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STUDIES ON ENDOPHYTIC *Burkholderia* sp. FROM SUGARCANE AND ITS SCREENING FOR PLANT GROWTH PROMOTING POTENTIAL

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ABSTRACT

The occurrence of *Burkholderia* genus as plant endophyte indigenous to locally cultivated sugarcane crop was studied. Totally 22 endophytic isolates were obtained from TB-T medium plates of which 10 were found to be putatively endophytic in sugarcane. The morphological, biochemical and MALDI-TOF MS analysis were done and 5 endophytic *Burkholderia* sp. isolates were identified. Among them, the isolate *Burkholderia* sp. ES4 shown maximum; diazotrophic activity (total nitrogen: 11.9 mg g⁻¹ of malate; nitrogenase activity: 578.3 n mol C₂H₄ released h⁻¹ mg⁻¹protein), mineral solubilization activity (10.2mm, 8.2mm and 3.2 mm solubilization zone of P, ZnO and CaCO₃ respectively; available phosphorous: 0.72mg ml⁻¹), antagonistic activity (54.56 percent inhibition of *Colletotrichum falcatum* mycelium) and phytohormone production (IAA (16.09 µg ml⁻¹); GA (10.54 µg ml⁻¹)), thereby revealing its plant growth promoting potential. The 16S r RNA gene sequence of the isolate *Burkholderia* sp. ES4 was sequenced and its phylogenetic analysis shown that the isolate forms a monophyletic subclade within the plant-beneficial-environment' (PBE) *Burkholderia* group. Thus a potent native plant endophytic strain, *Burkholderia* sp. ES4 that could be developed as biofertilizer inoculum for sugarcane crop, was obtained in this study.

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1 Introduction

Sugarcane is grown in over 110 tropical and subtropical countries with 50 percent of global production from Brazil and India. The sugarcane rich in sucrose is harvested and to maximize yields high rates of nitrogen (N) fertilizer are often applied. High cost of N fertilizers and off-site N losses becomes a problem and in spite of high agronomic input, there is decline in sugarcane yields (Bell et al., 2007). While N fertilizer used is in the range of ~120 to 300 kg N ha⁻¹, the global average indicated that only 50 per cent of N fertilizer applied is used by crops. Reasons of the low fertilizer use efficiency include high soil nitrification rates and weather extremes that promote N leaching and denitrification (Robinson et al., 2011). In addition, manufacture of synthetic fertilizers uses ≈ 10 per cent of global energy consumption. One of the methods that can remediate the problems associated with synthetic fertilizers is the use of microorganisms capable of supporting crop growth by their plant growth promoting activities. There are evidences for plant endophytic bacteria enabling to fix nitrogen, promote mineral solubilization, produce plant phytohormones and possess antagonistic property against plant pathogens; without provoking any host plant defence mechanisms by their colonization.

Burkholderia sp. was first described by Walter H. Burkholder in 1950, as *Pseudomonas cepacia* causing sour skin disease in onions and colonising the rhizosphere of many plant species. The *Burkholderia* genus comprises human, plant pathogenic and non-pathogenic strains and some species are found to be abundant as endophytes within plants. Initially biological nitrogen fixation (BNF) was reported in *B. vietnamiensis* (Gillis et al., 1995) of all the species of the genus after this Estrada-de los Santos et al. (2001) reported that *Burkholderia* could be a genus rich in plant-associated N₂ fixers. *B. kururiensis* (Zhang et al., 2000) was identified as a diazotrophic species, and many N₂ fixing isolates recovered from different plants (maize, coffee and sorghum) have subsequently been classified within novel *Burkholderia* sp., including *B. unamae* (Caballero-Mellado et al., 2004), *B. xenovorans* (Goris et al., 2004) and *B. tropica* (Reis et al., 2004). In Tamil Nadu, India improved yield of micropropagated sugarcane by the inoculation of endophytic *B. vietnamiensis* was reported by Govindarajan et al. (2006). Further, many other studies established *Burkholderia* sp., as an important rhizospheric and endophytic bacterial species (Weilharter et al., 2011; Mitter et al., 2013; Zuniga et al., 2013). Some *Burkholderia* sp. showed high potential to increase plant nutrient availability by nitrogen fixation and/or phosphate solubilisation, e.g., *B. unamae* and *B. tropica*; (Reis et al., 2004), antibiotics or siderophores production (Vial et al., 2011).

Burkholderia sp. has been isolated from intercellular spaces, roots and rhizosphere of sugarcane (James & Olivares, 1998; Fischer et al., 2012). Paungfoo-Lonhienne et al. (2016) had summarized, that the endophytic association between *Burkholderia* sp. and sugarcane happens by substantial

molecular and morphological adaptations in both the partners, where the first step in the establishment of the association was the formation of a robust bacterial biofilm on roots, by a transition of *Burkholderia* from a motile state to non-motile multicellular aggregates, typical for bacterial cells within biofilms. The association between *Burkholderia* Q208 and plants required a significant amount of energy to support bacterial growth and function, including the energy-demanding process of BNF. *Burkholderia* Q208 activated several pathways by high level of cytochrome bd production leading to glycolysis in both aerobic and anaerobic conditions followed by citric acid cycle in aerobic condition and two pathways viz oxalate catabolism and arsinine deiminase pathway in anaerobic condition thus favouring energy production under both aerobic and microaerobic conditions. The bacteria thus experienced an energy-rich, oxygen-depleted environment, two features required for BNF while being an endophyte. This study was conducted to isolate and identify endophytic *Burkholderia* sp. from locally cultivated sugarcane plants and screen the isolates for their plant growth promoting activities.

2 Materials and Methods

2.1 Isolation and Enumeration

All the laboratory experiments were carried out in an aseptic manner in the Department of Agricultural Microbiology, Tamil Nadu Agricultural University (TNAU), Coimbatore and partly in the Department of Soil Science and Agricultural Chemistry, TNAU, Trichy. Triplicate samples of sugarcane root and stem were obtained from fields of different regions of Tamil Nadu, India. The plant samples stored in polyethylene bags were washed thoroughly in tap water to remove the adhering soil particles. Root and stem pieces of 0.5 cm length were cut, surface sterilized by sequential washing in 70 per cent ethanol for 1 min, 2 per cent (v/v) sodium hypochlorite for 3 minutes and 70 per cent ethanol for 30 s and 2 rinses with ample sterilized distilled water. Surface sterilization was verified by plating aliquots (100µl) of the sterile distilled water used in the final rinse onto the plated media. The surface sterilized root and stem segments were separately grounded using a sterile pestle and mortar by using 2ml of PBS buffer and 1 ml aliquots were plated following dilution plating (Allen, 1953) on the media. Plates were incubated at 28°C and the colonies were observed after 48 h. Total gram-negative bacteria was enumerated on a 10% tryptic soy agar (TSA) medium containing tryptic soy agar (4g/l), agar (15 g/l) and crystal violet (0.005 g/l) to reduce the growth of gram-positive bacteria (Gould et al., 1985). The *Burkholderia* sp. isolation medium (TB-T) contained the following components (per liter): 20g of agar, 2 g of glucose, 1 g of L-asparagine, 1 g of NaHCO₃, 500 mg of KH₂PO₄, 100 mg of MgSO₄·7H₂O, 50 mg of trypan blue (TB), and 20 mg of tetracycline (T) (Hagedorn et al., 1987). The pH of the medium was adjusted to 5.5 with 10% phosphoric acid (4 ml/l), and the filter-sterilized tetracycline was added to the autoclaved medium. Total *Burkholderia* sp. count was enumerated and the bacterial colonies obtained from TB-T medium plates were purified by

streak plate technique and stored for further studies. They were numerically numbered, with the prefix as ER (Endophytic isolate from Root) and ES (Endophytic isolate from Stem). The isolates were subjected to putative endophytic authentication by inoculating the gnotobiotically (Figure 1) grown sugarcane plantlet from apical meristem explant that shown nil contamination when grown in shoot apex medium (White, 1963). The bacteria re-isolated from the inoculated plantlet by plating the macerated tissue were used for further analysis.

2.2 Characterization of *Burkholderia* sp.

Observations of colony morphology (Gerhardt et al., 1981); motility (Skerman, 1967); growth on differential carbon substrate utilization using BMGM medium (Paulina et al., 2001) one lacking azelaic acid but supplemented with a single carbon source (0.5% fructose, glucose, sucrose, mannitol, glycerol, malate, succinate); catalase (Smibert & Krieg, 1981); oxidase (Collins & Lyne, 1970); citrate utilization (Huhtanen et al., 1972); starch hydrolysis (Seeley & van Dekark, 1981); cellulase activity (Rautela & Cowling, 1966) and pectinase activity (Albersheim, 1966) were undertaken to understand the bio-chemical characteristics of the isolates.

2.3 Identification of the genus *Burkholderia* sp.

Isolates that were characterized to be *Burkholderia* sp., were identified by the method of matrix-assisted laser desorption ionization - time of flight (MALDI-TOF) mass spectrometry and MALDI Biotyper. The Bruker Biflex IV MALDI-TOF spectrometer (equipped with a UV nitrogen laser [337 nm] and a dual microchannel microplate detector) and MALDI Biotyper 2.0 software (Bruker Daltonics, Bremen, Germany) were used. Samples for the analysis were prepared according to manufacturers' recommendations: after 24–48 h of cultivation of an isolate on LB medium at 28°C, a single colony was transferred with a sterile tip onto the MALDI target in triplicates, drizzled with 1 µL of a saturated solution of α -cyano-4-hydroxycinnamic acid (Sigma-Aldrich) in organic solution (50% acetonitrile, 2.5% trifluoroacetic acid), and directly screened. The measurement of the spectra was performed as described by Uhlik et al. (2011). The matching of unknown spectra to the reference database is based on dedicated score (point) values. This value is used for calculating

the final score, according to which the identification results are evaluated as follows: if the logarithmic value of the final score is between 2.3 and 3, the isolate is identified at the level of species; for values between 2 and 2.3, the identification is secure at the level of genus; for values between 1.7 and 2, the identification at the level of genus is probable and for values lower than 1.7, the identification is not successful.

2.4 Diazotrophic activity of *Burkholderia* sp.

The nitrogen fixing ability of the identified *Burkholderia* isolates was estimated by their growth in N-free medium (Burris & Wilson, 1946) followed by estimation of total nitrogen (Humphries, 1956) and estimation of ammonia excretion (Cappuccino & Sherman, 1992). The nitrogenase activity of the isolates was evaluated by conducting Acetylene Reduction Assay (ARA) by Gas Chromatography (GC) (Chemito GC 7610) following the standard procedure given by Burris (1974) using NfB semi solid medium. After completion of the ARA, the cells were pre-digested by adding 10% SDS and sonicated briefly. Protein concentration in the resulting distributed mixture of suspension was determined (Lowry et al., 1951). The nitrogenase activity of the bacterial isolate was expressed as n mol C₂H₄ released h⁻¹ mg⁻¹ protein.

2.5 Mineral solubilisation potential of *Burkholderia* sp.

The extent of the solubilisation of insoluble phosphates by the identified *Burkholderia* isolates was assessed under *in vitro* condition. The bacterial isolates were screened for phosphate solubilisation as per the methodology followed by Gupta et al. (1994) on modified Pikovskaya medium. Quantitative assay of available phosphorous estimation was carried out using the method described by Olsen et al. (1954) and the phosphatase activity was done by the method described by (Morton, 1952). Solubilisation potential of zinc oxide by the isolates was examined by observing the ability of the cultures to grow on Tris minimal salt medium supplemented with 0.1 per cent zinc oxide for 10 percent glucose (di Simine et al., 1998), following the agar well diffusion assay. Solubilisation potential of calcium carbonate by the isolates was examined by observing the ability of the cultures to grow on modified Aleksandrov medium (Aleksandrov et al., 1967) substituted with soluble form of potassium and 3 per cent calcium carbonate, following the agar well diffusion assay.



Figure 1 Gnotobiotic studies to identify the putative endophytic bacteria

2.6 Antagonistic activity of *Burkholderia* sp. on sugarcane red rot pathogen *Colletotrichum falcatum*

Antagonistic activity of all the identified *Burkholderia* isolates was tested against fungal pathogen, *C. falcatum* (Cf671), the red rot casual organism in sugarcane obtained from red rot type culture collections in the plant pathology section of the Sugarcane Breeding Institute, Coimbatore, maintained in oat meal agar. The pathogenic fungal disc (9.0 mm) was placed in the centre of Petri dish containing sterile agar medium. The 48 h old bacterial isolate to be tested was streak lined at 2 cm away from the fungal disc on either side (Dennis & Webster, 1971). The plates were incubated at room temperature ($30 \pm 2^\circ\text{C}$) for four days and the radial mycelial growth (mm) of the pathogen was recorded and per cent inhibition of the mycelial growth was calculated.

2.7 Analysis of siderophore production

Production of siderophores by the identified *Burkholderia* isolates was performed qualitatively by plate assay (Schwyn & Neilands, 1987). The Chrome Azurol S (CAS) agar plates were used to check the production of siderophores by bacteria. It is based on the high affinity of siderophores towards ferric iron (Fe^{3+}) whereby ferric iron bound to dye, is complexed and released from the dye. The ternary complex chrome azurol S / Fe^{3+} / hexadecyl trimethyl ammonium bromide served as an indicator. The 48 h old bacterial isolates were streaked individually on to the agar plates amended with indicator dye prepared. The formation of bright zone with yellowish fluorescence in the dark blue coloured medium indicated positive for siderophore production. The result was scored either positive or negative, based on the colour change of the medium from blue to fluorescent yellow while no colour change marked the absence of siderophore production. Quantification of the siderophore production was done as followed by (Reeves et al., 1983). The culture supernatant was used for the estimation of catecholate type and salicylate type siderophores, by using Hathway reagent. The absorbance was read at 560 nm (UV-VIS spectrophotometer) and a standard curve was prepared using sodium salicylate for the estimation of salicylate type siderophore and 700 nm (UV – VIS spectrophotometer) with 2, 3-dihydroxybenzoic acid as standard to measure catechol type siderophore. The quantity of siderophore synthesized was expressed as $\mu\text{g ml}^{-1}$ of culture filtrate.

2.8 IAA and GA production by *Burkholderia* sp.

The quantity of IAA produced by each isolate was estimated by the spectrophotometric technique using van Urk Salkowski's reagent (1 ml of 0.5 N FeCl_3 mixed in 50 ml of 35 per cent perchloric acid) following the Salkowski's method (Ehmann, 1977; Ahmad et al., 2005). The quantity of IAA was expressed in $\mu\text{g ml}^{-1}$ by referring to a standard graph of IAA prepared from a series of IAA solutions of known concentrations. HPLC (Spectra System P1000, Thermo Electron Corporation) with UV detector and C18 column (4.6x150mm, Nacalai Tesque, Inc. Japan) was used to study the IAA fractions of the isolate that shown maximum IAA production

(Sarwar et al., 1992). The quantity of GA produced by each isolate was estimated by the spectrometric method followed by Mahadevan & Sridhar (1986). The quantity of GA was expressed in $\mu\text{g ml}^{-1}$ by referring to a standard graph of GA prepared from a series of GA solutions of known concentrations. The isolate that showed maximum GA production was subjected to HPLC. Hundred ml of culture broth was centrifuged (Remi, India) at 2817 xg for 15 min, and supernatant was used for extraction of gibberellic acid. The pH of supernatant was adjusted to 2.8 with 1 N HCl and gibberellic acid was extracted with an equal volume of ethyl acetate. This ethyl acetate extract was evaporated to dryness at 35°C , and the residue was dissolved in 500 μl of pure methanol. The samples were analyzed on HPLC (Spectra System P1000, Thermo Electron Corporation) with UV detector and C18 column (4.6x150mm, Nacalai Tesque, Inc. Japan) by the method followed by Li et al. (1994).

2.9 Phylogenetic analysis of the endophytic isolate *Burkholderia* sp. ES4

The genomic DNA from the endophytic isolate *Burkholderia* sp. ES4, which showed maximum plant growth promoting potential, was extracted using the standard protocol of hexadecyl-trimethyl ammonium bromide (CTAB) method as given by Melody (1997) with minor modifications and the isolated genomic DNA was stored at -20°C for further studies. Full length 16S rRNA gene (1500bp) was amplified from the stored genomic DNA using the universal eubacterial forward primer FD1(5' AGAGTTGA TCGTGGCTCAG 3') and the reverse primer RP2 (5' ACGGCTACCTTGTTACCACTT 3') (Weisburg et al., 1991). The PCR amplification was conducted in a PCR thermal cycler (MyCycler, Bio-Rad Laboratories Inc., USA). The amplified products were purified and sequenced by Bioserve Biotechnologies (India) Pvt. Ltd. (Hyderabad, India). The identity of 16S rRNA gene sequence was performed by similarity search using BLAST tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). To identify the bacterial strain, 16S rRNA gene sequence was compared with published sequence of closely relates species, strain on NCBI GenBank (Altschul et al., 1997). The phylogenetic tree was constructed using existing 16S rRNA gene sequence database of few other *Burkholderia* sp. strains in Genbank. The 16S rRNA gene sequences were aligned using CLUSTAL W (Thompson et al., 2013). A phylogenetic tree was constructed by using MEGA 6.0 (Tamura et al., 2013), in which an evolutionary distance matrix was generated as described by Jukes & Cantor (1969). The evolutionary tree for the dataset was inferred from the neighbor-joining method of Saitou & Nei (1987). The stability of relationships was assessed using bootstrap analysis of the neighbor-joining data based on 1000 resamplings.

2.10 Statistical analysis

All the data given in the tables were the mean of triplicate values obtained. Basic statistical parameters were calculated using AGRESS in Microsoft Excel of Windows 2010.

Table 1 Enumeration of endophytic gram negative bacterial colonies from sugarcane

Sample No. (S)	Plant part (P)	Count of bacteria recovered from		% of <i>Burkholderia</i> sp. isolates recovered
		10% TSA X10 ⁶ (C1)	TB-T X10 ⁵ (C2)	
1	Root(P1)	13.3	2.7	20.3
	Stem(P2)	9.7	4.3	44.3
2	Root	10.7	2.0	18.7
	Stem	6.7	1.3	19.4
3	Root	8.0	3.0	37.5
	Stem	6.7	0.7	10.4
4	Root	12.3	1.7	13.8
	Stem	5.0	0.3	6.0
5	Root	12.3	3.0	24.4
	Stem	7.0	1.7	24.3
6	Root	9.3	3.0	32.3
	Stem	4.0	2.0	50.0
7	Root	11.0	1.0	9.1
	Stem	6.0	1.0	16.7
8	Root	12.0	1.0	8.3
	Stem	8.7	1.0	11.5
9	Root	12.0	2.0	16.7
	Stem	8.7	1.7	19.5
10	Root	11.3	1.7	15.0
	Stem	8.0	2.0	25.0
SEd	0.86	0.38	0.86	1.22
CD(0.05)	1.71	0.77	1.71	2.42

Statistical significant differences among the treatments were analysed using ANOVA (Analysis of Variance) and multiple comparison test was done using LSD (Least Significance Difference) where the significance was evaluated at $p < 0.05$ for all tests.

3 Results and Discussion

In the present study, sugarcane root and stem samples were collected in triplicates from 10 different regions of sugarcane fields in Tamil Nadu, India. Gram negative bacterial colonies and *Burkholderia* sp. were enumerated using 10%TSA medium and TB-T medium respectively (Table 1). Among the 10 sample sets collected, sample 1 root showed maximum ($13.3 \times 10^6 \text{ ml}^{-1}$) gram negative bacterial count and it was followed by samples 4 and 5 roots ($12.33 \times 10^6 \text{ ml}^{-1}$) and minimum count was observed in sample 6 stem ($4.0 \times 10^6 \text{ ml}^{-1}$). On statistical analysis sample 1 root emerged to be the single sample with maximum gram negative bacterial population while all the other sample sets remained on par. Among the 10 sample sets collected, sample 1 stem showed maximum ($4.3 \times 10^5 \text{ ml}^{-1}$) *Burkholderia* sp count followed by samples 3,5 and 6 roots ($3.0 \times 10^5 \text{ ml}^{-1}$) and minimum count was observed in sample 4 stem ($0.3 \times 10^5 \text{ ml}^{-1}$). On statistical analysis it was found that mostly root samples harboured more *Burkholderia* sp. population than stem except the sample 1 stem population. Also the

percentage of *Burkholderia* sp. isolates recovered was found to be highest (50%) from sample 6 stem followed by sample1, stem (44.3%), least (6%) in sample 4 stem. Also for each sample set, bacterial counts with the selective medium (TB-T) were much lower than the count of total gram negative colonies. On TB-T medium the *Burkholderia* sp. colonies appeared to be white or yellow and the proportion of pigmented colonies differed among samples. Hagedorn et al. (1987) have shown that the TB-T medium provided high degree of selectivity for and detection of *Burkholderia* sp. upon initial plating from soil samples.

The other most commonly used PCAT medium was not completely specific for *Burkholderia* sp. since some gamma-proteobacteria episodically grew in this medium (Pallud et al., 2001). Species abundance and diversity of *B. cepacia* complex in the environment was studied by Ramette et al. (2005) in which TB-T was chosen over other *B.* sp. semi-selective media, because all known *Burkholderia* species can grow on it and it yields more *Burkholderia* isolates than other media. Isolation and identification of *Burkholderia* sp. from onion rhizosphere by Jacobs et al. (2008) shown that *B. cepacia* and *B. ambifaria* were preferentially recovered on TB-T medium, with 79% and 80% of the total isolates, respectively.

Table 2 Cultural morphology of bacterial endophytes isolated from sugarcane

Isolate	Colony morphology				Motility
	Colour	Elevation	Margin	Texture	
ER2	Creamy white	Raised	Smooth	Dry	+
ER5	Dull white	Flat	Smooth	Dry	+
ER8	Creamy white	Raised	Smooth	Mucous	+
ER9	Creamy white	Raised	Wavy	Dry	+
ER11	Pale yellow	Flat	Smooth	Dry	+
ES2	Dull yellow	Flat	Smooth	Dry	+
ES3	Dull yellow	Flat	Smooth	Dry	+
ES4	Dull yellow	Flat	Smooth	Dry	+
ES8	Dull yellow	Flat	Smooth	Dry	+
ES9	Creamy white	Raised	Smooth	Dry	+

Table 3 Differential biochemical characteristics of bacterial endophytes isolated from sugarcane

Isolate	Catalase	Oxidase	Cellulase	Starch hydrolysis	Citrate utilization	Pectinase
ER2	+	+	+	-	+	-
ER5	+	+	+	+	+	+
ER8	+	+	+	+	+	-
ER9	+	+	+	+	+	+
ER11	+	+	+	+	+	+
ES2	+	+	+	-	+	-
ES3	+	+	+	+	+	-
ES4	+	+	+	+	+	+
ES8	+	+	+	-	+	-
ES9	+	+	+	+	+	-

Table 4 Differential carbon substrate utilization of the isolated bacterial endophytes from sugarcane

Isolate	Glucose	Fructose	Sucrose	Mannitol	Glycerol	Malate	Succinate
ER2	+	+	+	+	+	+	+
ER5	+	+	+	-	-	+	+
ER8	+	+	+	-	+	-	+
ER9	+	+	+	+	-	+	+
ER11	+	+	+	+	+	+	+
ES2	+	+	+	+	-	+	+
ES3	+	+	+	+	+	-	+
ES4	+	+	+	+	+	+	+
ES8	+	+	+	+	+	+	+
ES9	+	+	+	+	+	+	+

Totally 22 endophytic bacterial isolates were obtained from TB-T medium plates, 12 from root samples and 10 from stem samples. The isolates re-isolated from, the macerated tissue culture plant samples raised gnotobiotically and inoculated with the corresponding isolate, were confirmed to be putative endophytic bacterial isolates, which reduced the isolate collection to 10 in number, viz ER2, ER5, ER8, ER9, ER11, ES2, ES3, ES4, ES8 and ES9. There are only a handful number of gnotobiotic studies on

plant endophytic *Burkholderia* sp. interactions with sugarcane (Compant et al., 2005; Paungfoo-Lonhienne et al., 2014; Paungfoo-Lonhienne et al., 2016). These studies indicated that large numbers of bacteria dwell at the surface of roots, and some occur inside root cortex cells. Movement of bacterial cells with cytoplasmic streaming had confirmed their localization inside living root cells. These results indicated that *Burkholderia* sp. had endophytic associations with sugarcane.

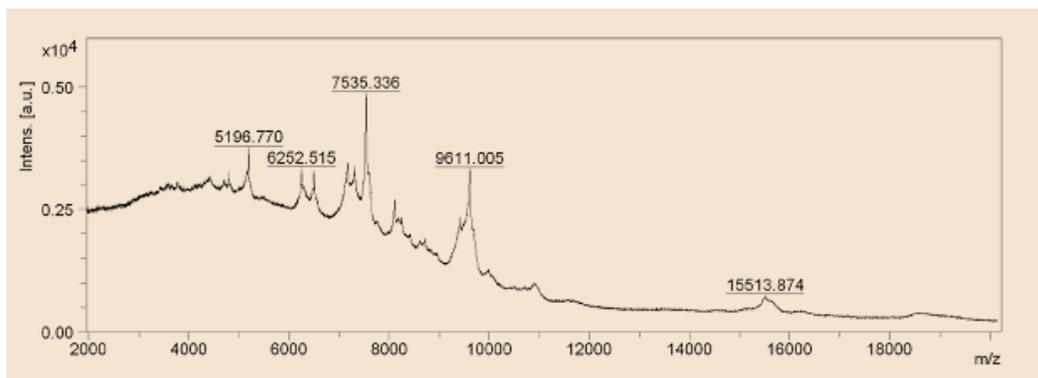


Figure 2 Representative spectra profile (with score values > 2.0) of the endophytic isolate *Burkholderia* sp. ES4 associated with sugarcane

The 10 putative endophytic *Burkholderia* sp. isolates were mostly white or yellow in color with mostly flat, smooth and dry colony characteristics as described by Hagedorn et al. (1987) (Table 2). All the 10 shown motility, catalase and oxidase positive. Cellulose and citrate utilization was found in all the 10 isolates. Only few isolates (ER5, ER9, ER11 and ES4) were found to be pectinase positive and few more isolates shown starch hydrolysis (Table 3).

Table 5 Identification of the genus *Burkholderia* sp. using Matrix-Assisted Laser Desorption/Ionization – Time of Flight Mass Spectrometer (MALDI-TOF MS)

S. No.	Isolate Code	Identification using MALDI-TOF MS*	Score value	Plant sample
1	ER2	<i>Bacillus</i> sp.	2.081	Root
2	ER5	<i>Burkholderia</i> sp.	2.064	Root
3	ER8	Not identified	-	Root
4	ER9	Not identified	-	Root
5	ER11	Not identified	-	Root
6	ES2	<i>Burkholderia</i> sp.	2.073	Stem
7	ES3	<i>Burkholderia</i> sp.	2.011	Stem
8	ES4	<i>Burkholderia</i> sp.	2.176	Stem
9	ES8	<i>Burkholderia</i> sp.	2.059	Stem
10	ES9	Not identified	-	Stem

Reis et al. (2004) had observed that *B. tropica* sp. nov. showed catalase, oxidase positive and no starch hydrolysis. The differential carbon substrate utilization pattern was studied and almost all the isolates grew in most of the C source tested (Table 4). Only few isolates shown nil growth in mannitol (ER5 and ER8), glycerol (ER5, ER9 and ES2) and malate (ER8 and ES3). Bio-chemical characterization of endophytic plant growth promoting *Burkholderia* sp. MSSP from root nodules of *Mimosa pudica* was done by Pandey et al. (2005) and found to have similar results like this study. The phenotypical and biochemical characters of our isolates showed similarity with that of most of plant endophytic *Burkholderia* sp. The method of Matrix-Assisted Laser Desorption/Ionization – Time Of Flight Mass Spectrometer

(MALDI-TOF MS) was used to identify the *Burkholderia* sp. by the protein profiling of intact bacterial colonies (Table 5, Figure. 2). Among the 10 endophytic bacterial isolates only 5 isolates (ER5, ES2, ES3, ES4 and ES8) shown the score values >2 corresponding to the genus *Burkholderia* sp. This technique is based on detection of molecular weight of most abundant cell molecules (presumably proteins and peptides) and using the mass spectra information as a fingerprint for a particular organism (Sauer & Kliem, 2010). Minimal sample preparation and the speed of data acquisition combined with the high throughput and sample processing automation, make MALDI-TOF MS a valuable screening and identification method (de Bruyne et al., 2011). Isolate V9, *B. ambifaria* was identified using MALDI-TOF MS, from a collection of maize rhizospheric soil isolates by Kifle & Laing (2016) to study its plant growth promoting potential. This study had shown that even though isolated using a selective medium, followed by phenotypic and bio-chemical characterization, not all the 10 isolates had the MALDI-TOF MS spectra corresponding to the genus *Burkholderia* sp.

The 5 endophytic *Burkholderia* sp isolates were tested for their ability to grow in N-free medium as qualitative evidence for their diazotrophic potential. All the 5 isolates showed growth in the N-free medium with the isolate ES4 showing maximum growth. The isolates were further analysed for total nitrogen and ammonia production, in which isolates ER5 and ES4 (11.9 mg g⁻¹ of malate) showed maximum total nitrogen production and isolate ES4 (3.1 mg ml⁻¹) showed maximum ammonia production followed by the isolate ER5 (2.5 mg ml⁻¹). The ARA results obtained showed that the nitrogenase enzyme activity of the isolate ES4 (578.3 n mol C₂H₄ released h⁻¹ mg⁻¹protein) was the highest among the other isolates. The statistical analysis inferred that the isolate ES4 showed results on par with the standard culture *Gluconacetobacter diazotrophicus* PAL5 (obtained from standard culture collection, Dept. of Agricultural Microbiology, TNAU, Coimbatore) (Table 6).

Studies on nitrogen fixing endophytic *Burkholderia* sp. associated with maize plants cultivated in Mexico by Estrada et al. (2002) showed that all the *Burkholderia* isolates could reduce acetylene

and also showed the presence of *nifH* gene in two of the isolates. Reis et al. (2004) described a novel nitrogen fixing sugarcane plant associated bacteria, *B. tropica* sp. nov. that shown positive result in acetylene reduction assay. Govindarajan et al. (2006) had studied the performance of a local isolate, *B. vietnamiensis* MG43, an endophytic nitrogen fixing bacterium from sugarcane, under field conditions and shown increase in sugarcane root colonization and biomass. They have also discussed that the locally isolated bacterium was capable of by-passing the initial competition phase in the plant colonization when compared to the standard strains used. Paungfoo-Lonhienne et al. (2014) described a new species *B. australis* sp. nov. which shown biological nitrogen fixation in sugarcane plantlets grown in axenic conditions. Increased nitrogen concentration in roots of rice was seen when inoculated with *B. cepacia* CS5 previously isolated as an endophyte from the rice plant (Hongrittipun et al., 2014). Also, De Oliveira Silva et al. (2016) had obtained two endophytic *Burkholderia* sp. (UAGC76 and UAGC78) from sugarcane roots that shown positive growth in N-free medium. Results of present study also show that the endophytic isolate *Burkholderia* sp. ES4 have prudent potential of biological nitrogen fixation that could be utilized in combination

with lesser quantity of inorganic N fertilizer application and also it can be noticed that the genus *Burkholderia*, is one among the predominant endophytic bacteria, found distinctly in sugarcane irrespective of geographical location and crop variety.

The 5 endophytic *Burkholderia* sp. isolates were tested for their mineral solubilization potential. All the 5 isolates showed phosphate and zinc solubilisation, among them isolate ES4 showed maximum phosphate (10.2mm) and zinc (8.2mm) solubilisation zone (Figure. 3). The available P was estimated and found to be produced maximum (0.72mg ml^{-1}) by the isolate ES4.

The isolates were further analysed for phosphatase activity, in which the isolate ES4 showed maximum ($5.5\ \mu\text{g}$ of PNP released $\text{ml}^{-1}\ \text{day}^{-1}$) activity. The calcium carbonate solubilisation was showed by only 3 (ER5, ER3 and ER4) of the 5 isolates in which isolate ES4 showed maximum (3.2mm) solubilisation zone. The statistical analysis inferred that the isolate ES4 had significantly increased mineral solubilization potential when compared to that of standard culture *Gluconacetobacter diazotrophicus* PAL5 (Table 7).

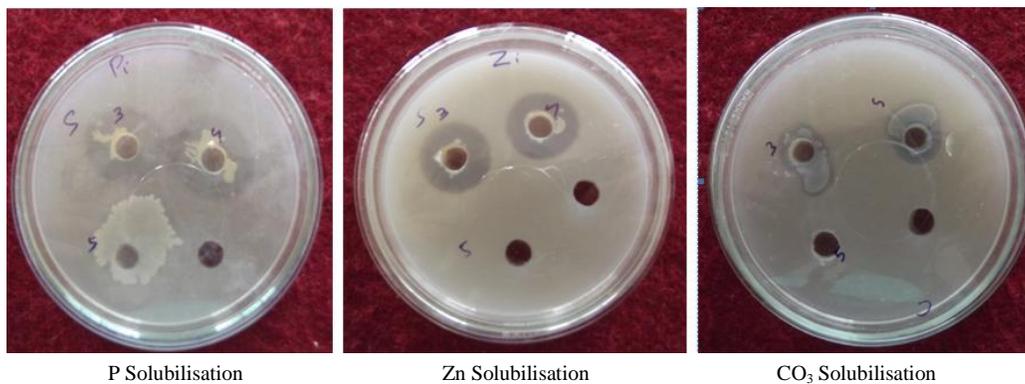


Figure 3 Mineral solubilisation potential of the endophytic isolate *Burkholderia* sp. ES 4

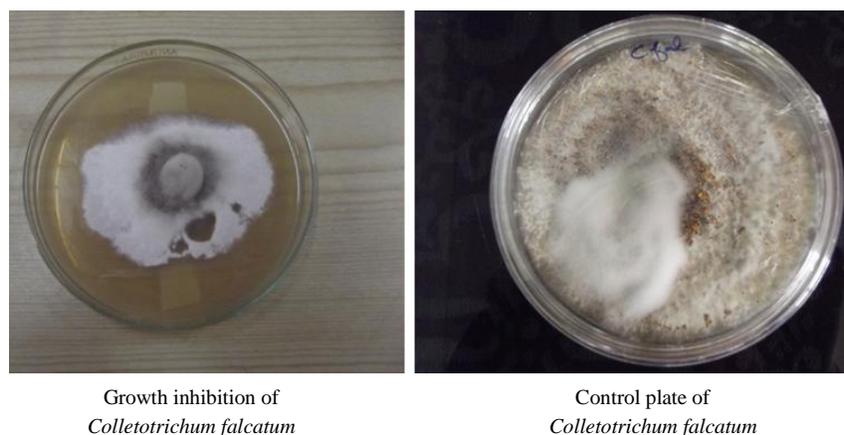
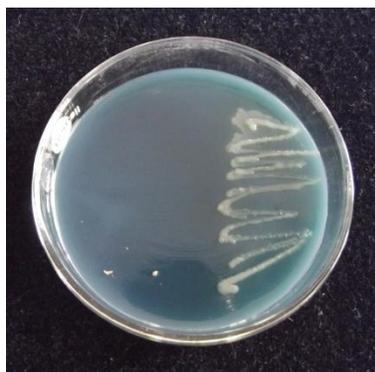


Figure 4 Antagonistic activity of the endophytic isolate *Burkholderia* sp. ES4 on sugarcane red rot pathogen *Colletotrichum falcatum*

Figure 5 Siderophore production of the endophytic isolate *Burkholderia* sp. ES 4Table 6 Assessment of the diazotrophic potential of the endophytic *Burkholderia* sp.

S. No.	Isolate Code	Isolate	Growth (A_{660nm})	Total nitrogen (mg g^{-1} of malate)	Ammonia (mg ml^{-1})	Nitrogenase activity (n mol C_2H_4 released $h^{-1} mg^{-1} protein$)
1	ER5	<i>Burkholderia</i> sp.	+++	11.9	2.5	426.7
2	ES2	<i>Burkholderia</i> sp.	+	07.2	1.8	131.3
3	ES3	<i>Burkholderia</i> sp.	++	05.9	2.2	206.7
4	ES4	<i>Burkholderia</i> sp.	+++	11.9	3.1	578.3
5	ES8	<i>Burkholderia</i> sp.	+	05.6	1.4	215.0
Std	PAL5	<i>Gluconacetobacter diazotrophicus</i>	+++	12.6	2.7	577.0
SEd				0.57	0.17	7.13
CD(0.05)				1.25	0.37	15.53

No growth (<0.10 OD); + (0.1-0.3 OD); ++ (0.3-0.5 OD), +++ (>0.5 OD)

Table 7 Estimation of solubilization potential of the endophytic *Burkholderia* sps.

S. No.	Isolate Code	Isolate	Solubilization zone (mm)	Available P (mg ml^{-1})	Phosphatase activity (μg of PNP released $ml^{-1} day^{-1}$)	ZnO Solubilization zone (mm)	$CaCO_3$ Solubilization zone (mm)
1	ER5	<i>Burkholderia</i> sp.	5.1	0.35	3.3	6.3	1.8
2	ES2	<i>Burkholderia</i> sp.	4.3	0.13	2.1	4.2	-
3	ES3	<i>Burkholderia</i> sp.	4.0	0.34	2.5	3.8	2.1
4	ES4	<i>Burkholderia</i> sp.	10.2	0.72	5.5	8.2	3.2
5	ES8	<i>Burkholderia</i> sp.	7.2	0.50	2.8	2.2	-
Std	PAL5	<i>Gluconacetobacter diazotrophicus</i>	5.1	0.42	5.1	5.2	-
SEd			0.18	0.02	0.30	0.21	0.14
CD(0.05)			0.40	0.05	0.63	0.50	0.35

In calcareous soils, P is absorbed on the surface of calcium carbonate (Mattingly, 1975), since few of our isolates were obtained from sugarcane plants those cultivated in calcareous soils, we intended to study the calcium carbonate biodissolution potential of the isolates. The endophytic bacteria, during the process of

plant colonization, enabling to solubilize the fixed phosphate in the soil and thereby increase its availability to the host plant would be considered as a plant growth promoting potential. In case of sugarcane grown in tropical soils, the inorganic P fertilizer applied during the time of planting, becomes rapidly fixed.

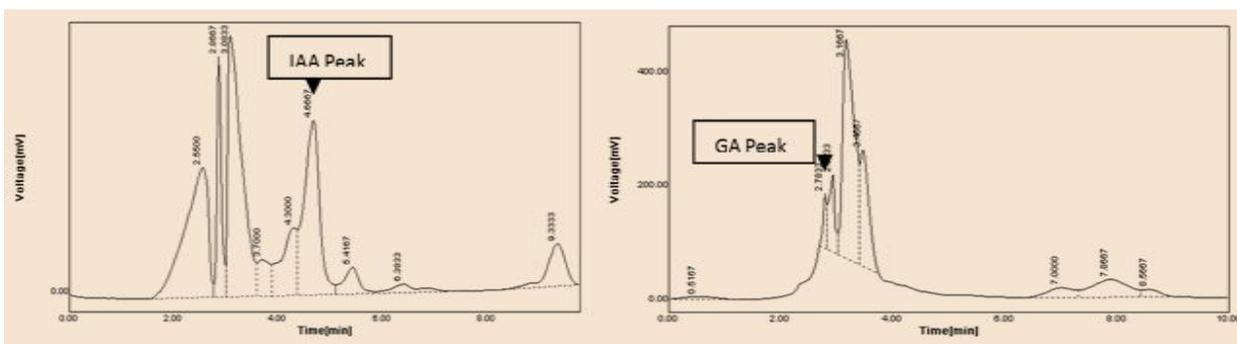


Figure 6 HPLC Chromatograms of IAA and GA production by the endophytic isolate *Burkholderia* sp. ES 4

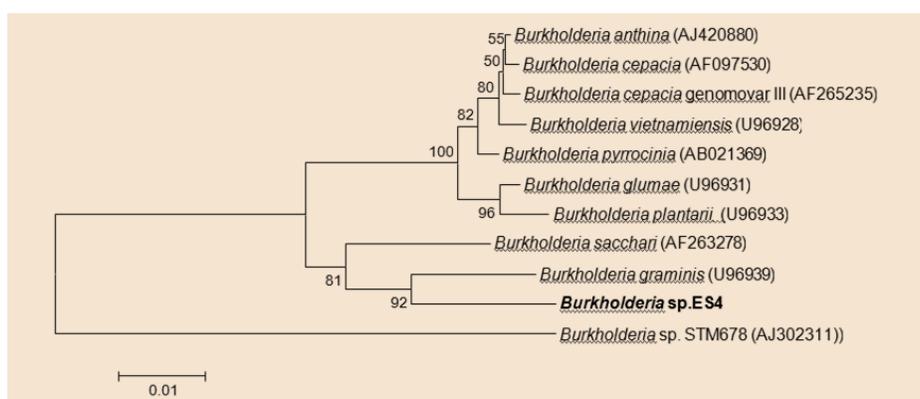


Figure 7 Phylogenetic tree for *Burkholderia* sp. ES4 based on partial 16S rRNA gene sequences. The tree was generated using the Neighbor-Joining method (Saitou and Nei, 1987) from Jukes–Cantor calculated distances (Jukes and Cantor, 1969); percentage of 10,000 bootstrap replicates are shown at each node; GenBank sequences of type strains of closely related species are included as references

One way of minimizing this loss is by application of bacterial inoculant or inoculants that can solubilize this P and transform it as for plant assimilation resulting in better use and crop yield (Wakelin et al., 2004; Canbolat et al., 2006; Dias et al., 2009). Castro-González et al. (2011) have given culturable *Burkholderia* sp. associated with sugarcane capable of mineral phosphate solubilisation. Studies on *Burkholderia* sp. showing zinc solubilization was conducted by Vaid et al. (2014) and thereafter increase in zinc uptake by the rice plant was also proven.

The antagonistic activity of the 5 endophytic *Burkholderia* sp. isolates was determined based on the per cent of mycelial growth inhibition of *Colletotrichum falcatum*, the red rot pathogen of sugarcane (Table 8). In this study, isolate ES4 shown maximum inhibition per cent against *C. falcatum* (54.56) (Figure. 4), which was found to be 44.03 per cent more than that of standard culture *Gluconacetobacter diazotrophicus* PAL5 (10.53) and 4.23 per cent more than that of the standard *Bacillus amyloliquefaciens* strain (50.33), obtained from the Department of Plant Pathology, TNAU, Coimbatore. One of the means to examine mechanisms by which the bacteria elicit induced systemic resistance of plants is to search for bacterial determinants of elicitation.

In our study the 5 endophytic *Burkholderia* sp. isolates were tested for siderophore production (Figure. 5) and the estimation of the production was also done. The results in Table 8 indicated that the isolate ES4 have given maximum ($14.30 \mu\text{g mg}^{-1}$ of cells) salicylate type and ($12.53 \mu\text{g mg}^{-1}$ of cells) catechol type siderophores, which were significantly more than that of the standards compared. The secreted siderophores chelate ferric ion (Fe^{3+}) with have high affinity, making Fe available to plant hosts and depriving a pathogen of iron (Schippers et al., 1987).

The roles of siderophores and other antagonistic mechanisms have been poorly described for the genus *Burkholderia*, except for the isolate *B. vietnamiensis*, for which pyochelin and salicylic acid (catechol type) have been identified by Bevivino et al. (1994). The ability of *Burkholderia* species to inhibit the growth of multiple soil-borne pathogens on different crops by different antagonistic mechanisms, such as production of antibiotics or siderophores were described by Compant et al. (2008) and Jamalizadeh et al. (2011).

Table 8 Assessment of antagonistic potential of the endophytic *Burkholderia* sp.

S. No.	Isolate Code	Isolate	Antifungal activity Per cent inhibition	Siderophore type	
				Salicylate ($\mu\text{g mg}^{-1}$ of cell)	Catechol ($\mu\text{g mg}^{-1}$ of cell)
1	ER5	<i>Burkholderia</i> sp.	50.11	10.08	6.38
2	ES2	<i>Burkholderia</i> sp.	49.34	5.16	-
3	ES3	<i>Burkholderia</i> sp.	34.44	10.04	7.39
4	ES4	<i>Burkholderia</i> sp.	54.56	14.30	12.53
5	ES8	<i>Burkholderia</i> sp.	40.12	11.60	8.20
6	PAL5	<i>Gluconacetobacter diazotrophicus</i>	10.53	7.53	5.32
7	Std	<i>Bacillus amyloliquefaciens</i>	50.33	14.07	12.14
SEd			0.10	0.11	0.20
CD(0.05)			0.21	0.23	0.44

Table 9 Quantification of IAA and GA production by the endophytic *Burkholderia* sps.

S.No.	Isolate Code	Isolate	IAA ($\mu\text{g ml}^{-1}$)	GA ($\mu\text{g ml}^{-1}$)
1	ER5	<i>Burkholderia</i> sp.	11.21	6.32
2	ES2	<i>Burkholderia</i> sp.	9.32	5.35
3	ES3	<i>Burkholderia</i> sp.	10.50	7.08
4	ES4	<i>Burkholderia</i> sp.	16.09	10.54
5	ES8	<i>Burkholderia</i> sp.	12.18	9.28
Std	PAL5	<i>Gluconacetobacter diazotrophicus</i>	16.12	10.11
SEd			0.17	0.17
CD(0.05)			0.36	0.37

Production of plant growth promoting phytohormones (IAA and GA) by the 5 endophytic *Burkholderia* sp. were estimated spectrophotometrically (Table 9). It was observed that the isolate ES4 shown maximum IAA ($16.09 \mu\text{g ml}^{-1}$) and GA ($10.54 \mu\text{g ml}^{-1}$) production. The IAA production by the isolate ES4 was found to be on par with that of standard culture *Gluconacetobacter diazotrophicus* PAL5 and GA production was significantly higher than that of standard culture *G. diazotrophicus* PAL5. Further, qualitative estimation of the IAA and GA produced by the isolate ES4 were observed from the HPLC chromatograms (Figure. 6), in comparison with the standard IAA and GA peaks. The chromatograms shown that the IAA (4.6667 min) and GA (2.9333 min) peaks obtained for the isolate ES4 were found at the same retention times as that of the IAA and GA standards. In our present study the indigenous endophytic *Burkholderia* sp. were found capable of synthesising IAA and GA. This biosynthesis has been reported to play a major role in the growth and development of the plant including cell division, elongation, differentiation and vascular bundle formation. Higher amount of IAA production increases lateral and adventitious root formation but inhibits the primary root growth (Xie et al., 1996), which is exactly what is needed for root development from sugarcane sett when planted. The production of IAA in *B. vietnamiensis* has been described by Govindarajan et al. (2008) and in *B. kururiensis* (rice plant

endophyte) by Mattos et al. (2008). Production of GA by *Burkholderia* sp. has been reported by Joo et al. (2009). Production of GA may modulate the hormonal balance involved in plant defence, including the jasmonate and salicylic acid pathways (Pieterse et al., 2009). The plant hormones IAA and GA, induced by *Burkholderia phytofirmans* PsJN, had the ability to promote lateral root formation and/or extension in *Arabidopsis thaliana* (Zhao et al., 2016).

The 16S rRNA gene sequence based phylogenetic tree analysis involving nucleotide sequences of *Burkholderia* species obtained from NCBI, revealed that the isolate *Burkholderia* sp. ES4 belonged to the family Burkholderiaceae (Figure. 7). The *Burkholderia* genus is divided into two main groups (Suárez-Moreno et al., 2012). The first group contains *Burkholderia* species that are pathogens in human, animals and plants. The second group includes non-pathogenic species mostly reported to be associated with and beneficial to plants. The latter group, to which *Burkholderia* sp. ES4 belongs, is referred to as the 'plant-beneficial-environment' (PBE) *Burkholderia* group because most have useful properties as antagonists to plant pests, as PGPR, and as organisms that degrade toxic substances (Chiarini et al., 2006). *Burkholderia* sp. ES4 forms a monophyletic subclade within the PBE group on the tree together with *Burkholderia graminis*

(U96939) which is a maize rhizospheric isolate (Veronique et al., 1998). The position of *Burkholderia* sp. ES4 in the PBE cluster makes it potentially a non-pathogenic bacterium, but this should be evaluated in more detail.

Results of present study showed that sugarcane has been a host plant for endophytic colonization of the genus *Burkholderia*. Among the 5 endophytic *Burkholderia* sp., the isolate *Burkholderia* sp. ES4 has shown maximum plant growth promoting activity like biological nitrogen fixation, mineral solubilisation, antifungal activity against red rot pathogen, siderophore production, IAA and GA production. There are many research papers supporting present work, the main breakthrough of this work was that these isolates were indigenous to the native sugarcane varieties grown in Tamil Nadu, thus could easily colonize the host plant when applied as biofertilizer inoculum.

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Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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