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Study of Siderophore Formation in Nodule-Forming Bacterial Species

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Abstract

Siderophores are potent ferric ion chelators produced by microbes like bacteria and fungi during iron stress. The objective of this study was to verify the siderophore-production in nodule- forming bacteria and then to compare them with pure cultures of Rhizobium meliloti bacteria. It was achieved by inoculating nodule-forming bacteria in YEM media followed by Chrome Azurol Sulfonate (CAS) assay, a universal siderophore detection method. Formation of orange halos in blue agar plates confirmed the CAS assay. Comparison of diameter of halos with the orange halos of pure strain revealed that the former were less effective in siderophore production. The detergent HDTMA (Hexadecyltrimethylammonium bromide) also appeared to be toxic for some isolates as indicated by negligible growth of the culture. This specificity of different bacterial strains towards siderophore formation and further chelation can be used as a model for identification studies of many human pathogenic bacteria and further to find a classic microbial species for iron-related poisoning.

Keywords: Siderophore, *Rhizobium meliloti*, YEM media, CAS assay.

Introduction

Siderophores are relatively low molecular weight, ferric ion specific chelating agents produced by bacteria and fungi growing under low iron stress. These compounds scavenge iron from the environment and make the mineral, available to the microbial cell[1]. Siderophores have been related to virulence mechanisms in microorganisms pathogenic to both animals and plants. In addition, they have clinical applications and are possibly important in agriculture[1]. Microorganisms growing under aerobic conditions needs iron for a variety of functions including reduction of oxygen for synthesis of ATP, reduction of ribonucleotide precursors of DNA, for formation of heme, and for other essential purposes. A level of at least 1 µM iron is needed for optimum growth. These environmental restrictions and biological imperatives have required that microorganisms form specific molecules that can compete effectively with hydroxyl ion for the ferric state of iron, a nutrient which is abundant but essentially unavailable[1].

Detection of siderophores is most readily achieved in iron limited media, which generally means either a synthetic recipe or introduction of a complexing agent that will render the iron selectively unavailable[1]. Although most siderophores are either hydroxamates or catechols, earlier tests based on such functional groups proved unreliable since they are absent from a few siderophores[1, 2]. The Chrome Azurol Sulfonate (CAS) assay has become widely used since it is comprehensive, exceptionally responsive, and more convenient than microbiological assays which, although sensitive, may be rigidly specific. The CAS assay may be applied on agar surfaces or in solution[3]. It is based on the color change that accompanies transfer of the ferric ion from its intense blue complex to the siderophore. A detergent must be present in order to achieve the intense color[1]. Gram-negative bacteria are impervious to detergents and hence the CAS reagent can be incorporated in the agar media where it has the potential for isolation of biosynthetic, regulatory, and transport mutants. For Gram-positive bacteria and fungi, the toxicity of the reagent must be mitigated in some way.

Identification of Rhizobial strains and especially of indigenous isolates continues to be one of the major shortcomings associated with competition studies. Because there is no universally accepted method, the method of choice depends on preference, experience, and equipment[4]. Here, an agar plate technique was used to distinguish strains and field isolates of Rhizobium to provide a basis for identifying nodule occupants in further competition studies. A rapid plate technique, based on differential growth characteristics, complements other techniques such as serological reactions, particularly when antisera cross-react with non-homologous strains. The technique involves culturing strains and isolates on chrome azurol S agar[4].

Siderophores produced by root nodule bacteria include carboxylates such as rhizobactin, citrate, anthranilate and catecholsoxamate. The ability to synthesize siderophores appears to be restricted to a limited range of strains rather than widely distributed in root nodule bacteria[5, 6].

Materials and Methods

Isolation of siderophore producing bacteria: Root tips from two leguminous plants present in two different farm locations in Raipur were taken. After washing the root system of the test

leguminous plant in running water, a well-formed, healthy pinkish nodule on the tap root was carefully cut out with a portion of the root attached to the nodule. The nodules were surface-sterilized for 5 minutes in 0.1% mercuric chloride in water and repeatedly washed with sterile water to get rid of the chemical[7].

The nodules were then washed in 70% ethyl alcohol for 3 minutes followed by more washing with sterile water followed by crushing with a sterile glass rod in a small aliquot of sterile water. Serial dilutions of the suspension were made to obtain sparse and distinct colonies when an aliquot of the appropriate dilution was inoculated on Yeast Extract Mannitol (YEM) agar medium (0.5g/L K_2HPO_4, 0.2g/L $MgSO_4$,0.1g/L NaCl, 0.5g/L yeast extract, 10g/L Mannitol, 15g/L agar)[7]. After a week incubation time, distinct colonies of bacteria were picked up and transferred to agar slants for further identification.

Chrome Azurol Sulfonate (CAS) assay: CAS dye measuring 60.5 mg was dissolved in 50 ml DI water (Millipore™ sterilized) and mixed with 10 ml iron (III) Solution (1 mM $FeCl_3.6H_2O$ and 10 mM HCl). Under stirring the solution was added to 72.9 mg HDTMA (Hexadecyltrimethylammonium bromide) dissolved in 40 ml DI water. The resultant dark blue solution was autoclaved. A mixture of 750 ml DI water, 100 ml 10X MM9 salts (60 g/L Na_2HPO_4, 30g/L KH_2PO_4, 5g/L NaCl, 10g/L NH_4Cl, 2ml of 1 M $MgSO_4$, 20ml of 20% glucose and 100 µl of 1M $CaCl_2$), 15 g agar and 0.1 M 10.29 g of Tris-HCl was made having pH of the solution 6.8. After cooling to 50°C, 30 ml of Tryptone as carbon source specific to Rhizobium species was added. Finally the dye solution added along the glass wall with enough agitation to achieve mixing without foaming[8,9].

The Rhizobial strains from the YEM media plates were taken and transferred to centrifuge tubes containing 5 ml saline solution and centrifuged at 5000 rpm for 10 minutes. The culture supernatant was collected and used further for CAS assay. For CAS assay the Blue agar plates were made with bored-wells in which 200 µl of culture supernatant was seeded. The plates were incubated in the dark at 28°C for 48h and examined for growth and production of orange halos surrounding the colonies[9, 10]. The CAS assay for siderophore production was compared with the halo formation in the pure culture of *Rhizobium meliloti* which was taken as standard for comparative analysis.

Results and Discussion

Twelve isolates were obtained when the samples were grown in YEM media. These isolates were then sub-cultured for isolation in pure culture form and CAS assay was performed using these different strains and thus compared with the reference *Rhizobium meliloti* strain shown in figure-1. When strains or isolates were incubated on CAS agar plates the following three responses were observed: no growth, growth but no halos surrounding colonies, and growth and small to large orange halos surrounding the colonies illustrated in figure-3. Results were visually distinct in terms of halo formation, because there was a contrast of orange halos against the blue medium. Response was consistent over all replicates when identical media were used, indicating that this technique is extremely reliable. Consequently in the presence of HDTMA, CAS is competitive in chelating the metal below neutral pH, while ferric hydroxide seems to have a higher stability at pH values above 7.

When orange halos from isolates were compared with pure strain of Rhizobium meliloti it was found that the diameter of orange halos from all isolates were less than the pure strain as shown in table-1.

Table-1
Study of Siderophore production by nodule-forming bacteria using CAS assay. In this table (+) means CAS assay was positive and orange halos were formed in blue agar plates while (-) means no growth and no halo formation. *Rhizobium meliloti* gave positive CAS assay with zone width of halo 1.70 ± 0.3. Some isolates showed no growth while some few formed small halos

Isolate	CAS assay (halo formation in blue agar)	Diameter of halo (in cm)
1	+	1.50 ± 0.2
2	+	1.40 ± 0.1
3	-	-
4	+	0.78 ± 0.2
5	+	1.30 ± 0.3
6	+	0.60 ± 0.4
7	+	0.80 ± 0.1
8	-	-
9	+	0.90 ± 0.4
10	-	-
11	+	1.10 ± 0.5
12	+	1.30 ± 0.1
R.meliloti	+	1.70 ± 0.3

Since the assay is based on the competitive exchange of iron (III), potential chelators are detectable corresponding to their affinity for the metal i.e., strong chelators like siderophores react in a 1: 1 ratio, while weaker ones need to be present in an excess. Hence, it is conceivable that, at least at higher concentrations, transition metal binding metabolites, especially antitumor antibiotics such as bleomycin, Adriamycin, and streptonigrin could also be detected.

However, it is difficult to grow fastidious microorganisms on the CAS agar plate and some ingredients of the CAS agar have innate antibacterial activity. The detergent HDTMA used in the preparation of CAS medium proved to be toxic to some bacteria as indicated by small halo formation in case of mixed rhizobial culture.

Conclusion

We successfully cultured nodule-forming bacteria particularly of Rhizobium species by using their growth specific YEM media. The orange halos formed by the isolates confirmed the universal CAS assay for siderophore production. While some of the isolates formed halos, others failed to do so because the growth media proved to be inhibitory to their growth. This was further confirmed by the smaller diameter of halos from all isolates when compared to the pure culture counterpart. We suggest that after optimizing every component of culture media the growth of every species can be studied and further siderophore formation can be assayed.

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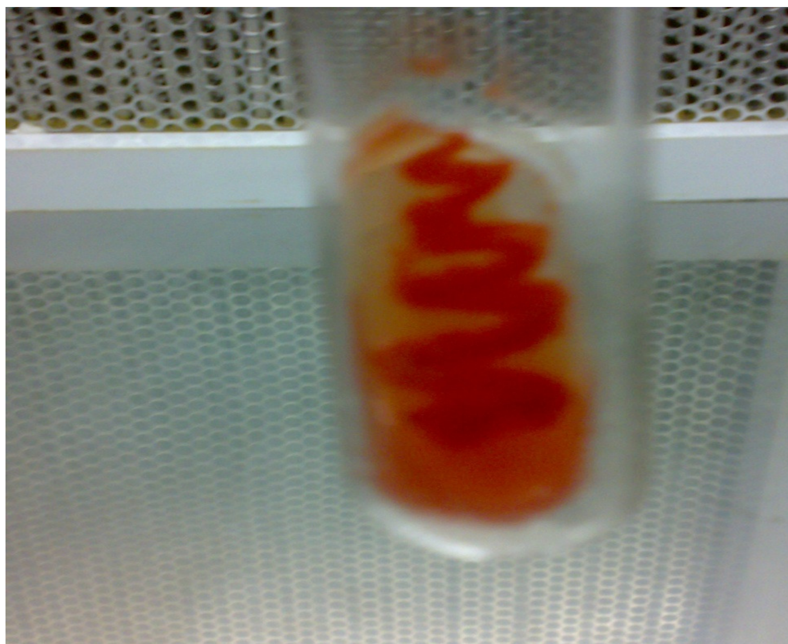

Figure-1
Pure culture of *Rhizobium meliloti*

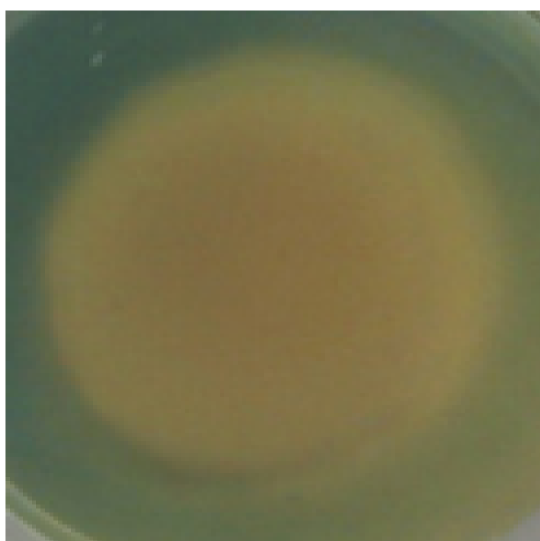

Figure-2
Orange halo formation of *Rhizobium meliloti*

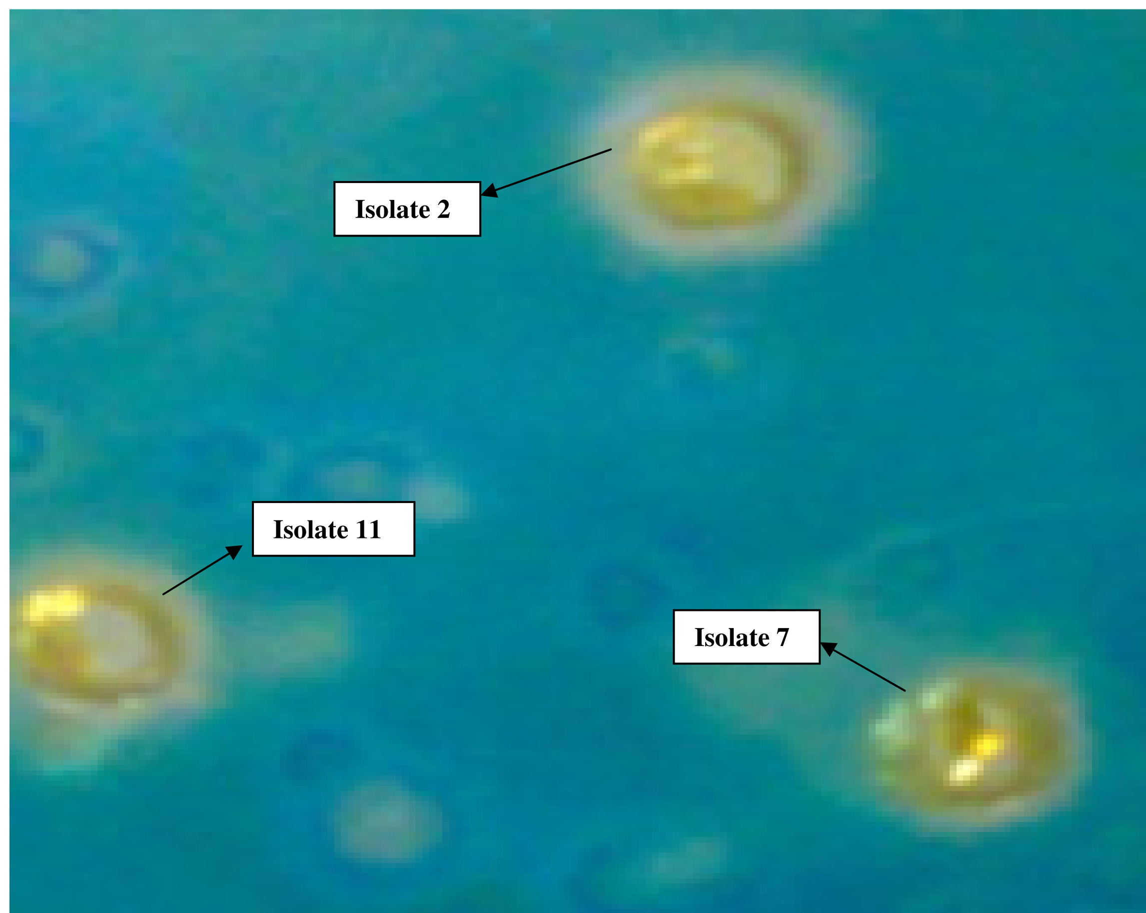

Figure-3
Formation of orange halo from isolates

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