

## Hydroxamate Siderophores of Endophytic Bacteria *Gluconacetobacter Diazotrophicus* Isolated from Sugarcane Roots

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**Abstract:** Isolates of *Gluconacetobacter diazotrophicus* (L1, L2, L3, L4, L5, L6, L7, L8 and L9) from sugarcane roots and standard isolate of *G. diazotrophicus* PAL5 were tested for the siderophores production. Methods like FeCl<sub>3</sub> test, Chrome Azural S (CAS) plate assay, Hydroxamate test, Carboxylate test were used. In Ferric chloride test, all of the *G. diazotrophicus* isolates produced purple color, In Chrome Azural S (CAS) plate assay, the formation of bright zone with yellowish fluorescent color in the dark colored medium was well documented in *G. diazotrophicus* L5 followed by L3 and L7, light yellowish fluorescent color was produced by the remaining isolates. In Hydroxamate assay, *G. diazotrophicus* local isolate L5 and L3 recorded the highest value when compared to the other local isolates and standard isolate *G. diazotrophicus* PAL5. Neither of the isolates recorded positive for Carboxylate test which means the isolates does not produce carboxylate type of siderophores. Experiments for type of siderophores revealed that *G. diazotrophicus* local isolate L5 produced higher amount of salicylate and catecholate type siderophores when compared to the other isolates.

**Key words:**

### INTRODUCTION

*Gluconacetobacter diazotrophicus* an endophytic diazotroph also encountered as rhizosphere bacterium is reported to possess different plant growth promoting characteristics. *Gluconacetobacter diazotrophicus* originally isolated from sugarcane (Cavalcante and Döbereiner, 1988) was considered to be an endophytic diazotroph. Since then reports on *G. diazotrophicus* from the rhizosphere soils of sugarcane, coffee, ragi and rice reveals it as a prominent rhizosphere dwelling organism (Li and Mac Rae, 1991; Jimenez-Salgado et al., 1997; Loganathan et al., 1999; Muthukumarasamy et al., 2002). Moreover, the detection of *Gluconacetobacter azotocaptans*, a close relative of *G. diazotrophicus* only in the rhizosphere soil of corn strongly suggests its occurrence and survival in soil (Mehnaz et al., in press). Iron is an essential element required by almost all microorganisms except for some bacteria of the genera *Lactobacillus* and *Streptococcus* (Guisseppi and Fridovich 1982; Archibald, 1983). Microorganisms growing under aerobic conditions require iron for a variety of function including reduction of ribotide precursors of DNA, synthesis of ATP via electron transport chain, synthesis of heme and many other vital processes (litqin and Calderwood, 1993; Neilands 1995). Fe<sup>3+</sup> is chelated using nitrogen atoms of thiazoline and oxazoline rings in hydroxamate-type siderophores (Crosa and Walsh 2002). Ferrichrome is the classic hydroxamate-type siderophore. It is produced by a number of fungi including *Ustilago sphaerogena*. Although produced by fungi, ferrichrome is used by a number of bacterial species with the appropriate receptor protein (Höfte 1993). Aerobactin is another hydroxamate-type siderophore that is produced by many bacteria including *E. coli* (Buyer et al. 1991).

### MATERIAL AND METHODS

#### **Bacterial strains:**

*G. diazotrophicus* PAL5 was obtained from Dobereiner Lab, Brazil and another native isolate *G. diazotrophicus* L1, L2, L3, L4, L5, L6, L7, L8 and L9 were isolated from sugarcane roots.

#### **Media:**

The *G. diazotrophicus* strains were cultured in LGI medium (g l<sup>-1</sup> composition: sucrose 100, K<sub>2</sub>HPO<sub>4</sub> 0.2,

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$\text{KH}_2\text{PO}_4$  0.6,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.2,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  0.02,  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  0.002,  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  0.018, bromothymol blue, 0.5% in 0.2 M KOH 5 ml  $\text{l}^{-1}$ , pH 5.5) routinely (Cavalcante and Döbereiner, 1988).

#### Chrome Azurol S (CAS) Agar:

Production of siderophore by bacterial antagonist was assayed by plate assay. The tertiary complex Chrome Azurol S (CAS) /  $\text{Fe}^{3+}$  / hexadecyl trimethyl ammonium bromide served as an indicator. Forty eight hour old culture of the bacterial isolates was streaked to the succinate medium (Succinic acid- 4.0g,  $\text{K}_2\text{HPO}_4$ - 3.0g,  $(\text{NH}_4)_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$ - 0.2g, Distilled water- 1 liter, pH- 7.0) amended with indicator dye. To prepare one liter of blue agar, 60.5 mg of CAS was dissolved in 50 ml of distilled water and mixed with 10ml of iron (III) solution (1mM  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  in 10 mM HCl). While constantly stirring, this solution was slowly added to 72.9mg of hexadecyl trimethyl ammonium bromide (HDTMA) dissolved in 40ml of water. The resultant dark blue liquid was observed for the formation of bright zone with yellowish fluorescent color in the dark colored medium. It was the indication of production of siderophore. The result was scored either positive or negative to this test (Schwyn and Neilands, 1987).

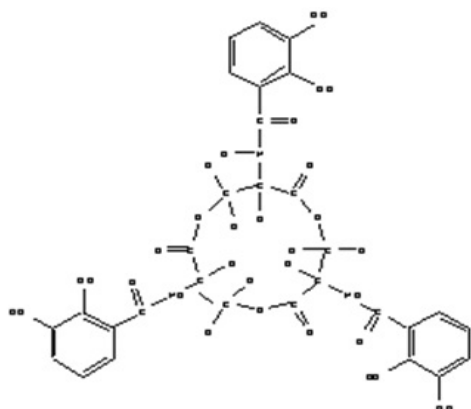
#### $\text{FeCl}_3$ test:

To 1 ml of culture filtrate 1-5 ml of ferric chloride solution was added. The formation of purple color indicated the presence of siderophores. (Neilands, 1981a)

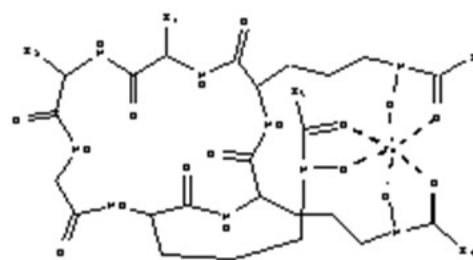
## RESULTS AND DISCUSSION

Siderophores play a vital role in the suppression of plant pathogen by chelation of Fe thereby creating competition for iron. Siderophore production under iron stress conditions confers upon these antagonistic organisms as an added advantage, resulting in exclusion of pathogens due to iron starvation. Although various bacterial siderophores differ in their abilities to sequester iron, in general, they deprive pathogenic fungi of this essential element since the fungal siderophores have lower affinity (Loper and Henkels, 1997; O'Sullivan and O'Gara, 1992).

Siderophores are classified on the basis of the chemical functional groups they use to chelate iron. Catecholate-type (phenolate) siderophores bind  $\text{Fe}^{3+}$  using adjacent hydroxyl groups of catechol rings.  $\text{Fe}^{3+}$  is chelated using nitrogen atoms of thiazoline and oxazoline rings in hydroxamate-type siderophores (Crosa and Walsh 2002). Ferrichrome is the classic Hydroxamate-type siderophore. The local isolate of *G. diazotrophicus* L5 was found to be the best in production of hydroxamate, salicylate and catecholate type of siderophores.



Enterobactin (catecholate type)



Ferrichrome (hydroxamate type)

Bano and Musaurat (2003) reported that *Pseudomonas aeruginosa* strain NJ-15 produced hydroxamate type of siderophores only. Although diverse group of siderophores are produced by many soil microbes in culture media only Schizokinen, a citrate-hydroxamate siderophore produced by *Bacillus megaterium* and *Anabaena* sp., has been purified and identified chemically from the soil (Akers, 1983).

*G. diazotrophicus* type strain PAL5 and all the local isolates recorded the production of siderophore on CAS agar plates. Appearance of yellow colored zone surrounding the growth on CAS agar plates and also the positive results of  $\text{FeCl}_3$  test unequivocally suggests the siderophore production. The *G. diazotrophicus* local isolate L5 recorded an instant appearance of deep red color by the addition of tetrazolium salt to siderophore

sample indicating that the nature of siderophore is hydroxamate type, followed by L3, L7, L9, L6, L4, L2, L8, reference strain PAL 5 whereas *G. diazotrophicus* local isolate L1 recorded slow appearance of red colour (Table 1).

*G. diazotrophicus* strains were plated in CAS medium and the production of siderophore was detected. Both the reference strain of *G. diazotrophicus* PAL5 and native isolates produced siderophores of hydroxamate type of siderophores. Among these cultures, the local isolate of *G. diazotrophicus* L5 was found to produce deep red colour, where as the type strain PAL5 produced light red colour in the tetrazolium salt test. The local isolate L5 produced  $133 \mu\text{g ml}^{-1}$  of salicylate type and  $126 \mu\text{g ml}^{-1}$  of catecholate type.

Carboxylate nature was determined by the disappearance of pink color on the addition of phenolphthalein to siderophore sample. All the strains recorded the appearance of pink color only. Hence there is no carboxylate nature of siderophore was produced by *G. diazotrophicus* strains.

Among the local isolates of *G. diazotrophicus* isolates, L5 recorded the highest production of salicylate type of siderophore ( $133 \mu\text{g ml}^{-1}$ ) followed by L3, L7, L9, L6, L4, L2, L8 (Table 2). When compared to the reference strain of *G. diazotrophicus* PAL and local isolates, catecholate type of siderophore was also produced in higher amounts by local isolate L5 ( $126 \mu\text{g ml}^{-1}$ ) (Table 2).

Chincholker *et al* (2000) reported that hydroxamate type of siderophore is produced by both antagonistic fungi and bacteria. Iron being a compound of cells, its deficiency can cause growth inhibition, decrease in nucleic acid synthesis, inhibition of sporulation and change in cell morphology. In addition, it also regulates the metabolic processes such as TCA cycle, electron transport chain, oxidative phosphorylation and photosynthesis. The deficit of available iron to pathogens might have resulted in death of the pathogenic organisms.

**Table 1:** Siderophore production by *G. diazotrophicus* cultures

S.No	Siderophore assay	G. diazotrophicus native isolates									G. diazotrophicus reference strain PAL5
		L1	L2	L3	L4	L5	L6	L7	L8	L9	
1.	FecI3 test	+	+	+	+	+	+	+	+	+	+
2.	CAS plate assay	+	+	++	+	+++	+	++	+	+	++
3.	Hydroxamate test	+	++	+++	++	+++	++	++	++	++	++
4.	Carboxylate test	-	-	-	-	-	-	-	-	-	-

(+) Positive reaction for the assay; (++) Appearance of light red color; (+++) Appearance of deep red color; (-) Appearance of pink color.

**Table 2:** Siderophore production (Salicylate and catecholate type) by *G. diazotrophicus* local isolates and reference strain.

G. diazotrophicus cultures	Salicylate type siderophore ( $\mu\text{g ml}^{-1}$ )	Catecholate type siderophore ( $\mu\text{g ml}^{-1}$ )
L5	133	133
L3	108	112
L7	89	97
L9	77	83
L6	68	71
L4	53	66
L2	51	62
L8	46	60
PAL5	40	57
L1	29	43

## REFERENCE

- Akers, H.A., 1983. Isolation of siderophore schizokinin from soil of rice fields. *Applied Environmental Microbiology*, 45: 1704-1706.
- Archibald, F., 1983. *Lactobacillus plantarum*, an organism not requiring iron. *FEMS Microbiol Lett.*, 19: 29-32.
- Bano, N. and J. Musarrat, 2003. Characterization of new *Pseudomonas aeruginosa* strain NJ-15 as a potential biocontrol agent. *Current Microbiology*, 46: 324-328.
- Buyer, J.S., V. de Lorenzo, and J.B. Neilands, 1991. Production of the siderophore aerobactin by a halophilic pseudomonad. *Applied Environmental Microbiology*, 57: 2246-2250.
- Cavalcante, V.A. and J. Dobereiner, 1988. A new acid-tolerant nitrogen-fixing bacterium associated with sugarcane. *Plant Soil*, 108: 23-31.
- Chincholker, S.B., B.L. Choudhari, S.K. Talegaenkar and R.M. Kothari, 2000. Microbial chelators, a sustainable tool for the biocontrol of plant diseases. In: *Biocontrol potential and its exploitation in sustainable*

agriculture vol. I, Crop disease, weeds and nematodes. (Eds.) Upadhyay, R.K., K.G. Mukerji and B.P. Chamola, Kluwen Academic/ Plenum Publishers, New York.

Crosa, J.H. and C.T. Walsh, 2002. Genetics and assembly line enzymology of siderophore biosynthesis in bacteria. *Microbiology and Molecular Biology Reviews*, 66: 223-249.

Guiseppi, J.D. and I. Fridovich., 1982. Oxygen toxicity in *Streptococcus sanguis*. *J Biol Chem*, 257: 4046-4051.

Höfte, M., 1993. Classes of microbial siderophores. In *Iron Chelation in Plants and Soil Microorganisms*. Barton, LB and Hemming, BC (Eds.). Academic Press, Inc.

Jimenez-Salgado, T., L.E. Fuentes-Ramirez, A. Tapia-Hernandez, M.A. Mascarua, E. Martinez-Romero and J. Caballero-Mellado, 1997. *Coffea arabica* L., a new host plant for *Acetobacter diazotrophicus* and isolation of other nitrogen fixing acetobacteria. *Applied Environmental Microbiology*, 63: 3676-3683.

Li, R.P. and I.C. Mac Rae, 1991. Specific association of diazotrophic acetobacters with sugarcane. *Soil Biology and Biochemistry*, 23: 999-1002.

Litwin, C.M. and S.B. Calderwood, 1993. Role of iron in the regulation of virulence genes. *Clin Microbiol.*, rev 6: 137-149.

Loganathan, P., R. Sunitha, A.K. Parida and S. Nair, 1999. Isolation and characterization of two genetically distant groups of *Acetobacter diazotrophicus* from a new host *Eleusine coracana* L. *Journal of Applied Microbiology*, 87: 167-172.

Loper, J.E. and M.D. Henkels, 1997. Utilization of heterologous siderophores enhances levels of iron available to *Pseudomonas putida* in the rhizosphere. *Applied Environmental Microbiology*, 65: 5357-5363.

Neilands, J.B., 1981. Iron absorption and transport in microorganisms. *Annu. Rev. Nutr.*, 27-46.

Neilands, J., 1995. Siderophores: Structure and function of microbial iron transport compounds. *J Biol Chem.*, 45: 26723-76726.

O'Sullivan D.J. and F. O'Gara, 1992. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. *Microbiology Review*, 56: 662-676.

Schwyn, B. and J.B. Neilands, 1987. Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry*, 160: 47-56.