Phylogenetic Analysis of Endophytic Bacteria from Nakshtra Trees

Priya Dnyandeo Kakade¹* and Sushma Ravindra Chaphalkar²

Vidya Pratishthan’s School of Biotechnology, Vidyanagar, Baramati, Pune-413133, India

*Corresponding author:

A B S T R A C T

Endophytic bacteria were inhabitant microflora of plant part. This association was presumably long term and they were mostly commensalisms. The microorganisms which were living within the plant cell without causing any harmful effect on the plant tissue. These bacteria were secrets the plant growth promoting hormones like indole acetic acid which helps for growth of plant. In the present study the endophytic bacteria was isolated from medicinal plants of nakshtra udyan and screened out for the different characteristics of these microorganisms. The isolates were identified by 16s r DNA technique and the phylogenetic analysis was done for the 96 isolates from Nakshtra Udyan plant. The isolates were grouped into 23 different types of group of species and subspecies of microorganisms in which Bacillus species are more predominant than Pseudomonas sp, Enterobacter sp, Alcaligene spp, Arthrobacter sp, Micrococcus sp, Klebsiella pneumoniae and kocuria sp. These bacteria were important for different properties like antimicrobial peptides, plant growth promoting hormones and used as biocontrol agent.

Keywords: Endophytic bacteria, 16 s rDNA technique, Phylogenetic analysis, Antimicrobial Peptides, Biocontrol agent.

Article Info

Accepted: 18 November 2016
Available Online: 10 December 2016

Introduction

A niche environment previously untapped has been explored for study of bacterial population within leaf portions of huge medicinal trees. This study has yielded into isolation of a few peculiarly characterized as endophytic bacteria hosting an unique culture collection at VSBI. This culture collection of endophytic bacteria has yielded into phylogenetic patterns.

They are known to secrete different types of secondary metabolites like antimicrobial peptides, growth promoting hormones and bioactive compounds which are used as biocontrol agent, in pharmacology used as antimicrobial drugs preparation, in food industry and agriculture (Dhanya et al., 2013 Susheel kumar et al., 2013). These endophytic bacteria play an important role for improvement in growth and health of plant and it has several mechanisms for it (Taghavi et al., 2009). Endophytes may directly produce chemical defense in plants through the production of secondary compounds which inhibit insects and pathogenic organisms. The in vitro secretion of substances by endophytes that limit the growth of other microbial species, including pathogens (Vania Specian et al., 2012). The many of endophytes re poorly investigated group of microorganisms that produces secondary metabolites which is used in modern medicine and about 40% of the prescription of drugs are based on them.
The endophytic bacteria produce hormones such as Indole Acetic acid (IAA) (Pedraza et al., 2004), cytokinin (Ergun et al., 2002) and Gibberellic acid (GA) (Kharwar et al., 2008) required for plant growth. In the 70's, endophytes were considered neutral, neither causing benefits nor showing detrimental influence on plants, but from the results of more recent studies, it has been possible to show that in many cases, they have an important role in plant protection against pathogens. Several studies have shown that the interaction between plants and endophytic bacteria is associated with beneficial effects such as plant growth promotion and biocontrol potential against plant pathogens (Lalande et al., 1989; Bashan et al., 1990; Chen et al., 1995; Hallmann et al., 1997).

Materials and Methods

Selection of medicinal plants and Explants collection

For the isolation of bacteria, the explants were collected from different parts of medicinal plants from Nakshtra Udyan located at Vidya Pratishthan’s school of biotechnology. The cultivation of these plants was strictly maintained in organic package and the age of plants was fourteen years.

Pretreatment and surface sterilization of explants

The explants used for the isolation were leaves, stem, and root of the plants. The collected explants were brought to the laboratory and washed under running tap water. After this, they were thoroughly washed with distilled water. Surface sterilization protocol was standardized which contained surface sterilizing agents like 1% phenolic compounds containing solution for 5 min followed by 0.1% sodium hypochlorite treatment for 5 min. Afterwards, the explants were washed with sterile distilled water.

Isolation of endophytic bacteria

The samples were aseptically ground in a motor and pestle in potassium dihydrogen phosphate buffer (pH 6.8) and inoculated into sterile nutrient broth medium with negative and positive control i.e. sterilized medium without inoculation of explants and uncrushed surface sterilized explants. The broth was incubated for 24 hrs at 30°C on rotary shaker incubator at 120 rpm. The grown culture was plated onto sterile nutrient agar plates. The plates were incubated at 37°C for 24 hrs. The isolated bacteria were plated onto selective medium after their morphological, biochemical and molecular identification and preserved by lyophilization.

Biochemical characterization of isolates

The standard tests for the characterization were done according to the Beregey’s Manual of Determinative Bacteriology. The isolates were characterized for colony characterization which includes size, shape, color, consistency, opacity, Gram’s nature, Capsule staining and presence of endospores. The biochemical characterization was done for IMViC test, starch hydrolysis, gelatin liquefaction, and different sources of carbon utilization, Oxidase and catalase tests by standard methods.

Molecular Characterization of isolates

The isolated strains were identified by using 16 s r DNA techniques. The genomic DNA of endophytic bacteria was isolated by using,
CTAB method. The amplification of template DNA was done by using universal primes, R1 forward (5’AGTTTGAT CCTGGCTCAG 3’) and R2 reverse (5’ GGACTACCAGGGTATCTAAT3’). The 50 ul PCR reaction contains Mgcl₂ (0.45mM), dNTPs (0.2mM), forward primer (10 pmol), reverse primer (10pmol), Taq polymerase (0.5U) having 10X assay buffer (1X), genomic DNA(1 ug/ul) and sterile MilliQ water is used. Amplification of DNA was done in automated thermocycle machine provided by applied biosystem and product was checked on 1% agarose gel. The gel was eluted by using SIGMA gel elution kit.

The sequencing reaction was carried out in 3130 genetic analyzer at VSCT. The BLAST of the sequences was done for sequences of bacteria to NCBI GeneBank.

**Phylogenetic tree analysis**

The phylogenetic tree was constructing by using MEGA software. The neighbor-joining method was used to construct the phylogenetic tree. The bootstrap resampling test with 100 replications was also applied.

**Results and Discussion**

These observation form one of the most significant and comparable account of endophytic bacteria in tropical tree species (Table.1). This high through put isolation followed by classification and identification of endophytic bacteria using biochemical and 16 s rDNA techniques has culminated into reliable unique culture collection center at our center. Biochemical preferences of these bacteria has shown acetate as preferred carbon source only to put forth a heavy duty of acetate scavenging function in tree species in longest longitude. The phylogeny study specifies an almost ubiquitous presence of *Bacillus sp*. Among 96 endophytic bacterial isolates 34 were from the same group i.e. *Bacillus sp.* (Table.2).

**Table.1** Plants used for isolation of endophytic bacteria.

| Sr. No. | Name of the plant | Botanical Name | Plant family | Image of plant |
|---------|-------------------|----------------|--------------|----------------|
| 1       | Adulsa            | *Adhatoda vasica* | Acanthaceae  | ![Image](image1.png) |
| 2       | Arjun             | *Terminalia arjuna* | Combretaceae | ![Image](image2.png) |
|   |   | Scientific Name | Family          |
|---|---|-----------------|-----------------|
| 3 | Awla | Emblica officinalis | Euphorbiaceae |
| 4 | Bakul | Mimusops elengi | Sapotaceae |
| 5 | Bel | Aegle marmelos | Rutaceae |
| 6 | Chandan | Santalum album | Santalaceae |
| 7 | Jai | Jasminum auriculatum | Oleaceae |
| 8 | Jamun | Eugenia jambolina | Myrtaceae |
|   |   |                         |                         |
|---|---|------------------------|------------------------|
| 9 |   | **Kadamb**             | **Anthocephalus kadamba** |
| 10|   | **Khair**              | **Acacia catechu**     |
| 11|   | **Kuchala**            | **Strychnos nuxvomica**|
| 12|   | **Mango**              | **Mangifera indica**   |
| 13|   | **Moha**               | **Madhuca indica**     |

*Family:*  
- Rubiaceae  
- Mimosaceae  
- Loganiaceae  
- Anacardiaceae  
- Sapotaceae
|   |   |   |   |
|---|---|---|---|
| 14 | Nagkeshar | *Messua ferrea* | Clusiaceae |
| 15 | Nagchapha | *Michelia Champaka* | Mangoleaceae |
| 16 | Palas | *Butea frondosa* | Fabaceae |
| 17 | Payar | *Ficus infectoria* | Aricaceae |
| No. | Name   | Scientific Name                  | Family   |
|-----|--------|----------------------------------|----------|
| 18  | Phanas | Artocarpus iniegrifolia         | Moraceae |
| 19  | Pimpal | Ficus religiosa                  | Articaceae|
| 20  | Raal   | Veteria indica                   | Dipterocarpaceae |
| 21  | Rui    | Calotropis gigantea              | Asclepiadiaceae |
| 22  | Savar  | Salmalia malabarica              | Malvaceae |
|    |    |                                |                                |
|----|----|--------------------------------|--------------------------------|
| 23 | Shami | *Prosopis spicigera* | Mimoceae |
| 24 | Umbar | *Ficus racemosa* | Articaceae |
| 25 | Vad | *Ficus bengahalensis* | Articaceae |
| 26 | Velu | *Bambusa arundinacea* | Garminae |
| 27 | Vet | *Calamus roteng* | Palmae |
Table 2 Classification of endophytic bacteria on the basis of identification:

| Name of isolated bacterial species | Total number of new strains | Code of isolate | Endo Code | Name of plant and plant part used | Sequence length |
|-----------------------------------|----------------------------|-----------------|-----------|-----------------------------------|----------------|
| Bacillus subtilis                 | 02                        | VCC.16.SB       | Endo_25   | Messua ferrea Stem                | 776            |
|                                   |                           | VCC.5.S         | Endo_1    | Acacia catechu Stem               | 776            |
| Bacillus megaterium              | 03                        | VCC.4.RA,       | Endo_7    | Eugenia jambolina Stem,           | 789            |
|                                   |                           | VCC.22.LD       | Endo_35   | Anthocephalus kadamba leaf        | 799            |
|                                   |                           | VCC.4.SB        | Endo_21   | Eugenia jambolina Stem            | 793            |
| Bacillus licheniformis           | 02                        | VCC.8.SA        | Endo_12   | Ficus religiosa Stem              | 701            |
|                                   |                           | VCC.15.LE       | Endo_82   | Terminalia arjuna leaf            | 754            |
| Bacillus axarequiensis           | 01                        | VCC.22.LC       | Endo_81   | Anthocephalus kadamba leaf        | 773            |
| Bacillus safensis                | 02                        | VCC.7.LC        | Endo_71   | Bambusa arundinacea leaf          | 751            |
|                                   |                           | VCC.21.L        | Endo_83   | Prosopis spicigera leaf           | 766            |
| Bacillus firmus                  | 01                        | VCC.26.LB       | Endo_86   | Calamus roteng leaf               | 768            |
| Bacillus pumilus                 | 01                        | VCC.22.LG       | Endo_50   | Anthocephalus kadamba leaf        | 700            |
| Bacillus aryabhattai             | 01                        | VCC.16.SC       | Endo_9    | Messua ferrea Stem                | 780            |
| Bacillus niacin                  | 01                        | VCC.16.SA       | Endo_20   | Messua ferrea Stem                | 786            |
| Bacillus cereus                  | 20                        | VCC.8.SB        | Endo_19   | Ficus religiosa Stem              | 778            |
|                                   |                           | VCC.22.LF       | Endo_45   | Anthocephalus kadamba leaf        | 736            |
|                                   |                           | VCC.15.LG       | Endo_41   | Terminalia arjuna leaf            | 780            |
|                                   |                           | VCC.25.LF       | Endo_80   | Madhuca indica leaf               | 774            |
|                                   |                           | VCC.25.LE       | Endo_75   | Madhuca indica                    | 762            |
| Microorganism                  | Collection Code | Culture Code | Species                          | Part     | Plate Number |
|-------------------------------|-----------------|--------------|----------------------------------|----------|--------------|
| Klebsiella pneumonia          | VCC.18.RT       | Endo_16      | Salmalia malabarica              | Root     | 648          |
| Alcaligens species           | VCC.15.LB       | Endo_93      | Terminalia arjuna                | Leaf     | 773          |
|                               | VCC.25.LB       | Endo_94      | Madhuca indica                   | Leaf     | 763          |
|                               | VCC.24.LA       | Endo_96      | Azadirachta indica               | Leaf     | 660          |
|                               | VCC.20.LC       | Endo_67      | Calotropi gigantean              | Leaf     | 778          |
| VCC.27.LD                     | Endo_74         | Arthrocarpus integrifolia         | Leaf     | 775          |
| VCC.27.LC                     | Endo_69         | Arthrocarpus integrifolia         | Leaf     | 771          |
| VCC.27.LB                     | Endo_73         | Arthrocarpus integrifolia         | Leaf     | 769          |
| VCC.14.LC                     | Endo_76         | Aegle marmelos                     | Leaf     | 775          |
| VCC.26.LD                     | Endo_88         | Calmus roteng                           | Leaf     | 769          |
| VCC.26.LC                     | Endo_87         | Calmus roteng                           | Leaf     | 769          |
| VCC.26.LE                     | Endo_89         | Calmus roteng                           | Leaf     | 688          |
| VCC.18.L                      | Endo_84         | Salmalia malabarica                 | Leaf     | 776          |
| VCC.2.LB                      | Endo_51         | Emblica officinalis                | Leaf     | 791          |
| VCC.24.LD                     | Endo_54         | Aegle marmelos                      | Leaf     | 786          |
| VCC.24.LE                     | Endo_59         | Azadirachta indica                 | Leaf     | 780          |
| VCC.23.LD                     | Endo_60         | Mangifera indica                  | Leaf     | 770          |
| VCC.7.LB                      | Endo_66         | Bambusa arundinacea                | Leaf     | 703          |
| VCC.1a.LB                     | Endo_72         | Adhatoda vasica                    | Leaf     | 783          |
| Enterobacter spp | 02 | VCC.19.S, Endo_2 | VCC.8.RA, Endo_30 | F. religiosa Stem, 677 |
|-----------------|----|-----------------|------------------|----------------------|
| Arthrobacter globiformis and Arthrobacter protophormiae | 02 | VCC.15.SA, Endo_23 | VCC.24.LC, Endo_449 | T. arjuna Stem, 751 |
| Kocuria sediminis and Kocuria rosea | 02 | VCC.4.SD, Endo_91 | VCC.4.RB, Endo_21 | E. jambolina Stem, 749 |
| Micrococcus luteus Micrococcus luteae and Micrococcus sp | 03 | VCC.11.SC, Endo_14 | VCC.13.SA, Endo_15 | J. auriculatum Stem, 724 |
| Pantoea dispersa | 01 | VCC.15.SB, Endo_10 | VCC.13.RB, Endo_31 | T. arjuna Stem, 724 |
| Psychrobacter spp | 02 | VCC.2.LC, Endo_56 | VCC.14.LB, Endo_52 | E. marmelos leaf, 768 |
| Ochrobacterium spp | 04 | VCC.2.LA, Endo_46 | VCC.23.LB, Endo_57 | M. indica leaf, 872 |
| Pseudomonas spp | 01 | VCC.3.RC, Endo_22 | VCC.22.LB, Endo_58 | A. kadamba leaf, 704 |
| Staphylococcus hemolyticus | 01 | VCC.CLIED, Endo_92 | VCC.27.LE, Endo_79 | A. integrifolia leaf, 720 |
| No significant sequence matching | 17 | VCC.5.R, Endo_3 | VCC.15.SC, Endo_6 | T. arjuna Stem, 764 |
| Strain ID | Endo Code | Organism Name       |
|-----------|-----------|---------------------|
| VCC.16.R, | Endo_8    | *Messua ferrea* root, |
| VCC.13.RA, | Endo_11  | *Jasminum auriculatum* Root, |
| VCC.3.RB, | Endo_18   | *Ficus racemosa* root, |
| VCC.3.RA, | Endo_29   | *Ficus racemosa* root, |
| VCC.11.SB, | Endo_33  | *Butea frondosa* stem, |
| VCC.1a.LA | Endo_38   | *Adhatoda vasica* leaf, |
| VCC.25.LI, | Endo_44  | *Madhuca indica* leaf, |
| VCC.23.LC, | Endo_55  | *Mangifera indica* leaf, |
| VCC.25.L, | Endo_63   | *Madhuca indica* leaf, |
| VCC.25.LC, | Endo_65  | *Madhuca indica* leaf, |
| VCC.27.LA | Endo_68   | *Artocarpus integrifolia* root, |
| VCC.20.L, | Endo_78   | *Calotropis gigantean* leaf, |
| VCC.13.SB, | Endo_90  | *Jasminum auriculatum* stem, |
| VCC.4.SD, | Endo_91   | *Eugenia jambolina* Stem, |
| VCC.24.LB | Endo_95   | *Azadirachta indica* leaf |

| Strain ID | Endo Code | Organism Name       |
|-----------|-----------|---------------------|
| Unculturable bacteria | 02 | VCC.4.SC, | Endo_17 | *Eugenia jambolina* Stem, |
| VCC.14.L | Endo_42   | *Aegle marmelos* leaf |

**Terribacillus saccharophilus** 01 | VCC.4.SA | Endo_7 | *Eugenia jambolina* Stem |

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Table 3: No significant sequence matching

| Code of endophytic bacteria | Name of the plant and plant part used | Gram’s Nature | Capsule | Endospore |
|-----------------------------|--------------------------------------|---------------|---------|----------|
| VCC.5.R                     | *Acacia catechu* root,               | Gram positive cocci | +       | -        |
| VCC.15.SC                   | *Terminalia arjuna* Stem,            | Gram positive cocci | _       | _        |
| VCC.16.R                    | *Messua ferrea* root,               | Gram negative cocci | +       | _        |
| VCC.13.R                    | *Jasminum auriculatum* Root,        | Gram positive cocci | +       | _        |
| VCC.3.RB                    | *Ficus racemosa* root,              | Gram positive cocci | _       | _        |
| VCC.3.RA                    | *Ficus racemosa* root,              | Gram positive cocci | +       | _        |
| VCC.11.SB                   | *Butea frondosa* stem,              | Gram negative cocci | +       | _        |
| VCC.1a.LA                   | *Adhatoda vasica* leaf,             | Gram negative rod  | +       | _        |
| VCC.25.LI                   | *Madhuca indica* leaf,              | Gram positive rod  | +       | _        |
| VCC.23.LC                   | *Mangifera indica* leaf,            | Gram positive rod  | +       | +        |
| VCC.25.L                    | *Madhuca indica* leaf,              | Gram negative rod  | +       | _        |
| VCC.25.LC                   | *Madhuca indica* leaf,              | Gram positive rod  | +       | +        |
| VCC.27.LA                   | *Artocarpus iniegrifolia* root,     | Gram negative rod  | +       | +        |
| VCC.20.L                    | *Calotropis gigantean* leaf,        | Gram negative rod  | +       | +        |
| VCC.13.SB                   | *Jasminum auriculatum* stem,        | Gram positive cocci | +       | +        |
| VCC.4.SD                    | *Eugenia jambolina* Stem,           | Gram positive cocci | +       | +        |
| VCC.24.LB                   | *Azadirachta indica* leaf           | Gram positive rod  | +       | +        |

(Note: +=Present, _= Absent)
Fig. 1 PCR amplification for 16 s rDNA

Lane L Marker, lane 1: VCC.5.S., lane 2: VCC.19.S., lane 3: VCC.5.R, lane 4: VCC.4.RA, lane 5: 11.SA, lane 6: VCC.15.SC, lane 7: VCC.4.SA, lane 8: VCC.16.R, lane 9: VCC.16.SC, lane 10: VCC.15.SB, lane 11: VCC.13.RA, lane 12: VCC.8.SA, lane 13: VCC.3.SA, lane 14: VCC.11.SC, lane 15: VCC.13.SA, lane 16: VCC.18.RT, lane 17: VCC.4.SC, lane 18: VCC.3.RB, lane 19: VCC.8.SB, lane 20: VCC.16.SA, lane 21: VCC.4.SB, lane 22: VCC.3.RC, lane 23: VCC.15.SA, lane 24: VCC.4.SD, lane 25: VCC.9.SB, lane 26: VCC.27.S, lane 27: VCC.27.R, lane 28: VCC.3.SB, lane 29: VCC.3.RA, lane 30: VCC.8.RA, lane 31: VCC.13.RB, lane 32: VCC.4.RB, lane 33: VCC.11.SA, lane 34: VCC.25.LG, lane 35: VCC.22.LD, lane 36: VCC.15.LF, lane 37: VCC.26.L, lane 38: VCC.1a.LA, lane 39: VCC.25.LH, lane 40: VCC.22.LE, lane 41: VCC.15.LG, lane 42: VCC.14.L, lane 43: VCC.15.L, lane 44: VCC.25.LI, lane 45: VCC.22.LF, lane 46: VCC.2.LA, lane 47: VCC.23.LA, lane 48: VCC.15.LC.

Lane L Marker, lane 49: VCC.24.LC, lane 50: VCC.22.LG, lane 51: VCC.2.LB, lane 52: VCC.1.4.LB, lane 53: VCC.22.LA, lane 54: VCC.24.LD, lane 55: VCC.23.LC, lane 56: VCC.2.LC, lane 57: VCC.23.LB, lane 58: VCC.22.LB, lane 59: VCC.24.LE, lane 60: VCC.23.LD, lane 61: VCC.7.LA, lane 62: VCC.23.LB, lane 63: VCC.25.L, lane 64: VCC.12.L, lane 65: VCC.25.LC, lane 66: VCC.7.LB, lane 67: VCC.20.L, lane 68: VCC.27.LC, lane 69: VCC.27.LC, lane 70: VCC.25.LD, lane 71: VCC.7.LC, lane 72: VCC.1a.LB
Fig. 2 Phylogenetic tree analysis
Other genera found to contribute to these humongous tree genomes are *Klebsiella pneumoniae*, *Alcaligens* species, *Enterobacter* spp *Arthrobacter globiformis* and *Arthrobacter protophormiae*, *Kocuria sediminis* and *Kocuria rosea*, *Micrococcus luteus*, *Micrococcus lalae* and *Micrococcus sp*, *Pantoea dispersa*, *Psychrobacter* spp, *Unculturable bacteria*, *Terribacillus saccharophilus* in tree *Salmalia malabarica* root, *Terminalia arjuna* leaf, *Madhuca indica* leaf, *Azadirachta indica* leaf, *Calotropis gigantea* leaf, *Vetiveria indica* Stem, *Ficus religiosa* Stem, *Terminalia arjuna* Stem, *Azadirachta indica* leaf, *Eugenia jambolina* Stem, *Eugenia jambolina* Root, *Butea frondosa* Root, *Jasminum auriculatum* Stem, *Jasminum auriculatum* Root, *Strychnos mujvoica*, *Eugenia jambolina* Stem, *Aegle marmelos* leaf, *Eugenia jambolina* Stem. An interesting finding of these endophytic bacterial isolation is as many as 17 isolates are amongst those which could not have significant matching with any of the known sequences on NCBI. Further biochemical investigation to characterize them shall provide a novel appendage to the present culture collection. Bioprospecting through these endophytic bacteria producing useful metabolites would make a valid value addition to the whole process of culture collection.

Acknowledgment

The authors are very thankful to Department of Biotechnology, New Delhi for financial support and also to VSBT for providing laboratory for experiments.

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How to cite this article:

Priya Dnyandeo Kakade and Sushma Ravindra Chaphalkar. 2016. Phylogenetic Analysis of Endophytic Bacteria from Nakshtra Trees. Int.J.Curr.Microbiol.App.Sci. 5(12): 565-582. doi: http://dx.doi.org/10.20546/ijcmas.2016.512.062