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Bacillus manliponensis – A new member of *Bacillus cereus* group isolated from marine algae *Enteromorpha intestinalis* (L) Nees (Chlorophyceae)

Emeemal Sharon .S[1] and Rani.G[2]

[1,2]*Department of Plant Biology and Plant Biotechnology, S.D.N.B.Vaishnav College for women Chrompet, Chennai, India*

Abstract

New Bacillus sps, strain BL4-6^T was isolated from the green marine algae Enteromorpha intestinalis (L) Nees (Chlorophyceae). BL4-6^T cells are oxidase-positive, catalyse-positive, ammonia production-positive. Based on the comparative 16S rRNA gene sequence analysis, the isolate belongs to Bacillus cereus group and it is closely related to Bacillus cereus(97.99%), Bacillus anthracis(97.99%), Bacillus thuringiensis(97.81%), Bacillus toyonensis(97.81%), Bacillus mycoides(97.45%), Bacillus weihenstephanensis(97.45%). The growth of this nitrogen fixing bacteria is proved by the formation of pellicle in nitrogen deficient NFB medium.The utilization of different carbon substrates by the bacterium and the effect of various antibiotics on the bacterium is also studied.This is the first report on the occurrence of Bacillus manliponensis as an endophytic nitrogen fixing bacteria in marine algae Enteromorpha intestinalis (L) Nees (Chlorophyceae).

Keywords: Bacillus manliponensis, Nitrogen fixing bacteria, Bacillus cereus group, Liquid Sea weed fertilizer.

I. INTRODUCTION

The *Bacillus cereus* group, also known as *B. cereus* sensu lato, consists of Gram-positive, rod-shaped, aerobic bacteria that are wide spread in natural environments. The bacteria of the *B. cereus* group produce various valuable enzymes and metabolites, (Nilegaonkar et al., 2007) degrade different types of pollutants and promote growth of both animals and plants when used as probiotic (Guinebretiere, M.H et al., 2013; Hong, H.A.,Duc, L.H & Cutting, S.M 2005). In light of the significance of the *B. cereus* group, the identification and taxonomy of the isolates within the group are of fundamental importance, and therefore have been extensively studied using various typing methods from phenotype to genotype. In the past, the bacteria of this group were classified into different species according to 16S rRNA gene sequences and characteristics such as the presence or absence of virulence plasmids (*B. anthracis* and *B. thuringiensis*), colonial morphology (*B. mycoides* and *B. pseudomycoides*), psychrophilic or thermotolerant ability (*B. weihenstephanensis* and *B. cytotoxicus*) (Yang Liu, 2015).

Bacillus cereus strain RS87 was previously studied for plant growth enhancement in several plant families and examined in both greenhouse and field experiments (jetiyanon, 2002; jetiyanon & plianbangchang, 2010; jetiyanon & plianbangchang, 2012). Multiple mechanisms of plant growth promotion by strain RS87 have been reported including indole-3-acetic acid (IAA) production, phosphate solubilization, siderophore production and nitrogen fixation(jetiyanon et al.,2008; jetiyanon & plianbangchang,2010; jetiyanon,2015). Xie et al. (1998) reported that the following species were nitrogen-fixing bacteria based on nitrogenase activity: *Bacillus megaterium, Bacillus cereus, Bacillus pumilus, Bacillus circulans, Bacillus licheniformis, B. subtilis, Bacillus brevis and Bacillus firmus*. However nifH gene was only detected in the following species: *Paenibacillus azotofixans, P.macerans, P. polymyxa, P. graminis and P. odorifer* (Achouaket al.,1999; Berge et al.,2002). *Bacillus anthracis, Bacillus cereus, Bacillus thuringiensis, Bacillus mycoides, Bacillus*

pseudomycoides, and *Bacillus weihenstephanensis* are members of the *B. cereus* group (Ash et al., 1991; Drobniewski, 1993; Lechner et al., 1998). Sequence comparison of the 16S rRNA gene within this group has shown that they are very closely related and it is not easy to differentiate the taxa due to their high genetic homology (Kaneko et al., 1978; Ash et al., 1991; Henderson et al., 1994; Nakamura,1998; Kim et al., 2008). There have been reports documenting that the only established difference between *B. cereus* and *B. thuringiensis* strains is the presence of genes coding for insecticidal toxins, usually present in plasmids (Thorne, 1993; Helgason et al., 2000). *B. thuringiensis* can no longer be distinguished from *B. cereus* when these plasmids are lost (Thorne, 1993). Helgason et al., (2000) contended that *B. anthracis*, *B. thuringiensis*, and *B. cereus* should be considered as belonging to the same species due to the close similarity of the genomes. Recently Jung et al., (2011) reported a new *Bacillus* species within the *Bacillus cereus* group. Millons of microbes inhabit the root zone of plants, some plants are capable of exploiting these micro organisms to meet their hormonal and nutritional needs to meet the demand for increasing productivity of economically important crop plants, several new plant microbe associations have been reported. (Kloepper et al., 1980, Malik et al., 1999, Goel et al., 2001, Tilak et al ., 2005).

Endophytic interaction of marine algae and microorganisms has also been observed in some seaweeds which provides an interesting biotic environment for these bacterial communities (se-kwon kim., 1807). The seaweed surface provides a suitable substratum for the settlement of microorganisms and also secretes various organic substance that function as nutrients for multiplication of bacteria and the formation of microbial biofilms (Steinberg et al., 2002; Staufenberger et al., 2008; Singh, 2013). Some water-soluble monosaccharides such as rhamnose, xylose, glucose, mannose and galactose are part of algal polysaccharides that constitute part of the cell wall (Popper et al., 2011) and the rest storage material (Lahaye & Axelos, 1993; Michel et al., 2010a, b). These algal polysaccharides are a potential source of carbon and energy for numerous marine bacteria (Hehemann et al., 2012) that produce specific molecules, which in turn facilitate seaweed–bacterial associations (Steinberg et al., 2002; Lachnit et al., 2013). Therefore, these interactions between seaweeds and bacteria have fascinated and attracted the attention of many researchers worldwide.

In the present study, we report a novel species of *Bacillus manoliponensis* as a new member isolated from the *Bacillus cereus* group as an endophytic nitrogen fixing bacteria from marine green algae *Enteromorpha intestinalis* (L) Nees *(Chlorophyceae*).

II. MATERIALS AND METHODS

2.1 SAMPLE COLLECTION

The marine alga *Enteromorpha intestinalis* (L) Nees (Chlorophyceae) was collected from the Kovalam coast 25 kms from Chennai (Fig: 1).The alga was identified with"The Structure and Reproduction of the Algae" (F.E.Fritsch 1967). The samples were collected in sterile plastic bags, stored and transported aseptically to the laboratory for further processing.

2.2 ISOLATION OF MARINE ENDOPHYTES

The marine algae were washed with sterile seawater, followed by two minutes wash in 70% ethanol and one-minute wash in 2% sodium hypochlorite. The samples were then rinsed with sterile seawater for five minutes with shaking and dried with tissue papers (Denise et al., 2002). The samples were cut into 2- 3 cm long segments using a sterile scalpel. The cut segments were then macerated using pestle and mortar, serially diluted and it is then inoculated in the (NFB) nitrogen free semisolid bromothymol blue malate medium(Dobereiner,J. and J.M.Day.1976).

The morphological characters such as colony size, pigmentation, edge and margin of the bacterial culture were recorded. The effect of Indole, Methyl red, Voges-Proskauer test, Citrate utilization, Lactose, Sucrose, Glucose fermentation, Triple sugar iron agar, Mannitol salt tolerance, Urease test, Nitrate reduction test, Starch, Casein hydrolysis, Pectinase, Cellulose assay, Production of HCN, and inorganic calcium phosphate on the nitrogen fixing bacteria was performed (Challa Krishna kumara et al., 2013). 1% concentration of twenty-two different carbon sources such as

glucose, sucrose, malic acid, arabinose etc., were added separately to the NFB medium to study their effect on bacteria. Different amino acids such as alanine, valine, arginine, etc, were added separately to the NFB medium to study their effect on bacteria (Cavalcante; & Do¨bereiner, J. 1988). The isolates were cultured overnight in Mueller Hinton broth prepared using seawater. The plates were incubated by inverting for 24 hours at 37°C and the zones of inhibition were noted and recorded by disc diffusion method (Atlas, 1993).

2.3 DNA EXTRACTION AND PURIFICATION

Genomic DNA was extracted and purified by using the method of Marmur (1961) with some modification.

2.4 16S rRNA AMPLIFICATION AND SEQUENCING

Universal eubacterial 16S rDNA primers, fD1 - 5'-GAG TTT GAT CCT GGC TCA-3' and rP2 - 5'-ACG GCT AAC TTG TTA CGA CT-3' (Weisburg et al., 1991). The PCR cycling conditions were as follows: an initial denaturation for 5 min at 95 ºC, followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 59ºC for 1 min and extension at 72 ºC for 2 min and then a final extension for 5 min at 72ºC.

The rDNA amplification reaction mixture (30µl) consists of 2X Amplicon Red master mixes (amplicon®) with 10 ng of total genome of each isolate, 10 pmol of each forward and reverse primer. The amplified PCR products were electrophoresed on 1% agarose gel. The gel was stained in ethidium bromide. The amplified fragments were purified and sequenced by Eurofins Scientific (Bangalore). For species level identification, sequences were compared with the Genbank database using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

III. RESULTS AND DISCUSSION

3.1 ISOLATION OF NITROGEN FIXING BACTRIA

The diluted seaweed extract which was inoculated in the NFB medium clearly showed the pellicle formation thus proving the presence of nitrogen fixing bacteria. This was subcultured to obtain pure isolates and stored at 4^0C for further morphological and biochemical studies (Fig. 2).Maximum number of colonies were observed in the dilution 10^{-6} number of colonies observed were 150 X 10^{-6} CFU/ml. The colony characterization showed the diameter ranging from 0.1 - 0.2cm, yellowish pigmentation with smooth margin.

3.2 BIOCHEMICAL ANALYSIS AND 16S r RNA SEQUENCING

The biochemical reactions of the bacteria showed the presence of oxidase enzyme by the change of oxidase disc to blue colour. Addition of hydrogen peroxide on the culture drop showed the effervescence thus showing the presence of catalyse enzyme in the bacteria. The bacteria also showed the production of ammonia with Nesseler's reagent. The isolate showed the positive results for Methyl red, Citrate utilization, glucose fermentation test, Triple sugar iron Test, Starch and Casein hydrolysis. The isolated endophytic bacteria was treated with various antibiotics of which, it was susceptible to Amikacin Chloramphenicol, Ciprofloxacin, Gentamicin, Streptomycin, Tetracycline and resistant to Penicillin-G, and Ampicillin. (Fig:3). The growth of bacteria was not observed in the medium supplemented with aminoacids Arginine, Valine, Leucine, Phenyl-alanine, cysteine, histidine, serinine, showing that the bacteria does not utilize these aminoacids for their growth, however abundant growth was observed in the NFB medium supplemented with the aminoacids Amino-butryic acid, Tryptophan, Lycine, Methionine.

Bacteria was grown in NFB medium supplemented with twenty-two carbon sources of which the growth was not seen in malonic acid, oxalic and oxo-glutaric acid showing that the bacteria does not utilize these carbon sources for their growth. Other carbon sources namely Azelic acid, Fructose, Galactose, Glucose, Lactose, Mannitol, Meso-erythriol Meso-inositol, Malic acid, Maltose, Mannose, Rhamnose, Sorbitol, Sucrose, Xylose supported the growth of bacteria. As pointed out by Popper et al., (2011), some of the above mentioned carbon sources such as rhamnose, xylose, glucose, mannose and galactose which are the algal polysaccharides that constitute the part of

cellwall, may promote the growth of these bacteria. There are reports on algal polysaccharides as a potential source of carbon and energy for numerous marine bacteria (Hehemann et al., 2012), that produce specific molecules, which in turn facilitate seaweed–bacterial associations (Steinberg et al., 2002; Lachnit et al., 2013). Therefore, these interactions between seaweeds and bacteria have fascinated and attracted the attention of many researchers worldwide.

Figure 1

Figure 2

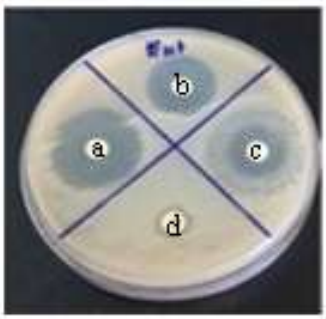

Figure 3

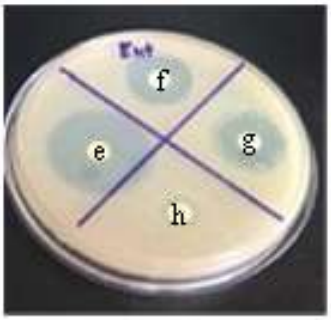

Figure 4

Fig.1. *Enteromorpha intestinalis*- Habit, Fig.2. Nitrogen fixing bacterial Culture and Fig.3 & 4. Effect of antibiotics on Nitrogen fixing bacteria

a) Amikacin, b) Ciprofloxacin, c) Chloramphenicol, d) Ampicillin, e) Tetracycline f) Gentamicin, g) Streptomycin , h) Penicillin-G

Based on the comparative 16srRNA gene sequence analysis, the isolate belong to the genus *Bacillus cereus* group and it is closely related to *Bacillus cereus* (97.99%), *Bacillus anthracis*(97.99%), *Bacillus thuringiensis*(97.81%), *Bacillus toyonensis*(97.81%), *Bacillus mycoides*(97.45%),*Bacillus weihenstephanensis*(97.45%).

sample E (548bp)=

>GGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGG
AAACCGGGGCTAATACCGGATAGCATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGC
TTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCT
CACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAG
TCTGACGGAGCAACGCCGCGTGAACGATGAAGGCCTTCGGGTCGTAAAGTTCTGTTGTT
AGGGAAGAACAAGTACAAGTTGAATAAGCTTGTACCTTGACGGTACCTAACCAGAAAG
CCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGA
ATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGC
TCAACCGTGGAGGGTCATTGGA

Identification of diazo1 Edit

Length of sequence :548bp View query sequence

Completeness :37.2% (75 - 623)

Tasks	Rank	Name	Strain	Authors	Pairwise Similarity (%)
	1	Bacillus manliponensis (Invalid name)	BL4-6(T)	Jung et al. 2011	100.00
	2	Bacillus cereus	ATCC 14579(T)	Frankland and Frankland 1887	97.99
	3	Bacillus anthracis	ATCC 14578(T)	Cohn 1872	97.99
	4	Bacillus anthracis	Ames	Cohn 1872	97.81
	5	Bacillus thuringiensis	ATCC 10792(T)	Berliner 1915	97.81
	6	Bacillus toyonensis	BCT-7112(T)	Jiménez et al. 2014	97.81
	7	Bacillus mycoides	DSM 2048(T)	Flügge 1886	97.45
	8	Bacillus pseudomycoides	DSM 12442(T)	Nakamura 1998	97.45
	9	Bacillus weihenstephanensis	NBRC 101238(T)	Lechner et al. 1998	97.45
	10	Bacillus bingmayongensis (Invalid name)	FJAT-13831(T)	Liu et al. 2013	97.45
	11	Bacillus cytotoxicus	NVH 391-98(T)	Guinebretière et al. 2013	97.08
	12	Bacillus gaemokensis (Invalid name)	BL3-6 KCTC 13318(T)	Jung et al. 2010	96.69

The 16SrRNA gene sequences of strain BL4-6^{T}is available in the Gene Bank data base under the accession number (KY847541).

Bacillus manliponensis isolated from marine algae *Enteromorpha intestinalis* (L) Nees (Chlorophyceae) formed pellicle in NFB showing that it is a nitrogen fixing bacteria. Seaweeds are generally used as liquid seaweed fertilizers for many crop plants (Abtez.P. 1980, Bhosle et al., 1975, Rajkumar and S.K.Subramanium 1999). Gandhiyappan,K and Perumal(2001) have studied the effect of liquid seaweed fertilizer of *Enteromorpha intestinalis* (L) Nees (Chlorophyceae) as a growth promoting substance. However though there are many reports on liquid seaweed fertilizer obtained from various marine algae as growth promoting fertilizers for various crop plants, the present work substantiate that the growth promoting characteristics of liquid seaweed fertilizer could be due to the presence of nitrogen fixing endophytic bacteria of *Bacillus cereus* group. The present work is the first report on the isolation of *Bacillus manliponensis* from the green algae *Enteromorpha intestinalis* (L) Nees (Chlorophyceae).

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