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Research Article

AMINO ACID CONJUGATED HYDROXAMATE TYPE OF SIDEROPHORE PRODUCTION IN METHYLOBACTERIUM PHYLLOSPHAERAE MB-5

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ABSTRACT
Methylobacterium phyllosphaerae MB-5 and CBMB-27, as members of pink pigmented facultative methylotrophs (PPFM), were screened for their ability to produce siderophores under *in vitro* condition. Both these strains were found to be positive for hydroxamate type of siderophore production and negative for catecholate type of siderophores. Thin layer chromatographic analyses (TLC) of the purified siderophores of these strains confirmed the presence of hydroxamate type of siderophores. The bidimensional paper chromatographic analyses revealed that the amino acids *viz*., tryptophan and tyrosine were the conjugates of the siderophores produced by *Methylobacterium phyllosphaerae* MB-5 and CBMB-27. It was concluded that *Methylobacterium phyllosphaerae*, as a coherent member of PPFM, produced hydroxamate type of siderophores during iron starvation and tryptophan and tyrosine were found to be the conjugated amino acids of the same.

Key Words: *PPFM, Methylobacterium Phyllosphaerae, Hydroxamate Type of Siderophore, Amino Acid Conjugates*

INTRODUCTION
Iron is a vital nutrient and an essential element for the life of most microorganisms, including bacteria (Loper and Buyer, 1991). It also plays a crucial role in plant-microbe interactions (Mila *et al.*, 1996). This essential trace element is often used as a cofactor in key metabolic processes, including deoxyribonucleotide synthesis, oxidative phosphorylation, and electron transport (Briat, 1992). It is known as one of the factors that limits the bacterial growth in plants (Expert *et al.*, 1996). However, it is often sparingly available for the living organisms, such as, bacteria and made biologically available by iron-chelating compounds, such as, siderophores that are synthesized and secreted by many bacteria and fungi under the conditions of iron limitation (Neiland, 1995) in order to scavenge iron from the environment.

Bacteria have commonly developed extensive and efficient siderophore-mediated iron acquisition strategies to colonize habitats and to satisfy their requirements (Braun 1997). Bacterial siderophores are low-molecular weight compounds with high Fe3+ chelating affinity (Sharma and Johri, 2003) responsible for the solubilization and transport of this element into bacterial cells. Some bacteria produce hydroxamate-type siderophores while others produce catecholate-types (Neilands and Nakamura, 1991). In a state of iron limitation, the siderophore producing microorganisms are also able to bind and transport the iron-siderophore complex by the expression of specific proteins (Nachin *et al.*, 2001; Nudel *et al.*, 2001). The production of siderophores by microorganisms is beneficial to plants, because it can inhibit the growth of plant pathogens (Sharma and Johri, 2003).

Pink pigmented facultative methylotrophs (PPFM) are a coherent group of aerobic, gram-negative, free-living, phyllosphere microorganisms. Some representatives of this family can produce associative growth but not symbiotic growth with higher plants (Thompson and Skerman, 1979). PPFM are considered to be agriculturally important one because of their plant growth promoting characteristics *viz*., N_2 fixation, hormonal interaction and siderophore production.

The genus *Methylobacterium*, as a member of PPFM, has ubiquitous occurrence in the environment and plays an important role in iron acquisition. Holland (1997) reported the role of PPFM in iron nutrition of

Vicia faba. *Methylobacterium mesophilicum* was found to produce siderophores with methanol or galactose, as sole carbon source, under iron-limited condition. Silva-Stenico *et al*., (2005) reported the production of siderophores by *Methylobacterium extorquens* under *in vitro* condition. However, there were no earlier reports, available, on the isolation and characterization of siderophores produced by *Methylobacterium phyllosphaerae* MB-5, an ubiquitous phyllosphere microorganism, in lowland rice ecosystem.

Hence, the present investigation has been undertaken with an aim to explore the isolation and characterisation of siderophores produced by the phyllosphere PPFM of lowland rice crop *viz*., *Methylobacterium phyllosphaerae* MB-5 and CBMB-27 under *in vitro* condition.

MATERIALS AND METHODS

Methylobacterium phyllosphaerae MB-5, an efficient isolate from the phyllosphere of lowland rice cv. ADT-43, grown at Parangipettai, Cuddalore district, Tamil Nadu state and *Methylobacterium phyllosphaerae* CBMB-27 (reference strain) were used for the present study. The isolate, MB-5, was obtained by imprint method as described by Holland *et al*., (2000) in methanol mineral salts (MMS) medium (Green and Bousefield, 1983) and phenotypically characterised as *Methylobacterium phyllosphaerae* as per the description of Madhaiyan *et al*., (2006). The two *Methylobacterium phyllosphaerae* strains were maintained in MMS medium at 28±2˚C with weekly transfer.

Isolation and Estimation of Siderophore

Iron deficient MMS medium was prepared, pH of the same was adjusted to 7.0, dispensed in 100 ml aliquots in 250 ml Erlenmeyer flasks and sterilised. After the sterilisation, the medium was inoculated with 1 ml (10^7 CFU/mL) log phase culture of *Methylobacterium* MB-5 and CBMB-27, separately, and incubated for 7 days at 28 ±2˚C.

Extraction of Siderophores from Medium (Modi et al., 1985)

The spent culture fluid was separated from cells by centrifugation at 7000 x g for 20 min. The supernatant was concentrated to one-fifth of the original volume by flash evaporation at 45˚C.

Catechol type phenolates were extracted on ethyl acetate extracts of the culture supernatant twice with an equal volume of solvent at pH 2.0. The ethyl acetate layer was removed and evaporated to dryness and the residues were dissolved in a minimum quantity of distilled water while hydroxamate types were measured from the untreated culture supernatant of *Methylobacterium* isolates.

Qualitative Estimation of Siderophore

One volume of the Hathway's reagent (Reeves *et al*., 1983) was added to 1 volume of the sample and the development of wine colour showed the presence of phenolate like siderophores while the development of orange colour showed the presence of hydroxamate type of siderophores.

Quantification of Hydroxamate Siderophores by Methylobacterium MB-5 and CBMB-27

The production of hydroxamate type of siderophores by *Methylobacterium* MB-5 and CBMB-27 was estimated quantitatively according to Gibson and Mcgrath (1969). Hydroxylamine hydrochloride was used as standard and 1.0 µ mole of compound gave the absorbance of 0.1 at 526 nm.

Characterisation of Siderophore

A sample of approximately 1 mg of the siderophores was hydrolysed with 6 M-HCl. After neutralisation with 1 M NaOH, amino acids were separated and identified by employing paper chromatography.

Qualitative Analyses of Amino Acids Present in the Siderophores of Methylobacterium Phyllosphaerae MB-5 and CBMB-27

Bidimensional paper (Whatman No. 1) chromatography was carried out to separate the amino acid conjugates presented in pure hydroxamate siderophore from acid hydrolysed, untreated culture supernatant of the *Methylobacterium* isolates, namely, MB-5 and CBMB-27. Identification of the amino acids in the sample was done along with four different standard amino acids *viz*., tryptophan, tyrosine, lysine and ornithine using the solvent system of Butanol: acetic acid: water (4:1:5 v/v) and phenol: water (80:20 v/v) and sprayed with ninhydrin (0.25% w/v) in acetone.

Qualitative Analysis of Hydroxamate Present in the Siderophores of Methylobacterium Isolates

Thin layer chromatography (TLC) on silica gel G plates was carried out to separate the hydroxamate siderophores presented in the acid hydrolysed, untreated culture supernatant of the *Methylobacterium* isolates, namely, MB-5 and CBMB-27, using the solvent system n-butanol: acetic acid: distilled water (12:3:5, v/v). The spots were developed and compared with reference chromatograms of authentic samples of hydroxylamine hydrochloride in the same solvent system and under identical conditions.

RESULTS AND DISCUSSION

In the present study, the siderophore production of the *Methylobacterium* isolates, namely, MB-5 and CBMB-27, their qualitative and quantitative analyses were studied under *in vitro* condition.

Table 1: Siderophore production by *Methylobacterium* isolates

Methylobacterium Phyllosphaerae* Isolate	**Siderophore Production (µM/L)**		
	2,3-DHBA	Salicyclic Acid	Hydroxylamine Nitrogen
MB-5	-	-	4.95
CBMB-27	-	-	4.89

** -at 1 x 10^7 CFU/mL inoculum level*

It was observed that the *Methylobacterium* isolates *viz*., MB-5 and CBMB-27 showed positive results for hydroxamate type of siderophores and negative for catecholate type of siderophores. The isolate MB-5 recorded the maximum hydroxamate type of siderophore production *viz*., 4.95 µM L^{-1} while the CBMB-27 recorded the lowest production of siderophore (4.89 µM L^{-1}) (Table 1).

The acid hydrolysed, untreated culture supernatant, obtained from *Methylobacterium* isolates, namely, MB-5 and CBMB-27 were subjected to thin layer chromatography (TLC) on silica gel G plates for the qualitative analyses of hydroxamate type of siderophore. The hydroxamate compounds were separated on the basis of RF values in comparison with authentic purified samples of hydroxylamine hydrochloride and the results presented in Table 2.

Table 2: Identification of hydroxamate in the siderophore of *Methylobacterium* MB-5 and CBMB-27

Component	**RF Value**
Hydrolysate of siderophore MB-5	0.56
Hydrolysate of siderophore CBMB-27	0.52
Hydroxylamine hydrochloride	0.54

The production of various microbial siderophores and their qualitative and quantitative analyses has been reported by many workers (Ito and Neilands, 1958: Corbin and Bulen, 1969: Korth, 1970: O'Brien and Gibson, 1970: Pollack and Neilands, 1970: Neilands, 1981). Lacava *et al*., (2008) observed that members of the genus *Methylobacterium* which was frequently isolated as endophytes from citrus plants with CVC symptoms are able to produce siderophores. Silva Stenico *et al*., (2005) also reported that a strain of *Methylobacterium extorquens* isolated from *Citrus sinensis* was able to produce hydroxamate type of siderophore but negative for catechol type.

In the present study, the siderophores of *Methylobacterium* isolates *viz*., MB-5 and CBMB-27 were analysed separately with thin layer chromatography, and two separate spots were recorded with RF values *viz*., 0.56 and 0.52 for MB-5 and CBMB-27, respectively. As these values were relatively similar with the RF values of authentic hydroxylamine hydrochloride, the production of hydroxamate type of siderophores by *Methylobacterium* isolates MB-5 and CBMB-27 was confirmed.

Identification of Amino Acid in the Siderophore of Methylobacterium Isolates

The acid hydrolysed, untreated culture supernatant of *Methylobacterium* isolates, namely, MB-5 and CBMB-27 were subjected to bidimensional paper chromatography. The amino acids were separated on the basis of RF values in comparison with authentic samples amino acids *viz*., tryptophan, tyrosine, Lysine and ornithine and the results presented in Table 3.

Table 3: Identification of amino acid conjugates of siderophores produced by *Methylobacterium* isolates

Amino Acid		RF Value
Tryptophan		0.46
Tyrosine		0.34
Lysine		0.31
Ornithine		0.30
Hydrolysate Siderophore		
MB-5	Spot - (i)	0.45
	Spot - (ii)	0.35
CBMB-27	Spot - (i)	0.47
	Spot - (ii)	0.33

The authentic samples of tryptophan, tyrosine, lysine and ornithine recorded the RF values of 0.46, 0.34, 0.31 and 0.30, respectively. The hydrolysate of siderophore, obtained from the isolates *viz*., MB-5 and CBMB-27, recorded each two spots on paper chromatogram with RF values of 0.45 and 0.35; and 0.47, and 0.33, respectively. The results of the present study clearly revealed the hydroxamate type of siderophore production in *Methylobacterium* isolates *viz*., MB-5 and CBMB-27 and the same might be the conjugates of amino acids *viz*., tryptophan and tyrosine, as these values were relatively similar with RF values of authentic tryptophan and tyrosine samples. Sridevi and Mallaiah (2008) reported that the purified siderophores of *Rhizobium* sp. isolated from *Sesbania sesban*, were the conjugates of tryptophan and tyrosine amino acids. Simianato *et al*., (2006) also characterized the siderophores produced by *Methylobacterium mesophilicum* (ARS 1/5 and ARS 1/6 strains).

It was confirmed that the *Methylobacterium phyllosphaerae* isolates *viz*., MB-5 and CBMB-27 produced hydroxamate type of siderophore production during iron limitation. Moreover, the amino acids *viz*., Tryptophan and tyrosine were found to act as the amino acid conjugates of the same. However, this is a preliminary study on the topic and the role of iron concentration and medium composition on siderophore production, type of hydroxamate (di or Tri hydroxamate) production and time course on production maxima needs to be further exploited.

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