Studies on plasmids of the endophytic bacteria of rare endangered plant from Western Ghats of Maharashtra.

*Chanda V. Berde[1], Shrinath P. Kavade[2] and Vikrant B. Berde[3]

*Dr. Chanda V. Berde

Department of Biotechnology, Gogate Jogalekar College, Ratnagiri. Maharashtra. India.

Email: berdeparu@gmail.com

Phone: 09860360635

Dr. Shrinath P. Kawade

Department of Botany, Arts, Commerce and Science College, Lanja. Maharashtra. India.

Email: shrinathkavade@gmail.com

Phone: 09730156865

Dr. Vikrant B. Berde

Department of Zoology, Arts, Commerce and Science College, Lanja. Maharashtra. India.

Abstract – The present work was aimed at finding the application of plasmids of endophytic bacteria as transmission vectors. Endophytic bacteria were isolated from rare plants. Rare endangered plant leaf samples were collected from Chandoli National Park, Sangli District Maharashtra state, India. Bacterial endophytes were isolated from the leaves and used for plasmid analysis. Out of the 12 isolates, six were found to be plasmid bearers. Next the other criteria for use the use of these plasmids i.e the presence of antibiotic resistivity, was checked. Endophyte Br-7 was found to be resistant to four of the seven antibiotics used i.e. Penicillin G, Amphotericin B, Clotrimazole and Tetracycline. Br-7 was thus selected for plasmid curing studies. Using 0.5 μg/ml EtBr, the culture was mutated and 12 mutant colonies were selected. Absence of plasmid was confirmed and the mutants further checked for antibiotic sensitivity against the four antibiotics. All the mutants showed sensitivity to tetracycline and resistance to others. This confirmed the presence of the tetracycline gene on plasmid in Br-7. Thus, tetracycline gene can be used as selective marker for the plasmid. Thus, the plasmid of endophyte Br-7 is a potential candidate for use as vector in transformation studies.

Index Terms: rare endangered plants, endophytes, plasmids, antibiotic sensitivity

ISSN: 2278 – 7798

I. INTRODUCTION

Plasmids are circular molecules of DNA that lead an independent existence in the bacterial cell. The plasmids that have been constructed for molecular cloning are relatively small, replicate under relaxed control, carry genes specifying resistance to one or more antibiotics and contain number of conveniently located restriction endonucleases sites into which the DNA to be cloned may be inserted. Many plasmid vectors contain a strategically located short segment of DNA known as a polylinker that has been synthesized to contain a variety of restriction sites that are not present elsewhere in the plasmid. A DNA to be cloned is, in many cases, obtained as a defined fragment through the application of restriction endonucleases [1].

A cloning vector needs to be relatively small, ideally less than 10 kb in size, as large molecules tend to break down during purification, and are also more difficult to manipulate [2]. Two kinds of DNA molecule that satisfy these criteria can be found in bacterial cells: plasmids and bacteriophage chromosomes.

Endophytes refer to organisms that live within plants. Fungi and bacteria are the most common organisms associated with the term endophyte. Endophytes are also being investigated for roles in agriculture and biofuels production. Endophytic microorganisms have a rich potential as producers of bioactive compounds, as nitrogen fixers, IAA producers, plant toxicity reducers, etc. Endophytic bacteria can also be used as vectors for transferring genes of interest to plants. Plasmids of endophytic bacteria can be used as vectors instead of whole organism, for genetic studies [3].

II. MATERIALS AND METHODS

A. Study area: *Oberinia brunoniana* is a rare endangered plant species encountered at Chandoli National Park (17°11′30″N 73°46′30″E), along the Sahyadri which is spread over an area of 317.67 sq. Km in Sangli District Maharashtra state, India

B. Isolation of endophytic bacteria from leaves:

The leaves of the plant *Oberinia brunoniana* were washed with tap water to remove dust or soil. The leaves were then cut into 1 inch pieces and treated with 1% savlon for 10 minutes followed by treatment with 0.1% $HgCl_2$ for 10 minutes. The leaves were then washed with sterile water 5 times. One piece of leaf was kept on sterile nutrient media plate as control. The remaining pieces were taken in flame sterilised mortar and crushed with the help of pestle by adding 2-3 ml of saline. The extract thus obtained was plated onto

ISSN: 2278 – 7798

media plates and incubated at 37 °C for 24 hrs. The bacterial colonies obtained were studied for cultural characteristics of isolates and maintained on nutrient agar slants.

C. Plasmid isolation:

For plasmid isolation from the isolates, loopful of culture was inoculated in LB broth and incubated overnight at 37 °C. Plasmids were isolated by modifying the method given by Sambrook *et al*., [4]. After addition of 0.6 volume of isopropyl alcohol, it was kept at -20 ^{o}C overnight. After this step, instead of addition of 70% ethanol, absolute alcohol was added. After addition of 100 µl of gel loading buffer, 20 µl sample was loaded in 0.8% agarose gel and electrophoresis (Bangalore Genei, India) was carried out. Bands were observed under UV transilluminator (Unik Enterprises, India). Plasmid bearing isolates were selected for further studies.

D. Plasmid curing for culture Br-7 [5]:

Plasmid curing was carried out by growing culture in 5ml LB broth containing 0.5µg/ml ethidium bromide. After 24 hours incubation, serial dilutions were prepared up to 10^{-7} with sterile saline. Last three dilutions were plated on nutrient agar plates. After incubation, isolated colonies from dilution 10^{-7} were selected. These colonies of mutants were inoculated in LB broth. After incubation, absence of plasmid was checked by alkaline lysis method.

E. Plasmid analysis of mutants: After plasmid curing, 12 mutants of Br-7 culture were checked for presence of plasmid by using modified alkaline lysis method, followed by electrophoresis.

F. Antibiotic sensitivity of plasmid bearing isolates:

Cultures bearing plasmid were grown in 5ml LB broth and incubated for 24 hours at room temperature. After incubation, the grown cultures were spread plated on NA plates. Antibiotic discs (Hi media, India) of Penicillin G, Amphotericin B, Streptomycin, Clotrimazole, Chloramphenicol, Gentamycin and Tetracycline were placed on the inoculated plates and incubated for 24 hours at room temperature. Zones of clearance were measured in mm.

G. Antibiotic sensitivity of mutants: Cured culture mutants were tested for antibiotic sensitivity. Four antibiotics were used – Tetracycline, Clotrimazole, Penicillin G and Amphotericin B in the assay, as above.

ISSN: 2278 – 7798

III. RESULTS AND DISCUSSION

Endophytic bacteria are harmless that reside within the plant hosts and known to boost the growth and development of host plants [6]. Microbial endophytes are typically defined as microorganisms that do not visibly harm the host plant but can be isolated from surface disinfested plant tissues or the inner parts of plant organ [7]. Bacterial endophytes seem to be ubiquitous in plant tissues, having been isolated from flowers, fruits, leaves, stems, roots and seeds of various plant species [8]. Various plasmids were isolated from many bacteria. There are various types of plasmids- Cryptic plasmid, metabolic plasmid, suicide plasmid, mobilizable plasmid and virulence plasmid. Degradative plasmids allow the host bacterium to metabolize unusual molecules such as toluene and salicylic acid, an example being TOL of *Pseudomonas putida.* Virulence plasmids confer pathogenicity on the host bacterium; these include the Ti plasmids of *Agrobacterium tumefaciens,* which induce crown gall disease on dicotyledonous plants. RP4 plasmid isolated from *Escherichia coli* shows Kanamycin, Tetracycline and Ampicillin markers [9]. Only those *E. coli* cells that contain RP4 (or a related plasmid) are able to survive and grow in a medium that contains toxic amounts of one or more of these antibiotics.

Oberinia brunoniana, rare endangered plant (Fig. 1), from Chandoli National Park, is a shrub belonging to

orchid family. The brown coloured leaves of this rare plant were used for the present study.

Fig. 1 *Oberinia brunoniana* (Google image)

A total of 12 endophytes were isolated from the leaves of the plant. They were designated as Br 1-12. Most of the isolated colonies were 4-5 mm in size and cream in colour while one type of colonies was yellow in colour (Br-6) and another light orange (Br-8). All the colonies were with entire margin and varied consistency.

Presence of plasmid in the isolates was checked by alkaline lysis method. Out of the 12 endophytic isolates, 6 were plasmid bearers *i.e* Br-2, Br-4, Br-5, Br-7, Br-9, Br-12. Next, the potential of the plasmids for use as vector was checked. Plasmids play a role in horizontal gene transfer as well as in manipulating expression

ISSN: 2278 – 7798

levels of different genes [10]. There are three criterias for use of plasmids as vector: A) There should be an origin of replication. B) Antibiotic resistance gene should be present on plasmid DNA. C) Restriction sites should also be present on plasmid for insertion of gene of interest [11].

The plasmid bearing isolates were checked for antibiotic sensitivity towards the following antibiotics - Penicillin G, Chloramphenicol, Clotrimazole, Amphotericin B, Streptomycin, Gentamycin and Tetracycline. Endophytic bacterial isolate Br-7 was resistant to four antibiotics *i.e* Penicillin G, Amphotericin B, Clotrimazole and Tetracycline, while others were resistant to less number of antibiotics. Hence, Br-7 was selected for further work.

Table 1: Antibiotic sensitivity of wild type endophytic isolate Br-7

Antibiotic	Zone of Inhibition (mm)
Penicillin G (P2)	R
Amphotericin B (AP 50)	R
Streptomycin (S 10)	15
Clotrimazole (CC 10)	R
Penicillin G (P 10)	19
Chloramphenicol (C 10)	25
Gentamycin (GEN 30)	17
Tetracycline(TE 10)	R

The plasmid isolated from culture Br-7 thus satisfies the second most important criteria for use as vector i.e the presence of antibiotic resistance gene which can be used as marker during gene transfer via the vector. However, the antibiotic resistance gene should be present on the plasmid. In order to confirm the location of the resistance genes, the culture was mutated using EtBr as per the method described by Berde, *et al*., [10]. Plasmid curing was carried out by growing culture in media with 0.5µg/ml ethidium bromide. Twelve mutant colonies were picked and checked for presence of plasmid and antibiotic sensitivity.

Loss of plasmid was confirmed by plasmid extraction followed by electrophoresis and visualisation of gel under UV. All mutants showed absence of plasmid DNA (Fig.2).

ISSN: 2278 – 7798

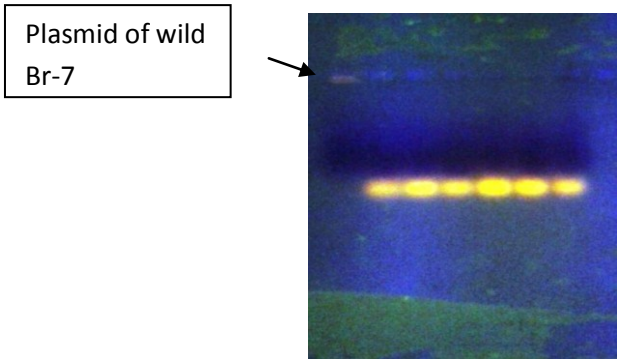

Fig. 2. Plasmid profile of mutants.

Upon plasmid curing, resistance to antibiotic tetracycline was lost showing the presence of this property on the plasmid. Loss of antibiotic resistance was concomitant with loss plasmid. All mutant cultures were found to be Tetracycline sensitive and resistant to Penicillin G, Amphotericin B and Clotrimazole (Table 1, Fig.32). This confirms the presence of Tetracycline marker on plasmid DNA while Penicillin G, Amphotericin B and Clotrimazole, to be present on chromosome. →The plasmid ofendophytic culture isolated from medicinal plant Kadamb, EC14, is reported to harbour three antibiotic resistance genes [10]. Plasmid isolated from endophytic culture Br-7 can be used as vector as it is having Tetracycline antibiotic marker. Presence of antibiotic resistance gene on plasmid, makes this plasmid a potential candidate as a vector. Further work involves molecular size estimation, restriction mapping and finding the transformation efficiency, needs to be carried out inorder to prove the efficiency of this plasmid as transformation vector.

Table 2: Antibiotic sensitivity of mutants of endophyte Br-7.

Mutants	Tetracycline (TE 10)	Amphotericin B (AP 50)	Penicillin G (P 2)	Clotrimazole (CC 10)
1	**12**	R	R	R
2	**12**	R	R	R
3	**11**	R	R	R
4	**13**	R	R	R
5	**14**	R	R	R
6	**12**	R	R	R
7	**12**	R	R	R
8	**14**	R	R	R
9	**10**	R	R	R
10	**13**	R	R	R
11	**12**	R	R	R
12	**12**	R	R	R

ISSN: 2278 – 7798

Wild	**R**	R	R	R

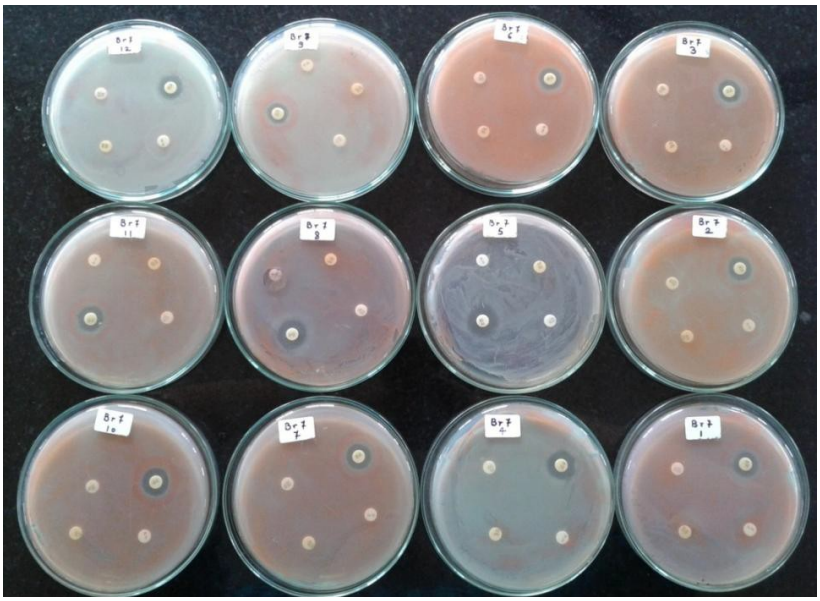

Fig 3: Antibiotic sensitivity of mutants of endophyte Br-7

IV. ACKNOWLEDGMENT

The authors are grateful to the Principal, Gogate Jogalekar College, Ratnagiri for providing support in terms of infrastructure.

V. REFERENCES

[1] http://nhjy.hzau.edu.cn/kech/zwswjs/kc/ziliao/cloning.pdf

[2] R. E. Procópio, W. Araújo, D. Fernando and J. L. Azevedo, "Characterization of a small cryptic plasmid from endophytic *Pantoea agglomerans* and its use in the construction of an expression vector," *Genetics and Molecular Biology*, vol. 3, pp. 103-109, 2011.

[3] http://xa.yimg.com/kq/groups/21666630/689296147/name/Ch-2-TA-Brown-gene_cloning_and_dna_analysis.pdf.

ISSN: 2278 – 7798

[4] J. Sambrook, E. F. Fritsch and T. Manniatis, *Molecular cloning: a laboratory manual*, 2nd Ed., Cold Spring Harbor Press, New York, 1989.

[5] M. Shahid, A. Malik and Sheeba, "Multi drug resistant Pseudomonas aeruginosa strains harbouring R plasmids and AmpC β-lactamases isolated from hospitalised burn patients in a tertiary care hospital of North India. *FEMS Microbiology Letters*, vol. 228, pp. 181-186, 2003.

[6] V. Pradeepa and M. Jennifer, "Screening and Characterization of Endophytic Bacteria Isolated from *Tabernaemontana divaricata* Plant for Cytokinin Production", *Advanced Biotechnology*, vol. 13, pp. 12-17, 2013.

[7] J. Hallmann and G. Berg, "Spectrum and Population Dynamics of Bacterial Root Endophytes", *Soil Biology*, Vol. 9, pp. 15-31, 2006.

[8] A. D. Altalhi, "Plasmids profiles, antibiotic and heavy metal resistance incidence of endophytic bacteria isolated from grapevine (*Vitis vinifera* L.)", *African Journal of Biotechnology*, vol. 8, pp. 5873-5882, 2009.

[9] H. Schwab, P. N. Saurugger and R. M. Lafferty, " Occurrence of deletion plasmids at high rates after conjugative transfer of the plasmids RP4 and RK2 from *Escherichia coli* to *Alcaligenes eutrophus* H16", *Archives of Microbiology,* Vol. 136, pp. 140-146, 1983.

[10] C. V. Berde, P. P. Bhosale and S. R. Chaphalkar, "Plasmids of endophytic bacteria as vectors for transformation in plants," *International Journal of Integrative Biology*, vol. 9, pp. 113-118, 2010.

[11] T. A. Brown, "The Basic Principles of Gene Cloning and DNA Analysis," *Gene cloning and DNA analysis - an introduction*, Sixth edition, A John Wiley & Sons, Ltd., Publication. London, pp. 13-25, 2010.

ISSN: 2278 – 7798