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Convergent evolution in bacteria from multiple origins under antibiotic and heavy metal stress, and endophytic conditions of host plant

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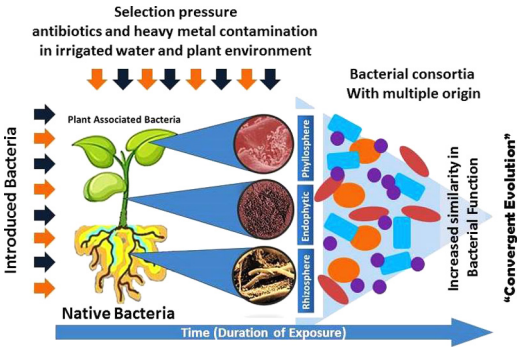
HIGHLIGHTS

- Multiple antibiotics and heavy metals resistance in endophytic bacteria were observed.
- The endophytic bacteria were found to be multiple origins.
- Native bacteria acquired antibiotic and heavy metal resistance.
- Introduced bacteria acquired plant growth promoting traits.
- Irrespective of origin, bacteria enhance the host plant fitness under abiotic stress.

GRAPHICAL ABSTRACT



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ABSTRACT

The focus of this work is to study the convergent evolution in bacteria from multiple origins under antibiotic and heavy metal stress, and endophytic conditions of host plant cultivated on the Yamuna river bank. Forty-one endophytic bacteria (EB) were isolated from green leafy vegetables (GLV's) and were found to be resistant to a wide range of antibiotics (AB) and heavy metals (HM) tested. Further, they showed susceptibility to Quinolones group of antibiotics, and the HM, Cadmium, Chromium, and Mercury. Twenty-seven percent of these bacteria endowed with Class I integron. The probability of co-existence of HM resistance with β lactams was higher, whereas quinolones group of AB recorded lesser values. These EB owned a wide array of beneficial traits, through which they improved the plant health under HM and salt stress conditions. Bacterial identity revealed the association of both plant beneficial and human pathogenic bacteria as an endophyte with GLV's. Principal component analysis showed a pattern of convergent evolution irrespective of their origin. In conclusion, under the selection pressure of AB and HM, the susceptible EB population may reduce with time and the resistant native/introduced bacteria might survive. The vertical and horizontal gene transfer between introduced and native bacteria is the crucial factor in enhancing their fitness along with the host plant to survive under abiotic stress conditions.

1. Introduction

Antibiotics (AB) and heavy metals (HM) are the most concerned emerging pollutants adversely affecting the environment at the global level. The constant release of these emerging contaminants to the water bodies leads to a long-term unfavorable effect on the aquatic and terrestrial organisms. In case of microbes, their response to these

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pollutants in order to survive leads to the development of AB and HM resistance. In recent years, river systems are becoming a hotspot for water pollution and acting as media for the dispersion of pollutants and pathogenic microbes in the environment. The Yamuna is one such holy Indian river which originates from Yamunotri glacier, 6387 m above mean sea level. Inside Delhi, the river runs about 22 KM from Wazirabad to Okhla barrage and is the major pollution donor to the river adding 80% of the contaminants (Jain, 2004).

The highest level of pollutants was seen in the floodplains which can be associated with treated and untreated effluents or with sewage flowing into the river. As per the study which was undertaken by The Energy and Resources Institute (TERI), the intensity of nickel (Ni), Manganese (Mn) and Lead (Pb) in Yamuna water were found elevated than the international water quality standards for fresh water. Additionally, the level of Ni, Mn, and Mercury (Hg) in the agricultural soil along the river was beyond the acceptable limit (Vodyanitskii, 2016). Likewise, the average HM concentration at different locations in the river water and soils varied in the order of Fe > Cr > Mn > Zn > Pb > Cu > Ni > Hg > As > Cd and Fe > Mn > Zn > Cr > Pb > Ni > Hg > Cu > As > Cd, respectively (Sehgal et al., 2012). Similarly, Yamuna river water collected at different sites recorded 0–13.75 μg l^{-1} of ampicillin contamination. A maximum concentration of 104.2 μg l^{-1} and 12.68 μg l^{-1} for ampicillin was recorded in the wastewater influents and effluents respectively on the river banks of Yamuna was recorded (Mutiyar and Mittal, 2014). Further, a research group from All Indian Institute of Medical science (AIIMS) in 2015, reported Fluoroquinolone, Macrolides, and Penicillin as common contaminants of Yamuna river water.

The existence of these pollutants in nature acts as a selection pressure on the living organisms. In order to survive under such conditions, the organism must develop a mechanism to handle these pollutants without harming themselves. “Bacteria” is one such organism that has mastered this art by surviving under such conditions and adopting various strategies to mitigate the adverse effects of AB/HM. As there is a possibility that the resistance genes for both AB and HM might be closely placed on the same plasmid, therefore they are more likely to be conveyed collectively in the environment (Calomiris et al., 1984). The combined effect of HM and AB may selectively increase the population of bacteria which are multidrug-resistant (MDR) posing a serious threat to the animal kingdom. In addition to this, MDR genes may transfer horizontally or vertically via several mechanisms including transformation, conjugation, transduction, integrin-mediated mobilization of gene cassettes, transposition, etc. This phenomenon plays a crucial function in contributing to the terrifyingly high pervasiveness of bacterial drug resistance.

Rhizosphere of a plant is considered as a rich source of nutrients and provides shelter to many beneficial bacteria which directly or indirectly improve the plant growth and health. Further, these bacteria may exist in lower population as endophytes and also in the phyllosphere. Endophytic microbes are ubiquitous found associated as obligate or facultative in almost all plants tested. Endophytes are host specific and within plants, their occurrence may be limited to specific tissue and their community varies with the age of the plant. The molecular mechanism of interaction between host and endophytic microbes remain elusive. In the early interactions, host plant tries to limit the growth of endophytic microbes through different structural and biochemical defense mechanism. Successful endophytes are endowed with counteracting mechanism which eases the process of survival and further colonization, and benefits to host plant (Lee et al., 2009; Dudeja et al., 2012: Kandel et al., 2017).

Because of AB and HM selection pressure, plants cultivated on the polluted river banks act as a site of harboring the bacteria which are resistant to multiple AB and HM. By considering these circumstances, it is hypothesized that the probability of occurrence of MDR (both native and introduced) associated with GLVs cultivated on the river banks of Yamuna is high. Also, with time, vertical and horizontal gene transfer between introduced and native bacteria lead to the convergent evolution resulting in plant growth promoting traits in introduced bacteria and AB and HM resistance in native bacteria. Here, we focused on EB as it is protected from the external environment and comparatively their chances of entering into the food chain are higher.

2. Materials and methods

2.1. Collection of plant materials

The green leafy vegetables (GLVs) viz., water spinach (*Ipomoea aquatic*), Malabar spinach (*Basella alba*), Red Amaranth (*Amaranthus* sp.), Radish (*Raphanus raphanistrum* subsp. *sativus*) and Methi (*Trigonella foenum-graecum*) were collected from lower region of the Yamuna river bank of Delhi from 3 different sites namely, Okhla barrage (Site-1); Madanpur Khadar colony (Site-2) and Jaitpur sector 12 (Site-3) (Suppl. Fig. S1). These fields have been irrigated using the Yamuna water at least for the past 5 years. The samples were labeled and placed separately in polyethylene bags, transported to the laboratory and processed immediately.

For laboratory experiments, methi seeds were obtained from National Seeds Corporation Limited (NSC, Pusa Complex, IARI). Seeds were surface sterilized with 70% ethanol (30 s) followed by 0.5% NaOCl (30 min) and were washed three times with sterile water, blot-dried and used throughout the experiment.

2.2. Isolation of endophytic bacteria (EB)

The collected samples were thoroughly washed under running tap water, blot-dried and were cut into small bits (≈2 cm for stem and 1 cm^2 for leaf). The sample pieces were surface sterilized by immersing consecutively in 70% ethanol (v/v) for 1 min and 3.5% sodium hypochlorite for 3 min and then rinsed thrice with sterile distilled water. Aseptically blot-dried samples (~1 g) were ground in 50 ml PBS using prechilled sterile mortar and pestle on ice flakes. The suspension was centrifuged at 3000 rpm at 4 °C for 8 min. The supernatant was serially diluted and 50 μl from each dilution was spread plated on Nutrient agar (NA) (supplemented with nystatin 500 mg/L). The plates were incubated at 35 ± 1 °C for 24–36 h. The plates were observed for the morphologically distinct bacterial colonies and were pure cultured on NA slants. Long-term storage of each bacterium was done in 40% glycerol at −86 °C and for regular use; these bacteria were routinely subcultured on NA medium.

2.3. Antibiotic resistance pattern analysis in EB

The endophytic bacterial isolates were screened against 47 AB belonging to 16 different classes which were frequently used to treat bacterial infection in human and animals. Standard disk diffusion method was followed as per The Clinical & Laboratory Standards Institute (CLSI, 2007). The AB tested were imipenem (IPM), meropenem (MRP), amoxicillin (AMX), penicillin-G (P), aztreonam (AT), cefotaxime (CTX), cefepime (CPM), cephalexin (CN), cefuroxime (CXM), cefadroxil (CFR), ceftazidime (CAZ), cefpirome (CFP), ceftriaxone (CTR), ceftizoxime (CZX), methicillin (MET), augmentin (AMC), cloxacillin (COX), cephradine (CH), ciprofloxacin (CIP), moxifloxacin (MO), norfloxacin (NX), ofloxacin (OF), sparfloxacin (SPX), levofloxacin (LE), gatifloxacin (GAT), tobramycin (TOB), gentamicin (GEN), amikacin (AK), streptomycin (S), isepamicin (IP), azithromycin (AZM), erythromycin (E), vancomycin (VA), doxycycline hydrochloride (DO), tetracycline (TE), nitrofurantoin (NIT), co-trimoxazole (COT), sulphafurazole (SF), trimethoprim (TR), chloramphenicol (C), polymyxin B (PB), colistin (CL), fusidic acid (FC), pristinamycin (RP), novobiocin (NV), lincomycin (L) and rifampicin (RIF) (Himedia, India).

The test bacteria were grown on NB for 12 h at 35 ± 1 °C on a rotary shaker (150 rpm). 100 μl of bacterial culture from log phase was spread plated on NA and plates were allowed to surface dry for 10 min