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RESEARCH ARTICLE

Insect Gut Bacteria: A Novel Source for Siderophore Production

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Abstract Insect gut microbial community is known to produce biotechnologically important products. Present study is focussed on isolation of bacteria and screening of siderophore producing strains of *Sathrophyllia femorata* collected from Western Ghats region near Pune, Maharashtra, India (19°20′N and 73°47′E). Total 207 bacteria were isolated from gut of 2 different specimens (DM and EM) of grasshopper belonging to *S. femorata*. Out of 207 bacterial isolates, 166 (79 %) isolates produced siderophore during their submerged growth in iron deficient succinic acid medium. Among these, 24 isolates produced copious amount of siderophore and hence selected for further study. Further analysis of these isolates showed absorption maxima either at 240 or at 250 nm depending on type of siderophore. Molecular identification of these siderophore producing bacteria based on 16S rRNA gene sequencing confirms their affiliation to 37 species representing 19 genera. Dominance of phylum Proteobacteria followed by Firmicutes was observed. Among 19 genera, gammaproteobacteria like *Acinetobacter*, *Klebsiella* and *Pseudomonas* showed abundance over other genera like *Serratia*, *Stenotrophomonas* and *Yokenella*. Some of these bacteria have been used in plant growth promotion and as biocontrol agents against insect pests of plants. Siderophore producing bacteria having insecticidal activity against the insect pests promoting plant growth can serve as green bioinoculants for sustainable agriculture.

Keywords Western Ghats · Grasshopper · Insect gut · Iron

Introduction

Insects are ubiquitous in nature, they are widely distributed and are found everywhere on earth. Insects are one of the diverse groups of invertebrates and are important part of an ecosystem. They play key role in pollination besides serving as primary or secondary host for many pathogens. Despite of their significant role, they have not been fully studied because of their microscopic nature, limited habitat and restricted diet. Their studies have been restricted to their behaviour, life cycle, usefulness and pathogenicity only. Diversity of microflora of insect body and its impact on insect's activity offers good scope of study.

Insects are known to harbour diverse microflora in and on their body. Variety of microorganisms live symbiotically within the insect [1], they provide nutrients to the insects [2] and get ample food resources and shelter in its gut [3]. Many microbes are known to provide iron nutrition to their host partner. Such microbes secrete biomolecules called siderophores that help in sequestration of iron. The siderophores are produced and excreted outside the microbial cell to form complex with iron and transport this complex to the insect [4].

Iron is an essential and vital component for all living forms including microorganisms. It plays a key role in various biochemical processes like synthesis and activity

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of DNA, RNA, enzyme proteins, electron transfer chain and host defence mechanisms, etc. [5]. Although iron is abundant in nature, it is not available in soluble form. To solubilise it the microorganisms have evolved iron solubilising ligands called siderophores. Excess intake of iron causes iron toxicosis and may lead to the death of the insect. Iron is present in regular diet of insects [5]. Symbiotic bacteria present in insect help the insect to overcome iron toxicosis by sequestering the iron from ingested food and reduce the iron load. There are some opportunistic pathogens present in the insect gut which required iron for pathogenicity. They chelate the iron from insect ingested food by producing siderophore. Such pathogens compete with insect's iron transporter proteins as well as with symbiotic microbes in insect gut for iron sequestration [6].

There are numerous reports on various aspects of siderophore production and their applications for iron nutrition in plants and pathogenic microbes. However, there are no reports available on screening of siderophore producing bacteria present as symbionts in insect gut. Hence, the present study was undertaken to isolate, screen and identify siderophore producing bacteria from the gut of grasshoppers of species *Sathrophyllia femorata* [7] collected from one of the biodiversity hotspots in India i.e. Western Ghats, Junnar, Maharashtra which is known to harbour 25 % of total biodiversity in India.

Material and Methods

Collection of Insects

Grasshopper, *S. femorata* was collected in triplicate from biodiversity hotspot i.e. Western Ghats, Junnar, Maharashtra, India with location 19°20′N and 73°47′E and brought to laboratory for further studies.

Isolation and Preservation of Bacterial Cultures

Grasshoppers were anesthetized using chloroform, and dissected under aseptic conditions; their guts were removed and separately suspended in phosphate buffer saline (pH 7.0). These guts were aseptically homogenised., serially diluted up to 10^{-6} dilution and 100 µl of dilution from 10^{-3} to 10^{-6} were individually spread on 30 different types of media HK1-HK30 (Hi-Media, Mumbai, India). These media are used to cover maximum bacterial diversity. Plates were incubated for 48–72 h at 30 °C and observed for the appearance of bacterial colonies. Any bacterial colony appearing was picked up, purified and preserved in phosphate buffer saline containing 20 % glycerol [8].

Screening for Siderophore Production

Siderophores are produced under low stress of iron and hence siderophore production under laboratory conditions was carried out in iron deficient succinic acid media. For this purpose, all the isolates were initially revived and separately grown in 24 deep well plate containing 3 ml of succinic acid medium (SM). Chemical composition of SM was K_2HPO_4 (17.22 mM) or, KH_2PO_4 (14.69 mM), $MgSO_4.7H_2O$ (0.81 mM), NH_4SO_4 (8.76 mM), succinic acid (33.87 mM) at pH 6.8 [9]. Wells were sealed using breathable septa and kept in shaking incubator with 150 rpm at 28 ± 2 °C for 48 h.

Qualitative and Quantitative Estimation of Siderophore

After 48 h of incubation plates were centrifuged at 3800 rpm for 5 min to pellet down the cell. Cell free supernatant was used for siderophore assay by using Chrome Azurol S (CAS) test [10]. For this, 100 µl of cell free supernatant was dispensed into 94 well microtiter plates with equal quantity of liquid CAS reagent and observed for colour change. Quantitative estimation was done by CAS shuttle assay [11] in which 1 ml of cell free supernatant was mixed with 1 ml of CAS reagent and the absorbance was recorded at 630 nm.

Spectrophotometric Analysis

One ml of supernatant was scanned in the range of 200–1000 nm on UV–Vis spectrophotometer (Shimadzu, Japan, model Spectramax plus 384) and peaks at different wavelength were recorded [12].

Type Determination of Siderophore

Bacteria produce hydroxamate, catecholate or mixture of these two types of siderophores. In order to characterize the type of siderophore produced by isolates under study two tests were carried out. Csaky test [11] to detect presence of hydroxamate type of siderophore and Arnow test [11] to detect catecholate nature of siderophores.

Identification of Isolates by 16S rRNA Gene Sequencing

Siderophore producing bacterial isolates were identified using 16S rRNA gene sequencing. DNA was isolated using standard phenol Chloroform method [13]. PCR reaction was set using 27F and 1492R universal 16S primers with PCR conditions as follows; initial denaturation at 94 °C for 5 min, 35 cycles for 94 °C for 1 min, 55 °C for 1 min and

72 °C for 1 min with final extension at 72 °C for 7 min having final hold at 20 °C. PCR amplified products (>1400 bp) were purified and sequenced with ABI 3730xl automated sequencer using 'ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit' (Perkin Elmer, Applied Biosystems Division, Foster City, CA). The sequences were edited using ChromasPro version 1.34 and counting was done. The fasta sequences were used for identification. NCBI and Eztaxon database were used for sequence similarity.

Results and Discussion

Isolation and Preservation of Bacterial Cultures

A total of 207 isolates including 113 and 94 from gut of *S. femorata* strain id DM and EM respectively were obtained and purified. Pure cultures of these isolates were preserved in 20 % glycerol with phosphate buffer saline.

Screening for Siderophore Production

Out of 207 isolates, 166 (79 %) isolates showed ability to produce siderophore in iron free succinic acid medium (Fig. 1) detected using CAS assay. Cell free supernatant of siderophore rich broth of these isolates when mixed with CAS reagent changed the blue colour of CAS to orange red indicating the chelation of iron by siderophore present in supernatant. All these isolates produced siderophore in varying amount. However, 24 isolates produced copious amount of siderophore and thus were selected as potent siderophore producers (Table 1).

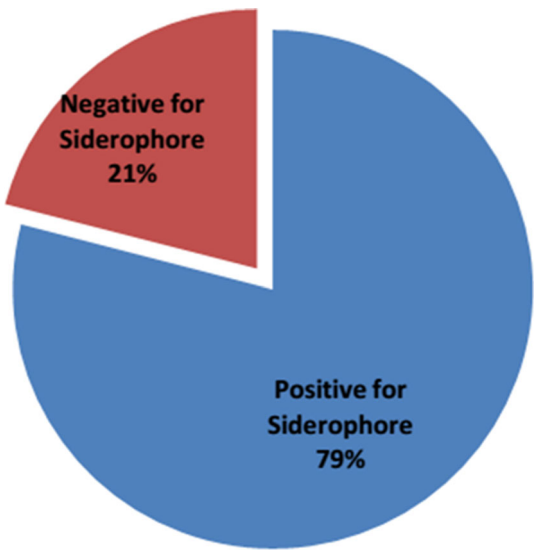

Fig. 1 Screening for siderphore production

Quantitative Estimation of Siderophore by Spectrophotometric Analysis

Siderophore rich cell free supernatant of isolates DM040, DM044, DM049, DM052, DM060, DM074, DM088, DM090, DM091 and DM 101 from grasshopper strain id DM showed maximum absorbance at wavelength between 240 and 250 nm (Fig. 2a) while the isolates EM003, EM004, EM005, EM007, EM011, EM015, EM017, EM034, EM037, EM048, EM056, EM067, EM068, EM075, EM077, EM081 and EM091 from grasshopper strain id EM showed maximum absorbance at 240 nm (Fig. 2b). The absorbance depends on type of siderophore present in the respective supernatant. Results showed that cultures from grasshopper strain id DM have diverse type of siderophore as compared to cultures from grasshopper strain id EM which has only one type of siderophore as it showed only single absorption maxima.

Type Determination of Siderophore

Siderophore rich cell free supernatant of isolates DM040, DM044, DM049, DM052, DM060, DM074, DM088, DM090, DM091 and DM 101 from grasshopper strain id DM yielded positive Csaky test and Arnow test indicating the presence of mixture of hydroxamate and catecholate types of siderophores. While the isolates EM003, EM004, EM005, EM007, EM011, EM015, EM017, EM034, EM037, EM048, EM056, EM067, EM068, EM075, EM077, EM081 and EM091 from grasshopper strain id EM yielded only Csaky positive test indicating presence of only hydroxamate type of siderophore. This can be correlated with the absorption maxima of sample containing mixture of siderophore (240–250 nm) and single type of siderophore absorbing at 240 nm. Since hydroxamate type of siderophores are comparatively stable with high iron chelating ability [14] imparting suppressiveness to soil which is important for preventing the growth of phytopathogens [14], its production has potential advantage for its exploitation in agriculture.

Identification of Isolates by 16S rRNA Gene Sequencing

All 166 siderophore producing bacterial isolates were identified belonging to 19 genera with 37 different species. The percentage similarity of all the isolates was between 99 and 100 %. Sequence analysis showed the dominance of phylum Proteobacteria followed by Firmicutes. Among 19 genera, gammaproteobacteria like *Acinetobacter*, *Klebsiella* and *Pseudomonas* showed dominance are genus *Serratia*, *Stenotrophomonas* and *Yokenella*. Gene sequences of these isolates were submitted to genebank with

Table 1 16S rRNA identification of siderophore producing isolates with genebank accession number and % siderophore unit

Strain ID	Length (bp)	Closest match	Similarity	Completeness (%)	Genebank accession number	% Siderophore unit
DM040	1437	*Klebsiella michiganensis* W14(T)	99.86	93.9	KT750842	29.58
DM044	1390	*Klebsiella michiganensis* W14(T)	99.85	93.9	KT750843	26.15
DM049	1320	*Serratia nematodiphila* DSM 21420(T)	99.62	100	KT750844	24.54
DM052	1373	*Serratia nematodiphila* DSM 21420(T)	99.85	100	KT750845	23.29
DM060	1408	*Klebsiella pneumoniae subsp. pneumoniae* DSM 30104(T)	99.5	100	KT750846	54.47
DM074	1359	*Pantoea eucrina* LMG 2781(T)	99.92	91.9	KT750847	29.04
DM088	1241	*Acinetobacter pittii* CIP 70.29(T)	100	100	KT750848	25.31
DM090	1181	*Klebsiella pneumoniae subsp. pneumoniae* DSM 30104(T)	99.49	100	KT750849	25.05
DM101	1312	*Delftia lacustris* DSM 21246(T)	100	100	KT750850	23.25
EM003	940	*Acinetobacter soli* CIP 110264(T)	99.79	100	KT750851	45.96
EM011	1411	*Pantoea eucrina* LMG 2781(T)	99.93	91.9	KT750852	39.31
EM015	1409	*Citrobacter freundii* ATCC 8090(T)	99.79	100	KT750853	32.48
EM017	1416	*Serratia marcescens subsp. sakuensis* KRED(T)	99.86	100	KT750854	31.15
EM034	1200	*Acinetobacter guillouiae* CIP 63.46(T)	99.25	100	KT750855	28.21
EM037	1250	*Pseudomonas hunanensis* LV(T)	99.68	97.7	KT750856	28.92
EM048	1320	*Exiguobacterium acetylicum* DSM 20416(T)	99.85	100	KT750857	31.52
EM055	1410	*Stenotrophomonas maltophilia* MTCC 434(T)	99.22	100	KT750858	52.58
EM056	1350	*Acinetobacter haemolyticus* CIP 64.3(T)	99.85	100	KT750859	32.93
EM057	1410	*Pseudomonas otitidis* MCC10330(T)	99.79	100	KT750860	52.06
EM067	1374	*Escherichia marmotae* HT073016(T)	99.42	100	KT750861	27.74
EM068	1369	*Lactococcus garvieae* ATCC 49156(T)	99.63	100	KT750862	40.04
EM075	907	*Pseudomonas oryzihabitans* NBRC 102199(T)	99.67	100	KT750863	33.07
EM077	1340	*Bacillus megaterium* NBRC 15308 = ATCC 14581(T)	100	100	KT750864	51.22
EM081	1285	*Acinetobacter johnsonii* CIP 64.6(T)	99.61	100	KT750865	43.70

respective gene bank accession number (Table 1). Proteobacteria, Bacteroidetes and Actinobacteridae are representative phyla of bacteria which are easy to culture as compare to other phyla like Firmicutes.

Isolate EM068 showing closest (99.22 %) similarity (99.6 %) with *Lactococcus garvieae* has so far been reported from fish [15]. *L. garvieae* is reported as fish pathogen but its virulence is negligible in human beings. This is the first report of this bacterium in insect and no other evidence was found to prove its pathogenicity in insect host. Another bacterial species, *Stenotrophomonas maltophilia* (isolate EM055) from this study is reported for the first time from the gut of grasshopper and it is well established as plant growth promoting bacteria from rhizospohere [16]. However its exact role in the gut of grasshopper has to be explored.

Insect pests are the major threats to agriculture crops causing pre-harvest and post harvest losses to agriculture crops. These insects have been traditionally controlled by using hazardous chemicals that poses additional damage to the agro-ecosystem. Use of insect antagonists against insect pests has been seen as an alternative and eco-friendly strategy [17]. Antagonistic insects carry pathogenic bacteria in their gut that scavenge iron by producing siderophore and thus prevent iron nutrition of insect subsequently restricting its growth. Choosing and employing microbes from insect gut microflora that is harmless to human being against insect pest are expected to suppress their population.

Primary screening of siderophore showed that 79 % of total gut bacterial population was positive for siderophore production. All potent siderophore producing bacteria were dominant as well as rare bacterial genera as compared to total insect gut microbial diversity. Most of the genera as presented in Table 1 are pathogenic to plants or animals. Siderophore production is one of the important features of virulent bacteria [18]. Siderophore also helps the bacteria to colonise in insect gut [19].

Acinetobacter and *Klebsiella* were found as dominant siderophore producing bacteria. They have been previously

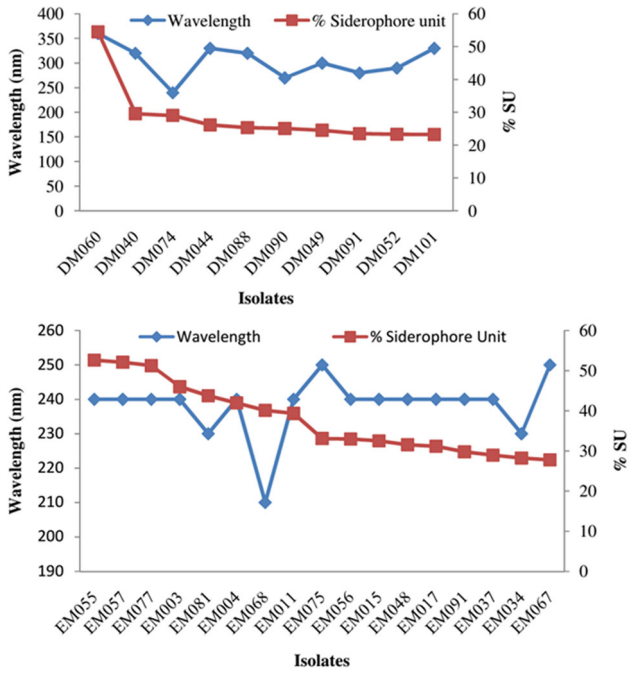

Fig. 2 Quantititive estimation of sideophore and estimation of λ-max by spectrophotometric assay

reported for siderophore production. Their siderophore production has been correlated with their growth and pathogenicity. Higher number of these genera in insect gut may help the insect in its iron nutrition.

Bacteria that are pathogenic to insects and help in plant growth promotion can be considered as biocontrol agent and eco-friendly alternative to synthetic chemical insecticides. Genus *Serratia* is well known example of insect pathogen [20, 21] having protease, gelatinase, DNase, chitinase activity along with siderophore production [20]. Siderophore producing bacteria isolated from rhizosphere are known for their biocontrol activity [6, 22, 23]. This study will lead to the emergence of biocontrol agents from insect gut microflora.

Conclusion

Abundance of siderophore producing bacteria in grasshopper gut, their species biodiversity and diversity of siderophore production can be taken as a good indication for the further search of siderophore producing bacteria in other insects from Western Ghats region. These diverse genera included pathogenic as well as non pathogenic bacteria from phylum Proteobacteria and Firmicutes. Proteobacteria are larger in number as compared to Firmicutes. Among these 79 % of bacteria produced more or less same amount of siderophore. The result in this study indicated that siderophores production may be useful for iron acquisition in gut and also may contribute to antagonistic effect against pathogenic bacteria as siderophore acts to prevent the iron nutrition of other microbes. Siderophore production is one of the virulence characters of bacteria. Thus siderophore producing bacteria are helpful in biocontrol of insect pests.

Insect pests are the major threats to agriculture crops causing pre-harvest and post harvest losses to agriculture crops. Application of siderophore based insect antagonists against insect pests can be employed as one of the best alternative and eco-friendly strategy to control insect pests and prevent plant diseases [19], provided that such antagonists are not pathogenic to humans. Choosing and employing microbes from insect gut microflora that are harmless to human beings against insect pests are expected to decrease their population.

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Compliance with Ethical Standards

Conflict of interest Authors do not have any conflict of interest.

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