Characterization, phylogenetic distribution and evolutionary trajectories of diverse hydrocarbon degrading microorganisms isolated from refinery sludge

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Abstract

Phylogenic association between bacteria living under harsh conditions can provide important information on adaptive mechanism, survival strategy and their potential application. Indigenous microorganisms isolated from toxic refinery oily sludge with ability to degrade a diverse range of hydrocarbons were identified and characterized. The strains including *Pseudomonas aeruginosa* RS1, *Microbacterium* sp. RS2, *Bacillus* sp. RS3, *Acinetobacter baumannii* RS4 and *Stenotrophomonas* sp. RS5 could utilize *n*-alkanes, cycloalkanes, polynuclear aromatic hydrocarbons (PAHs) with 2–4 rings and also substituted PAHs as sole substrate. The phylogenetic position of *Bacillus* sp. RS3 and *Pseudomonas* sp. RS1 was tested by applying the maximum likelihood (ML) method to the aligned 16S rRNA nucleotide sequences of PAH and aliphatic hydrocarbon degrading strains belonging to the corresponding genus. The base substitution matrix created with each set of organisms capable of degrading aromatic and aliphatic hydrocarbons showed significant transitional event with high values of transition: transversion ratio (R) under all conditions. The guanine-cytosine (GC) content of the hydrocarbon degrading test strains was also found to be highest for the clade which harbored them. The test strains consistently occupied a distinct terminal end within the phylogenetic tree constructed by ML analysis. This study reveals that the refinery sludge imposed environmental stress on the bacterial strains which possibly caused significant genetic alteration and phenotypic adaptation. Due to the divergent evolution of the *Pseudomonas* and *Bacillus* strains in the sludge, they appeared distinctly different from other hydrocarbon degrading strains of the same genus.

Keywords Refinery oily sludge · Aliphatic and aromatic hydrocarbons · Bacteria, phylogeny · Maximum likelihood method · MEGA-6

Debdeep Dasgupta and Jublee Jasmine contributed equally to this work.

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Introduction

Understanding physiology and survival of microorganism under harsh environmental conditions is an interesting topic of research for environmental microbiologists. The influence of environment on the fitness of a microorganism solely depends on genes and functional elements such as regulatory regions and they are governed by a process of natural selection (Doolittle 2013). Microorganisms living in harsh conditions may exhibit traits that are beneficial for their survival in such environments through a process of natural selection. For example, microorganisms in oily sludge exposed to various hazardous and toxic chemicals (Li et al. 2014) may develop the ability to utilize these hydrocarbons and survive through a process of natural selection. Various researchers have isolated oil/hydrocarbon degrading organisms from oily sludge (Joseph and Joseph 2009; Calvo et al.

2004) where they have evolved specific mechanisms for hydrocarbon uptake, such as production of biosurfactant. In one study, *Pseudomonas* sp. and *Bacillus* sp. isolated from oil sludge were reported to reduce total petroleum hydrocarbons (TPH) from 63 to 84% within 6 weeks (Ayotamuno et al. 2007). A microbial consortium composed of *Pseudomonas aeruginosa* and *Rhodococcus* sp. isolated from soil polluted with oily sludge demonstrated 90% degradation of hydrocarbons within six weeks (Cameotra and Singh 2008). However, none of these studies emphasized the potential of microbial strain isolated from oily sludge in degradation of diverse hydrocarbons and possible adaptive machinery due to prolonged exposure to hazardous toxic chemicals.

In most of the cases evolutionary theory assumes that mutations are random with respect to the adaptive values, however, this does not always hold true in all respects (Keller et al. 2007). For instance, the percentage of mutation in species with large genome size is more than in species with small genome (Zhang and Zhao 2004; Gojobori et al. 1982; Bulmer 1986; Hess et al. 1994). To minimize these confounding effects of natural selection, the non-coding sequences such as pseudogenes (Petrov and Hartl 1999; Weinert et al. 2009; Case et al. 2007) and copies of transposable elements (Bensasson et al. 2001) are being studied by several groups. Among bacteria, the evolution and diversity of Rickettsiales was investigated by finding substitution of base using the 16S rRNA sequence (Perna and Kocher 1996). The sequence of 16S rRNA gene was used as a phylogenetic marker at the subspecies level by maximum likelihood method in *Escherichia coli* and *Shigella flexneri* (Lassalle et al. 2015). This approach supports the process of molecular evolution as it is freed from selective constraints of natural selection and elucidate equal substitution rate at all three codon positions, frameshift mutation and the stop codon positions (Vasileva-Tonkova et al. 2001). Interestingly, a substitutional bias toward transition was observed in several housekeeping genes sequenced in multiple strains of *E. coli* and *S. enterica*. The ratio of transitions to transversions was found to be approximately 2.5:1 (Francino et al. 1996). Another study by Casane et al. (1997) showed that as in genomic DNA of the nucleus, the newly inserted sequences also tend to evolve towards higher guanine-cytosine (GC) content. Recent studies by Lassalle et al. (2015) suggested a mysterious force that favors high GC content in bacteria. A positive correlation between GC content and intra genic recombination were observed in most bacterial species. The possible mechanism was documented as GC biased gene conversion over the course of genome evolution. This phenomenon was also observed in yeast during the process of meiosis where it actively promoted fixation of deleterious alleles (Galtier et al. 2009; Necşulea et al. 2011). Selection of organisms is promoted by favoring GC nucleotides at polymorphic sites during the conversion of

intermediates of recombination (Webster and Hurst 2012). In contrast, Hildebrand et al. (2010) reported excess of G+C to adenine + thymine (A+T) mutations even when datasets with evidence of recombination was removed. Interestingly, various factors, such as growth temperature, environment, availability of oxygen and nitrogen affect the genomic GC content in bacteria and therefore the mutational bias (Foerster et al. 2005; Sueoka 1988). Although the exact nature of selective advantage conferred is unclear, one of the major benefits is facilitation of more complex gene regulation (Smardaa et al. 2014; Rocha and Feil 2010; Raghavan et al. 2012).

The current study reports isolation of bacterial strains from oily sludge with ability for degradation of diverse hydrocarbons. Subsequently, the phylogenetic distribution, nucleotide base substitution matrix and GC content evolution of two such bacterial strains isolated from refinery sludge were examined in comparison to strains with similar features reported earlier.

Materials and methods

Source of oily sludge

Oily sludge was obtained from the weathering pit in a petroleum refinery, Bharat Petroleum Corporation Limited (BPCL), Mumbai, India in August, 2010. The oily sludge was stored at 4 °C and the sludge samples were dried, homogenized and sieved manually before use.

Isolation and characterization of indigenous cultures from oily sludge

1 g of the oily sludge was mixed with 9 mL of distilled water and subjected to repeated tenfold serial dilutions followed by spread plating on sterile nutrient agar plates so as to determine the microbial load present in oily sludge. Preliminary biodegradation studies indicated microbial activity in uninoculated control flasks containing oily sludge suspended in mineral media possibly due to the presence of indigenous cultures present in the oily sludge (Jasmine and Mukherji 2015). Aliquots withdrawn from such control flasks over time plated on nutrient agar plates consistently demonstrated presence of colonies (Jasmine and Mukherji 2015). The predominant and distinct colonies that consistently appeared in these plates were isolated through multiple transfers and characterized. The strains were classified by differential Gram staining method (Cappuccino and Sherman 2007), 3% KOH test (Haleblian et al. 1981) followed by characterization based on the presence or absence of catalase and oxidase enzymes, carbohydrate fermentation tests using phenol red broth and triple sugar iron (TSI) agar. Metal tolerance

levels of the indigenous strains were also performed using a modified Kirby–Bauer disc diffusion method (Molina et al. 2011). Identification of the indigenous strains was further performed through 16S rRNA sequencing using universal primers 518F (5'-CCAGCAGCCGCGTAATACG-3') and 800R (5'-TACCAGGGTATCTAATCC-3') (Macrogen, Inc. South Korea). The forward and reverse sequences of all the selected indigenous strains were identified using Basic Local Alignment Tool (BLAST) that showed maximum percent homology match with existing sequences in the NCBI database. Evolutionary analysis for each of the strains was performed using MEGA ver.5 (Tamura et al. 2011) and bootstrap consensus phylogenetic trees for each of the strains were constructed. The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). The evolutionary distances were computed using the p-distance method (Nei and Kumar 2000) and were expressed in units of the number of base differences per site. The analysis involved 23, 13, 16, 13 and 21 nucleotide sequences for strains RS1, RS2, RS3, RS4 and RS5, respectively. All ambiguous positions were removed from each sequence pair. There were a total of 1569, 1906, 1876, 1866 and 1931 positions for RS1, RS2, RS3, RS4 and RS5, respectively, in the final dataset.

Growth studies of indigenous cultures from oily sludge on various hydrocarbons

Growth studies of the indigenous microorganisms isolated from oily sludge was performed using various hydrocarbons, i.e., *n*-hexadecane, *n*-octadecane, *n*-nonadecane, docosane, the 2-ring polynuclear aromatic hydrocarbons (PAHs), naphthalene and 1-methyl naphthalene, the 3-ring PAHs, anthracene and phenanthrene and the 4-ring PAHs, fluoranthene and pyrene as sole source of carbon and energy. Separate flasks were kept for each of the substrates and for each of the strains. Solid hydrocarbons (100 mg/L each of *n*-octadecane, *n*-nonadecane, docosane, naphthalene, fluoranthene, anthracene, phenanthrene and pyrene) and 200 mg/L of liquid hydrocarbons (*n*-hexadecane, cyclohexane, 1-methyl naphthalene, decalin) were added as sole source of carbon and energy in separate 500 mL flasks containing 100 mL mineral media. The choice of hydrocarbon concentration and experimental set-up for the growth studies were as discussed by other researchers (Jeswani and Mukherji 2013; Ghosh et al. 2014). The aqueous solubility of these hydrocarbons is so low that they cannot support microbial growth when present below their solubility limit. At a higher concentration, the surface area of the hydrocarbon present as insoluble solid or non-aqueous liquid is higher. This facilitates uptake of hydrocarbons and microbial growth by enhancing the

dissolution rate for microbes that take up hydrocarbons from the aqueous phase after dissolution. For microbes that utilize hydrocarbons via direct interfacial uptake, a higher concentration provides greater area on which microbes can attach and thus enhances the growth rate (Mohanty and Mukherji 2012). The mineral media was prepared as described by Mukherji et al. (2004). The mineral media was composed of (mg/L): KH_2PO_4 (170), K_2HPO_4 (435), Na_2HPO_4 (668), NH_4Cl (850), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (22.5), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (27.5), and FeCl_3 (0.25), CoCl_2 (0.0382), H_3BO_3 (0.0618), Na_2MoO_4 (0.0254), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.0392), ZnCl_2 (0.1363), NiCl_2 (0.013), $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (0.7016), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.2807), and Na_2SO_4 (0.142). The experimental flasks containing 100 mL mineral media were inoculated with the desired strain grown up to late log phase (5 mL of inoculum adjusted to unit absorbance). Two set of controls were maintained, un-inoculated controls and controls without the hydrocarbon substrate. The un-inoculated controls were not inoculated and only contained the hydrocarbons added to mineral media while the controls without substrate had only inoculum added to the mineral media. The experimental and control flasks were incubated in a rotary shaker operated at 150 rpm and 35 °C. At regular intervals, bacterial growth was monitored by recording the increase in absorbance at 600 nm using a visible spectrophotometer and the increase in colony forming units (CFU)/mL was determined by plating on nutrient agar plates.

Molecular phylogenetic analysis of bacterial strains

Among the various strains identified, one Gram negative organism, i.e., *Pseudomonas aeruginosa* and one Gram positive organism, i.e., *Bacillus* sp. were chosen for further bioinformatics study since aliphatic and aromatic hydrocarbon degraders in these two genus are widely reported in the literature. An attempt was made to compare the strains isolated from oily sludge in this study with other strains in each of these genus reported to degrade aliphatic hydrocarbon (*n*-hexadecane) and the high molecular weight (HMW) PAH (pyrene) with respect to phylogenetic positioning, sequence substitution divergence and GC content evolution. 16S rRNA sequence of seven previously reported *Bacillus* sp. and five *Pseudomonas* sp. with potential for pyrene degradation were collected from the Genbank (Tables 1, 2). Similarly, sequence of four previously reported *Bacillus* sp. and five *Pseudomonas* sp. with potential for *n*-hexadecane/oil degradation were also obtained (Tables 1, 2). The 16S rRNA sequence of the first 3–5 neighboring hits for each strain mentioned in Tables 1 and 2 were obtained by BLAST analysis (Identity match > 99%). Two most widely studied and cited strains namely *Pseudomonas aeruginosa* PAO1 and *Bacillus subtilis* subsp. 168 (Vasileva-Tonkova et al. 2001; Stover et al. 2000) were selected as outgroup and

Table 1 Distribution, isolation site, growth condition and carbon source of *Bacillus* sp.

Sole carbon source	Strain	Isolation site	Growth substrate	References
Reference strain	<i>Bacillus subtilis</i> subsp. 168	Not known (no report of HMW-PAH degradation)	Tryptophan requiring auxotroph, strain 168	Kunst et al. (1997) Zeigler et al. (2008)
Strain isolated from sludge	<i>Bacillus</i> sp. RS3	Oily sludge from a refinery in Mumbai, India	Diverse hydrocarbon (Figs. 1, 2)	This study
HMW-PAH degrader	<i>Bacillus</i> sp. PK-12	Crude oil-contaminated soil, Patiala, Punjab (India)	Pyrene (50 µg ml ⁻¹)	Khanna et al. (2012)
	<i>Bacillus subtilis</i> strain BMT4i	Soil sample from automobile workshop, Srinagar, Uttarakhand, India	Pyrene (50 µg ml ⁻¹)	Lily et al. (2010)
	<i>Bacillus</i> sp. PK-14	Crude oil-contaminated soil of Patiala, Punjab (India)	Pyrene (50 µg ml ⁻¹)	Khanna et al. (2012)
	<i>Bacillus</i> sp. PK-13	Crude oil-contaminated soil of Patiala, Punjab (India)	Pyrene (50 µg ml ⁻¹)	Khanna et al. (2012)
	<i>Bacillus vallismortis</i> strain JY3A	Soil samples from Oil Refinery Factory, Jinan, China	Pyrene (1 g l ⁻¹)	Ling et al. (2011)
	<i>Bacillus subtilis</i> strain: PK6	Coal tar contaminated site (Rakhra village, Patiala city, Punjab, India)	Pyrene (50 µg ml ⁻¹)	Khanna et al. (2012)
	<i>Bacillus</i> sp. ISTPY1	Soil samples from Okhla landfill site, Delhi	Pyrene (1 mg ml ⁻¹)	Swati et al. (2014)
Aliphatic hydrocarbon degrader	<i>Bacillus subtilis</i> strain ustb-2	Tissue sample from <i>Amorphophallus konjac</i> calli	<i>n</i> -Hexadecane (1% v/v)	Yu et al. (2013); Deng et al. (2011)
	<i>Bacillus subtilis</i> BSn5	Tissue sample from <i>Amorphophallus konjac</i> calli	<i>n</i> -Hexadecane (1% v/v)	Yu et al. (2013); Deng et al. (2011)
	<i>Bacillus cereus</i> strain ACE4	Corrosion products of diesel-transporting pipelines in a northwestern India	Hexadecane (1%)	Rajasekar and Ting (2010)
	<i>Bacillus subtilis</i> strain 20B	Fermented food (rice) sample—idly batter, Baroda, India	Heptane, kerosene and paraffin oil, cotton seed oil	Joshi et al. (2008)

were used as root for constructing the trees. The nucleotide sequences of each set were aligned using DNA Clustal X (2.1) and gap filling was done manually using SEAVIEW (64bit) (Gouy et al. 2010).

Maximum likelihood (ML) phylogenetic criteria

Out of the six models available in MEGA-6, the model with the lowest Bayesian Information Criterion (BIC) score (Tamura–Nei Model) was selected with the best considered substitution pattern for construction of the phylogenetic tree (Tamura et al. 2011). A discrete Gamma distribution (Γ) with 5 rate categories to model the non-uniformity of evolutionary rates was used and all positions containing gaps and missing data were completely eliminated. A fraction of the sites was assumed to be invariable during evolution (+I) and the total sum of base substitution was made equal to 1 for each computation. The ML tree was constructed with 1000 bootstrap replicates in MEGA6. The tree was rooted with the outgroup reference strain (R). All pyrene and hexadecane degrading strains are designated with “T” as type strain

within the phylogenetic tree and laboratory isolate *Bacillus* sp. RS3 and *P. aeruginosa* RS1 are referred as test strain.

Estimation of transition/transversion bias and determination of GC content

The rate of substitution among the retrieved sequences or transition/transversion bias in the two bacterial genera for degradation of the two hydrocarbons along with their neighbors was estimated in MEGA6 using maximum composite likelihood (MCL) method (Tamura et al. 2011) by fitting the Tamura–Nei model. The probability of substitution (r) of one base with another yielded by the software was tabulated for each set of sequence and % substitution by transition and transversion were calculated and expressed in the form of pie charts.

Measuring the variation in GC content is a major issue in genetics as genomic base composition contributes to the fitness of organisms such that characterizing the base composition is essential for reliable detection of selection (Lassalle et al. 2015). For estimation of GC content in various clades

Table 2 Distribution, isolation site, growth condition and carbon source of *Pseudomonas* Sp

Sole carbon source	Strain	Isolation site	Growth substrate	References
Reference strain	<i>Pseudomonas aeruginosa</i> PAO1	Wound isolate	<i>n</i> -Alkane	Stover et al. (2000); Holloway (1955); Smits et al. (2003)
Strain isolated from sludge	<i>Pseudomonas aeruginosa</i> RS1	Oily sludge from a refinery in Mumbai India	Diverse hydrocarbon (Figs. 1, 2)	Ghosh et al. (2014)
HMW-PAH degrader	<i>Pseudomonas aeruginosa</i> strain SP4	Not known (Tehran University of Medical Sciences)	Pyrene (200 µg ml ⁻¹)	Jorfi et al. (2013)
	<i>Pseudomonas aeruginosa</i> strain M	Crude petroleum oil-contaminated soil sample from north-east India	Pyrene (250 µg 100 ml ⁻¹)	Das and Mukherjee (2007)
	<i>Pseudomonas</i> sp. Pyr41	Sludge obtained from anoxic reactor of Shao-guan Coking Wastewater Treatment system in Guangdong, China	Pyrene (50 mg l ⁻¹)	Ping et al. (2011)
	<i>Pseudomonas putida</i> strain PL2	Soil samples from the surface layer at a garage in Hang Zhou City, Zhejiang Province of China	Pyrene (50 mg l ⁻¹)	Deng et al. (2012)
Aliphatic Hydrocarbon degrader	<i>Pseudomonas fluorescens</i> strain 29L	Not known	Pyrene (50 mg l ⁻¹)	Husain (2008)
	<i>Pseudomonas aeruginosa</i> strain SR17	Soil samples from local vehicle garage, situated in Guwahati, Assam, India	Crude oil (2% w/v)	Patowary et al. (2016)
	<i>Pseudomonas aeruginosa</i> strain CPCL	Petroleum sediments near Manali, Chennai	Hexadecane (2%, w/v)	Sakthipriya et al. (2016)
	<i>Pseudomonas</i> sp. P-1-6	Heavily petroleum hydrocarbon-contaminated soil	Hexadecane (27, 39, 27 and 13%)	Pacwa-łociniczak et al. (2014)
	<i>Pseudomonas aeruginosa</i> strain RS29	Crude oil-contaminated soil from the Lakowa oil fields of Assam, India	Crude oil (2% w/v)	Saikia and Deka (2013)
	<i>Pseudomonas nitroreducens</i> strain TSB.MJ10	Sediment samples collected from the estuarine mangrove ecosystem	0.5% glucose or sodium benzoate	De Sousa and Bhosle (2012)

generated by the ML tree, the average %GC content of all the members in a clade was determined. The ratio of GC content with respect to the reference strain were calculated by first subtracting the clade with minimum GC content from each clade and also from that of the reference strain.

$$\text{Ratio} = \frac{\text{Mean GC of a clade} - \text{Mean GC of clade with least GC}}{\text{GC of reference strain} - \text{Mean GC of clade with least GC}} \quad (1)$$

Results

Identification and characterization of indigenous strains isolated from oily sludge

The refinery oily sludge revealed presence of a wide variety of microorganisms with an initial microbial load of 6.12×10^9 CFU g⁻¹. The microbes associated with the sludge were capable of degrading the oil associated with

sludge under favorable conditions (Jasmine and Mukherji 2015). Some of the microorganisms were selectively isolated for their consistent re-occurrence during oily sludge biodegradation studies. These isolated bacterial strains were grown and maintained in 0.5% (w/v) extracted oil

derived from the sludge. Characterization of the strains, RS1, RS2, RS3, RS4 and RS5 was performed based on colony morphology, size and shape, sugar assimilation characteristics and catalase and oxidase reaction. The results are shown in Table S1 & S2. All the indigenous bacterial strains showed good tolerance to a wide variety of metals, such as, Al, Mg, K, Na, Ca, Zn, Ni, Cu, Co, Bi, Fe and Pb (5–500 ppm) (Table S3). Sequence analysis of 16S rRNA gene followed by NCBI-BLAST revealed the strains RS1,

RS2, RS3, RS4 and RS5 as *Pseudomonas aeruginosa*, *Microbacterium* sp., *Bacillus* sp., *Acinetobacter baumannii* and *Stenotrophomonas* sp., respectively, with NCBI

accession number of KF751345, KF751343, KF751344, KF751346, KF751347. The phylogenetic tree of all strains is shown in Fig. 1. The percentage of replicate trees in

Fig. 1 Phylogenetic tree for **a** RS1, **b** RS2, **c** RS3, **d** RS4 and **e** RS5

Fig. 1 (continued)

which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The trees were drawn to scale, with branch lengths in the same units as those of evolutionary distances used to infer the phylogenetic tree.

Growth studies of indigenous bacterial strains on various hydrocarbons

The ability of indigenous bacterial strains to utilize specific petroleum hydrocarbons belonging to various groups as sole substrate, i.e., *n*-alkanes, cycloalkanes and PAHs was

Fig. 1 (continued)

evaluated (Figs. 2a–f and 3a–f). Since the mineral media did not contain any carbon source, it is not expected to support growth of these heterotrophic bacteria. Thus, growth in the experimental flasks is indicative of the ability of the strains to utilize the hydrocarbon provided as both electron donor and carbon source. No increase in absorbance at 600 nm was observed in any of controls devoid of hydrocarbon substrate. All the strains could readily utilize *n*-hexadecane and *n*-octadecane as sole substrate and end of log phase was reached within 8 days. The extent of growth on *n*-alkanes was much higher than on the cycloalkane, cyclohexane. Similarly, although the strains could grow on 1-methyl naphthalene, the extent of growth was low in comparison with other substrates. Previous studies with oil extracted from oily sludge had also confirmed the ability of these strains to grow on and degrade oil present in oily sludge as sole substrate (Jasmine and Mukherji 2015). The hydrocarbons used in this study are present as components in the oil extracted from sludge as confirmed through GC–MS analysis (Jasmine and Mukherji 2015). *Pseudomonas aeruginosa*, *Bacillus* sp., *Acinetobacter baumannii* and *Stenotrophomonas* sp., i.e., all strains other

than the *Microbacterium* sp. showed remarkable ability for uptake of a wide range of compounds, i.e., 2-, 3- and 4-ring PAHs (naphthalene, anthracene, phenanthrene, fluoranthene and pyrene); cycloalkanes (decalin and cyclohexane) and substituted naphthalenes (1-methylnaphthalene). These strains could also utilize crude oil and extracted oil from sludge as sole substrate (Jasmine and Mukherji 2014).

Construction of evolutionary phylogenetic tree and clade analyses

Phylogenetic tree was constructed with known sequence of hydrocarbon degrading microorganisms capable of utilizing either the PAH, pyrene, or the aliphatic hydrocarbon *n*-hexadecane as sole source of carbon. This analysis was done to determine the relatedness amongst various strains of *Pseudomonas* sp. and *Bacillus* sp. reported to degrade pyrene or *n*-hexadecane. One Gram positive *Bacillus* sp. and one Gram negative *Pseudomonas* sp. were chosen to understand the systematic distribution and evolutionary distance

Fig. 2 Growth profiles of strains isolated from refinery sludge on *n*-alkanes and cycloalkanes, **a** *n*-hexadecane, **b** *n*-octadecane, **c** *n*-nonadecane, **d** docosane, **e** cyclohexane and **f** decalin. Here, continuous line with diamond *Pseudomonas aeruginosa*, continuous line with square *Acinetobacter baumannii*, dashed-dotted line with triangle *Bacillus* sp., dashed lines with asterisk *Microbacterium* sp., and continuous line with cross *Stenotrophomonas* sp.

between strains isolated from sludge and known strains with potential for utilizing similar hydrocarbons.

The phylogeny of the 16S rRNA gene sequence of 52 *Bacillus* strains were placed in five different clusters (Fig. 4a). The strains included seven pyrene degrading strains, one outgroup reference strain *Bacillus subtilis* 168, the test strain *Bacillus* sp. (Table 1) and 43 neighboring strains obtained from the Genbank. The tree was rooted with the outgroup reference strain *Bacillus subtilis* 168. It is interesting to note that most of the PAH degrading strains were isolated from crude oil-contaminated soils. Clade I and II were further segregated into two sub-clades resulting in a total of seven clusters. The pyrene degrading strains were distributed across distinctly separate clades. The test strain *Bacillus* sp. RS3 was placed in the terminal end among all clades and it showed the most divergent sequence compared to others in the tree. The closest strain *Bacillus* sp. PK-12 was found to be placed in a separate sub-clade showing the unique characteristic of *Bacillus* sp. RS3 among pyrene degrading *Bacillus* sp. For the *n*-hexadecane degrading strains, slightly different distribution was observed. 16S

rRNA gene sequence of 30 *Bacillus* strains were plotted to construct the phylogenetic tree. The strains were found in two different clusters (Fig. 4b). The strains include *n*-hexadecane degrading *Bacillus* strains, one outgroup reference strain, *Bacillus subtilis* 168, one test strain *Bacillus* sp. RS3 (Table 1) and 23 neighboring strains obtained from the Genbank. The tree was rooted with the outgroup reference strain, *Bacillus subtilis* 168. It is interesting to note that in case of *Bacillus* sp., all *n*-hexadecane degrading organisms were found to exist within two clades as opposed to the set of pyrene degrading *Bacillus* strains. Notably similar to the tree for the set of pyrene degrading *Bacillus* sp., the test strain *Bacillus* sp. RS3 was also found in the terminal position in the tree for *n*-hexadecane degrading strains.

Similarly, the phylogeny of the 16S rRNA gene sequence of 36 *Pseudomonas* strains were placed in five different clusters (Fig. 5a). The strains include the five pyrene degrading strains, one outgroup reference strain *Pseudomonas aeruginosa* PAO1, one test strain *Pseudomonas aeruginosa* RS1 (Table 2) and 35 neighboring strains obtained from the Genbank. The tree was rooted

Fig. 3 Growth profiles of strains isolated from refinery sludge on 2, 3 and 4 ring PAHs, **a** naphthalene, **b** 1-methyl naphthalene, **c** phenanthrene, **d** anthracene, **e** fluoranthene, and **f** pyrene. Here, continuous line with diamond *Pseudomonas aeruginosa*, continuous line with square *Acinetobacter baumannii*, dashed-dotted line with triangle *Bacillus* sp., dashed lines with asterisk *Microbacterium* sp., and continuous line with cross *Stenotrophomonas* sp.

with the outgroup reference strains *Pseudomonas aeruginosa* PAO1. Here the Clade II was further segregated into two sub-clades resulting in a total of five clusters. The PAH degrading strains were distributed across the various clades except *Pseudomonas aeruginosa* strain SP4, *P. aeruginosa* strain M and *Pseudomonas* sp. Pyr41 were found to be present in Clade I. Strikingly similar to *Bacillus* sp. RS3, the test strain *P. aeruginosa* RS1 was also found to be placed at the terminal end of the phylogenetic tree and showed maximum divergence. The sequence of hexadecane/oil degrading *Pseudomonas* strains was placed in four different clades (Fig. 5b). The strains include six *n*-hexadecane degrading strains, one outgroup reference strains *P. aeruginosa* PAO1, one test strain *P. aeruginosa* RS1 (Table 2) and 30 neighboring strains obtained from the Genbank. The tree was rooted with the outgroup reference strain, *P. aeruginosa* PAO1. The Clade I and III were further segregated into two sub-clades resulting in a total of six clusters. *P. aeruginosa* RS1 was also found at the terminal end among the aliphatic hydrocarbon degrading strains of *Pseudomonas* sp., however, the bootstrap support was relatively low.

Maximum likelihood matrix of substitution matrix

Maximum likelihood matrix of substitution was obtained using MEGA6 where each entry is the probability of substitution (r) of one base (row) with another expressed as percentage. Substitution pattern and probabilities were estimated using the Tamura–Nei (1993) model (Tamura et al. 2011; Tamura and Nei 1993). A discrete Gamma distribution was used to model the evolutionary rate differences among the sites (5 categories, +G). For the combination of strains harboring *Bacillus* sp. RS3 capable of degrading pyrene and their neighboring strains, the nucleotide frequencies were $A = 24.50\%$, $T/U = 20.80\%$, $C = 21.54\%$, and $G = 33.16\%$ (Table S4). The estimated transition/transversion bias (R) is 1.29 (Fig. 6). The maximum log likelihood for this computation was -1127.292 . The analysis involved 52 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 336 positions in the final dataset. In contrast, for the combination of strains including the test strain *Bacillus* sp. RS3 with its neighbors comprising of aliphatic hydrocarbon/oil degraders the change in nucleotide frequencies were $A = 24.74\%$,

Fig. 4 a Phylogenetic tree constructed with 16S rRNA sequence of 52 strains of *Bacillus* sp. based on a maximum likelihood analysis. The pyrene degrading type strains are marked with superscript “T” and reference strain as “R”. **b** Phylogenetic tree constructed with 16S rRNA sequence of 30 strains of *Bacillus* sp. based on a maximum likelihood analysis. The *n*-hexadecane degrading type strains are marked with superscript “T” and reference strain as “R”

$T/U = 21.53\%$, $C = 24.06\%$, and $G = 29.67\%$ (Table S4). The maximum log likelihood for this computation was -443.875 . The analysis involved 30 nucleotide sequences. There were a total of 272 positions in the final dataset. Notably the estimated transition/transversion bias (R) was 3.08 (Fig. 6).

Similarly for the PAH degrading *Pseudomonas* sp. RS1 and neighboring strains, the nucleotide frequencies were $A = 25.51\%$, $T/U = 20.77\%$, $C = 22.72\%$, and $G = 30.99\%$ (Table S4). The maximum log likelihood for this computation was -1455.758 . The analysis involved 36 nucleotide sequences. There were a total of 827 positions in the final dataset. The estimated transition/transversion bias (R) was 1.44 (Fig. 6). In contrast, with aliphatic hydrocarbon degrading neighbors of the same strain, the nucleotide frequencies were $A = 25.99\%$, $T/U = 21.77\%$, $C = 21.43\%$, and

$G = 30.82\%$ (Table S4). The maximum log likelihood for this computation was -1255.652 . The analysis involved 37 nucleotide sequences. There were a total of 385 positions in the final dataset. Here the estimated transition/transversion bias (R) is 1.42 (Fig. 6).

GC content evolution

The GC content of individual clades harboring the test strains were calculated in comparison to reference strain. The ratio of GC content with respect to the reference strain were calculated by first subtracting the clade with minimum GC content from each clade and also from that of the reference strain as shown in Eq. 1. In case of the combination of HMW-PAH degrading *Bacillus* sp. the GC content was found highest (55.55%) in Clade I holding the test strain *Bacillus* sp. RS3. The ratio of

Fig. 4 (continued)

Fig. 5 a Phylogenetic tree constructed with 16S rRNA sequence of 36 strains of *Pseudomonas* sp. based on a maximum likelihood analysis. The pyrene degrading type strains are marked with superscript “T” and reference strain as “R”. **b** Phylogenetic tree constructed with 16S rRNA sequence of 37 strains of *Pseudomonas* sp. based on a maximum likelihood analysis. The *n*-hexadecane degrading type strains are marked with superscript “T” and reference strain as “R”

Fig. 5 (continued)

GC content of the same clade with respect to reference strain was also the highest (1.76) in comparison to other clades (Fig. 7). Similarly for the aliphatic hydrocarbon/oil degrading *Bacillus* sp. and its neighbors, the GC content was found higher in the clade harboring the *Bacillus* sp. RS3 (55.27%) (Fig. 7). The GC content ratio for the *Bacillus* sp. RS3 and its neighboring strains could not be determined since the strains were only distributed in two clades.

However, for the HMW-PAH degrading *Pseudomonas* sp., the GC content of the clade holding the test strain *Pseudomonas* sp. RS1 was comparable with its neighboring clade (54.27%). The ratio of GC content with respect to reference strain was also higher at (1.06) among these clades (Fig. 7). In contrast, for the combination of aliphatic hydrocarbon degrading *Pseudomonas* sp., the GC content for the clade harboring the test strain *Pseudomonas* sp. RS1 was found to be the highest (55.2%). The ratio of GC content with respect to reference strain was also found to be the highest at 1.52.

Discussion

Over the past few decades, evolution of bacterial populations has been detected and documented in various ways. The advancement in sequencing technologies and computational

bioinformatics has made it feasible to study variation in the whole-genome sequence and detect all changes by position within the genome from a single nucleotide change to large-scale insertions and deletions (Bryant et al. 2012). Change in environmental condition may lead to evolution of the bacterial genome by altering the mutation rate (Carja et al. 2014). In bacteria, stress can increase the mutation rate/frequency by inducing mutagenic mechanisms, such as the SOS transcriptional response or contingency loci (Bjedov et al. 2003). Interestingly, point mutation results in selective advantage in bacteria and can easily be compared with genomes of neighboring strains identified by constructing phylogenetic relationships and measuring the evolutionary pressures (Bryant et al. 2012). A probabilistic model for evolution of DNA sequence data leads to the process of branching and construction of evolutionary tree which also measures the changes in DNA sequence along the tree (Felsenstein 1981). Maximum likelihood approach based on MEGA-6 facilitates sequence alignment, phylogenetic tree reconstruction and visualization for testing an array of evolutionary hypotheses as desired by researchers (Bryant et al. 2012). In the current study, phylogenetic relationship of diverse hydrocarbon degrading bacterial strains isolated from refinery sludge were compared with known test strains with potential for degradation of either aliphatic hydrocarbon/oil or

Fig. 6 Percentage of transition and transversion base substitution measured by maximum likelihood estimate of substitution matrix pattern using the Tamura–Nei (1993) model constructed with 16S rRNA nucleotide sequence of polynuclear aromatic and aliphatic hydrocarbon degrading strains. Here **a** PAH degrading *Bacillus* sp., **b** Aliphatic hydrocarbon degrading *Bacillus* sp., **c** HMW-PAH degrading *Pseudomonas* sp., **d** Aliphatic hydrocarbon degrading *Pseudomonas* sp. A discrete Gamma distribution was used to model evolutionary rate differences among sites [five categories, +G]. For estimating ML values, a tree topology was automatically computed. All positions containing gaps and missing data were eliminated. All analyses were conducted in MEGA6. Here, stripes represents % transition and dashes represents % transversion

HMW PAHs. The refinery sludge was found to contain an active indigenous microbial community that could achieve $45 \pm 17\%$ of degradation of oil over 30 days when nutrients were added (Jasmine and Mukherji 2015). Presence of such intrinsic hydrocarbon degrading community in sludge has been widely reported in the literature, however, potential for degradation of hydrocarbons belonging to diverse groups by individual bacterial strains isolated from oily sludge/oil-contaminated sites is less commonly reported (Jeswani and Mukherji 2013; Mishra et al. 2001; Tahhan et al. 2011).

In the present study, active indigenous microorganisms that showed significant degradation of oil associated with oily sludge were isolated and characterized. The predominant strains in the oily sludge were identified as *Pseudomonas aeruginosa* (RS1), *Microbacterium* sp. (RS2), *Bacillus* sp. (RS3), *Acinetobacter baumannii* (RS4) and *Stenotrophomonas* sp. (RS5). All the indigenous strains demonstrated remarkable ability for uptake of a wide range of compounds, *n*-alkanes, cycloalkanes (decalin and cyclohexane) and substituted naphthalenes (1-methylnaphthalene); 2-, 3- and 4-ring PAHs (naphthalene, anthracene, phenanthrene, fluoranthene and pyrene). These cultures could also utilize crude oil and extracted oil from sludge as

sole substrate. Several strains of the genera *Pseudomonas*, *Acinetobacter*, *Stenotrophomonas* and *Bacillus* isolated from hydrocarbon-contaminated environments are reported to grow on and degrade aliphatic and aromatic hydrocarbons (Cameotra and Singh 2008; Mishra et al. 2001; Vasudevan and Rajaram 2001; Assih et al. 2002; Verma et al. 2006; Gallego et al. 2007; Thavasi et al. 2011; Cerqueira et al. 2011). However, the role of *Stenotrophomonas* sp. and *Microbacterium* sp. on hydrocarbon degradation has not been extensively reported. Since not many hydrocarbon degrading strains of *Stenotrophomonas* sp. and *Microbacterium* sp. have been reported, DNA sequence data for strains of *Stenotrophomonas* sp. and *Microbacterium* sp. with hydrocarbon degradation ability in the NCBI database is limited such that evolutionary analysis could not be performed by construction of phylogenetic tree using ML method.

The phylogenetic analysis of isolated diverse hydrocarbon degrading bacterial strains with the known PAH or aliphatic hydrocarbon degraders presented relative distribution and unique characteristics of each combination under study. In case of the first combination of strains capable of utilizing PAHs, it was observed that the clade holding the Gram

Fig. 7 GC Content of housekeeping gene (16S rRNA) constructed based on a maximum likelihood analysis for **a** PAH degrading *Bacillus* sp., **b** aliphatic hydrocarbon/oil degrading *Bacillus* sp., **c** HMW-PAH degrading *Pseudomonas* sp. and **d** aliphatic hydrocarbon/oil

degrading *Pseudomonas* sp. GC content ratio of individual clades with respect to the reference strain for **e** PAH degrading *Bacillus* sp., **f** HMW-PAH degrading *Pseudomonas* sp. and **g** aliphatic hydrocarbon degrading *Pseudomonas* sp.

positive *Bacillus* sp. RS3 was isolated from the other clades harboring known PAH degraders. In contrast, the same strain was found to coexist with the clade holding known aliphatic degraders in the second combination. Interestingly, in both the cases the test strain *Bacillus* sp. RS3 was found positioned at the terminal end of the phylogenetic tree indicating its unique characteristics. The maximum log likelihood value among the PAH degrading strains were much higher (-1127.29) compared to the aliphatic degrading strains

(-443), indicating that the sequences are better conserved in the latter compared to the former. Therefore, the numbers of clades harboring all the aliphatic hydrocarbon/oil degrading strains were only two indicating that base substitution rate and sequence divergence rate is much lower among aliphatics/oil degraders. In contrast, for the combination of PAH degrading Gram negative *Pseudomonas* sp., the maximum log likelihood was high (-1455) for both the conditions and the strains were found to be distributed across the various

clades. However, the sequence was found less conserved in case of *Pseudomonas* sp. compared to *Bacillus* sp. which could be due to low bootstrap score among these combinations. Notably for both groups, i.e., aliphatic hydrocarbon and PAH degrading strains, the test strain *P. aeruginosa* RS1 was situated in the terminal end and was characterized by the most divergent sequence indicating its unique phenotypic features.

The base substitution among the various sequences was further tested by maximum likelihood for substitution using the software MEGA-6. Interestingly in early works, the base substitution by transition and transversion was correlated with degree of sequence divergence (Stoltzfus and Norris 2016). The transitional bias due to mutation occurs at instantaneous rates (Vogel and Kopun 1977) and acts on DNA observed in introns, pseudogenes, and other non-coding regions, such that this is evident across the entire genome (Li et al. 1985). In the current study, the maximum number of base substitution sites was found among the combination of PAH degrading *Pseudomonas* sp. and their neighboring strains (827 sites) with a transition/transversion bias (R) of 1.4. Whereas for the combination of PAH degrading *Bacillus* sp., the number of sites where base substitution occurred was lower (336) and the transition to transversion ratio was 1.28 indicating that sequence divergence was comparable with *Pseudomonas* sp.; however, sites where mutation by substitution took place was significantly lower. Strikingly for the combination of *n*-hexadecane degrading *Bacillus* sp. the number of sites where substitution took place was lower compared to the PAH degraders; however, most of the substitution was of transition type (76%) and transition/transversion ratio was 3.04. This clearly suggests that the sequence of this particular set was better conserved as also evident from the fact that all strains were grouped in only two clades. Therefore, for all the combinations studied, *Pseudomonas* sp. was found to show the highest rate of base substitution by transition and unique phylogenetic position. Interestingly, for all the combinations, the rate of transition was higher compared to the rate of transversion; however, sequence divergence may also be affected by the number of sites where substitution took place. The results were further tested by quantifying the GC content in each cluster harboring the type strains (marked with “T” in the phylogenetic tree). Notably the sub-clade harboring the test strain *Bacillus* sp. RS3 revealed the highest GC content and higher GC ratio compared to the reference strain and other strains containing the PAH degrading *Bacillus* sp. Similar result was observed for the combination of aliphatic hydrocarbon degrading *Pseudomonas* sp. where the clade holding the test strain *P. aeruginosa* RS1 showed the highest GC content. However, among the combination of PAH degrading *Pseudomonas* sp. the clade harboring the test strain *Pseudomonas aeruginosa* RS1 showed relatively comparable GC content with other

clades. In higher organisms, the evolution of GC content is driven by recombination as reported for analysis of polymorphism data and substitution patterns along genomes which tends to increase the probability of fixation of AT>GC mutations (Spencer et al. 2006). Therefore, it is likely that in case of bacteria too, stress-induced mutation results in high rate of recombination as reflected in the 16S rRNA sequences of the strains isolated from oily sludge and such recombinations are possibly distributed throughout their entire genome as a result of evolution.

Conclusions

The indigenous microorganisms isolated from oily sludge showed tolerance to heavy metals predominantly found in oily sludge and could grow on a wide range of aliphatic and aromatic hydrocarbons present in oily sludge indicating remarkable degradation ability. The survival strategy of bacteria with environmental change results in adaptive evolution with dynamic change in phenotype and genotype. The toxic refinery sludge imposed environmental stress on the bacterial strains and this possibly caused significant genetic alteration and phenotypic adaptation. This resulted in divergent evolution of the *Pseudomonas* and *Bacillus* strains such that they appeared to be distinctly different from other hydrocarbon degrading strains of the same genus.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

References

- Assih EA, Ouattara AS, Thierry S, Cayol JL, Labat M et al (2002) *Stenotrophomonas acidaminiphila* sp. nov., a strictly aerobic bacterium isolated from an upflow anaerobic sludge blanket (Uasb) reactor. *Int J Syst Evol Micr* 52:559–568
- Ayotamuno M, Okparanma R, Nweneka E, Ogaji S, Probert S (2007) Bio-remediation of a sludge containing hydrocarbons. *Appl Energy* 84:936–943
- Bensasson D, Zhang D-X, Hartl DL, Hewitt GM (2001) Mitochondrial pseudogenes: Evolution’s misplaced witnesses. *Trends Ecol Evol* 16:314–321
- Bjedov I, Tenaillon O, Gérard B, Souza V, Denamur E et al (2003) Stress-induced mutagenesis in bacteria. *Science* 300:1404–1409
- Bryant J, Chewapreecha C, Bentley SD (2012) Developing insights into the mechanisms of evolution of bacterial pathogens from whole-genome sequences. *Future Microbiol* 7:1283–1296

- Bulmer M (1986) Neighboring base effects on substitution rates in pseudogenes. *Mol Biol Evol* 3:322–329
- Calvo C, Toledo F, González-López J (2004) Surfactant activity of a naphthalene degrading *Bacillus pumilus* strain isolated from oil sludge. *J Biotechnol* 109:255–262
- Cameotra SS, Singh P (2008) Bioremediation of oil sludge using crude biosurfactants. *Int Biodeterior Biodegrad* 62:274–280
- Cappuccino JG, Sherman N (2007) *Microbiology: a laboratory manual*, 7th edn. Pearson Education, New York
- Carja O, Liberman U, Feldman MW (2014) Evolution in changing environments: modifiers of mutation, recombination, and migration. *Proc Natl Acad Sci USA* 111:17935–17940
- Casane D, Boissinot S, Chang BH-J, Shimmin LC, Li W-H (1997) Mutation pattern variation among regions of the primate genome. *J Mol Evol* 45:216–226
- Case RJ, Boucher Y, Dahllöf I, Holmström C, Doolittle WF, Kjelleberg S (2007) Use of 16S rRNA and rpoB genes as molecular markers for microbial ecology studies. *Appl Environ Microbiol* 73:278–288
- Cerqueira VS, Hollenbach EB, Maboni F, Vainstein MH, Camargo FAO et al (2011) Biodegradation potential of oily sludge by pure and mixed bacterial cultures. *Bioresour Technol* 102:11003–11010
- Das K, Mukherjee AK (2007) Differential utilization of pyrene as the sole source of carbon by *Bacillus subtilis* and *Pseudomonas aeruginosa* strains: role of biosurfactants in enhancing bioavailability. *J Appl Microbiol* 102:195–203
- De Sousa T, Bhosle S (2012) Isolation and characterization of a lipopeptide bioemulsifier produced by *Pseudomonas nitroreducens* tsb.mj10 isolated from a mangrove ecosystem. *Bioresour Technol* 123:256–262
- Deng Y, Yhu YG, Wang PX, Zhu L, Zheng JS et al (2011) Complete genome sequence of *Bacillus subtilis* bsn5, an endophytic bacterium of *Amorphophallus konjac* with antimicrobial activity for the plant pathogen *erwinia carotovora* subsp. *carotovora*. *J Bacteriol* 193:2070–2071
- Deng L, Ren Y, Wei C (2012) Pyrene degradation by *Pseudomonas* sp. and *Burkholderia* sp. Enriched from coking wastewater sludge. *J Environ Sci Heal A* 47:1984–1991
- Doolittle WF (2013) Is junk DNA bunk? A critique of ENCODE. *Proc Natl Acad Sci USA* 110:5294–5300
- Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood. *Approach J Mol Evol* 17:368–376
- Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39(4):783–791
- Foerster KU, von Mering C, Hooper SD, Bork P (2005) Environments shape the nucleotide composition of genomes. *EMBO Rep* 6:1208–1213
- Francino MP, Chao L, Riley MA, Ochman H (1996) Patterns of substitutions in enterobacterial genes are generated by transcription-coupled repair. *Science* 272:107–109
- Gallego JLR, Garcia-Martinez MJ, Llamas JF, Bellosch C, Pelaez AI et al (2007) Biodegradation of oil tank bottom sludge using microbial consortia. *Biodegradation* 18:269–281
- Galtier N, Duret L, Glémin S, Ranwez V (2009) GC-biased gene conversion promotes the fixation of deleterious amino acid changes in primates. *Trends Genet TIG* 25:1–5
- Ghosh I, Jasmine J, Mukherji S (2014) Biodegradation of pyrene by a *Pseudomonas aeruginosa* strain RS1 isolated from refinery sludge. *Bioresour Technol* 166:548–558
- Gojobori T, Li WH, Graur D (1982) Patterns of nucleotide substitution in *Pseudogenes* and functional genes. *J Mol Evol* 18:360–369
- Gouy M, Guindon S, Gascuel O (2010) SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol* 27:221–224
- Halebian S, Harris B, Finegold SM, Rolfe RD (1981) Rapid method that aids in distinguishing Gram-positive from Gram-negative anaerobic bacteria. *J Clin Microbiol* 13(3):444–448
- Hess ST, Blake JD, Blake RD (1994) Wide variations in neighbor-dependent substitution rates. *J Mol Biol* 236:1022–1033
- Hildebrand F, Meyer A, Eyre-Walker A (2010) Evidence of selection upon genomic GC-content in bacteria. *PLoS Genet* 6:e1001107
- Holloway (1955) Genetic recombination in *Pseudomonas aeruginosa*. *J Gen Microbiol* 13:572–581
- Husain S (2008) Effect of ferric iron on siderophore production and pyrene degradation by *Pseudomonas fluorescens* 291. *Curr microbiol* 57:331–334
- Jasmine J, Mukherji S (2014) Evaluation of bioaugmentation and biostimulation effects on the treatment of refinery oily sludge using 2ⁿ full factorial design. *Environ Sci Process Impacts* 16:1889–1896
- Jasmine J, Mukherji S (2015) Characterization of oily sludge from a refinery and biodegradability assessment using various hydrocarbon degrading strains and reconstituted consortia. *J Environ Manage* 149:118–125
- Jeswani H, Mukherji S (2013) Batch studies with *Exiguobacterium aurantiacum* degrading structurally diverse organic compounds and its potential for treatment of biomass gasification wastewater. *Int Biodeter Biodegrad* 80:1–9
- Jorfi H, Rezaee A, Ali GAM, Jaafarzadeh NA (2013) Application of biosurfactants produced by *Pseudomonas aeruginosa* sp4 for bioremediation of soils contaminated by pyrene. *Soil Sediment Contam* 22:890–911
- Joseph P, Joseph A (2009) Microbial enhanced separation of oil from a petroleum refinery sludge. *J Hazard Mater* 161:522–525
- Joshi S, Bharucha C, Desai AJ (2008) Production of biosurfactant and antifungal compound by fermented food isolate *Bacillus subtilis* 20b. *Bioresour Technol* 99:4603–4608
- Keller I, Bensasson D, Nichols RA (2007) Transition-transversion bias is not universal: a counter example from grasshopper pseudogenes. *PLoS Genet* 3:e22
- Khanna P, Goyal D, Khanna S (2012) Characterization of pyrene utilizing *Bacillus* spp. from crude oil contaminated soil. *Braz J Microbiol* 43:606–617
- Kunst F, Ogasawara N, Moszer I, Albertini AM, Alloni G et al (1997) The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature* 390:249–256
- Lassalle F, Périán S, Bataillon T, Nesme X, Duret L et al (2015) GC-content evolution in bacterial genomes: the biased gene conversion hypothesis expands. *PLoS Genet* 11(2):e1004941
- Li W-H, Luo C-C, Wu C-I (1985) Evolution of DNA sequences. In: MacIntyre RJ (ed) *Molecular evolutionary genetics*. Plenum, New York
- Li SJ, Hua ZS, Huang LN, Li J, Shi SH et al (2014) Microbial communities evolve faster in extreme environments. *Sci Rep* 27:6205
- Lily MK, Bahuguna A, Dangwal K, Garg V (2010) Optimization of an inducible, chromosomally encoded benzo [a] pyrene (bap) degradation pathway in *Bacillus subtilis* bmt4i (mtcc 9447). *Ann Microbiol* 60:51–58
- Ling J, Zhang G, Sun H, Fan Y, Ju J et al (2011) Isolation and characterization of a novel pyrene-degrading *Bacillus vallismortis* strain JY3A. *Sci Total Environ* 409:1994–2000
- Mishra S, Jyot J, Kuhad RC, Lal B (2001) In situ bioremediation potential of an oily sludge-degrading bacterial consortium. *Curr Microbiol* 43:328–335
- Mohanty S, Mukherji S (2012) Alteration in cell surface properties of *Burkholderia* spp. during surfactant aided biodegradation of petroleum hydrocarbons. *Appl Microbiol Biot* 94:193–204
- Molina TL, Patel R, Molina DD, Persans MW, Lowe KL (2011) Isolation of a naturally-occurring nickel resistance plasmid from a rare hypersaline estuary (Laguna, Madre, Texas, USA) for potential

- use as a bio-indicator of metal contamination. *World J Microb Biot* 27:2163–2171
- Mukherji S, Jagadevan S, Mohapatra G, Vijay A (2004) Biodegradation of diesel oil by an Arabian Sea sediment culture isolated from the vicinity of an oil field. *Bioresour Technol* 95:281–286
- Necşulea A, Popa A, Cooper DN, Stenson PD, Mouchiroud D, Gautier C, Duret L (2011) Meiotic recombination favors the spreading of deleterious mutations in human populations. *Hum Mutat* 32:198–206
- Nei M, Kumar S (2000) *Molecular evolution and phylogenetics*. Oxford University Press, New York
- Pacwa-płociniczak M, Plaza GA, Poliwoda A, Piotrowska-seget Z (2014) Characterization of hydrocarbon-degrading and biosurfactant-producing *Pseudomonas* sp. P-1 strain as a potential tool for bioremediation of petroleum-contaminated soil. *Environ Sci Pollut R* 21:9385–9395
- Patowary R, Patowary K, Kalita MC, Deka S (2016) Utilization of paneer whey waste for cost-effective production of rhamnolipid biosurfactant. *Appl Biochem Biotechnol* 180:383–399
- Perna NT, Kocher TD (1996) Mitochondrial DNA: molecular fossils in the nucleus. *Curr Biol* 6:128–129
- Petrov DA, Hartl DL (1999) Patterns of nucleotide substitution in *Drosophila* and mammalian genomes. *Proc Natl Acad Sci USA* 96:1475–1479
- Ping L, Zhang C, Zhu Y, Wu M, Hu X, Li Z et al (2011) Biodegrading of pyrene by a newly isolated *Pseudomonas putida* pl2. *Biotechnol Bioprocess Eng* 16:1000–1008
- Raghavan R, Kelkar YD, Ochman H (2012) A selective force favoring increased G+C content in bacterial genes. *Proc Natl Acad Sci* 109:14504–14507
- Rajasekar A, Ting YP (2010) Microbial corrosion of aluminum 2024 aeronautical alloy by hydrocarbon degrading bacteria *Bacillus cereus* ace and *Serratia marcescens* ace2. *Ind. Eng Chem Res* 49:6054–6061
- Rocha EPC, Feil EJ (2010) Mutational patterns cannot explain genome composition: are there any neutral sites in the genomes of bacteria? *PLoS Genet* 6:e1001104
- Saikia RR, Deka S (2013) Removal of hydrocarbon from refinery tank bottom sludge employing microbial culture. *Environ Sci Pollut R* 20:9026–9033
- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4(4):406–425
- Sakthipriya N, Doble M, Sangwai JS (2016) Systematic investigations on the biodegradation and viscosity reduction of long chain hydrocarbons using *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*. *Environ Sci Process Impacts* 18:386–397
- Smardaa P, Bures P, Horová L, Leitchb I, Mucinac L, Pacinie E, Tichýa L, Grulich V, Rotreklová O (2014) Ecological and evolutionary significance of genomic GC content diversity in monocots. *PNAS* 111(39):E4096–E4102
- Smits TH, Witholt B, van Beilen JB (2003) Functional characterization of genes involved in alkane oxidation by *Pseudomonas aeruginosa*. *Antonie Van Leeuwenhoek* 84:193–200
- Spencer CC, Deloukas P, Hunt S, Mullikin J, Myers S, Silverman B et al (2006) The influence of recombination on human genetic diversity. *PLoS Genet* 2(9):e148
- Stoltzfus A, Norris RW (2016) On the causes of evolutionary transition:transversion bias. *Mol Biol Evol* 33:595–602
- Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrenner P et al (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406:959–964
- Sueoka N (1988) Directional mutation pressure and neutral molecular evolution. *Proc Natl Acad Sci USA* 85:2653
- Swati GP, Das MT, Thakur IS (2014) In vitro toxicity evaluation of organic extract of landfill soil and its detoxification by indigenous pyrene-degrading *Bacillus* sp. *Int Biodeter Biodegrad* 90:145–151
- Tahhan RA, Ammari TG, Goussous SJ, Al-Shdaifat HI (2011) Enhancing the biodegradation of total petroleum hydrocarbons in oily sludge by a modified bioaugmentation strategy. *Int Biodeter Biodegrad* 65:130–134
- Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol* 10:512–526
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M et al (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731–2739
- Thavasi R, Jayalakshmi S, Banat IM (2011) Effect of biosurfactant and fertilizer on biodegradation of crude oil by marine isolates of *Bacillus megaterium*, *Corynebacterium kutscheri* and *Pseudomonas aeruginosa*. *Bioresour Technol* 102:772–778
- Vasileva-Tonkova E, Sotirova A, Galabova D (2001) The effect of rhamnolipid biosurfactant produced by *Pseudomonas fluorescens* on model bacterial strains and isolates from industrial wastewater. *Curr Microbiol* 62:427–433
- Vasudevan R, Rajaram P (2001) Bioremediation of oily sludge-contaminated soil. *Environ Int* 26:409–411
- Verma S, Bhargava R, Pruthi V (2006) Oily sludge degradation by bacteria from Ankleswar, India. *Int Biodeterior Biodegradation* 57:207–213
- Vogel F, Kopun M (1977) Higher frequencies of transitions among point mutations. *J Mol Evol* 9:159–180
- Webster MT, Hurst LD (2012) Direct and indirect consequences of meiotic recombination: implications for genome evolution. *Trends Genet* 28:101–109
- Weinert LA, Werren JH, Aebi A, Stone GN, Jiggins FM (2009) Evolution and diversity of *Rickettsia* bacteria. *BMC Biol* 2:7–16
- Yu C, Yao J, Cai M, Wang F, Masakorala K et al (2013) Functional gene expression of oil-degrading bacteria resistant to hexadecane toxicity. *Chemosphere* 93:1424–1429
- Zeigler DR, Prágai Z, Rodriguez S, Chevreux B, Muffler A et al (2008) The origins of 168, w23, and other *Bacillus subtilis* legacy strains. *J Bacteriol* 190:6983–6995
- Zhang F, Zhao Z (2004) The influence of neighboring-nucleotide composition on single nucleotide polymorphisms (SNPs) in the mouse genome and its comparison with human SNPs. *Genomics* 84:785–795