Chapter - I

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INTRODUCTION

Environment offers specific alcove for the omnipresent microorganisms, which are conferred with unique physiological, genetic and metabolic features of adaptive value. Bacteria represent an elite group which can adapt to the changing environmental conditions faster than others. An ‘extreme’ environment is a place where living conditions are severe and are challenging to life forms. Such environments include extreme temperatures, radiation, pressure, acidity, alkalinity, air, water, salt, sugar, carbon dioxide, hydrocarbons and the like. Extremophiles are microorganisms that withstand such conditions. They are mainly unicellular and prokaryotic. Petrophilic bacteria are part of the extremophiles and can withstand high concentration of petroleum oil and the constituent hydrocarbons prevalent thereof in the contaminated site. The flouting and fracas of environment by humans has resulted in the alteration of ecological niches in which the microorganisms face a new blend of stress, which in turn tune them with adaptive virtues. Humans have altered and contaminated the ecological niches by releasing the effluents of hydrocarbons, heavy metals, organic solvents, tannery, textile and other xenobiotics into soil, water and air.

1.1 Petroleum Spill

Petroleum oil is a complex mixture of hydrocarbons, organic solvents and heavy metals. Petroleum oil spills occur in many ways. There is a low level spillage of oil from storage tanks, ship lanes and pipe lines. Major oil leakage happens from tankers, pipe lines and storage tanks into terrestrial and aquatic environments. The colossal release of oil from large marine ships and industrial effluents occurs while transporting bulk volumes. Petroleum spill causes immense pollution in soil and water, and propose to be major environmental pollutants. Petroleum contaminated soils are characterized by high salt content, heavy metals, polycyclic aromatic hydrocarbons, organic solvents, and are loaded with extreme pH. Petroleum spill exhibits significant challenges to the native microbes in the contaminated site for the
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following reasons: the lower molecular weight hydrocarbons exhibit toxicity owing to
the solvent effects on bacterial cell membranes; most of hydrocarbons present in the
oil exhibit limited solubility in water and hence are resistant to biodegradation; and
heavy metals such as vanadium, arsenic and lead challenge the bacterial growth by
inhibiting the enzymes.

1.2 Organic Solvents and Heavy Metals

Petroleum contaminated soils often consist of high concentration of organic solvents
and heavy metals, which challenge the survival of the native microorganisms. Since
organic solvents and heavy metals are toxic to microbes even at low concentrations,
unique bacterial extremophiles tend to withstand the harmful effects of the organic
solvents and heavy metals. Organic solvents can be degraded easily by the microbes
whereas the immutable nature of the heavy metals makes them persist in the soil. To
survive under the dual stress, bacteria prefer to undergo adaptation mechanisms at the
molecular as well as physiological levels. The latter encompasses efflux of solvents
and metal ions out of the cell, degradation of the toxic solvents, accumulation and
inactivation of the metal ions inside the cell, and reduction of the heavy metal ions to
less toxic states (Nies, 1999). Since the composition of petroleum oil varies from site
to site, the complex organic and heavy metals constituents alter the composition of the
soil, air and water in many ways. The surviving organisms could be classified under
polyextremophiles since they face a blend of the stress.

1.3 Bacterial Adaptation in Petroleum Spills

Oily sludge is a complex mixture of total petroleum hydrocarbon, water, and soil
particles. Total petroleum hydrocarbon is primarily composed of alkane, aromatics,
nitrogen-, sulfur-, and oxygen-containing compounds, and asphaltene fractions
(Lazaroiae, 2010). The alteration of environmental conditions by petroleum
contamination may generate a new reliable stress on native bacteria which it never
faced before. Bacteria surviving the petroleum contamination tend to show diverse
adaptations mechanisms at the molecular as well as cellular level by altering the
echelon of gene expression patterns (Chowdhury et al., 1996). This results in rapid
changes in bacterial interior chemistry, surface chemistry and genetic information.
Bacteria tend to alter physiological and/or genetic process in response to extreme environmental conditions and accumulate it over a course of time through generations. Such process of adaptation could be of either intrinsic or acquired. Some bacteria undergo intrinsic adaptation without altering the genetic constituents (Normark and Normark, 2002).

Intrinsic adaptive mechanisms of bacteria could be of changes in structure, behaviour or physiology. Structural adaptations help with alterations that minimize contact with the stressful environment. Behavioral adaptation results in the aggregation of individual cells to adhere to a hydrophobic surface and maintain a mixed socio-community of different bacterial cells. Physiological adaptation of bacteria involves adjustment in metabolic and genetic mechanisms according to the fulsome environments. Some bacteria acquire adaptation leading to acquiring genetic traits from other bacteria that enable them to withstand extreme conditions. This is in contrast to acclimation, which refers to short-term physiological adjustments that occur during a lifetime in response to transitory changes in environmental conditions.

To successfully colonize petroleum contaminated environments, bacteria have evolved a number of strategies that range from molecular to whole cell to ecosystem levels. Normally the bacteria tackle the petroleum contamination in two distinct modes of behaviour - the freely existing independently surviving planktonic cells and the aggregated cells attached to the hydrophobic surfaces in biofilm encased in extracellular polymeric substances.

1.4 Planktonic Cells

Planktonic cells are free-living cells which divide rapidly, have a high rate of metabolism, carry genes for numerous protective stress responses and are readily overwhelmed by strong environmental and antimicrobial challenges. In planktonic cell population, chemical signals produced by one cell will not be enough to induce genetic changes in neighbouring cells and hence these cells die before stress responses can be activated. They exhibit significant phenotypical, biochemical and morphological differences with bacteria that form biofilm aggregation. Planktonic cells encounter petroleum contamination physiologically by resorting to a number of strategies. They
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include: altering the cell surface hydrophobicity and charges, minimizing contact with the hydrocarbons, producing bioemulsifiers and exopolysaccharides to emulsify and degrade the hydrocarbon constituents, aggregating among themselves or co-aggregate with other bacteria, coating themselves with exopolymeric substances (EPS), siphoning out the harmful organic solvents and heavy metals by efflux pumps, excluding themselves from the contaminated site by chemo-sensing the pollutants, altering the lipid content of plasma membrane - thereby excluding the entry of solvents into the cytoplasm, and floating on the oil. The accumulation of hydrocarbons results in the disruption of bilayer stability of the membrane structure. This causes impaired membrane function, thereby leading to leakage of RNA, phospholipids and proteins resulting in cell death.

1.5 Biofilm Community

Under critical environmental situations, bacteria prefer to form biofilm aggregates as a means of protection against nutrient deprivation, pH changes, oxygen radicals and antimicrobials. Biofilm is a communal interaction of bacteria that attach to hydrophobic surface. The matrix which holds bacterial biofilms together is a complex mixture of exopolymeric substances with polysaccharides, proteins and extracellular DNA. Biofilm shows high tolerance and resistance, coupled with slow growth, persistors and secretion of EPS, which protects the embedded bacteria from the harmful effects of the organic solvents, antibiotics, heavy metals, detergents (Stewart et al., 2002; Kazy et al., 2002). In biofilms, bacteria produce enough chemical signals that can reach and cause changes in cellular behaviour by switching ON/OFF their genes by responding to environmental changes. Apart from tackling environmental challenges, biofilm also provides an ideal niche for the exchange of genetic traits among commonly dwelling bacteria to resist environmental challenges.

1.6 Lateral Gene Transfer

Bacteria possess genome plasticity and are genetically diverse, and hence are very efficient in acquiring novel genetic traits required to colonize new environment (Steinert et al., 2000). Highly controlled genetic networks and metabolic regulation are responsible for the ability of bacteria to live and survive under constantly
changing environmental conditions. Genetically, bacteria respond to petroleum contamination by several mechanisms such as induction of appropriate genes to resist or degrade the critical hydrocarbon compounds, single nucleotide changes or rearrangements in DNA and acquiring genetic information laterally. Among these mechanisms, bacteria prefer to adapt through acquiring new genes via horizontal gene transfer. Unable to reproduce sexually, the bacterial species embedded in the matrix evolve this mode of gene transfer.

The adaptation mechanisms of both the biofilm and planktonic cells are underpinned by new gene cassettes from other bacteria. Adaptation mechanisms such as catabolism of pollutants, altering the surface hydrophobicity and surface charges, bioemulsifiers, autoaggregation / coaggregation, efflux pumps, biofilm formation could be undertaken by bacteria only if the mobile elements recruit appropriate gene cassettes. The cassettes encode for the proteins/enzymes needed for the adaptive mechanisms.

1.7 Mobile genetic elements - Integrons and Gene Cassettes

Mobile genetic elements are jumping genes that can translocate and move within the genome. There are classes of MGE that contribute to the transfer of genes such as plasmids, transposons and bacteriophages. One of these elements, the integrons, is the main subject of the present research, since they maintain a platform for essential genes for the deprived bacteria to acquire and survive the imposing environmental conditions. An integron is a two-component gene capture and dissemination system found in plasmids, chromosomes and transposons. The presence of the genes encoding site specific recombinase and integrase in the integron helps in acquiring and integrating the gene cassettes of relevance to the host genome without affecting its native gene content (Michael et al., 2004). Gene cassettes contain functional genes, which may encode proteins or enzymes conferring resistance to antimicrobial substances and quaternary ammonium compounds. The ability of the free planktonic cells and the biofilm community to adapt to petroleum contamination is attributed to the integrons and their associated gene cassettes.
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STATE OF THE ART OF THE RESEARCH TOPIC

Petroleum spills remain a huge problem of contaminating land and waters, and the associated environments. Petroleum hydrocarbons are a multifarious concoction of organic solvents, hydrocarbons and heavy metals. The contaminated soils provide us with an environment ideal to investigate bacterial adaptation, in which the role of integrons is critical. Bacteria tackle petroleum contamination by independent life forms of planktonic cells as well as cell associations, called biofilm. Planktonic bacteria normally seek to “fish out” external gene cassettes from relevant bacteria. This trap is aided by integrons. Integrons could also be a backbone of the extracellular DNA (eDNA) present in the biofilm EPS, helping preserving the three dimensional structure of the biofilm. The overall process runs concertedly with changes in the interior chemistry, cell surface chemistry and genetic combination. Together they confer the cells with the ability to withstand the harmful effects of organic solvents and heavy metals.

DEFINITION OF THE PROBLEM

Bacteria usually react to the hydrocarbon contamination by inducing adaptation mechanisms such as acquiring catabolic genes to degrade the hydrocarbons, producing bioemulsifiers, aggregation and co-aggregation between bacteria, efflux pumps, chemotaxis, floating behaviour and altering the membrane chemistry (Edward et al., 2011). These changes go hand in hand with recruiting and exchanging essential genes via horizontal gene transfer. The biofilm forming aggregation is encased in matrix of EPS.

Apart from the eDNA, the extracellular matrix consists of a gene pool for horizontal gene transfer among bacteria. Under critical situations, bacteria prefer biofilm over planktonic cells. The former exhibits higher resistance to organic solvents and heavy metals than the latter, and does not suffer from growth inhibition and killing by antimicrobials (Lima et al., 2011). The integrons cushion the backbone of the eDNA, and stabilize the structure of the biofilm while performing the role of mobile element to recruit gene cassettes. Bacteria recruit exogenous gene cassettes essential for the survival of both free planktonic cells and biofilm associations. The integrons provide
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bacteria with a “gene-fishing” system, which endows the recipient bacteria with the capacity to mobilize adaptation-related genes to circumvent the environmental pressure (Cambray et al., 2011). The integrons have been reported to acquire gene cassettes essential for exhibiting antibiotic resistance (Guo et al., 2011) and similar environmental stress (Elsaied and Maruyama, 2011) in planktonic cells and biofilm. This study is an attempt to find out the role of Class I integron and their gene cassettes in tackling the petroleum contamination.

SCOPE OF THE RESEARCH WORK

The investigation aims at isolating bacterial populations from petroleum contaminated soils and studying the molecular basis of adaptation mechanisms. We have made attempts to study the role of the integrons in recruiting gene cassettes for planktonic cells to encode proteins/enzymes for adaptive process and for maintaining the integrity of the biofilm architecture. This research is expected to throw light in elucidating the molecular tools and mechanism that are operating amongst the planktonic cells and biofilm forming bacterial communities of pathogenic bacteria as well, since they also face similar situations and exhibit similar adaptation mechanisms. Thus, the findings of the research are intended to make use of early prophylactic measures to control the disease causing pathogens.

AIMS AND OBJECTIVES OF THE PRESENT STUDY

Integrons and their associated gene cassettes were believed to underpin dynamics of the bacterial population in their adaptation to the ingredients of the stressful environment such as the petroleum contaminated soils. For, little is known about the potential contribution of these genetic elements in driving the adaptive mechanisms. There is dearth of significant information with regard to (i) classes of integrons involved in the process of recruiting gene cassettes against antimicrobials, and (ii) the adaptation mechanisms of the isolates of the contaminated site. The scope of the investigation was to focus on vital information to close the gaps in the areas of application of the research.
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**Objectives**

(i) To profile the physico-chemical characters of contaminated soil in order to understand the types of ingredients that contribute to the stress;

(ii) To isolate and identify by biochemical and 16S rRNA sequences of the bacterial species that have successfully inhabited the soil;

(iii) To determine the resistance/susceptibility of the isolates to a range of antimicrobials;

(iv) To investigate how the cells react to contaminants in the environment – by studying the cell surface hydrophobicity and surface charges; the induction of efflux pump activity, its influence on cell surface hydrophobicity and surface charges; and to carry out PCR-detection of \( qacE \Delta 1 \) efflux pump gene;

(v) To assay the auto-aggregation and co-aggregation of bacterial cells and floating behaviour of isolates towards hydrocarbons;

(vi) To determine the secretion of exopolysaccharides and bioemulsifiers; and

(vii) To investigate the culture dependent screening of integrons and their associated gene cassettes.

Besides, it was also aimed at searching for the genetic basis of the biofilm and the eDNA. The vital data on these parameters of the bacterial isolates would explain the significance of the presence of similar mechanisms among the pathogens. The later part of the study was to assess the evolutionary adaptation of biofilm association. This was done by:

(i) Evaluating the biofilm formation;

(ii) CLSM analysis of the biofilm architecture;

(iii) Determination of antimicrobial susceptibility of the biofilm;

(iv) Evaluation of the effect of matrix degrading enzymes on the biofilm;

(v) Determination of the role of efflux pumps in biofilm formation;

(vi) Characterization of exopolymeric substances;

(vii) Characterization of extracellular DNA; and determining the effect of exogenous DNA on cell surface hydrophobicity, surface charges and aggregation; and finally

(viii) PCR detection of Class I integrons in the eDNA of biofilm