while *Sphingomonas* prevailed among the clones.

Long *et al.* (2008) isolated 77 endophytic bacterial isolates from roots, stems and leaves of black nightshade plants (*Solanum nigrum*) grown in two different native habitats in Jena, Germany .

Aravind *et al.* (2009) isolated 80 isolates of endophytic bacteria from different varieties of black pepper (*Piper nigrum* L.) grown at different locations in India. Another 30, isolates were obtained from tissue cultured black pepper plants. These isolates were tentatively grouped into *Bacillus* sp. (32 strains), Pseudomonads (26 strains), *Arthrobacter* sp. (20 strains), *Micrococcus* sp. (10 strains), *Curtobacterium*sp. (1 strain), *Serratia* (1strain) and 20 unidentified strains based on morphology and biochemical tests.

Seo *et al.* (2010) isolated a total of 264 colonies of endophytic bacteria from the interior of young radish leaves and roots. Endophytic bacteria from the phylum *Proteobacteria* were predominant in the leaf (61.3%) and root (52.1%) samples.

Thamizh Vendan *et al.* (2010) investigated the diversity of bacterial endophytes associated with ginseng plants of varying age levels in Korea. Although a mixed composition of endophyte communities was recovered from ginseng based on the results of 16S rDNA analysis, bacteria of the genus *Bacillus* and *Staphylococcus* dominated in 1-yearold and 4-year-old plants. Phylogenetic analysis revealed four clusters: *Firmicutes*, *Actinobacteria*, α-*Proteobacteria*, and *γ-Proteobacteria*, with *Firmicutes* being predominant.

Sadananda *et al* (2011) isolated 13 different endophytic fungi from different parts of *Tabebuia argentea*. The endophytic fungal extracts were prepared using ethyl acetate and evaluated for their phytochemical constituents. *Aspergillus niger* and *Alternaria alternata* yielded saponins, phenolic compounds, anthraquinones, steroids, cardiac glycosides and tannins. Other endophytes yielded less phytochemical compounds compared to plant extracts. Naphthoquinone (natural lapachol) was identified in *A. niger* and *A. alternata*. These two endophytes also exhibited significant antimicrobial activity against an array of pathogenic fungi and bacteria. Endophytic isolates of *A. niger* and *A. alternata* were of particular interest because they showed significant antagonistic activity against all tested bacteria and fungi at different range. The fungal culture, endophytes, *A. niger* and *A. alternata* showed strongest antioxidant capacity, having the highest levels of phenolics. This was the first report of lapachol (naphthoquinone) producing endophytes and their antimicrobial and antioxidant activities. The investigation revealed that the metabolites produced by a variety of

endophytic fungi can be a potential source of novel natural antimicrobials, antioxidants and anticancer agents.

Pereira *et al.* (2011) investigated bacterial diversity associated with the roots of maize through the use of culture-dependent and culture-independent methods and showed that γ-Proteobacteria within *Enterobacter*, *Erwinia*, *Klebsiella*, *Pseudomonas*, and *Stenotrophomonas* genera were the predominant groups. The culturable component of the bacterial community revealed that the predominant group was Firmicutes, mainly of *Bacillus* genus, while *Achromobacter*, *Lysinibacillus*, and *Paenibacillus* genera were rarely found in association with the roots. Only two genera within γ-Proteobacteria, *Enterobacter* and *Pseudomonas*, were found in the culture collection.

Compant *et al* (2011) found that endophytic bacteria colonize various plants and organs. Endophytes colonizing flowers as well as berries and seeds of grapevine plants grown under natural conditions was investigated by cultivation as well as by fluorescence *in situ* hybridization. For comparison, bacteria were additionally isolated from other plant parts and the rhizosphere and was characterized. Flowers, fruits, and seeds hosted various endophytic bacteria. Some taxa were specifically isolated from plant reproductive organs, whereas others were detected in the rhizosphere, endorhiza or grape inflorescence/infructescence stalk at the flowering or berry harvest stage. Microscopic analysis by fluorescence *in situ* hybridization of resin-embedded samples confirmed the presence of the isolated taxa in plant reproductive organs and enabled us to localize them within the plant. *Gamma proteobacteria* (including *Pseudomonas spp*.) and *Firmicutes* (including *Bacillus spp.*) were visualized inside the epidermis and xylem of ovary and/or inside flower ovules. *Firmicutes,* mainly *Bacillus spp.* were additionally visualized inside berries, in the intercellular spaces of pulp cells and/or xylem of pulp, but also along some cell walls inside parts of seeds. Analysis of cultivable bacteria as well as microscopic results indicated that certain endophytic bacteria could colonize flowers, berries, or seeds.

2.2 16S rRNA AND RIBOTYPING

16S ribosomal RNA (or 16S rRNA) is the component of the 30S small subunit of a prokaryotic ribosome that binds to the Shine-Dalgarno sequence. The genes coding for it are referred to as 16S rRNA gene and are used in reconstructing phylogenies, due to

the slow rates of evolution of this region of the gene. Woese and Fox (1990) were two of the people who pioneered the use of 16S rRNA in phylogenies.

It has several functions such as:

- Like the large (23S) ribosomal RNA, it has a structural role, acting as a scaffold defining the positions of the ribosomal proteins.
- The 3' end contains the anti-Shine-Dalgarno sequence, which binds upstream to the AUG start codon on the mRNA. The 3' end of 16S RNA binds to the proteins S1 and S21 known to be involved in initiation of protein synthesis.
- It interacts with 23S and aids in the binding of the two ribosomal subunits i.e., 50S+30S
- It stabilizes correct codon-anticodon pairing in the A site, via a hydrogen bond formation between the N1 atom of Adenine residues and the 2'OH group of the mRNA backbone.

HYPERVARIABLE REGIONS

The bacterial 16S gene contains nine hypervariable regions (V1-V9) ranging from about 30-100 bps long and are involved in the secondary structure of the small ribosomal subunit (Gray *et al*, 1996). The degree of conservation varies widely between hypervariable regions, with more conserved regions correlating to higher-level taxonomy and less conserved regions to lower levels, such as genus and species. While the entire 16S sequence allows for comparison of all hypervariable regions, at approximately 1500 base pairs long it can be prohibitively expensive for studies seeking to identify or characterize diverse bacterial communities. These studies commonly utilize the Illumina platform, which produces reads at rates 50-fold and 12,000-fold less expensive than 454 pyrosequencing and Sanger sequencing, respectively. While cheaper and allowing for deeper community coverage, Illumina sequencing only produces reads 75-150 base pairs long, and has no established protocol for reliably assembling the full gene in community samples. Full hypervariable regions can be assembled from a single Illumina run, however, making them ideal targets for the platform.(Gray *et al*, 1984; Bartram *et al*, 2011; Yang *et al*, 2016)

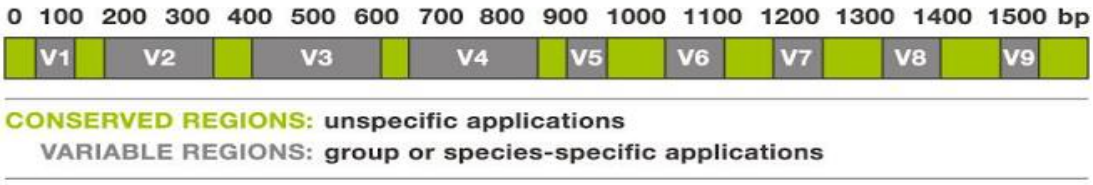


Figure 2.1 Schematic representation of the hypervariable regions of the 16S rDNA.

16S hypervariable regions can vary dramatically between bacteria, the 16S gene as a whole maintains greater length homogeneity than its Eukaryotic counterpart, which can make alignments easier (Burke *et al*, 2016) Additionally, the 16S gene contains highly conserved sequences between hypervariable regions, enabling the design of universal primers that can reliably produce the same sections of the 16S sequence across different taxa. Although no hypervariable region can accurately classify all bacteria from Domain to Species, some can reliably predict specific taxonomic levels. Many community studies select semi-conserved hypervariable regions like the V4, as it provides resolution at the phylum level as accurately as the full 16S gene. While lesser-conserved regions struggle to classify new species when higher order taxonomy is unknown, they are often used to detect the presence of specific pathogens.

Chakravorty *et al* (2007) characterized the V1-V8 regions of a variety of pathogens to determine which hypervariable regions was most useful to include for disease-specific and broad assays. Amongst other findings, they noted that the V3 region was best at identifying the genus for all pathogens tested, and that V6 was the most accurate at differentiating species between all pathogens tested, including Anthrax (Větrovský and Baldrian , 2013).

The families *Enterobacteriaceae, Clostridiaceae*, and *Peptostreptococcaceae*, species share up to 99% sequence similarity across the full 16S gene whereas the V4 sequences can differ by only a few nucleotides, leaving reference databases unable to reliably classify the bacteria at lower taxonomic levels. By limiting 16S analysis to select hypervariable regions, these studies fail to observe differences in closely related taxa and group them into single taxonomic units, therefore underestimating the total diversity of the sample. Furthermore, bacterial genomes house multiple 16S genes, with the V1, V2, and V6 regions containing the greatest intraspecies diversity. While not the most precise method of classifying bacterial species, analysis of the hypervariable

regions remains one of the most useful tools available to bacterial community studies. (Van de Peer Y., Chapelle S., De Wachter R.,1996).

Sequence based analysis of the 16S rDNA gene is also referred to as ribotyping. Ribotyping is a molecular technique for bacterial identification that uses information from rRNA-based phylogenetic analyses. It is rapid and specific method widely used in clinical diagnostics and analysis of microbial communities in food, water, and beverages.

UNIVERSAL PRIMERS

The most common primers suitable for annealing with the highly conserved regions of 16S rRNA gene are listed below (table 2.1) (Weisburg *et al* ,1991;Eden *et al*,1991; Weidner *et al*, 1996).

Table 2.1: List of universal primers

Primer name	Sequence (5'-3')
8F	AGA GTT TGA TCC TGG CTC AG
U1492R	GGT TAC CTT GTT ACG ACT T
928F	TAA AAC TYA AAK GAA TTG ACG GG
336R	ACT GCT GCS YCC CGT AGG AGT CT
1100F	YAA CGA GCG CAA CCC
1100R	GGG TTG CGC TCG TTG
337F	GAC TCC TAC GGG AGG CWG CAG
907R	CCG TCA ATT CCT TTR AGT TT
785F	GGA TTA GAT ACC CTG GTA
805R	GAC TAC CAG GGT ATC TAA TC
533F	GTG CCA GCM GCC GCG GTA A
518R	GTA TTA CCG CGG CTG CTG G
27F	AGA GTT TGA TCM TGG CTC AG
1492R	CGG TTA CCT TGT TAC GAC TT

Although the applicability of 16S rRNA sequences for bacterial dassification is now well accepted, the general use of these molecules had been hindered by the technical difficulty of obtaining their sequences. Lane *et al* (1985) described a protocol for rapidly generating large blocks of 16S rRNA sequence data without isolation of the16S rRNA or cloning of its gene. The 16S rRNA in bulk cellular RNA preparations was selectively targeted for dideoxy nucleotide terninated sequencing by using reverse transcriptase and synthetic oligo deoxy nucleotide primers complementary to universally conserved 16S rRNA sequences. Three particularly useful priming sites, which provide access to the three major 16S rRNA structural domains, routinely yielded 800-1000 nucleotides of 16S rRNA sequence. The method was evaluated with respect to accuracy, sensitivity to modified nucleotides in the template RNA, and phylogenetic usefulness, by examination of several 16S rRNAs whose gene sequences were known. The relative simplicity of this approach facilitated a rapid expansion of the 16S rRNA sequence collection available for phylogenetic analyses.

Bacterial endosymbionts of insects have long been implicated in the phenomen on of cytoplasmic incompatibility, in which certain crosses between symbiont infected individuals lead to embryonic death or sex ratio distortion. The taxonomic position of these bacteria has, however, not been known with any certainty. Similarly, the relatedness of the bacteria infecting various insect hosts has been unclear. The inability to grow these bacteria on defined cell free medium has been the major factor underlying these uncertainties. O'neill *et al* (1991) circumvented this problem by selective PCR amplification and subsequent sequencing of the symbiont 16S rRNA genes directly from infected insect tissue. Maximum parsimony analysis of the sequences indicated that the symbionts belonged in the α-subdivision of the Proteobacteria, where they were most closely related to the *Rickettsia* and their relatives. They were all closely related to each other and were assigned to the type species *Wolbachia pipientis*. Lack of congruence between the phylogeny of the symbionts and their insect hosts suggested that horizontal transfer of symbionts between insect species may occur. Comparison of the sequences for *W. pipientis* and for *Wolbachia persica*, an endosymbiont of ticks, showed that the genus *Wolbachia* is polyphyletic. A PCR assay based on 16S primers was designed for the detection of *W.pipientis* in insect tissue, and initial screening of insects indicated that cytoplasmic incompatibility was a general phenomenon in insects.

Schmidt *et al* (1991) examined the phylogenetic diversity of an oligotrophic marine *picoplankton* community by analyzing the sequences of cloned ribosomal genes. Bulk genomic DNA was isolated from *picoplankton* collected in the north central Pacific Ocean by tangential flow filtration. The mixed population DNA was fragmented, size fractionated, and cloned into bacteriophage lambda. Thirty eight clones containing 16S rRNA genes were identified and portions of the rRNA gene were amplified by PCR and sequenced. The resulting sequences were used to establish the identities of the *picoplankton* by comparison with an established database of rRNA sequences. Fifteen unique *eubacterial* sequences were obtained, including 4 from *cyanobacteria* and 11 from *proteobacteria*. A single eukaryote related to *dinoflagellates* was identified; no archaebacterial sequences were detected. The cyanobacterial sequences were all closely related to sequences from cultivated marine *Synechococcus* strains and with cyanobacterial sequences obtained from the Atlantic Ocean (Sargasso Sea). Several sequences were related to common marine isolates of the sub division of *proteobacteria*. In addition to sequences closely related to those of described bacteria, sequences were obtained from two phylogenetic groups of organisms that are not closely related to any known rRNA sequences from cultivated organisms. Both of these novel phylogenetic clusters are *proteobacteria*, one group within the α subdivision and the other distinct from known proteobacterial subdivisions. The rRNA sequences of the α-related group are nearly identical to those of some Sargasso Sea *picoplankton*, suggesting a global distribution of these organisms.

Ueda *et al* (1998) investigated the frequency of heterogeneity among the multiple 16S rRNA genes within a single microorganism by direct determination of the 120-bp nucleotide sequences containing the hypervariable α region of the 16S rRNA gene from 475 *Streptomyces* strains. Display of the direct sequencing patterns revealed the existence of 136 heterogeneous loci among a total of 33 strains. The heterogeneous loci were detected only in the stem region designated helix 10. All of the substitutions conserved the relevant secondary structure. The 33 strains were divided into two groups: one group, including 22 strains, had less than two heterogeneous bases; the other group, including 11 strains, had five or more heterogeneous bases. The two groups were different in their combinations of heterogeneous bases. The former mainly contained transitional substitutions, and the latter was mainly composed of transversional substitutions, suggesting that at least two mechanisms, possibly

misincorporation during DNA replication and horizontal gene transfer, cause rRNA heterogeneity.

Kattar *et al* (1999) reported the first case of fatal septicemia caused by *Bordetella hinzii*. The causative organism exhibited a biochemical profile identical to that of *Bordetella avium* with three commercial identification systems (API 20E, API 20 NE, and Vitek GNI card). However, its cellular fatty acid profile was not typical for either *B. avium* or previously reported strains of *B. hinzii*. Presumptive identification of the patient's isolate was accomplished by traditional biochemical testing, and definitive identification was achieved by 16S rRNA gene sequence analysis. Phenotypic features useful in distinguishing *B. hinzii* from *B. avium* were production of alkali from malonate and resistance to several antimicrobial agents.

Barney *et al* (2000) ribotyped a total of 46 brewery and 15 ATCC *Pediococcus* isolates using a Qualicon RiboPrinter. Of these, 41 isolates were identified as *Pediococcus damnosus* using *Eco*RI digestion. 3 ATCC reference strains had patterns similar to each other and matched 17 of the brewery isolates. 6 other brewing isolates were similar to ATCC 25249. The other 18 *P. damnosus* brewery isolates had unique patterns. Of the remaining brewing isolates, one was identified as *P. parvulus*, two were identified as *P. acidilactici*, and two were identified as unique *Pediococcus* species. The use of alternate restriction endonucleases indicated that *Pst*I and *Pvu*II could differentiate some strains having identical *Eco*RI profiles. An acid resistant *P. damnosus* isolate could be distinguished from non-acid-resistant varieties of the same species using *Pst*I instead of *Eco*RI. 16S rRNA gene sequence analysis was compared to riboprinting for identifying *pediococci*. The complete 16S rRNA gene was PCR amplified and sequenced from 7 brewery isolates and 3 ATCC references with distinctive riboprint patterns. The 16S rRNA gene sequences from 6 different brewery *P. damnosus* isolates were homologous with a high degree of similarity to the GenBank reference strain but were identical to each other and one ATCC strain with the exception of 1 bp in one strain. A slime-producing, beer spoilage isolate had 16S rRNA gene sequence homology to the *P. acidilactici* reference strain, in agreement with the riboprint data. Although 16S rRNA gene sequencing correctly identified the genus and species of the test *Pediococcus* isolates, riboprinting proved to be a better method for subspecies differentiation.

The *Lactobacillus acidophilus* complex includes *Lactobacillus acidophilus, Lactobacillus amylovorus, Lactobacillus crispatus, Lactobacillus gallinarum, Lactobacillus gasseri* and *Lactobacillus johnsonii*. Kullen *et al* (2000) developed a rapid and definitive DNA sequence- based identification system for unknown isolates of the *L. acidophilus* complex. A 1500 bp region of the 16S rRNA gene, which contained the V1 and V2 variable regions, was amplified from the isolates by the polymerase chain reaction. The sequence of this region of the 16S rRNA gene from the type strains of the *L. acidophilus* complex was sufficiently variable and allowed for clear differentiation amongst each of the strains. As an initial step in the characterization of potentially probiotic strains, the technique was successfully used to identify a variety of unknown human intestinal isolates. The approach described in the study represented a rapid and definitive method for the identification of *L. acidophilus* complex members.

Pediococci are among the most prevalent microbial contaminants in breweries and they can cause ropiness and the accumulation of high levels of diacetyl in beer. Satokari *et al* (2000) first identified 18 *Pediococcus* strains, mainly of brewery origin, using phenotypical characterization and then ribotyped them. Six *Pediococcus* type strains and 3 other *Pediococcus* strains were used as references. Ribotyping showed higher discriminative capacity compared to phenotypical identification methods. Strains could be identified to species level and differentiated even at strain level using this genetic fingerprinting method. The identifications performed by ribotyping were confirmed by 16S rDNA sequencing of selected strains. Automated ribotyping was found to be a rapid and reliable method for identifying pediococci.

Small *et al* (2001) reported the development and validation of a simple microarray method for the direct detection of intact 16S rRNA from unpurified soil extracts. Total RNAs from *Geobacter chapellei* and *Desulfovibrio desulfuricans* were hybridized to an oligonucleotide array consisting of universal and species-specific 16S rRNA probes. PCR- amplified products from *Geobacter* and *Desulfovibrio* were easily and specifically detected under a range of hybridization times, temperatures, and buffers. However, reproducible, specific hybridization and detection of intact rRNA was accomplished only by using a chaperone-detector probe strategy. With this knowledge, assay conditions were developed for rRNA detection using a 2-h hybridization time at room temperature. Hybridization specificity and signal intensity were enhanced using fragmented RNA. With the chaperone detection strategy, they were able to specifically hybridize and detect *G. chapellei* 16S rRNA directly from a total-RNA soil extract,

without further purification or removal of soluble soil constituents. The detection sensitivity for *G. chapellei* 16S rRNA in soil extracts was at least 0.5 g of total RNA, representing approximately $7.5x10^6$ *Geobacter* cell equivalents of RNA. These results suggested that it is now possible to apply microarray technology for the direct detection of microorganisms in environmental samples, without using PCR.

Schwudke *et al* (2001) aimed to obtain data for the molecular characterization of *Bdellovibrio* bacteria, which were recently split into the genus *Bdellovibrio* and the newly designated genus *Bacteriovorax.* They determined the 16S rDNA sequences of five reference strains and performed a phylogenetic analysis including published 16S rRNA sequences of *Bdellovibrios*, the comparison of the secondary structure showed significant differences in two regions of the 16S rRNAs of the species *Bdellovibrio bacteriovorus*, *Bacteriovorax starrii,* and *Bacteriovorax stolpii.* Also ribotyping techniques gave specific hybridization patterns and revealed that two rRNA operons were present in the investigated strains. The hybridization probe derived from the genetic locus *hit*, associated with the host independent (HI) phenotype of *B. bacteriovorus*, was found to be specific for the species. Sequence comparison of the *hit* locus revealed few base pair changes between host independent (HI) and host dependent (HD) strains. Ribotyping and hybridization experiments using the *hit* probe were applied to characterize *Bdellovibrio* strains isolated from the gut of animals and humans and one isolate from sewage.

Nilsson *et al* (2002) studied the sequence polymorphism of the 16S rRNA gene of *Vibrio vulnificus* and said that it is a possible indicator of strain virulence**.** *Vibrio vulnificus* exhibits considerable strain-to-strain variation in virulence. Attempts to associate phenotypic or genotypic characteristics with strain virulence were largely unsuccessful. Based on a 17-nucleotide difference throughout the sequence of the small subunit 16S rRNA gene, there were two major groups of *V. vulnificus* designated types A and B. In a survey of the 16S rRNA genotype in 67 *V. vulnificus* human clinical and nonclinical strains, they determined that the majority of nonclinical isolates were type A (31 of 33) and that there was a statistically significant association between the type B genotype and human clinical strains (26 of 34).

Ennahar *et al* (2002) classified a total of 161 low-G C-content gram-positive bacteria isolated from whole-crop paddy rice silage and subjected to phenotypic and genetic analyses. Based on morphological and biochemical characters, these presumptive Lactic Acid Bacterium (LAB) isolates were divided into 10 groups that

included members of the genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Weissella*. Analysis of the 16S ribosomal DNA (rDNA) was used to confirm the presence of the predominant groups indicated by phenotypic analysis and to determine the phylogenetic affiliation of representative strains. The virtually complete 16S rRNA gene was PCR amplified and sequenced. The sequences from the various LAB isolates showed high degrees of similarity to those of the GenBank reference strains (between 98.7 and 99.8%). Phylogenetic trees based on the 16S rDNA sequence displayed high consistency, with nodes supported by high bootstrap values. With the exception of one species, the genetic data was in agreement with the phenotypic identification. The prevalent LAB, predominantly homofermentative (66%), consisted of *Lactobacillus plantarum* (24%), *Lactococcus lactis* (22%), *Leuconostoc pseudomesenteroides* (20%), *Pediococcus acidilactici* (11%), *Lactobacillus brevis* (11%), *Enterococcus faecalis* (7%), *Weissella kimchii* (3%), and *Pediococcus pentosaceus* (2%). The study, the first to fully document rice-associated LAB, showed a very diverse community of LAB with a relatively high number of species involved in the fermentation process of paddy rice silage. The comprehensive 16S rDNA-based approach to describing LAB community structure was valuable in revealing the large diversity of bacteria inhabiting paddy rice silage and enabling the future design of appropriate inoculants aimed at improving its fermentation quality.

Kilic *et al* (2002) used ribotyping to examine 111 *Clostridium (Cl.) perfringens* isolates from industrially produced ground meat in order to collect a basis for a contamination survey. Among the 111 isolates 107 distinctly different ribopatterns were detected. In only four cases two *Cl. perfringens* isolates showed an identical ribopattern. The isolates gave identical ribotype patterns in three different runs, carried out 3–4 months apart from each other. The discriminatory index for *EcoRI* ribotyping of the *Cl. perfringens* isolates was 0Æ99. Results showed that ribotyping was suitable for subtyping *Cl. perfringens* isolates from raw meat. Ribotyping appeared to be a useful tool for profound epidemiologic studies of *Cl. perfringens*-contamination in food production and processing.

Montes *et al* (2003) clarified the taxonomic status of vibrio strains isolated from an aquaculture system and compared the results of the identifications made by phenotypic and molecular methods. 51 vibrio strains isolated from the aquaculture system were characterized by ribotyping and 16S rRNA gene sequencing. The strains were identified phenotypically as *Vibrio anguillarum, V. mediterranei, V. splendidus, V.*

aestuarianus, V. ordalii, V. fischeri and *V. scophthalmi*. Cluster analysis of ribotype patterns showed that the strains were separated into two main groups: *V. splendidus–V. lentus* and *V. scophthalmi* groups. The use of 16S rRNA gene sequence allowed differentiation among *V. splendidus* biovar I and *V. lentus* strains. The molecular methods identified strains of *V. splendidus* biovar I, *V. lentus* and *V. scophthalmi*, showing discrepancies with phenotypic characterization. The molecular methods, as 16S rRNA gene sequence analysis, were necessary for the identification of phenotypically close species to avoid mis-identifications. Interestingly, this is the first report of *V. lentus* strains associated to turbot culture.

Andollina *et al* (2004) studies aimed to evaluate the possibility to use automated EcoRI ribotyping to address, during the same analysis, both identification and genetic characterisation of 38 *Staphylococcus aureus* and 64 coagulase-negative *staphylococci* collected from surgical injuries. The ribotyping identification results confirmed those obtained using the API StaphÒ system for 96% of the isolates. All strains were successfully genotyped and the ribotyping discriminatory power was very high for both groups of *Staphylococci* tested. The same, as well as different biotypes were identified among isolates with the identical ribotyping profile.

Samadpour *et al* (2004) compared antibiotic resistance and ribotyping pattern ability to identify triplicate isolates sent from a group of 40 *Escherichia coli* taken from seven host sources. Of the 120 isolates, 22 isolates were resistant to ampicillin, streptomycin, tetracycline and trimethoprim and 98 isolates were susceptible. Antibiotic patterns identified 33 of the triplicates and three of the six groups had isolates from multiple hosts. Ribotyping divided the isolates into 27 ribotype groups with all triplicates grouped into the same ribotype group with one host per group. Antibiotic susceptibility pattern placed 98 of the isolates in a single group with 50% of the antibiotic susceptibility pattern groups containing multiple host species. Ribotyping groups were host specific with each host having one to seven ribotype groups.

Carretto *et al* (2005) showed that routine identification of coagulase-negative *Staphylococci* is problematic so the performance of automated ribotyping was evaluated for identification of coagulase-negative *Staphylococci* other than *Staphylococcus epidermidis*. In total, 177 isolates were tested, comprising 149 isolates from blood samples, 15 isolates that were not identified by internal transcribed spacer (ITS)-PCR and 13 reference strains. The identification results were compared with those obtained by the API 20 Staph system, with standard phenotypic and molecular methods. Most

(93.8%) isolates were identified correctly by automated ribotyping. Automated ribotyping was able to distinguish *Staphylococcus capitis* reliably from *Staphylococcus caprae*. The results demonstrated the value of automated ribotyping for identification of coagulase-negative *Staphylococcus* (CoNS) isolates from human sources and helped to clarify the clinical relevance of CoNS species. In addition, automated ribotyping was also able to detect polymorphisms that might be useful for epidemiological purposes within *S. capitis, Staphylococcus hominis, Staphylococcus haemolyticus, Staphylococcus simulans, S. caprae, Staphylococcus warneri, Staphylococcus lugdunensis, Staphylococcus schleiferi, Staphylococcus sciuri, Staphylococcus pasteuri* and *Staphylococcus xylosus*.

Reiter B. and Sessitsch A. (2006) investigated the presence and taxonomy of endophytic bacteria of the entire aerial parts of crocus (*Crocus albiflorus*), a wildflower native in the Alps,. A combination of plating of plant macerates, isolation and sequence identification of isolates, and direct 16S rDNA PCR amplification followed by whole-community fingerprinting (T-RFLP) and by construction of a bacterial clone library was used. The results clearly indicated that a wide range of bacteria from diverse phylogenetic affiliation, mainly *Firmicutes* and γ-*Proteobacteria*, live in association with plants of *C. albiflorus*. The community composition of the culturable component of the microflora was remarkably different from that of the clone library. Only 3 bacterial divisions were found in the culture collection, which represented 17 phylotypes, whereas 6 divisions were identified in the clonal analysis comprising 38 phylotypes. The predominant group in the culture collection was the low G + C Gram-positive group, whereas in the clone library, the γ-*Proteobacteria* predominated. Interestingly, the most prominent bacterium within the uncultured bacterial community was a *pseudomonas* closely related to a cold-tolerant *Pseudomonas marginalis* strain. The results suggested that *Crocus* supports a diverse bacterial microflora resembling the microbial communities that have been described for other plants and containing species that have not been described in association with plants.

Several characteristics of the 16S rRNA gene, such as its essential function, ubiquity, and evolutionary properties, have allowed it to become the most commonly used molecular marker in microbial ecology. However, one fact that has been overlooked is that multiple copies of this gene are often present in a given bacterium. These intragenomic copies can differ in sequence, leading to identification of multiple ribotypes for a single organism. To evaluate the impact of such intragenomic

heterogeneity on the performance of the 16S rRNA gene as a molecular marker, Case *et al* (2006) compared its phylogenetic and evolutionary characteristics to those of the single-copy gene *rpoB*. Full-length gene sequences and gene fragments commonly used for denaturing gradient gel electrophoresis were compared at various taxonomic levels. Heterogeneity found between intragenomic 16S rRNA gene copies was concentrated in specific regions of rRNA secondary structure. Such "heterogeneity hot spots" occurred within all gene fragments commonly used in molecular microbial ecology. This intragenomic heterogeneity influenced 16S rRNA gene tree topology, phylogenetic resolution, and operational taxonomic unit estimates at the species level or below. *rpoB* provided comparable phylogenetic resolution to that of the 16S rRNA gene at all taxonomic levels, except between closely related organisms (species and subspecies levels), for which it provided better resolution. It is particularly relevant in the context of a growing number of studies focusing on subspecies diversity, in which single-copy protein-encoding genes such as *rpoB* could complement the information provided by the 16S rRNA gene.

Wang *et al* (2007) investigated the structure and diversity of the bacterial communities in rhizosphere soils of native *Phragmites australis* and *Scirpus mariqueter* and alien *Spartina alterniflora* in the Yangtze River Estuary by constructing 16S ribosomal DNA (rDNA) clone libraries. The bacterial diversity was quantified by placing the clones into Operational Taxonomic Unit (OTU) groups at the level of sequence similarity of > 97%. Phylogenetic analysis of the resulting 398 clone sequences indicated a high diversity of bacteria in the rhizosphere soils of these plants. The members of *α-proteobacteria, β-proteobacteria, γ−proteobacteria,* and *δ-proteobacteria* of the phylum *Proteobacteria* were the most abundant in *rhizobacteria*. *Phragmites, Scirpus,* and *Spartina* rhizosphere soils contained 200, 668, and 382 OTUs, respectively. The bacterial communities in the *Spartina* and *Phragmites* rhizosphere soils displayed species dominance revealed by 1/D, whereas the bacterial community in *Scirpus* rhizosphere soil had uniform distributions of species abundance. Overall, analysis of 16S rDNA clone libraries from the rhizosphere soils indicated that the changes in bacterial composition occur concomitantly with the shift of species composition in plant communities.

Yu *et al* (2007) compared different hypervariable (V) regions of the archaeal 16S rRNA gene (*rrs*) to establish a preferred V region(s) for use in *Archaea*-specific PCR-denaturing gradient gel electrophoresis (DGGE). The PCR products of the V3

region produced the most informative DGGE profiles and permitted identification of common methanogens from rumen samples from sheep. The study also showed that different methanogens can be detected when different V regions were targeted by PCR-DGGE. Dietary fat appeared to transiently stimulate *Methanosphaera stadtmanae* but inhibit *Methanobrevibacter* sp. strain AbM4 in rumen samples.

Rodriguez *et al* (2009) showed that the growth and development of rice (*Oryzae sativa*) seedlings was regulated epigenetically by a fungal endophyte. In contrast to un-inoculated (nonsymbiotic) plants, endophyte colonized (symbiotic) plants preferentially allocated resources into root growth until root hairs were well established. During that time symbiotic roots expanded at five times the rate observed in non-symbiotic plants. Endophytes also influenced sexual reproduction of mature big sagebrush (*Artemisia tridentata*) plants. Two spatially distinct big sagebrush subspecies and their hybrids were symbiotic with unique fungal endophytes, despite being separated by only 380 m distance and 60 m elevation. A double reciprocal transplant experiment of parental and hybrid plants, and soils across the hybrid zone showed that fungal endophytes interact with the soils and different plant genotypes to confer enhanced plant reproduction in soil native to the endophyte and reduced reproduction in soil alien to the endophyte. Moreover, the most prevalent endophyte of the hybrid zone reduced the fitness of both parental subspecies. Because these endophytes were passed to the next generation of plants on seed coats, this interaction provided a selective advantage, habitat specificity, and the means of restricting gene flow, thereby making the hybrid zone stable, narrow and potentially leading to speciation.

Kumar *et al* (2009) determined the distribution of *Salmonella* serovars in seafood and examined the intraserovar genetic variations in *Salmonella enterica* subsp. *enterica* serovar *Rissen* and *Salmonella weltevreden* by polymerase chain reaction (PCR)-ribotyping and enterobacterial repetitive intergenic consensus (ERIC)- PCR methods. A total of 417 seafood samples collected over 2003–2006 from fishing harbours and fish markets of Cochin (India) were studied for presence of *Salmonella serovars*. Seafood samples were analysed for the presence of *Salmonella* by Bacteriological Analytical Manual (BAM), U.S. Food & Drug Administration (USFDA) method. The study indicated that 23.2% of the sea- food samples was positive for *Salmonella* and a total of 241 *Salmonella* isolates comprising of 27 different serovars were isolated from seafood. *S. weltevreden, Salmonella rissen, Salmonella typhimurium* and *Salmonella derby* were found to be the most predominant serovars in

seafood. PCR-ribotypes and ERIC- PCR profiles showed multiple genotypic profiles for *S. rissen* and *S. weltevreden* in seafood and the level of discrimination indices obtained was at 0.974 for *S. rissen* and 0.988 for *S. weltevreden,* respectively. The study highlighted the major *Salmonella* serovars in the sea- food of Cochin (India) and molecular fingerprinting pattern revealed genetic variation among *S. rissen* and *S. weltevreden*. Thus widespread occurrence of *Salmonella* contamination in seafood and multiple clones of *S. rissen* and *S. weltevreden* detected in seafood indicated the diverse routes of *Salmonella* contamination in seafood.

High-throughput molecular technologies profile microbial communities at high resolution even in complex environments like the intestinal microbiota. Recent improvements in next-generation sequencing technologies allow for even finer resolution. Claesson *et al* (2010) compared phylogenetic profiling of both longer (454 Titanium) sequence reads with shorter, but more numerous, paired-end reads (Illumina). For both approaches, they targeted six tandem combinations of 16S rRNA gene variable regions, in microbial DNA extracted from a human faecal sample, in order to investigate their limitations and potentials. *In silico* evaluations predicted that the V3/V4 and V4/V5 regions provide the highest classification accuracies for both technologies. Experimental sequencing of the V3/V4 region revealed significant amplification bias compared to the other regions, emphasising the necessity for experimental validation of primer pairs. The latest developments of 454 and Illumina technologies offered higher resolution compared to their previous versions, and showed relative consistency with each other. Nonetheless, with improved quality and longer reads, the far greater coverage of Illumina promises unparalleled insights into highly diverse and complex environments such as the human gut.

Guard *et al* (2012) compared two DNA-based methods for the ability to assign serotype to 139 isolates of *Salmonella enterica* ssp. I. Intergenic sequence ribotyping (ISR) evaluated single nucleotide polymorphisms occurring in a 5S ribosomal gene region and flanking sequences bordering the gene dkgB. A DNA microarray hybridization method that assessed the presence and the absence of sets of genes was the second method. Serotype was assigned for 128 (92.1%) of sub- missions by the two DNA methods. ISR detected mixtures of serotypes within single colonies and it cost substantially less than Kauffmann–White serotyping and DNA microarray hybridization.

CHAPTER 3

MATERIALS AND METHODS

3.1 ENDOPHYTES

Some endophytes were isolated from VL149 variety in the laboratory (Rani, 2016). These were maintained as glycerol stocks at -50°C. The glycerol stocks were thawed on ice and the culture was revived by streaking on appropriate medium. The medium was chosen based on the initial plate the endophyte had been isolated from (Rani, 2016). The plates were incubated at appropriate temperature.

Medium used for the revival of glycerol stocks (Table 3.1)

Table 3.1: List of selected endophytes.

Endophyte	Medium	Temperature Of Growth	Incubation Time
3	Nutrient Agar	37°C	23 Hrs
4	Nutrient Agar	37°C	23 Hrs
5	Nutrient Agar	37°C	48 Hrs
6	Mac Conkey Agar	37°C	73 Hrs
7	Potato Dextrose Agar	37°C	23 Hrs
8	Potato Dextrose Agar	37°C	23 Hrs
12	Nutrient Agar	28°C	48 Hrs
13	Nutrient Agar	28°C	48 Hrs
14	Nutrient Agar	28°C	73 Hrs
16	Mac Conkey Agar	28°C	20 Hrs
17	Nutrient Agar	37°C	20 Hrs
18	Luria Bertani	28°C	20 Hrs
19	Luria Bertani	28°C	44 Hrs
20	Nitrate Hi Veg Agar	37°C	66 Hrs

3.2 INOCULATION OF REVIVED CULTURE

The culture was inoculated in 1.5 ml of Luria Bertani (HiMedia) broth and was incubated at 37°C overnight. Endophyte no.7 was a fungal isolate. The powdered fungal colony was available and was used for DNA isolation.

3.3 ISOLATION AND PURIFICATION OF ENDOPHYTIC GENOMIC DNA

Two kits (HiMedia) were used for isolation of genomic bacterial and fungal DNA respectively. The protocol given by the manufacturer was followed.

3.3.1 Bacterial DNA isolation using Hi Pura Bacterial Genomic DNA purification kit

- 1.5 ml of overnight grown culture was harvested by centrifuging at 10,000 rpm (22.80VO2 rotor, Hermle table top centrifuge) for 2 mins. The culture medium was decanted by inverting the tube and adsorbing the droplets on tissue paper.
- The pellet was then resuspended completely in 200 µl of lysozyme solution and was incubated for 30 mins at 37˚C.
- 10 µl of Proteinase K solution (20 mg/ml) was then added to the sample. It was mixed and incubated for 30 mins at 55˚C and then cooled on ice.
- Thereafter, 10 µl of RNase A solution (DS0003) was added and mixed and incubated for 10 mins at room temperature.
- 200 µl of lysis solution (C1) (DS0010) was added to the sample and mixed thoroughly by vortexing for few seconds followed by incubation for 10 mins at 55˚C.
- 200 µl of absolute alcohol was added to the lysate and mixed thoroughly by vortexing for few seconds.
- The lysate was transferred onto Hi-elute miniprep spin column and was centrifuged at 10,000 rpm for 1 min. The flow through was discarded and the spin column was placed in the same 2.0 ml collection tube.
- 400 µl of pre wash solution was added to the column and centrifuged at 10, 000 rpm () for 1 min. The flow through was discarded.
- 500 µl of the diluted wash solution was added and centrifuged for 3 mins at 13,000 rpm.
- The flow through was discarded and the column was paced in the same collection tube and was centrifuged for additional 1 min at 13000 rpm.
- As a modification of the protocol the column was placed in the new collection tube and left open for drying for an additional 10 mins.

- 200 µl of elution buffer (ET) (DS0040) was directly added onto the column without spilling to the sides. After incubation for 5 mins at room temperature and the tubes were centrifuged at 10,000 rpm for 1 min to elute DNA.
- 100 µl of elution buffer (ET) (DS0040) was added again to increase the final DNA concentration in the eluate. After incubation for 1 min the tubes were centrifuged at 10000 rpm for 1 min.
- The eluate was stored at 4˚C for further analysis.

3.3.2 Fungal DNA isolation using Hi Pura Fungal Genomic DNA purification kit

- 150 mg of the powdered fungal tissue was taken 400 µl of lysis buffer (PL) (DS0016) was added to the mixture and vortexed vigorously
- 10 µl of RNase solution was added and kept at 15-25˚C at room temperature for 10 mins.
- The mixture was incubated at 65°C for 10 mins with intermittent mixing by inversion.
- 130 µl of precipitation buffer (PS) (DS0017) was added to the lysate and the contents were mixed and incubated on ice for 5 mins. The lysate was centrifuged at 14,000 rpm (22.80VO2 rotor, Hermle table top centrifuge) for 5 mins.
- The supernatant was loaded onto the Hi Shredder column placed in a 2.0 ml collection tube and was centrifuged for two minutes at 14,000 rpm.
- The flow through fraction was transferred carefully to a new collection tube without disturbing the cell debris pellet.
- 1.5 ml of the diluted binding buffer (BB) (DS0018) i.e. 675 µl was added to the clear lysate and was mixed by pipetting.
- 650 µl of the mixture was added to the Hi Elute Miniprep spin column placed in a 2.0 ml collection tube. It was centrifuged for 1 min at 8000 rpm. The flow through was discarded.
- The remaining solution was added to the column and centrifuged again for 1 min at 8000 rpm. The flow through was discarded.
- The column was replaced in the same 2 ml collection tube and diluted wash buffer (WSP) (DS019) was added to it. The tubes were centrifuged for 1 min at 8000 rpm. The flow through was discarded and the column was placed in the same collection tube.

- Another 500 µl of the diluted wash (WSP) was added to the column and centrifugation was performed for 1 min at 14,000 rpm. The flow through was discarded and the column was placed in the same tube.
- The tube with the column was centrifuged for 2 mins at 14,000 rpm to dry the membrane.
- The Hi Elute Miniprep spin column was transferred to a new collection tube and 200 µl of elution buffer (ET) (DS0040) was added directly into the column without spilling to the sides.
- After incubation for 5 mins at room temperature the tubes were centrifuged at 10,000 rpm for 1 min to elute DNA.
- Another 100 µl of elution buffer was added and incubated for 1 min for higher yield of DNA. It was centrifuged at 10,000 rpm.
- The eluate was stored at 4˚C for further analysis.

3.4 PCR amplification using universal primers

3.4.1 PCR amplification using universal bacterial primers

- Universal primer pairs (Table 3.3) were used for amplification are found to have sequences complimentary to the 16S rDNA of isolated bacterial genomes.

Table 3.2: List of universal bacterial primers

Primer Name	Sequence	No. of nucleotides	T_m (°C)
UF	5’ CAGAGTTTGATCCTGGCTCAG 3’	21	63.9
CD[R]	5’ CTTGTGCGGGCCCCCGTCAATTC 3’	23	62.4
533 (F)	5’ GTGCCAGCAGCCGCGGTAA 3’	19	57.6
UR	5’ AAGGAGGTGATCCAGCC 3’	17	60.2

[Weisburg *et al* (1991), Rudi *et al* (1997), Turner *et al* (1999)]

Table 3.3: Universal bacterial primer pairs

Primer Pair Name	Primer Combination	Expected Amplicon Size In bp (Variable Regions Included)
P3	UF with CD[R]	929(V3)
P7	533(F) with UR	1026(V3 and V6)

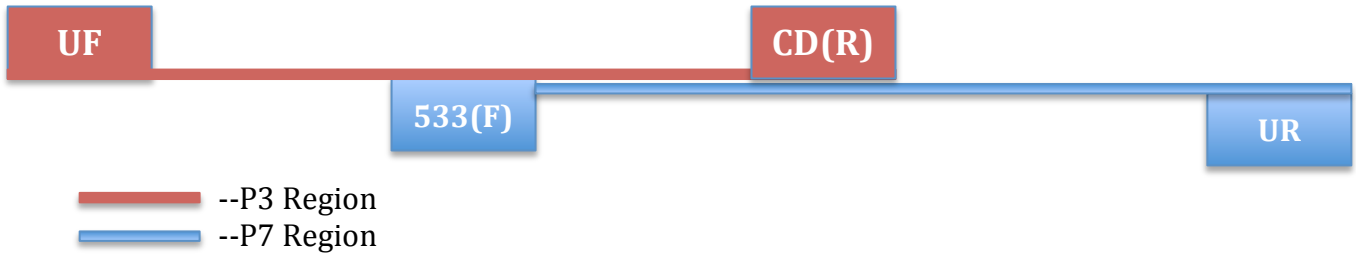


Fig.3.1: Diagrammatic representation of position of primer pairs in the 16S rDNA

- PCR was performed using these universal primers and taking the isolated bacterial endophytic genomes as template. Composition of PCR mix was as follows (Table 3.4)

Table3.4: Composition of PCR mix

PCR Components	Stock Concentration	Volume Taken Per Reaction (Final Concentration)	Volume Taken For 26 Reactions (Master Mix)
Template	50-100ng/ µl	1 µl	----
Green Taq Buffer	10X	1 µl (1X)	26 µl
dNTPs(Mix)	10mM	0.2 µl (200-250 µM)	5.2 µl
Taq DNA Polymerase	5U/µl	0.1 µl (0.5 U)	2.6 µl
Primer (F & R)	50 pmole/µl	0.1 µl + 0.1 µl (5 pmole/µl)	2.6 µl + 2.6 µl
Sterile Water	---	8.5 µl	221 µl
Total		10 µl	260 µl

- PCR conditions were as follows (Table 3.5)

Table 3.5: Conditions for PCR

Steps	Reactions	Condition
1	Initial Denaturation	94°C/4 mins
2	Denaturation	94°C/30s
3	Annealing	P3-55°C/30s P7-57°C/30s
4	Extension	72°C/30s
5	Final Extension	72°C/2 mins
6	Final Hold	4°C
Number Of Cycles		35

- Obtained PCR product was then checked by electrophoresis for correct fragment size in an agarose maxi gel (0.8 %) by using ΦX174/Hae III digest as marker.

3.4.2 PCR amplification using universal eukaryotic primers

- Universal eukaryotic primer pairs were also found which have sequences complimentary to the 16S rDNA of isolated fungal genomes. Universal primers pairs used are as follows (Table 3.7).

Table 3.6: list of universal eukaryotic primers

Primer name	Sequence
NS1	5’ GTAGTCATATGCTTGTCTC 3’
NS8	5’ TCCGCAGGTTCACCTACGGA 3’
ITS1	5’ TCCGTAGGTGAACCTGCGG 3’
ITS4	5’ TCCTCCGCTTATTGATATGC 3’

(White *et al*, 1990)

Table 3.7: Universal eukaryotic primer pairs

Primer Pair Name	Primer Combination
E2	NS1 and NS8
E5	ITS1 and ITS4

- A PCR was performed using these universal primers and taking the isolated fungal genome as template. Composition of PCR mix was as follows (Table 3.8).

Table 3.8: Composition of PCR mix

PCR Components	Stock Concentration	Volume Taken Per Reaction (Final Concentration)	Volume Taken For 2 Reactions (Master Mix)
Template	50-100ng/ µl	1 µl	----
dNTPs(Mix)	10mM	0.2 µl (200-250 µM)	0.4 µl
Taq DNA Polymerase	5U/µl	0.1 µl (0.5 U)	0.2µl
Primer (F & R)	50 pmole/µl	0.1 µl + 0.1 µl (5 pmole/µl)	0.2 µl + 0.2 µl
Sterile Water	---	8.5 µl	17 µl
Total		10 µl	20 µl

- PCR conditions were similar as described above except for annealing at 57°C.
- Obtained PCR product was then checked by electrophoresis for correct fragment size by using Low Range Plus DNA ruler as marker.

3.5 PCR PRODUCT PURIFICATION

The PCR product was purified with HiPurA^{TM} Minispin DNA clean up kit (HiMedia) as per the manufacturer's instruction. The steps were as follows:

- 5-volume binding buffer (100 µl) was mixed with 1 volume of PCR product (20µl). This mixture was transferred to HiElute Miniprep Spin Column and centrifuged at 13000 rpm for 1min.
- The flow through was discarded and the column was placed back in the collection tube.
- 700µl wash buffer was added to the column and centrifuged at 13000rpm for 1min.
- The flow through was discarded and the column was placed back in the collection tube and was centrifuged at 13000 rpm for 2 mins to remove traces of wash buffer.
- Next, the column was transferred to a clean collection tube and 25µl elution buffer was carefully added to the centre of the column.
- After incubation for 5 mins at room temperature the tubes were centrifuged at 10000 rpm for 1min.

- The elution step was repeated by adding another 25µl of elution buffer and incubation for 1min was given. Centrifugation was carried out at 10000 rpm for 1min.
- 50µl of PCR purified DNA fragment obtained as the eluate was stored at 4˚C for further use.

3.6 CLONING OF AMPLICONS

- The PCR generated fragments therefore have an A overhang at the 5' end, specialized vectors with 3'U or 3'T overhangs have to be used for efficient cloning.
- Two vectors viz. pGEMT (Promega) and pMD20 (Takara) were used in the present study. (Figure 3.2)

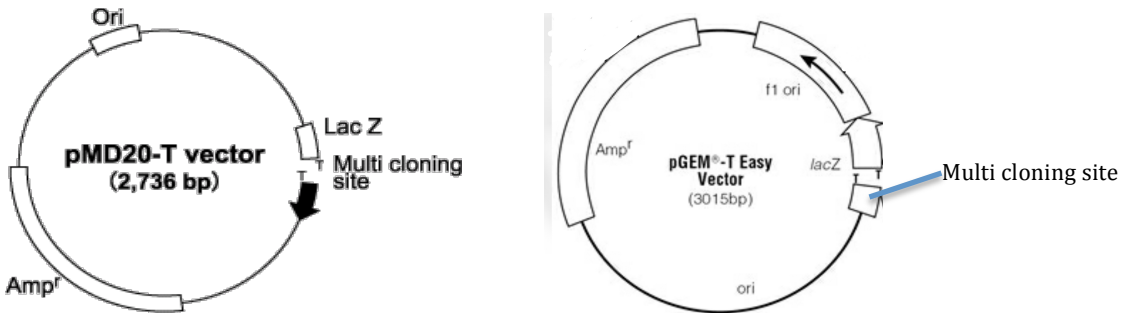


Figure 3.2: Structure of pMD20-T cloning vector and pGEM-T cloning vector.

- The PCR products were ligated to the vector in a 10µl ligation mix as per the manufacturer's instruction. The components were added in the following order (Table 3.9).

Table 3.9: Components of ligation reactions.

Components	Volume Taken	
	pMD20-T vector	pGEM-T vector
Sterile Water	2 µl	1 µl
Purified PCR Product (Fragment)	2 µl	2 µl
Ligation Buffer (2X)	5 µl	5 µl
Vector	1 µl	1 µl
Ligase (3U/ µl)	--	1 µl
Total	10 µl	10 µl

(Before adding ligase a brief spin was given).

- The ligation mix was then incubated at 16°C for 2 hrs and then overnight at 4˚C.

3.7 TRANSFORMATION AND SELECTION OF POSITIVE CLONES

3.7.1.Preparation of competent cells

- E.coli strain JM101 was grown in 5 ml LB broth at 37˚C. Of this 1 ml was used to inoculate 40 ml of fresh LB broth and was grown for 2.5-3 hrs with shaking at 100 rpm at 37°C to obtain cells in the log phase (O.D600nm=0.6). The density of the culture was ascertained visually.
- This culture was then transferred aseptically to fresh autoclaved Oakridge tubes and was centrifuged at 8000 rpm for 10 mins (SA300, Sorvall centrifuge). The supernatant was decanted and tubes were inverted on tissue paper to allow traces of medium to drain away.
- The pellet was then resuspended in 15 ml of icecold 100mM $CaCl_2$ and the cells were mixed by vortexing. This was then kept on ice for 30 mins.
- After 30 mins the tubes were recentrifuged at 10000 rpm for 10 mins. The supernatant obtained was decanted.
- 1.5 ml of ice-cold 100mM $CaCl_2$ was added to the pellet and the cells were resuspended by gentle pipetting.
- The competent cells thus prepared were stored as aliquots of 50 µl at 4˚C till further use.

3.7.2.Transformation of ligated sample

- 50 µl of competent cells were mixed with 5 µl of ligation reaction (Section 3.6) in a 1.5 ml microfuge tube and was mixed by gentle tapping. This was kept on ice for 30 mins.
- After 30 mins a heat shock was given at 42°C for 2 mins. The tubes were then immediately chilled on ice for 5 mins.
- 100 µl LB broth was added to each microfuge tube and was incubated at 37°C in a water bath for 30 mins without shaking.

3.7.3.Preparation of selective media plates

- LB powder (2%) and agar (2%) was dissolved in distilled water and was autoclaved at 15 Lbs for 15 mins. This was then allowed to cool and Ampicillin (Appendix) was added to it and was mixed evenly by swirling the flask.
- Medium was poured in autoclaved petriplates in laminar airflow and was allowed to solidify.
- 40 µl of X-Gal (Appendix) and 4 µl of IPTG (Appendix) was evenly spread onto each petriplate using sterile spreaders.
- Entire volume of transformed culture was then evenly spread with sterile spreaders and plates were kept for incubation overnight at 37°C.

3.7.4.Selection of positive clones and preparation for colony PCR

- PCR tubes were labeled and a gridded LB Amp plate was prepared accordingly.
- Distinct white colonies (4 white colonies for each endophyte) were directly picked using sterile toothpick and were damped in labeled PCR tube and same toothpick was then streaked in the respective labeled grid in the gridded plate.
- A PCR was done to check positivity of selected clones. Vector specific primers were used in this case to allow amplification of recombinant clones only. Vector specific primers used in this case was SP6 (forward) and T7 (reverse) primer.
  (* For endophyte #3 M13 (F and R) primer was used.)
- The PCR mix constituents were as follows (Table 3.10)

Table 3.10: Components of colony PCR master mix

PCR Components	Stock Concentration	Volume Taken Per Reaction (Final Concentration)	Volume Taken For 112 Reactions (Master Mix)
Green Taq Buffer	10X	1 µl (1X)	112 µl
dNTPs(Mix)	10mM	0.2 µl (200-250 µM)	22.4 µl
Taq DNA Polymerase	5U/µl	0.1 µl (0.5 U)	11.2 µl
Primer (F & R)	50 pmole/µl	0.1 µl + 0.1 µl (5 pmole/µl)	11.2 µl + 11.2 µl
Sterile Water	---	8.5 µl	952 µl
Total		10 µl	1120 µl

- PCR conditions were as follows:

Table 3.11: Conditions For colony PCR

Steps	Reactions	Condition
1	Initial Denaturation	94°C/6 mins
2	Denaturation	94°C/30s
3	Annealing	55°C/30s
4	Extension	72°C/30s
5	Final Extension	72°C/2 mins
6	Final Hold	4°C
Number Of Cycles		35

- PCR samples were then run on a 2% gel and were checked for correct band size using ΦX174/HaeIII digest as marker.

3.7.5.Inoculation Of Positive Clones

- Clones showing proper band size and optimum concentration were selected.
- Screw capped flasks containing LB broth (35ml) were autoclaved. Then 35µl ampicillin was added to each flask.
- Culture of selected clones was picked from grid plate and was inoculated in them.
- Culture flasks were then incubated at 37°C overnight with shaking (~100rpm)

3.8 PLASMID DNA ISOLATION

- Cultures grown were first transferred in Oakridge tubes. These tubes were then centrifuged at 8000 rpm for 10 mins (SA300, Sorwall centrifuge). Supernatant was discarded.
- 700 µl ice-cold solution I (Appendix) was added in each tube and was vortexed vigoursly so that the pellet gets suspended in the solution. This was then kept in ice for 10 mins.
- 1.4 ml freshly prepared solution II (Appendix) was added and was mixed gently by inversion. This was kept on ice for 10 mins.
- 1 ml ice-cold solution III (Appendix) was added and mixed gently by inversion. This was then kept on ice for 20 mins.

- Tubes were then centrifuged for 15 mins at 14000 rpm. Supernatant was then carefully transfered to fresh tube and 0.6 volume (3ml) of ice-cold isopropanol was added and kept at -20°C for 1 hr.
- Tubes were then centrifuged at 14000 rpm for 15 mins. Supernatant was carefully discarded and tubes were allowed to dry. Pellet was distinctly visible.
- 50 µl TE (Appendix) was added and tubes were vortexed so that pellet gets dispersed in TE. Entire sample was then transferred to respectively labeled 1.5 ml microfuge tubes.
- 3 µl RNase (Appendix) was added to each microfuge tube and left overnight.

3.9 EXTRACTION OF PLASMID DNA

- For extraction of plasmid DNA phenol chloroform treatment was given.
- For this 500 µl phenol : chloroform : isoamyl alcohol (25:24:1) was added. Tubes were vortexed and centrifuged.
- Upper phase was carefully transferred to fresh tubes and 500µl isoamyl alcohol was added to the tubes. Tubes were then vortexed and centrifuged. Upper aqueous phase was transferred to fresh tubes.
- Then 30 µl 3M sodium acetate and 350 µl isopropanol was added to each tube and were gently inverted and mixed and kept at -20°C for 15 mins for precipitation.
- Tubes were then centrifuged. Supernatant was drained out and 1ml 70% alcohol was added inverted once and kept for 10mins and then was centrifuged. Supernatant was drained out.
- A second wash was given by adding 500 µl of 70% alcohol, kept for 5mins and again was centrifuged. Supernatant was drained out and tubes were damped in fresh tissue paper to remove extracts of alcohol.
- Tubes were left for air drying.
- Pellet was then dissolved in 50 µl of sterile water by vortexing and also a brief spin was given.
- The clones were quantified by electrophoresis on a 1% Agarose gel along with λ/HindIII digest as DNA ladder.

3.10 CONFIRMATION OF POSITIVE CLONES BY PCR

A PCR was set in a 10µl system and all components were added to make the required master mix. 9.5µl master mix was distributed in each tube and also DNA template was added to each tube. PCR conditions were same as discussed earlier. Amplified product was then analysed on a 1.5% agarose gel along with ΦX174/HaeIII digest as DNA ladder.

3.11 COMMERCIAL SEQUENCING OF POSITIVE CLONES

The selected positive clones were then sent for sequencing.

3.12 ARRANGEMENT OF CONTIGS

The sequences obtained after commercial sequencing were in the form of contigs. These contigs were arranged according to the overlapping sequences.

3.13 BLAST ANALYSIS , IDENTIFICATION AND CLASSIFICATION

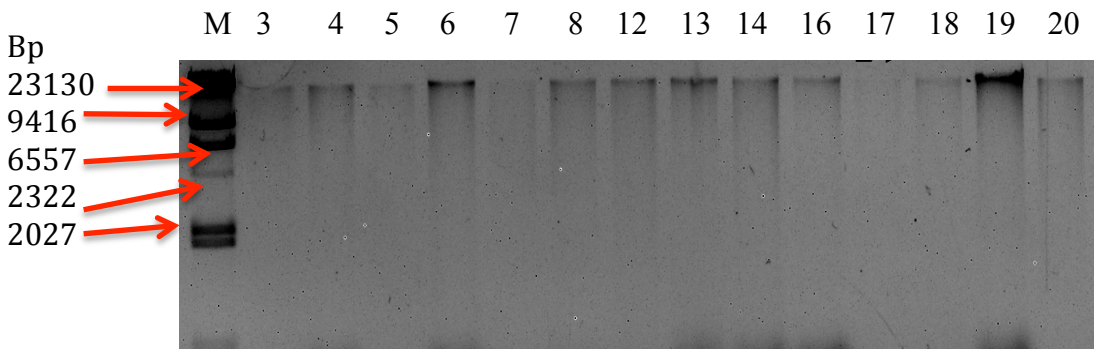
Arranged complete sequences were then analysed in public domain using BLAST according to which the isolated endophytes were identified and classified.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Quantification Of Isolated Genomic DNA By Gel Electrophoresis

The isolated genomic DNA was obtained as an eluate of 300µl. The quality and quantity of the genomic DNA was checked by running a 3µl aliquot on a 0.8% agarose gel (Plate 1). As seen in the gel photo the DNA obtained was of high molecular weight with minimal shearing. The concentration was adjusted to 50ng/µl, if required, with the addition of sterile water.

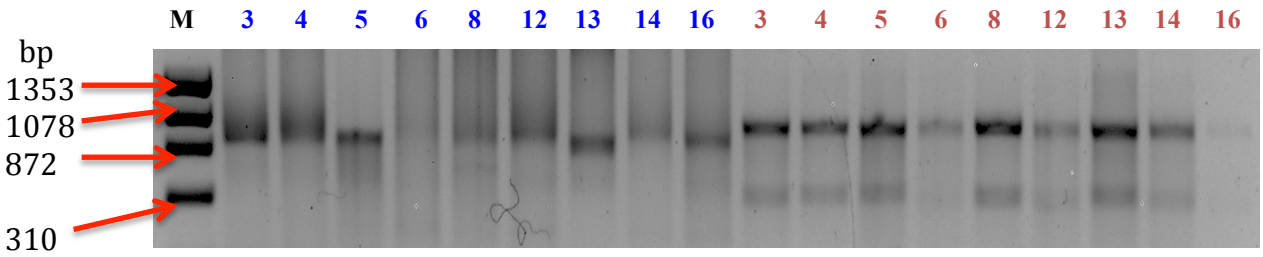


M- λ/HindIII digest

Plate 1: 0.8% agarose gel electrophoresis of endophytic genomic DNA.

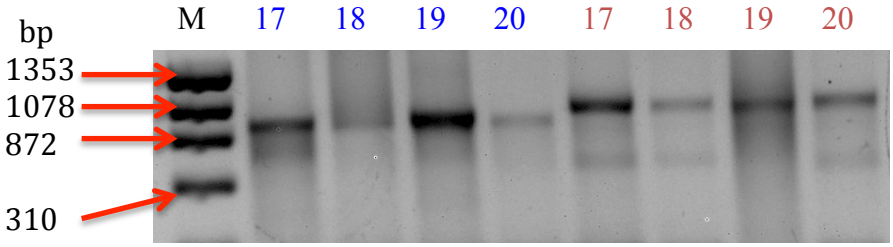
4.2 Amplification With Universal Primers.

The genomic DNA was amplified with sets of primer pairs P3 and P7 (Section 3.4.1). The amplicons obtained were checked on agarose gels (Plate 2 a and b)



(a) P3 amplicons #3 to #16 and P7 amplicons #3to#16

M - ΦX174/Hae III digest



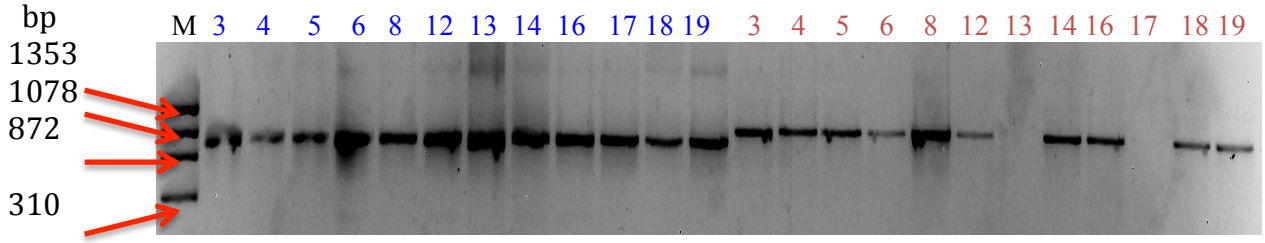
(b) P3 amplicons #17 to #20 and P7 amplicons #17 to #20

M - ΦX174/Hae III digest

Plate 2 : 0.8% agarose gel electrophoresis of amplicons obtained with universal primers.

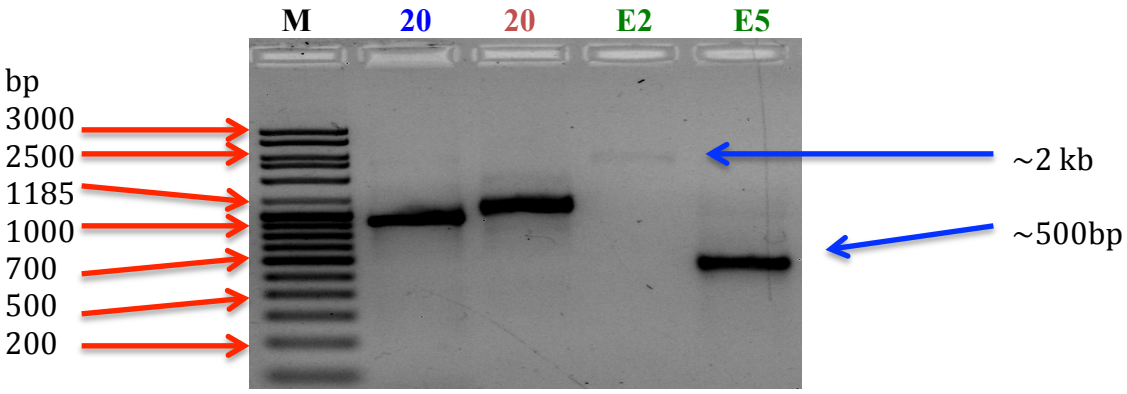
Proper amplification was obtained in each case. Correct sized fragments of P3 (1104 bp) and P7 (1201bp) were obtained. Endophyte 7 which is a fungus was amplified with sets of primers E2, E3 and E5 (Section 3.4.2). These were also checked on agarose gels and the correct sized fragments were obtained (data not shown). The fragments were then purified for cloning.

The purified PCR products were obtained as a 50µl eluate and an aliquot of 5 µl was electrophoresed (Plate 3 a and b). Poor concentration was seen in case of amplicons of endophyte #13 & 17 with primer set P7. The PCR was repeated to obtain a good concentration. The 2kb fungal amplicon also showed a low concentration. The PCR was repeated with primer set E2 and the amplicons obtained in both sets were pooled and then used for cloning. No amplification was seen with primer set E3 in the fungal endophyte 7.



(a) P3 amplicons #3 to #19 and P7 amplicons #3to#19

M - ΦX174/Hae III digest



(b) P3(20), P7(20) & fungal clones (E2 and E5) amplicons

M - Low Range Plus DNA ruler

Plate 3 : agarose gel (2%) electrophoresis of cartridge purified PCR amplicons.

In Plate 3 (b) proper concentration and fragment size of P3 and P7 of endophyte #20 was observed with a clear difference between their band size. Also fungal clones E2 and E5 were amplified and purified. The fungal clone E2 did not show proper amplification, hence was reamplified and purified. E5 showed proper results.

4.3 Cloning And Selection Of Recombinants.

The PCR purified product was ligated to specific vectors (Section-3.6). The ligated product was used to transform *E.coli* JM101 (Section 3.7.2) and was spread on selective media plates (Section- 3.7.3). Blue/white colonies along with some blue-centred colonies were observed (Plate 4).

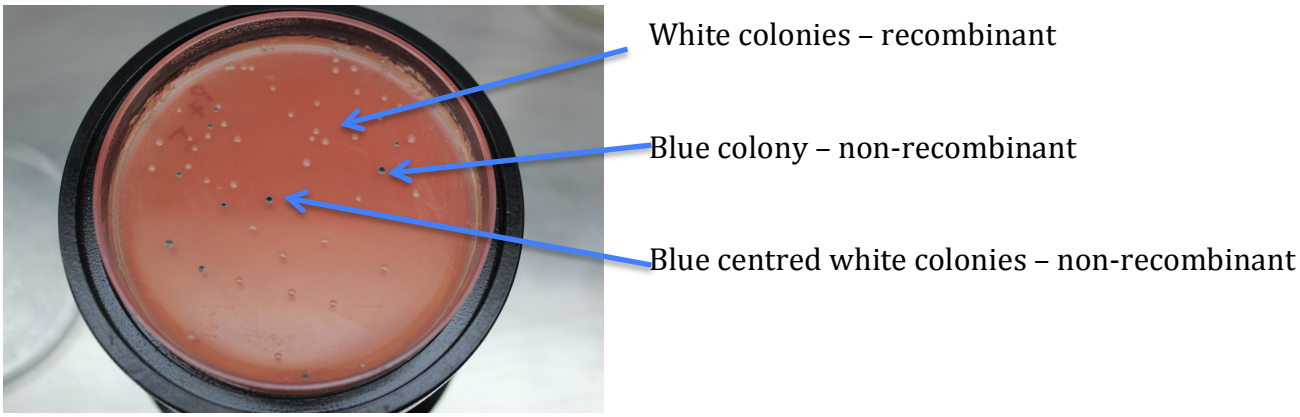


Plate 4: LB+Amp (X-gal+IPTG) plate with transformed *E.coli* JM101 colonies.

At least four white colonies were then subjected to colony PCR (Section-3.7.4). The product obtained was checked on an agarose gel (Plate 5).

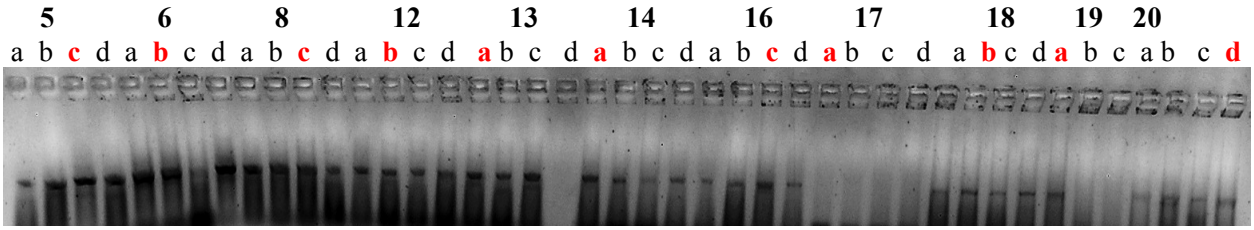
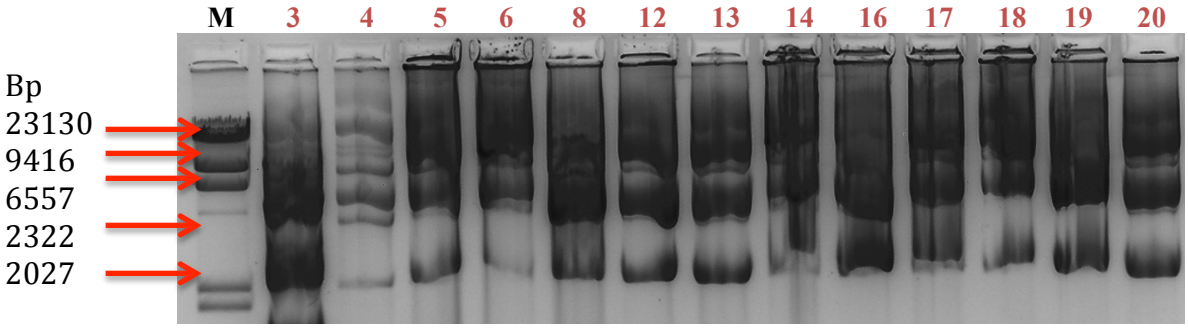


Plate 5: 2% agarose gel electrophoresis of selected white colonies.

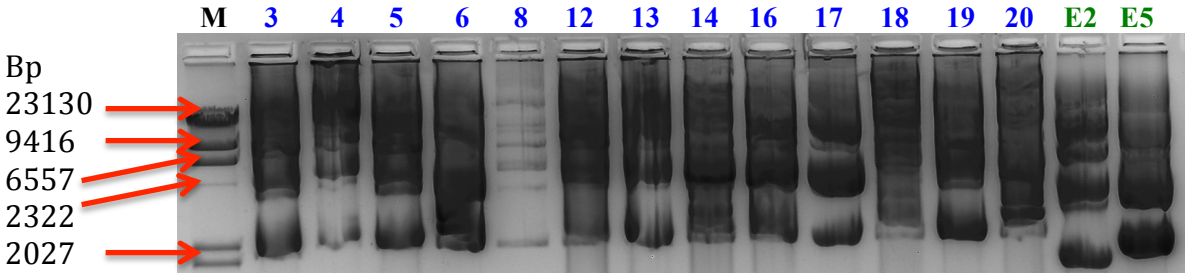
One positive clone was selected for each endophyte as depicted in Plate 5 in red colour. The colonies harbouring the positive clones were then grown on LB broth containing ampicillin (Section 3.7.5). The plasmid was isolated and purified (Section 3.8 and 3.9).

The final product was resuspended in 50 µl of sterile water. A 3µl aliquot mixed with loading dye (Appendix) was run on agarose gel (Plate 6 a, b).



(a) P7 amplicons

M- λ/HindIII digest

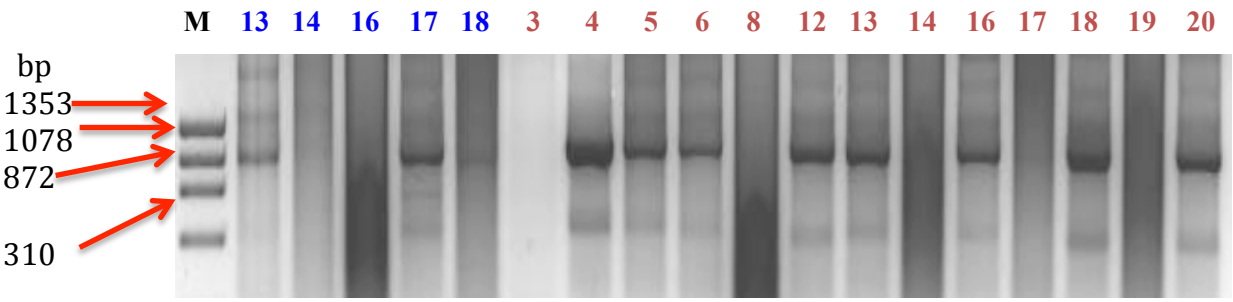


(b) P3 amplicons and fungal E2 & E5 amplicons

M- λ /HindIII digest

Plate 6: Agarose (1%) gel electrophoresis of purified recombinant plasmids.

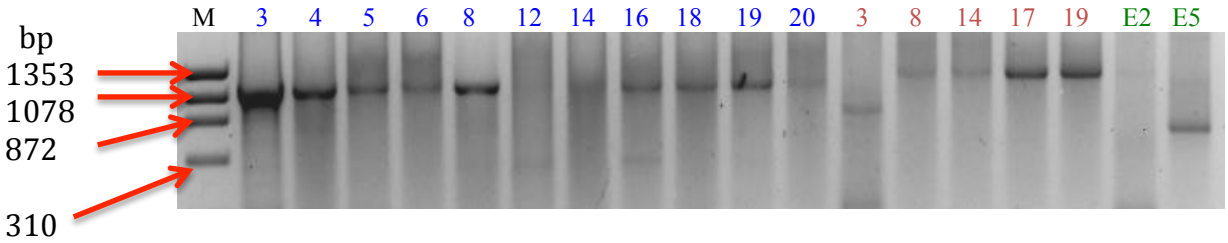
Before sending the samples for commercial sequencing the quality of isolated plasmid was also checked by performing a PCR of the samples. The clones were checked for the presence of the cloned fragment by using vector specific primers (Section- 3.10). The product was then run on an agarose gel (Plate 7a & 7b).



M- ΦX174/HaeIII digest

Plate 7a: 1.5% agarose gel electrophoresis of PCR product.

The clones that did not amplify or gave the incorrect size products were reisolated and reaccessed.



M- ΦX174/HaeIII digest

Plate 7b: 1.5% agarose gel electrophoresis of PCR product.

The quality and quantity of isolated plasmid was confirmed to be fine with a concentration of 250-400ng/µl and the isolated plasmids also showed good amplification. An aliquot of 10 µl out of the 50 µl eluate was taken in labeled 1.5 ml microfuge tubes and was sealed with parafilm properly and was sent for sequencing.

4.4 Sequences of the clones

Readable sequences were not obtained for all clones. Some partial sequences were obtained. The vector sequences were edited out and the clone sequences were arranged. The primer sequences helped to align the contigs and generate the complete sequence.

ENDOPHYTE 4

Endophyte 4 was a bacterium isolated from nutrient agar medium after growth at 37°C for 23 hrs. It was viscous, circular, raised, entire and transparent (Rani, 2016). A single colony from the pure culture plate was inoculated in LB broth. The genomic DNA isolated from this was further amplified (Section 3.4).

The sequences flanked by UF and CD[R] primers (henceforth referred to as P3) could not be obtained as the sequence reads were poor. However, the sequences for the latter part of 16S rRNA i.e., those flanked by 533(F) and UR primers (henceforth referred to as P7) could be obtained. The P7 sequence contains the V6 region, which is one of the most hypervariable regions and used for identification purposes (Větrovský and Baldrian, 2013). The complete read was of 1034 bp (Fig. 4.1).

Fig. 4.1: P7 sequence of endophyte 4

#4 P7 sequence

533(F)

GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTT
CTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAA
AGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAATACCAGTGGCGAAGGCGACTTTCTGGTCTGT
AACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTG
CTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGG
TTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCT
TACCAAATCTTGACATCCTTTGAAAACTCTAGAGATAGAGCCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGG
TTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAA
GTTGGGCACTCTAGGTTGACTGCCGGGTGACAAACCGGAGGAAGGTGGGGGATGACGTCAAATCATCATGCCCCTTATG
ATTTGGGCTACACACGTGCTACAATGGACAATACAAAGGGCAGCTAAACCGCGAGGTCATGCAAATCCCATAAAGTTG
TTCTCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGT
GAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGCCGGTGGAGTAACCAT
TTATGGAGCTAGCCGTCGAAGGTGGGACAAATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGG**AAGGTGCGG**
CTGGATCACCTCCTT **(1034 bp)**

UR

The P7 sequence was subjected to Basic Local Alignment Search Tool (BLAST) with the nucleotide sequences in the public domain of the National Centre for Biotechnology Information (NCBI). A strong match with *Staphylococcus saprophyticus* strain FDAARGOS_355*, Staphylococcus succinus subsp. succinus* strain SSY001 and *Staphylococcus xylosus* strain S170 and other *Staphylococcus* species was obtained. In the first three cases 99% identity and 100% query cover was seen.

Staphylococcus saprophyticus species are Gram positive, non-motile, non-spore forming cocci (Kloos and Bannerman, 1994). The characteristics reported by Kloos and

Bannerman and those documented for endophyte 4 (Rani, 2016) are compared in Table 4.1. The characters are in complete concurrence.

Table4.1: Comparison of characteristics of endophyte 4 with *Staphylococcus saprophyticus*

Characteristics	*Staphylococcus saprophyticus*	Endophyte 4
Temp of growth	30 – 37 °C	37 °C
Colony morphology	Irregular Viscous Slightly convex	Irregular Viscous Flat
Acetoin production, Urease production, Citrate utilisation	+	+
Ornithine, sorbitol, rhamnose and arabinose utilization	-	-

Though no information is available about *Staphylococcus saprophyticus* being an endophyte of finger millet, it has been reported in common bean (*Phaseolus vulgaris*) by Costa *et al* (2012). They isolated culturable endophytes from the leaves of *Phaseolus vulgaris*. Of the 158 total isolates, 36.7% belonged to the *Proteobacteria*, 32.9% to *Firmicutes*, 29.7% to *Actinobacteria*, and 0.6% to *Bacteroidetes*. The 3 *P. vulgaris* cultivars showed class distribution differences among *Actinobacteria, α-proteobacteria* and *Bacilli*. Based on 16S rDNA sequences, 23 different genera were isolated comprising bacteria commonly associated with soil and plants. The genera *Bacillus*, *Delftia*, *Methylobacterium*, *Microbacterium*, *Paenibacillus*, *Staphylococcus* and *Stenotrophomonas* were isolated from all three cultivars. This was the first report of endophytic bacteria from the leaves of *P. vulgaris* cultivars.

Staphylococcus saprophyticus has also been isolated as an endophyte from cashew leaves (Lins *et al*, 2014). However no other report of *Staphylococcus saprophyticus* as an endophyte is available. In contrast it has been found to be a frequent cause of urinary tract infection in the young women (Triveddi *et al*, 2015).

The P7 sequence of endophyte 4 had shown the same degree of match with *S. xylosus* and *S. succinus* as with *S. saprophyticus*. Though no result of *S. succinus* as an endophyte is available, *S. xylosus* has been identified as one. Reiter et al (2002) showed *S. xylosus* to be a part of the endophytic population of potato based on the 16S rRNA information.

The tentative classification of endophyte 4 based on the partial 16S sequence, its colony characteristics and biochemical traits is as follows:

Kingdom:	*Bacteria*
Phylum:	*Firmicutes*
Class:	*Bacilli*
Order:	*Bacillales*
Family:	*Staphylococcaceae*
Genus:	*Staphylococcus*
Species:	*S. saprophyticus*

However, the complete 16S sequence should be obtained for confirmation of the identity.

ENDOPHYTE 7

Endophyte 7 had been isolated from potato dextrose agar (PDA) medium after growth at 37°C for 23 hrs (Rani, 2016). The colony morphology indicated that it was a fungus (Plate 8).

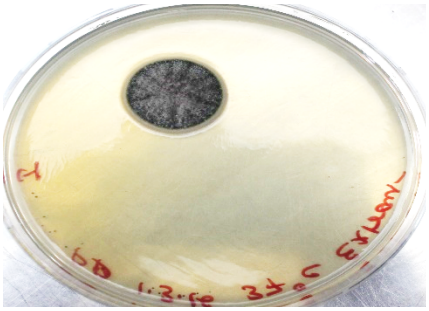


Plate 8 : Endophyte 7 (Rani, 2016)

The genomic DNA isolated was amplified with universal primers ITS 1 and ITS 4 (Section 3.4.2). The ITS region is popularly used for identifying fungal species as it shows higher degree of variation than the other genic regions of rDNA (White *et al*, 1990) The ITS region of the major rRNA transcript was sequenced. The sequence obtained was 551 bp long (Fig 4.2).

Fig. 4.2: ITS sequence of endophyte 7

Endophyte 7 sequence

ITS 1

TCCGTAGGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGTCTAACCACCGGGATGTTCATAACCCTTTGTTGTC
CGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAACTTTGCA
GTCTGAGTAAACTTAATTAATAAATTAAAACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGA
AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGTATTCCG
GGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGTATTGGGCAACGCGGTCCGCCGCGTGCCTCA
AATCGACCGGCTGGGTCTTCTGTCCCCTAAGCGTTGTGGAAACTATTCGCTAAAGGGTGTTCGGGAGGCTACGCCGTAA
AACAACCCCATTTCTAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAA**GCATATCAATAAGCGGAGGA**

(551 bp) **ITS 4**

When subjected to BLAST a 100% match was obtained with *Cladosporium cladosporoides*. *Cladosporium* colonies are olive-grey to dull green, velvety and tufted on PDA medium. The edges of the colony may be olive-grey to white, and feathery (Bensch *et al*, 2010). The colony characteristics of endophyte 7 had been documented in the laboratory as woolly, blackish with an outer slimy zone (Plate 8). Thus, dull green and blackish, tufted and woolly, white edge and slimy zone are all characteristics that match with each other.

Based on the colony appearance and and the ITS sequence endophyte 7 has been identified and classified as follows:

Kingdom:	*Fungi*
Division:	*Ascomycota*
Subdivision:	*Pezizomycotina*
Class:	*Dothideomycetes*
Order:	*Capnodiales*
Genus:	*Cladosporium*
Species:	*C. cladosporioides*

Cladosporium species have been shown to be present as an endophyte in pine. Paul *et al* (2008) discovered many *Cladosporium* species previously unreported in Korea from needles of pine trees (*Pinus spp.*). Their analyses was also based on the internal transcribed spacer gene sequence and cultural and micromorphological characteristics. They identified two species i.e., *C. oxysporum* and *C. sphaerospermum*. Neither species had ever been reported in Korea. However, no report of *Cladoporium* being as an endophyte in finger millet or any other plant is available.

It has been reported that *Cladosporium cladosporioides* produces antifungal metabolites targeted toward plant pathogens (Wang *et al*, 2013). Three different compounds isolated from *C. cladosporioides* (cladosporin, isocladosporin and 5′ hydroxyasperentin), as well as a compound (5′,6-diacetyl cladosporin) synthesized from 5′-hydroxyasperentin have antifungal properties. As these compounds are effective against different types of fungi, *C. cladosporioides* is an important species for potential treatment and control of various plant-infecting fungi. This species also produces calphostins A, B, C, D and I, which are protein kinase C inhibitors. These calphostins have cytotoxic activity due to their ability to inhibit protein kinase C activity.

Also an endophytic fungus strain LF70 isolated from the leaves of firmoss (*Huperzia serrate)* was identified as *Cladosporium cladosporioides* (Zhang et al, 2015). This analyses was also based on morphological characteristics and nuclear rDNA ITS sequence analysis. The strain produced Huperzine A (HupA) which is an acetylcholinesterase inhibitor. The amount of HupA produced by this endophytic fungus was quantified to be 56.84 μg/L by HPLC, which was higher than that of other reported endophytic fungi, *Acremonium* sp., *Blastomyces* sp., and *Botrytis* sp. Acetylcholinesterase inhibition activity of HupA produced by strain LF70 was also similar to authentic HupA *in vitro*. HupA is used in treating Alzheimer's disease and preventing further memory degeneration. Thus, the fungus has great commercial value.

This work is the first report for the presence of *Cladosporium cladosporoides* as an endophyte in finger millet. Further investigation and studies are required for exploitation of the potential of this fungus.

ENDOPHYTE 12

Endophyte 12 was a bacterium isolated from nutrient agar media which was grown at 28°C for 48 hrs. It was buttery, circular, raised, entire and light yellow in colour (Rani, 2016). Only the P7 sequence could be obtained for this endophyte and it was 1019 bp in length (Fig. 4.3).

Fig. 4.3: P7 sequence of endophyte 12

#12 P7 sequence

533(F)
CCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGG
CGGTAATGTAAGACAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGTGACTGCATTGCTGGA
GTGCGGCAGAGGGGGATGGAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACACCGATG
GCGAAGGCAATCCCCTGGGCCTGCACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCCTAAACGATGTCAACTGGTTGTTGGGTCTTCACTGACTCAGTAACGAAGCTAA
CGCGTGAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGC
ACAAGCGGTGGATGATGTGGTTTAATTCGATGCAACGCGAAAAACCTTACCCACCTTTGACATGTACGGA
ACCCTTTAGAGATAGAGGAGTGCTCGAAAGAGAGCCGTAACACAGGTGCTGCATGGCTGTCGTCAGCTCG
TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGCCATTAGTTGCTACGAAAGGGCACT
CTATGGGGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATAGGT
GGGGCTACACACGTCATACAATGGCTGGTACAGAGGGTTGCCAACCCGCGAGGGGGAGCTAATCCCATA
AAGCCAGTCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGGA
TCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGCGGGTTCT
GCCAGAAGTAGGTAGCCTAACCGTAAGGAGGGCGCTTACCACGGCAGGGTTCGTGACTGGGGTGAAGTC
GTAACAAGGTAGCCGTATCGGAAGGTGC**GGCTGGATCACCTCCTT** **(1019 bp)**
UR

The partial P7 sequence when subjected to BLAST, showed a match with *Acidovorax avenae subsp. avenae* ATCC 19860 and *Acidovorax citrulli* strain KACC17005 (100% query cover and 99% identity).

The colony characteristics and the biochemical characteristics documented in the laboratory (Rani, 2016) were compared with available information on *A. avenae* sub. *citrulli*. Fontana *et al* (2013) have described the colony characteristics whereas Choi *et al* (2010) studied the biochemical traits. Table 4.2 indicates a complete match between endophyte 12 and *Acidovorax* species.

Table4.2: Comparison of characteristics of endophyte 12 with *Acidovorax avenae*

Characteristics	***Acidovorax avenae subsp. citrulli***	**Endophyte 12**
Growth medium	Nutrient agar	Nutrient agar
Temperature of growth	30°C	28°C
Colony morphology	Circular Entire Raised White-cream or yellow	Circular Entire Raised Creamish white
Urease reduction, glucose utilization, arabinose utilization	+	+
Ornithine, sorbitol and rhamnose utilization	-	-

The genera *Acidovorax* are members of the β-*Proteobacteria* that primarily cause leaf spot and streak diseases of a variety of host plants .The most significant pathogens within the genus *Acidovorax* genus belong to the species *A. avenae.* Amongst them the *subsp. avenae* is a significant pathogen of *graminaceous* species and the *subsp. citrulli* is an important pathogen of cucurbit species, especially watermelon. The *subsp*. *cattleyae* causes disease of orchids and is of lesser significance that the other two subspecies (Horuz et al, 2014).

A. avenae was shown to be present as an endophyte in the seeds of rice plant (Mano *et al*, 2008) alongwith several other microbes.

Pereiera *et al* (2014) also identified Acidovorax as an endophyte in *Zea mays***.** They studied the phylogenetic diversity of culturable bacterial endophytes of *Zea mays* plants growing in an agricultural soil contaminated with Zn and Cd. Endophytic bacterial counts were determined in roots and shoots, and isolates were grouped by random amplified polymorphic DNA and identified by 16S ribosomal RNA (rRNA) gene sequencing. Endophytes were further characterized for the production of plant growth-promoting (PGP) substances. Phylogenetic analysis showed that endophytes belong to three major groups: α-*Proteobacteria* (31 %), γ-*Proteobacteria* (26 %) and *Actinobacteria* (26%). *Pseudomonas, Agrobacterium, Variovorax* and *Curtobacterium* were among the most represented genera. Strains *Ochrobactrum haematophilum* ZR 3-5, *Acidovorax oryzae* ZS 1-7, *Frigoribacterium faeni* ZS 3-5 and *Pantoea allii* ZS 3-6 were shown to effect an increased root elongation and biomass of maize seedlings grown in soil contaminated with Cd and Zn.

The tentative classification of endophyte 12 based on morphological and biochemical traits combined with the partial 16S sequence is as follows:

Domain:	*Bacteria*
Phylum:	*Proteobacteria*
Class:	*Betaproteobacteria*
Order:	*Burkholderiales*
Family:	*Comamonadaceae*
Genus:	*Acidovorax*
Species:	*A. avenae*

However, it is desirable to have the complete 16S sequence for confirmation of the identity of the endophyte.

ENDOPHYTE 13

Endophyte 13 was a bacterium isolated from nutrient agar medium grown at 28℃ for 48 hrs had viscous, circular, flat, entire, creamish white colony features (Rani, 2016). When the reads were arranged, a complete P3 and P7 sequence could be obtained but when these P3 and P7 sequences were aligned, many mismatches at the overlapping region were found. The forward and reverse reads matched completely for both P3 and P7. The supposed anomaly can be explained on the basis of the fact that there are multiple copies of the 16S rRNA gene present in a bacterium. The copies can differ in sequence, leading to identification of multiple ribotypes for a single organism (Case *et al*, 2006). It is probable that the P3 and P7 sequences obtained are from two different copies of the 16S rRNA.

The P3 sequence (873 bp) and P7 sequences (1028 bp) (Fig. 4.4) were subjected to BLAST separately.

Fig. 4.4: P3 and P7 sequence of endophyte 13.

#13 P3 sequence

UF

CAGAGTTTGATCCGGGCTCAGAACGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAACGCCCCGCAAGGGGAGT
GGCAGACGGGTGAGTAACGCGTGGGAACATACCCTTTTCTACGGAATAGCTCTGGGAAACTGGAATTAATACCGTATAC
GCCCTACGGGGGAAAGATTTATCGGGGAAGGATTGGCCCGCGTTGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAA
GGCGACGATCCATATCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACCCCTACGGGAGGC
AGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTTATTTATGAAGGCCTTAAGGTTGTAG
AGCTCTTCTCGGCGATAAGATAATGACGGTAGTCGGAGAAGAAGCCCCGGCTAACTTC**GTGCCAGCAGCCGCGGTAA**

533(F)

TACGAAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGATATTTAAGTCAGGGGTGAAGTC
CCAGAGCTCAACTCTGGAACTGCCTTTGATACTGGGTATCTTGAGTATGGAAGAGGTAAGTGGAATTGCGAGTGTAGAG
GTGAAATTCGTAGATATTCGCAGGAACACCAGTGGCGAAGGCGGCTTACTGGTCCATTACTGACGCTGAGGTGCGAAA
GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGTTAGCCGTCGGGCAGTATACT
GTTCGGTGGCGCAGCTAACGCATTAAACATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAG**GAATTGACG**
GGGGCCCGCACAAG **(873 bp)**

CD[R]

#13 P7 sequence

533(F)

GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTT
CTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAG
AGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGT
AACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTG
CTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGA
CTGAAACTCAAAG**GAATTGACGGGGGCCCGCACAAG**

CD[R]

CGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATA
GGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC
CGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAG
GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGG
CAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAG
CTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA
CGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCCAGCCGCCGAAGGTGGGACAGATGATTGGGGT
GAAGTCGTAACAAGGTAGCCGTATCGGAAGGTGC**GGCTGGATCACCTCCTT** **(1028 bp)**

UR

The P7 sequence containing the V6 region when subjected to BLAST showed a 100% match with *Bacillus amyloliquefaciens* strain GD-2*, Bacillus velezensis* strain SCGB 574 *and Bacillus subtilis* strain TLO3. The P3 sequence containing the V3 region when subjected to BLAST showed the closest match with *Bacillus subtilis.* Therefore, endophyte 13 is a species of *Bacillus.*

Bacillus subtilis, known also as the hay bacillus or grass bacillus, is a Gram-positive, catalase-positive bacterium, found in soil and the gastrointestinal tract of ruminants and humans. It is widely used for secreted enzyme production on an industrial scale (www.wikipedia.com).

The colony characteristics and the biochemical characteristics reported by different workers [Behera *et al*, 2014,; Saadabi *et al*, 2010] and those documented for endophyte 13 (Rani, 2016) are compared in Table 4.3.

Table4.3: Comparison of characteristics of endophyte 13 with *Bacillus subtilis*

Characteristics	***Bacillus subtilis***	**Endophyte 13**
Growth medium	Nutrient agar	Nutrient agar
Temperature of growth	25 -30 °C	28°C
Colony morphology	Circular or irregular Viscous Flat Cream-white or pale yellow	Circular Viscous Flat Creamish white
Nitrate reduction and arabinose utilization	+	+
Methyl test, lysine test, lactose utilization, Ornithine utilization and rhamnose utilization	-	-

Again there is concurrence between the traits of *B. subtilis* and endophyte 13.

Studies by Wilhelm *et al* (1998) reported the presence of *B. subtilis* as an endophyte of chestnut (*Castanea sativa*). During screening and testing bacterial isolates for antagonistic effects on the growth of *Cryphonectria parasitica in vitro* these bacteria were shown to have a potential use in the control of plant diseases. Liu *et al* (2009) described *B. subtilis* as an endophyte isolated from wheat roots, which exhibited high antifungal activity to *Gaeumannomyces graminis var. tritici* (Ggt). Ji *et al* (2008) reported *B. subtilis* as an endophytic strain that showed biological control against bacterial wilt colonization in mulberry. Their work was also based on bacteriological properties and 16S rRNA gene sequencing.

Also, cultures of *B. subtilis* were popular worldwide before the introduction of antibiotics as an immune stimulatory agent to aid treatment of gastrointestinal and urinary tract diseases. It was used throughout the 1950s as an alternative medicine, which upon digestion was found to significantly stimulate broad-spectrum immune activity including activation of secretion of specific antibodies IgM, IgG and IgA and release of CpG dinucleotides inducing INF A/Y producing activity of leukocytes and cytokines important in the development of cytotoxicity towards tumor cells. It was marketed throughout America and Europe from 1946 as an immune stimulatory aid in the treatment of gut and urinary tract diseases such as Rotavirus and Shigellosis (Ciprandi *et al*, 1986; Shylakhovenko *et al*, 2003).

On the basis of the colony and biochemical features combined with the 16S sequence a tentative classification of endophyte 13 is as follows:

Domain:	*Bacteria*
Phylum:	*Firmicutes*
Class:	*Bacilli*
Order:	*Bacillales*
Family:	*Bacillaceae*
Genus:	*Bacillus*
Species:	*B. subtilis*

ENDOPHYTE 16

Endophyte 16 was a bacterium isolated from nutrient agar media grown at 28°C for 20 hrs. It had tough, yellow, flowery, flat, papery outgrowths with a zone of clearance. Two single reads for the P7 region flanked by 533F(384 bp) and UR (743 bp) primers were obtained. As the read with 533F was small it did not overlap with the read with UR (Fig. 4.5).

When the sequences were subjected to BLAST individually the UR read showed 98% identity with 100% query cover with *Pantoea stewartii subsp. stewartii* DC283, *Pantoea deleyi* strain 3F and *Pantoea ananatis* strain BD 377. The 533F read showed 97% identity with 100% query cover with *P. ananatis* and *P. deleyi* strains. In an earlier study in the laboratory the BLAST analysis of an amplicon (350 bp) of finger millet variety VL149 showed that it had 96% homology with *Pantoea vagans* strain C9-1 chromosome. The

accession number of the sequence is KC020191 (Anita Pande and Kumari Aradhna, 2012). The translate also showed a 96% homology with the nitrate/nitrile sensor protein NarQ of *Pantoea sp.* (Aradhna, 2012).

Fig. 4.5: P7 sequence of endophyte 16

#16 P7 sequence

533(F)

GTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCT
GTTAAGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGAAACTGGCAGGCTTGAGTCTCGTAGAGGGGG
GTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAG
ACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGGTAGTCCACGCCGTAAAGGATGTCGA
CTTGAAGGTTGTTCCCTTGAGGAGCGTCTTCCGGAGCTAACGCATTAAGCTTCCGCTTTGTGAGGACGGCCG (384 bp)

----------no overlap----------

GGTAGTACACGCTGTAAACGATGTCGACTTGGAGGTTCTACCCTTGAGGAGTGGCTTCTGGAGCTAACGCGTTAAGTCG
ACCGTCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGT
TTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGTGAACTTGGCAGAGATGCCTTGGTGCCTTCGGG
AACGCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTTATCCTTTGTTGCCAGCGATTCGGTCGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGA
CGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGA
GAGCAAGCGGACCCCATAAAGTGCGTCGTAGTCCGGATCGGAGTCTGCAACTCGACTCCGTGAAGTCGGAATCGCTAGT
AATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGC
AAAAGAAGTAGGTAGCTTAACCCTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGAAGTCGTAACAAGGT
AACCGTAGGGG**AACCTGCGGCTGGATCACCTCCTT** **(743 bp)**

UR

Pantoea vagans is a Gram-negative enterobacterial plant epiphyte of a broad range of plants. The complete sequence (4.89-Mb) of *P. vagans* strain C9-1 (formerly *Pantoea agglomerans*) has been sequenced. It is commercially registered for biological control of fire blight, a disease of pear and apple trees caused by *Erwinia amylovora* (Smits *et al*, 2010).

Pantoea ananatis are Gram-negative; facultatively anaerobic (Walterson *et al*,2015). The colony characteristics reported by Walterson *et al* (2015) and the biochemical characteristics as reported in ABIS Encyclopedia and those documented for endophyte 16 (Rani, 2016) are compared in Table 4.4. The features match completely.

Sheibani-Tezerji *et al*, 2015 investigated three closely related *P. ananatis* strains (named S6, S7, and S8), isolated from maize seeds of healthy plants. Plant inoculation experiments revealed that each of these strains exhibited a different phenotype ranging from weak pathogenic (S7), commensal (S8), to a beneficial, growth-promoting effect (S6) in maize. They were the first to show that *P. ananatis* strains could colonize the same ecological niche but show distinct interaction strategies with the host plant. The comparative analysis revealed that genomes of these three strains were highly similar.

Table4.4: Comparison of characteristics of endophyte 16 with *Pantoea ananatis*

Characteristics	*Pantoea ananatis*	Endophyte 16
Temperature of growth	25 -30 °C	28°C
Colony morphology	Motile Circular Entire Convex Mucoidal	Motile Circular Entire Convex Viscous
Glucose utilization and arabinose utilization	+	+
Lysine test, urease production, acetoin production, ornithine utilization and nitrate reduction	-	-

Megias *et al* (2016) showed *Pantoea ananatis* AMG521 as the rice endophyte having several plant growth-promoting properties resulting in yield. Its draft genome was estimated at 4,891,568 bp with 4,704 coding sequences. The genome encoded genes for *N*-acylhomoserine lactone (AHL) synthases, AHL hydrolases, hyperadherence (*yidQ*, *yidP*, and *yidR*), fusaric acid resistance, and oxidation of lignin highlighting its biotechnological potential.

P. ananatis is capable of infecting humans and occurs in diverse ecological niches, such as part of a bacterial community contaminating aviation jet fuel tanks and contributing to growth promotion in potato and pepper. Coutinho *et al*, 2009 *P. ananatis* is also a common epiphyte. Some of its strains are ice-nucleating, a feature which has been used as a biological control mechanism against some insect pests of agricultural crops and by the food industry. *P. ananatis* has both antifungal and antibacterial properties. As a pathogen it infects both monocotyledonous and dicotyledonous plants. The symptoms are diverse depending on the host infected, and include leaf blotches and spots, die-back, and stalk, fruit and bulb rot.. *P. ananatis* causes disease symptoms in a wide range of economically important agricultural crops and forest tree species worldwide. It is regarded as an emerging pathogen based on the increasing number of reports of diseases occurring on previously unrecorded hosts in different parts of the world.

Though the complete 16S sequence would give a confirmation of the identity, endophyte 16 can be tentatively classified, on the basis of the partial 16S sequence combined with the colony and biochemical characteristics, as follows:

Domain: *Bacteria*
Phylum: *Proteobacteria*
Class: *Gammaproteobacteria*
Order: *Enterobacteriales*
Family: *Enterobacteriaceae*
Genus: *Pantoea*
Species: *P.ananatis*

ENDOPHYTE 17

Endophyte 17 was a bacterium isolated from nutrient agar media grown at 37°C for 20 hrs. It was irregular, flat, papery, white. Only the P3 sequence (940 bp) could be obtained for this endophyte (Fig. 4.6).

Fig. 4.6: P3 sequence of endophyte 17

#17 P3 sequence

UF
CAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTT
GCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACC
GGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACC
CGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCC
ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGAC
GGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAAT
AGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTAC**GTGCCAGCAGCCGCGGTAA**TACGTAGGTGG
CAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAAC
CGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGT
AGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGC
GAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCT
GCAGCTAACGCATAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAG**GAATTGACGGGGGCCCGCA**
CAAG (940 bp) **CD[R]**

P3 when subjected to BLAST showed a match with *Bacillus subtilis*. The colony characteristics and the biochemical characteristics reported by different workers [Behera *et al*, 2014,; Saadabi *et al*, 2010] and those documented for endophyte 17 (Rani, 2016) are compared in Table 4.5.

Thus, endophyte 13 and 17 apparently belong to *Bacillus* species. The importance of *Bacillus* species has already been discussed. Interestingly, in a parallel work in the laboratory (Personal Communication) a dendrogram created on the basis of RAPD markers showed that endophyte 13 and 17 were the most closely related and fell in one clade. This corroborates the result obtained in the present study and vice versa.

Tentatively, the broad classification of endophyte 17 would be the same as endophyte 13 (Page number 57).

Table4.5: Comparison of characteristics of endophyte 17 with *Bacillus subtilis*

Characteristics	*Bacillus subtilis*	Endophyte 13
Growth medium	Nutrient agar	Nutrient agar
Colony morphology	Circular or Irregular Flat Cream-white or pale yellow	Irregular Flat Creamish white
Nitrate reduction and arabinose utilization	+	+
Methyl test, lysine test, lactose utilization, Ornithine utilization and rhamnose utilization	-	-

ENDOPHYTE 18

Endophyte 18 was a bacterium isolated from LB medium incubated at 28°C for 20 hrs. It was dry, circular, flat, entire and creamy. In this case only one read of P7 was obtained (Fig 4.7). The reverse sequence was not available. The BLAST showed a match with *Staphylococcus saprophyticus* with 100% query cover and 98% identity.

Fig. 4.7: P7 sequence of endophyte 18

#18 P7 sequence

533(F)
GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTT
CTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAA
AGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGT
AACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGAGTG
CTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCTTTTT
TTGAAACTCAAAGGAACTGACGAGGGACCCGCACGAGGCGGTGGGAGCATG (442 bp)

The characteristics reported by Kloos and Bannerman (1994) and those documented for endophyte 18 (Rani, 2016) are compared in Table 4.6.

It can be tentatively classified as *Staphylococcus* species. The importance of *Staphylococcus* species has already been discussed with endophyte 4.

Table4.6: Comparison of characteristics of endophyte 18 with *Staphylococcus saprophyticus*

Characteristics	*Staphylococcus saprophyticus*	Endophyte 4
Colony morphology	Circular Flat Entire Slightly yellow	Circular Flat Entire Creamy
Urease production, glucose utilization and sucrose utilisation	+	+
Nitrate reduction, Ornithine, sorbitol, rhamnose and arabinose utilization	-	-

ENDOPHYTE 19

Endophyte 19 was a bacterium isolated from LB media incubated at 28°C for 44 hrs. It was buttery, circular, raised, entire and creamy in colour (Rani, 2016). A partial P7 forward sequence and a partial P7 reverse sequence was obtained for endophyte 19 (Fig. 4.8). Only a partial P7 sequence was arranged. They were subjected to BLAST individually. The 533(F) tagged sequence matched with *Enterobacter tabaci* strain YIM Hb-3 (96% query cover, 100% identity). The UR tagged sequence showed a similar match.

Fig. 4.8: P7 sequence of endophyte 19

#19 P7 sequence

533(F)
AAGGAGGTGATCCAGCCGCAGGTTCCCCTACGGTTACCTTGTTACGACTTCACCCCAGTCATGAATCACAAAGTGGTA
AGCGCCCTCCCGAAGGTTAAGCTAACTACTTCTTTTGCAACCCAGTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCC
GGGAACGTATTCACCGGAGCATTCTGATCTACAATTACCACCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCGAT
CCGGACTACGACGCAATTTATGAGGGCCGCTTGCTCTCGGGAGGTCGCTTCGCTTTGTATGCGCCATTGTA (307 bp)
UR
GTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCT
GTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGG
GGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAA
GACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGA
CTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGAGGAGTACGGGCGCTTTTT
TAAAACTCATATGAATTGACAGGGGCCCACACAAGCGGGGGAGAGAGAGCCCCGAGTCAAGGAGCAGAGAGAAGCTT
ACCTACGCTTGACATC (483 bp)

The colony characteristics (Duan *et al*, 2015) and biochemical characteristics reported by Tan *et al* (2014) and Cabral (2010) were matched with those documented for endophyte

19 (Rani, 2016) (Table 4.7). From this data it appears that endophyte 19 is an *Enterobacter sp.*

Table4.7: Comparison of characteristics of endophyte 19 with *Enterobacter tabaci*

Characteristics	*Enterobacter tabaci*	Endophyte 4
Temperature of growth	25 - 40 °C	28°C
Colony morphology	Motile Circular Raised Entire Creamy yellow	Motile Circular Raised Entire Cream
Urease production	+	+
Lactose utilization, indole test	-	-

Enterobacter is a genus of common Gram-negative, facultatively anaerobic, rod - shaped, non spore forming bacteria of the family *Enterobacteriaceae*. Several strains of these bacteria are pathogenic and cause opportunistic infections in immunocompromised hosts and in those who are on mechanical ventilation. The urinary and respiratory tracts are the most common sites of infection. The genus *Enterobacter* ferments lactose with gas production during a 48-hour incubation at 35-37 °C in the presence of bile salts and detergents (Tan *et al*, 2014).

Studies by Hinton and Bacon (1995) have described *Enterobacter* as an endosymbiont of corn. This was a first report of a strain of this bacterium as an endophytic symbiont of roots. Studies by McInroy and Kloepper (1995) described *Enterobacter* as an indigenous bacterial endophyte from cotton and sweet corn. Taghavi *et al* (2010) also reported *Enterobacter* to be an endophytic plant growth promoting γ-*proteobacterium* isolated from the stem of poplar (*Populus trichocarpa*). Witzel *et al* (2012) isolated *Enterobacter radicincitans* sp. nov. DSM16656^{T}, a new species of the genus *Enterobacter* that was a biological nitrogen-fixing endophytic bacterium with growth promoting effects on a variety of crop and model plant species. The presence of genes for nitrogen fixation, phosphorous mobilization, and phytohormone production reflected the microbe's potential plant growth-promoting activity.As an endophyte *Enterobacter* has also been reported in diseased pepper in Hong Kong. This was *E. cloacae subsp. Cloacae* strain ENHKU01 (Liu *et al*, 2012).

E. cloacae P101, an endophyte of switchgrass (*Panicum virgatum*) has been completely sequenced (Humann *et al*, 2014). *E. cloacae* strains have been used as biocontrol agents of fungal pathogens.

E. cloacae has also been shown to be an obligatory endophyte of mature pollen grains of Mediterranean pines (Madmony *et al*, 2005) *E. cloacae* isolated from pollen of *Pinus halepensis* was able to produce indolyl-3-acetic acid (IAA) from L-tryptophan in culture. It could also promote adventitious root formation in many bean (*Vigna radiata*) cuttings.

The partial 16S sequence and the matches in Table 4.7 shows that the probable classification 19 is:

Kingdom:	*Bacteria*
Phylum:	*Proteobacteria*
Class:	*Gammaproteobacteria*
Order:	*Enterobacteriales*
Family:	*Enterobacteriaceae*
Genus:	***Enterobacter***

ENDOPHYTE 20

Endophyte 20 was a bacterium isolated from LB medium incubated at 37°C for 66 hrs. It was viscous, circular, raised, entire and creamish yellow in colour. A complete P7 sequence (1451 bp) was obtained for this endophyte (Fig. 4.9).

Fig. 4.9: P7 sequence of endophyte 20

#20 P7 sequence

533(F)

GTGCCACGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTAATG
TAAGACAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGTGACTGCATTGCTGGAGTGCGGCAGAGGGGGATGGA
ATTCCGCGTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACACCGATGGCGAAGGCAATCCCCTGGGCCTGCACTGACGCT
CATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTGGTTGTCGGGTC
TTCACTGACTCAGTAACGAAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTG
ACGGGGACCCGCACAAGCGGTGGATGATGTGGTTTAATTCGATGCAACGCGAAAAACCTTACCCACCTTTGACATGTACGGA
ATCCTTTAGAGATAGAGGAGTGCTCGAAAGAGAGCCGTAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATG
TTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGCCATTAGTTGCTACGAAAGGGCACTCTAATGGGACTGCCGGTGACAAAC
CGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATAGGTGGGGCTACACACGTCATACAATGGCCGGTACAGAG
GGTTGCCAACCCGCGAGGGGGAGCTAATCCCATAAAGCCAGTCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGT
CGGAATCGCTAGTAATCGCGGATCAGAATGTCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGG
AGCGGGTTCTGCCAGAAGTAGGTAGCCTAACCGTAAGGAGGGCGCTTACCACGGCAGGGTTCGTGACTGGGGTGAAGTCGTA
ACAAGATAGCCGTATCGGAAGGTGC**GGCTGGATCACCTCCTT** **(1023 bp)**

UR

The P7 sequence when subjected to BLAST showed a match with *Acidovorax avenae subsp. citrulli*.

The colony characteristics reported by Fontana *et al* (2013) and the biochemical characteristics reported by Choi *et al* (2010) were compared with those documented for endophyte 20 (Rani, 2016) (Table 4.8).

Table4.8: Comparison of characteristics of endophyte 20 with *Acidovorax avenae*

Characteristics	*Acidovorax avenae*	Endophyte 20
Colony morphology	Circular Entire White cream or yellow	Circular Entire Creamish yellow
Urease reduction and nitrate reduction	+	+
Lactose, Ornithine, sorbitol and rhamnose utilization	-	-

The tentative classification of endophyte 20 based on the partial 16S sequence and the matches in Table 4.8 is the same as that for endophyte 12 (Page number 54).The importance of *Acidovorax* species has already been discussed.

The summary of the tentative classification deduced from the present study is given in Table 4.9.

Table 4.9: Tentative classification of isolated endophytes.

Endophyte no.	Tentative identification	Phylum	Class	Order	Family
4	*Staphylococcus sp.*	*Firmicutes*	*Bacilli*	*Bacillales*	*Staphylococcaceae*
7	*Cladosporium cladosporoides*	*Ascomycetes*	*Dothideomycetes*	*Capnodiales*	*Cladosporiumceae*
12	*Acidovorax sp.*	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*
13	*Bacillus sp.*	*Firmicutes*	*Bacilli*	*Bacillales*	*Bacillaceae*
16	*Pantoea sp.*	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*
17	*Bacillus sp.*	*Firmicutes*	*Bacilli*	*Bacillales*	*Bacillaceae*
18	*Staphylococcus sp.*	*Firmicutes*	*Bacilli*	*Bacillales*	*Staphylococcaceae*
19	*Enterobacter sp.*	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*
20	*Acidovorax sp.*	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*

Endophyte 4 and 18 were both *Staphylococcus* species. However, their colony features and biochemical traits were not exactly the same. This shows that they may be different strains of the same microbe with perhaps different functions. This kind of a scenario has already been reported (Sheibani-Tezerji *et al*, 2015). The same may be the case for endophyte 13 and 17, both of which have been identified as *Bacillus sp.* Likewise, 12 and 20 have been identified as *Acidovorax sp.*

The phylum distribution shows that 4 of 9 endophytes that could be analysed belonged to phylum *Firmicutes,* 4 belonged to *Proteobacteria* and one was a fungus of phylum *Ascomycetes.*

All 4 *Firmicutes* were from the class *Bacilli.* Two of the *Proteobacteria* belonged *γ-proteobacteria* and 2 to *β-proteobacteria.*

As already discussed the microbes identified as endophytes in the present study have never previously shown to be present in finger millet. They do, however, without exception, exist as endophyte in one or the other crop species. All of them have been shown to have a positive effect on the host plant which ranges from production of useful bioactive compounds, protection from pathogens, enhancement of growth and yield to tolerance to abiotic stresses.

The endophytes of finger millet, which is an inherently drought tolerant crop, assumes great importance in today's scenario of climatic upheavels. Definitely, indepth studies would yield a mine of information worth tapping.

The 16S sequences which were submitted to the NCBI GenBank were allotted accession numbers as given in Table 4.10.

Table 4.10: GenBank Accession Numbers

Fragment	Accession Number
Endophyte 4 P7 sequence	MG193754
Endophyte 12 P7 sequence	MG193755
Endophyte 13 P3 sequence	MG193756
Endophyte 13 P7 sequence	MG193757
Endophyte 17 P3 sequence	MG193758
Endophyte 20 P7 sequence	MG193759
Endophyte 7 ITS sequence	MG195949

CHAPTER 5

SUMMARY AND CONCLUSION

SUMMARY

The present study work titled "16S ribotyping and classification of endophytes of finger millet (E*leusine coracana* L. Gaertn) variety VL149 ." was conducted at the College of Biotechnology, Birsa Agricultural University, Ranchi. The results obtained have been summarized below.

1. Genomic DNA of 13 bacterial and 1 fungal endophyte was isolated and purified partial/complete sequences of 16S rDNA were obtained. Endophyte 7 was a fungus and ITS was obtained in this case.
2. Endophyte 7, which was a fungus was identified as *Cladosporium cladosporoides.*
3. The bacteria fell into two phyla- *Firmicutes* and *Proteobacteria.*
4. All *Firmicutes* belonged to class *Bacilli* [Endophyte – 4, 13, 17 and 18].
5. Endophyte 4 and 18 belonged to species *Staphylococcus,* whereas endophyte 13 and 17 belonged to *Bacilli* species.
6. The *Proteobacteria* were of two classes i.e., *β-proteobacteria* (endophyte 12 and 20) and *γ-proteobacteria* (endophyte 16 and 19).
7. Both endophytes 12 and 20 were identified as *Acidovorax sp.*
8. Endophyte 16 was identified as *Pantoea sp.* and 19 was shown to be an *Enterobacter.*

CONCLUSION

The study could collate information about the colony characteristics and biochemical traits with the 16S rDNA sequences/ITS sequence and tentatively identify 9 different endophytes. From the comparision and information in the public domain, all the 9 endophyte have positive effects on the other crop species they inhabit. Studies on their exact role in finger millet may help to develop a consortium / several consortia which can be introduced into other popular crop species, thus concomitantly transferring desirable traits.

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APPENDIX

Preparation of stock solution

Tris-Cl (1 M)

121.1 g of Tris base was dissolved in 800 ml of water. The pH was adjusted to 8 by adding 42 ml of concentrated HCL. The solution was allowed to cool to room temperature before making final adjustments. The volume of solution was adjusted to 1000 ml with H2O. It was dispensed into aliquots and sterilize by autoclaving.

EDTA (0.5M, pH 8.0)

186.1 g of Disodium EDTA to H_2O was added to 800 ml of H_2O. It was stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.0 with NaOH. It was dispensed in aliquot and sterilized by autoclaving.

NaCl (Sodium chloride 5 M)

292 g of NaCl was added in 800 ml of H_2O. The volume was adjusted to 1000 ml by addition of H_2O. It was dispensed into aliquots and followed by autoclaving. It was stored at room temperature.

TE buffer (Tris EDTA)

10 ml of 100 mM of Tris Cl (pH 8.0) and 2 ml of 10 mM EDTA (pH 8.0) was taken and the volume was made up to 100 ml. The solution was sterilized by autoclaving. It was stored at RT.

SDS (20%)

It is also called as sodium lauryl sulphate. 200 g of SDS was dissolved in 900 ml of H_2O. The solution was heated to 68°C and stirred with magnetic stirrer. The pH was adjusted to 7.2 by addition of HCl. The final volume was adjusted to 1000 ml.

TAE (Tris-acetate EDTA) buffer (50X, 1000ml)

242 g of Tris base was dissolved in 500 ml of distilled water. 100 ml of 0.5 M EDTA (pH 8.0) and 57.1 ml of glacial acetic acid was added and the volume made up to 1000 ml.

Gel loading buffer (6X)

The constituent of the gel-loading buffer was 0.25% bromophenol blue, 0.25% xylene cyanol FF and 1.5% ficol type 400, dissolved in distilled water. It was prepared as described by Sambrook and Russel, 2001. The solution was stored at RT.

DNAse free RNAse (10 mg/ml)

10 mg of RNAse was dissolved in 1 ml of 10 mM Tris Cl (pH 7.5) and 15 mM NaCl. The mixture were heated to 100°C for 15 min, and then allowed to cool to room temperature. It was distributed in aliquots of 100ml and stored at -20°C.

Ethidium bromide

10 mg of ethidium bromide was dissolved in 1 ml of sterile water. The tube was wrapped with aluminium foil.

Ampicillin(100µg/ml)

100 mg of ampicillin dissolved in 1 ml distilled water, mixed by vortexing and kept at - 20°C.

Xgal(800µg/plate)

20 mg of Xgal dissolved in 1 ml of Dimethyl formamide mixed it properly. The tube was wrapped with aluminium foil and kept at -20°C.

IPTG (800µg/plate)

200mg of IPTG dissolved in 1 ml distilled water, mixed it properly and kept at -20°C.

Solution I

Components	**Stock concentration**	**Volume taken**	**Final concentration**
EDTA	0.5 M	1.00 ml	10 mM
Glucose	20%	2.25 ml	50 mM
Tris	1 M	1.25 ml	25 mM
Distilled water		45.50 ml	
Total volume		**50 ml**	

Solution II

Components	Stock concentration	Volume taken	Final concentration
NaOH	10 N	0.4 ml	0.2 N
SDS	20%	1.00 ml	1%
Distilled water		18.60 ml	
Total volume		**20 ml**	

Solution III

Components	Stock concentration	Volume taken	Final concentration
K/Na Acetate	5 M	60.0 ml	3M
Glacial Acetic Acid		11.5 ml	5 M
Distilled water		28.5 ml	
Total volume		**100 ml**	