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Genome analysis of crude oil degrading *Franconibacter pulveris* strain DJ34 revealed its genetic basis for hydrocarbon degradation and survival in oil contaminated environment

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ABSTRACT

Franconibacter pulveris strain DJ34, isolated from Duliajan oil fields, Assam, was characterized in terms of its taxonomic, metabolic and genomic properties. The bacterium showed utilization of diverse petroleum hydrocarbons and electron acceptors, metal resistance, and biosurfactant production. The genome (4,856,096 bp) of this strain contained different genes related to the degradation of various petroleum hydrocarbons, metal transport and resistance, dissimilatory nitrate, nitrite and sulfite reduction, chemotaxy, biosurfactant synthesis, etc. Genomic comparison with other *Franconibacter* spp. revealed higher abundance of genes for cell motility, lipid transport and metabolism, transcription and translation in DJ34 genome. Detailed COG analysis provides deeper insights into the genomic potential of this organism for degradation and survival in oil-contaminated complex habitat. This is the first report on ecophysiology and genomic inventory of *Franconibacter* sp. inhabiting crude oil rich environment, which might be useful for designing the strategy for bioremediation of oil contaminated environment.

1. Introduction

Crude oil exploration, transportation, storing and refining processes generate considerable amounts of hazardous oily waste containing total petroleum hydrocarbons (TPH) which include aliphatics, aromatics, NSO compounds and asphaltenes [39]. Improper handling and disposal of such pollutants pose a serious threat to the environment [9,20]. Microbial bioremediation has been strongly recommended as an alternative cleanup technology, which is relying on the diverse metabolic capabilities of microorganisms to remove wide range of pollutants leading to environmental decontamination [12,51]. However there are few practical constrains in implementing efficient bioremediation technology due to the complex nature of contaminated sites and to the lack of knowledge about the adaptation and survival strategies of microorganisms and about the various interacting environmental factors regulating bioremediation processes in such adverse environments [23,28]. The prevailing physicochemical conditions of the contaminated site govern the survival and activity of the inhabiting microbial population. Therefore it is essential to understand the physiological, metabolic and genetic potential of the native microorganisms in order to design a bioremediation strategy [2,3,15]. Several reports are available on hydrocarbon/crude oil degrading bacteria from diverse environments belonging to the genera of *Pseudomonas*, *Rhodococcus*, *Bacillus*, *Paenibacillus*, *Stenotrophomonas*, *Alcaligenes*, *Enterobacter*, *Arthrobacter*, *Rhodococcus*, *Alcanivorax*, *Marinobacter*, *Cycloclasticus*, etc. [6,10,23,29,46,56]. Recent advances in genomics have offered valuable information about the genetic basis of microbial adaptation to the contaminated environments and performance for degradation/removal of pollutants. Genome sequencing projects have been undertaken to assess the genetic potential and survival mechanisms of oil-eating organisms in oil rich environments [6]. Several enzymes and pathways for the degradation of crude oil components have been identified, which include alkane monooxygenase (AlkB) and cytochrome P450 type metabolic routes for transformation of short to medium chain alkanes [4]. Genes involved in aromatic hydrocarbon degradation such as naphthalene dioxygenase (*ndo*B), catechol 2,3 dioxygenase, *nid*A and *nid*B have also been studied [50]. Previous studies have found that microbially produced biosurfactants can promote hydrocarbon degradation and genome scale analysis revealed the presence of genes responsible for biosurfactant production in many crude oil degrading organisms [10,34,44]. Genome analysis has also revealed that adaptation of the microorganisms in the polluted environments is ensured at the genetic level by two component systems, chemotaxis, thermosensing and nutrient

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uptake and detoxification for responding to the corresponding environmental condition [10].

Enterobacter helveticus and *Enterobacter pulveris*, isolated from fruit powder and its related environments, have been recently reclassified as *Franconibacter helveticus* and *Franconibacter pulveris* based on their biochemical traits, multi locus sequence alignments (MLSA) and genome scale analysis [48]. Till date no information is available on *Franconibacter* spp. that inhabits petroleum rich environments. In the present work, we have identified a crude oil degrading *Franconibacter pulveris* isolated from tank bottom sludge of Duliajan oil fields, Assam. Strain DJ34 was characterized in terms of its biochemical, metabolic and genomic properties. The genome contained different genes related to the degradation of petroleum hydrocarbons, metal transport and resistance, dissimilatory nitrate, nitrite and sulfite reduction, chemotaxis, biosurfactant synthesis, etc. Detailed COG analysis revealed the genomic capability of this organism for degradation and survival in petroleum contaminated complex niche. To our knowledge, this is the first report on the ecophysiology and genome analysis of *Franconibacter pulveris* inhabitant to crude oil rich environment, which might be useful for designing bioremediation strategy.

2. Materials and methods

2.1. Isolation, identification and characterization of Franconibacter pulveris strain DJ34

Strain DJ34 was isolated from petroleum hydrocarbon contaminated sludge of Duliajan oil fields, Assam, India on Reasoner's 2A (R2A) agar plate. Selected colonies were repeatedly subcultured on Luria-Bertani (LB) agar plates to obtain a single pure culture. The isolate was routinely grown in LB medium at 30 °C, maintained on LB agar, and preserved as glycerol stock (15% v/v) at −80 °C. Phylogenetic analysis was conducted based on the 16S rRNA gene sequence similarity. The representative 16S rRNA gene sequences of the most closely related strains were retrieved from NCBI and type strains from EzTaxon and the consensus tree was constructed using the neighbor-joining method with 500 bootstrap consensus values by using MEGA 5.0. Multi locus sequence alignment (MLSA) was performed based on the concatenated coding sequences of seven house-keeping genes (*atp*D, *fus*A, *gln*S, *glt*B, *gyr*B, *inf*B, *pps*) retrieved from the whole genome sequences of related organisms available in NCBI database [48].

Gram's reaction of strain DJ34 was determined by using gram staining kit (HIMEDIA). Catalase activity was confirmed by bubble production on addition of 3% H_2O_2 to freshly grown cells. Oxidase activity was checked by using oxidase discs (HIMEDIA). Biochemical tests were performed by following the methods of Stephan et al. [48]. The growth of the strain was monitored in individual substrate and by using BIOLOG GEN III microplate assay technique. The chemotaxonomic approach was undertaken for further assigning the strain by comparing it with the type strain *Franconibacter pulveris* 24057^T. Respiratory quinone was isolated and analyzed by reverse phase high-performance liquid chromatography (HPLC, Agilent) using Sorbax C18 column [22]. Methanol: isopropanol mixture (2:1, v/v), was used at a flow rate of 1 mL min^{-1} for elution of quinones, which were identified by Matrix-assisted laser desorption-ionization-time-of-flight mass spectrometry (MALDI-TOF MS, Alpha Xtreme, Bruker Daltonix) [21]. Polar lipids were extracted and identified by the method of [22]. Cellular fatty acids were analyzed using cells from the mid log phase of growth. The collected cell mass was saponified followed by methylation and esterification of fatty acids. Cellular fatty acid methyl esters (FAMEs) were detected by GC-FID (CLARUS 500, Perkin Elmer) installed with omega wax capillary column (30 mm × 0.25 mm, *df* 0.25 μ) supelco 24,136 by using bacterial FAME standard (BAME mix, Sigma Aldrich) [33]. Average Nucleotide Identity (ANI) analysis was conducted by using currently available online tools for ANI calculation (**http://enve-omics.ce.gatech.edu/ani/index**) [48].

The growth of strain DJ34 at various temperatures (4–50 °C), at different NaCl concentrations (0–10%) and at different pH range (1.0–11.0) was tested in mineral salt medium (MSM) [9]. Heavy metal sensitivity of the isolate was investigated following growth at 30 °C for 7 days on MSM agar plates with yeast extract as carbon source supplemented with different heavy metal salts ($CdNO_3$, $Pb(NO_3)_2$ and $NiCl_2$) at various concentrations ranging from 0.1 mM to 5 mM. Growth in anaerobic condition was tested on agar plates of anaerobic medium (HiMedia, India). Utilization of multiple electron acceptors during growth other than oxygen was investigated by providing nitrate-reducing, sulfate-reducing and iron-reducing conditions in anaerobic agar plates of MSM amended with various electron acceptors (sodium nitrate, sodium sulfate and ferric chloride; 20 mM each) and donors (glucose, 0.1% and yeast extract, 0.1%). Surfactant production ability of the isolate was examined by adding equal volume diesel with culture supernatant (2 mL) in a culture tube and vortexed vigorously to form an emulsion. The tube was allowed to stand of 24 h. The emulsion stability was determined after 24 h. The emulsification index (E24) was estimated as the percentage height of the emulsion layer to the total height of the liquid column [7,38].

The potential of the isolate to utilize various hydrocarbons as sole carbon source was evaluated in MSM. The culture was incubated at 30 °C for 3 days in presence of Benzene (B), Toluene (T), Ethyl Benzene (E) or Xylene (X) at concentration of 50 mg L^{-1} and BTEX mixture at 200 mg L^{-1}. Utilization of alkanes as sole source of carbon by the bacterium was carried out during growth in 100 mL Erlenmeyer flasks containing 20 mL MSM supplemented with pentadecane (C_{15}) or hexadecane (C_{16}) at a concentration of 250 mg L^{-1}. Inoculation was done from an overnight grown cell in the range of 10^7–10^8 CFU mL^{-1} and incubated at 30 °C with 150 rpm shaking. At 0 day and after every 7 days the flasks were sacrificed for determining the cell numbers and analyzing the residual *n*-alkane by gas chromatography. The residual *n*-alkane from the medium was extracted by adding equal volume of *n*-hexane and deep freezing the lower water layer to collect the upper organic phase. Remaining water is being absorbed by adding Na_2SO_4. The residual concentrations of the alkanes were analyzed using a GC (Agilent 7820A) equipped with a split/splitless injector, FID detector and a HP-5 column (30 m × 0.32 mm and i.d. 0.25 um thickness). Nitrogen was used as carrier gas (flow rate 25 mL min^{-1}). The oven program was set initially at 80 °C for 2 min, followed by increasing to 210 °C at 10 °C rise per minute. Crude oil and oil containing actual sludge were also used as sole carbon source (1% w/v) and growth was measured by corresponding CFU count. Reduction of Total Petroleum Hydrocarbon (TPH) of sludge was analyzed by gravimetric method [51]. During crude oil degradation initial inoculum of 10^7 cells mL^{-1} was used. At 0, 7, 14, 21 and 28 day the flasks were sacrificed for determining the cell numbers. Residual crude oil was determined by gas chromatography by following the method of Thavasi et al. [49].

Cell morphology in presence and absence of crude oil (1% v/v) was studied by electron microscopy. Cells from mid log phase were harvested, washed with normal saline (0.85%) and fixed with glutaraldehyde (0.2%, v/v). Fixed cells were dehydrated with increasing concentration of ethanol (30–100%, v/v) and placed on cover glass coated with Poly-L-lysine. Finally it was gold coated and examined under SEM (ZEISS EVO 60, Carl ZEISS SMT) as described by Mohapatra et al. [33].

2.2. Genome analysis

The genome sequence of the organism was obtained by using the Illumina HiSeq 2500 platform [35] and annotated through NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP). The Integrated Microbial Genome (IMG) server (http://img.jgi.doe.gov) was the primary source used for genome predictions and comparisons. The Cluster of Orthologous Groups (COG) of protein sequences were analyzed using the functional category comparison tool of IMG. Transport system was analyzed by using Transport Classification

Database (**http://www.tcdb.org//**). A circular map representing the genome of strain DJ34 was generated using the web based CGview program [17]. Chromosomal deviations in GC contents, also known to be genomic islands (GIs) were predicted by Island viewer 3 online tool [11]. One-sample *t*-test was done to asses possible differences of the gene abundances of each COG category between *Franconibacter pulveris* DJ34 and other hydrocarbon degrading bacteria such as *Acinetobacter baylyi* ADP1, *Acinetobacter lwoffii* SH145, *Alcanivorax borkumensis* SK2, *Bacillus thuringiensis* BT407, *Burkholderia cepacia* 383, *Desulfatibacillum alkenivorans* AK-01, *Desulfococcus oleovorans* Hxd3, *Geobacillus thermodenitrificans* NG80-2, *Marinobacter algicola* DG893, *Pseudomonas aeruginosa* PAO1, *Pseudomonas fluorescens* Pf-5, *Rhodococcus jostii* RHA1, *Polymorphum gilvum* SL003B-26A1T and *Pseudomonas aeruginosa* N002 as found in the IMG database. A similar test was also done to assess the difference in gene abundances of each COG category between *Franconibacter pulveris* DJ34 and two of its closely related strains *Franconibacter helveticus* 1159 and *Franconibacter pulveris* 601 (deposited in Belgian Co-ordinated Collection of Micro-organisms (BCCM) as *Franconibacter pulveris* 24057^T) [48].

3. Results and discussion

3.1. Isolation, identification and characterization of Franconibacter pulveris strain DJ34

Strain DJ34 was isolated from the hydrocarbon contaminated sludge of Duliajan oil fields, Assam, in R2A medium. Nearly complete 16S rRNA gene sequence (1486 bp) of the strain is submitted in the NCBI database (GenBank accession number KM054665). The closest BLAST match of the strain was *Franconibacter* sp. strain DL503 (Accession no. KX865161.1) with 99% sequence similarity. Table S1 represents the results of closest matches of *Franconibacter pulveris* DJ34 from BLAST and EzTaxon analysis. Results of EzTaxon analysis revealed that the closest match of strain DJ34 is the type strain *Franconibacter pulveris* 24057^T with 98.53% sequence similarity (Fig. 1a). MLSA of DJ34 with the seven housekeeping genes of other *Franconibacter* spp. showed the closest similarity to our strain with type strain *Franconibacter pulveris* 24057^T with 95% bootstrap value, which was clustered within a branch containing other strains of *Franconibacter pulveris* suggesting a strong phylogenetic relationship between the strain DJ34 and *Franconibacter* spp. [48] (Fig. 1b).

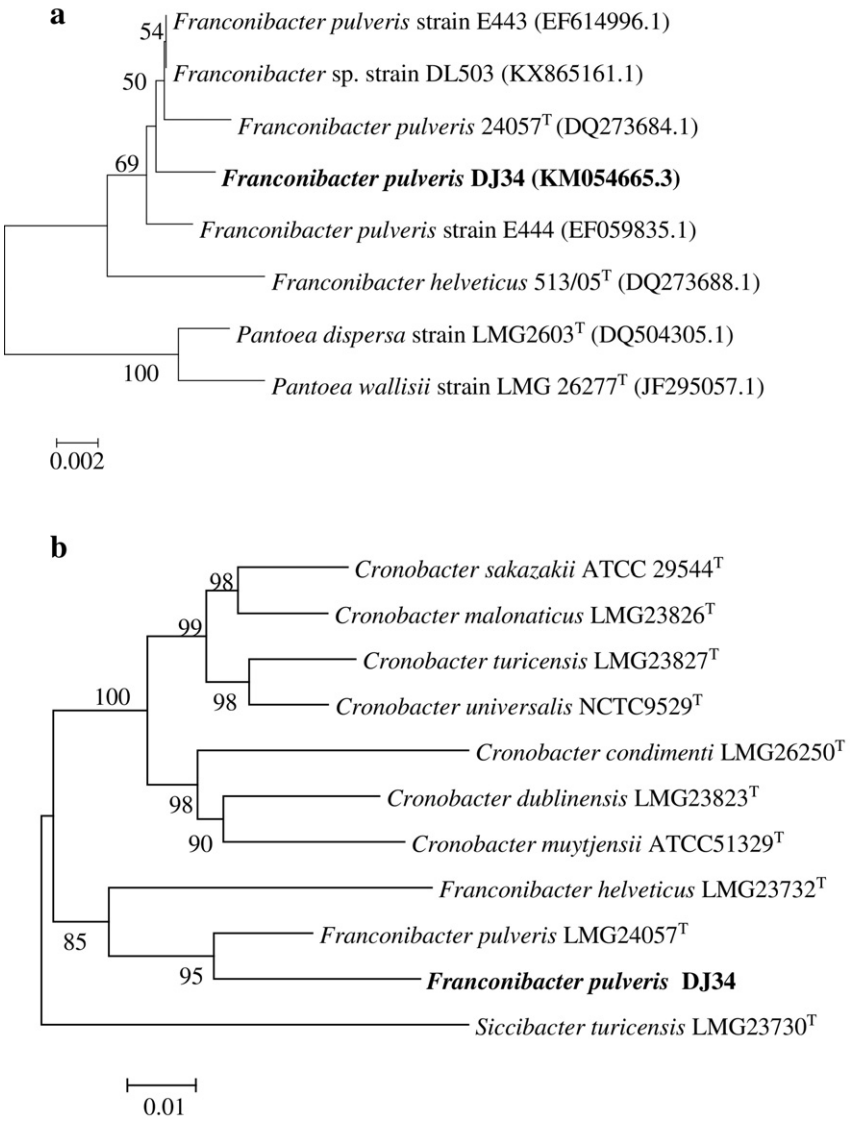

Fig. 1. (**a**): Neighbor-joining tree based on 16S rRNA gene sequences showing phylogenetic relationship between *Franconibacter pulveris* DJ34 and its closely related phylogenetic neighbors, constructed through MEGA version 5.0. Numbers at nodes represent bootstrap values obtained with 500 replications. *Bar* 0.02 indicates 2% nucleotide substitution; (**b**): Neighbor-joining tree based on Multi Locus Sequence Alignment (MLSA) showing phylogenetic relationship between *Franconibacter pulveris* DJ34 and its closely related members. Nodes represent bootstrap value obtained with 500 replications. Bar 0.01 represents 1% nucleotide substitution.

Table 1
Biochemical and metabolic characteristics of *Franconibacter pulveris* strain DJ34 under aerobic/anaerobic condition.

Growth substrate	Response	Growth substrate	Response	Growth condition/substrate	Response
Catalase	++	D-mannose	++	pH 1–2	−
Oxidase	−	Melibiose[a]	++	pH 3–7	++
Galactose	++	Sucrose	++	pH 9–11	−
Inositol	++	Turanose[a]	++	Temperature 4–15 °C	++
Lactose	++	Raffinose[a]	++	Temperature 15–25 °C	++
Mannitol	++	Maltose	++	Temperature 25–45 °C	++
Maltose	++	α-lactose[a]	++	Salinity 0–3 (%)	++
Sucrose	++	D-cellobiose[a]	++	Salinity 4–6 (%)	++
Starch	++	β-gentiobiose[a]	++	Salinity 7–10 (%)	++
Indole	−	α-L-rhamnose[a]	++	Anaerobic agar[b]	++
VP	−	D-arabitol	++	e^- acceptor (NO^{3-})[b]	++
MR	−	Glycerol	++	e^- acceptor (SO_4^{2-})[b]	++
Citrate	++	D-mannitol	−	e^- acceptor (Fe^{3+})[b]	++
Galacturonate[a]	++	D-saccharate[a]	++	Mixed e^- acceptor (NO^{3-}, SO_4^{2-},Fe^{3+})[b]	++
D-mannitol	−	L-malate[a]	++	Lead (1 mM)	++
Maltose	++	D-glucuronate[a]	++	Nickel (1 mM)	++
D-glucose	−	D-galacturonate[a]	++	Cadmium (1 mM)	+
Sucrose	++	*N*-acetyl-D-glucosamine[a]	++	Benzene (50 ppm)	++
D-arabitol	++	D-gluconate[a]	++	Toluene (50 ppm)	++
Trehalose[a]	++	L-aspartate[a]	++	Ethylbenzene (50 ppm)	++
L-rhamnose[a]	++	L-glutamate[a]	++	Xylene (50 ppm)	++
D-cellobiose[a]	++	L-alanine[a]	++	BTEX (200 ppm)	++
myo-inositol[a]	++	L-serine[a]	++	Pentadecane 250 ppm	++
D-sorbitol[a]	++	α-L-fucose[a]	−	Hexadecane 250 ppm	++
α-D-glucose	++	L-histidine[a]	−	Crude oil (1%)	++
β-D-fructose	++	DL-β-hydroxybutyrate[a]	−	Sludge (1%)	++
D-galactose	++	Propionate[a]	−	Tween 80 hydrolysis	−
Trehalose[a]	++	α-ketoglutarate[a]	−	Biosurfactant	++

++ very good growth, + good growth, − no growth e^- electron.
[a] Results obtained by BIOLOG GEN III microplate assay
[b] Growth under anaerobic condition

Cells of strain DJ34 were Gram negative, rod shaped and facultatively anaerobic. After 24 h of aerobic growth in LB agar at 30 °C colonies were yellow in color, while on R2A agar it produced circular white colonies with smooth and convex surfaces. The biochemical and metabolic characteristics of the strain were summarized in Table 1. Biochemical tests results of strain DJ34 showed strong similarity with that of the reference type strain *Franconibacter pulveris* LMG 24057^T [48]. Results for chemotaxonomic analysis were summarized in Table 2. The predominant respiratory quinone of strain DJ34 was identified as Ubiquinone-8 (Q-8), which was also similar to the reference strain *Franconibacter pulveris* LMG 24057^T [48]. The polar lipids (PL) detected in DJ34 strain were diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), unidentified aminolipid (AL), choline containing lipid (CL) and glycolipid (GL), which is consistent with the profile of *Franconibacter pulveris* 24057^T (Fig. S1). The fatty acids of strain DJ34 were determined to be $C_{16:0}$ (22.33%), $C_{18:1}\omega7c$ (21.71%), $C_{18:0}$ (16%), $C_{18:2}\omega7c10c$ (7.1%), iso$C_{15:0}$ (6.55%), $C_{14:0}$ (3.62%), $C_{12:0}$ 2-OH (1.06%), $C_{14:0}$ 2-OH (0.33%), $C_{11:0}$ (0.25%) and $C_{12:0}$ (0.18%). The profile of cellular fatty acids was also consistent with that of the *Franconibacter pulveris* LMG 24057^T, although the proportions of the fatty acids were different (Fig. S2). Average Nucleotide Identity (ANI) value of 95% was recommended as a species threshold corresponding to a DNA–DNA hybridization value of 70% [16]. ANI between the genomes of *Franconibacter pulveris* DJ34 and *Franconibacter pulveris* LMG 24057^T was 99.17% which also indicated a strong relationship between the strains.

Metabolic characteristics of the strain are presented in Table 1. The strain showed growth at 4–45 °C temperature, at pH range 3–9, in presence of heavy metals (Ni, Pb and Cd) at around 1 mM range and tolerance of NaCl upto 10%. The strain also showed confluent growth under nitrate-reducing, sulfate-reducing and iron-reducing anaerobic conditions. The E24 value for biosurfactant production was nearly 20%. CFU

Table 2
Chemotaxonomic comparison of *Franconibacter pulveris* strain DJ34 with type strain *Franconibacter pulveris* LMG 24057^T.

Fatty acids	*Franconibacter pulveris* LMG 24057^T	*Franconibacter pulveris* DJ34
Saturated		
11:0	–	0.25
12:0	0.63	0.18
14:0	0.36	3.62
16:0	18.48	22.33
18:0	10.48	16.0
19:0	2.12	1.7
20:0	−	0.16
iso 15:0	5.7	6.55
iso 17:0	11.11	17.66
Unsaturated		
18:1	16.2	22.61
18:2	7.17	7.1
Hydroxylated		
10:0 2-OH	−	0.15
12:0 2-OH	0.65	1.06
14:0 2-OH	0.21	0.33
14:0 3-OH	0.15	0.18
Major quinone	UQ-8	UQ-8
Polar lipids		
DPG	+	+
PG	+	+
PE	+	+
AL	+	+
CL	+	+
GL	+	+

+ detected; − not detected.
DPG diphosphatidylglycerol, *PG* phosphatidylglycerol, *PE* phosphatidylethanolamine, *AL* unidentified aminolipid, *CL* choline-containing lipid, *GL* glycolipid.

counts during growth in presence of each alkane increased during the period of 0 to 7 days and then it gradually decreased till 28th day. A remarkable increase in hydrocarbon degradation was observed during this period. Pentadecane proved to be the preferred choice for carbon source than hexadecane, probably due to shorter carbon chain length than the later [53]. Removal of hexadecane from growth medium was 86.33%, whereas pentadecane was degraded by more than 99% at the end of 28 days (Fig. S3a). Previous investigators have also demonstrated higher utilization of pentadecane and thus higher cell yield compared to hexadecane (C_{16}) [52]. Strain DJ34 encodes for alkane 1-monooxygenase which could initiate the degradation of alkanes to alkyl alcohol as suggested by Li et al. [27]. DJ34 also encodes alcohol dehydrogenase which is responsible for converting alkyl alcohol to alkyl aldehyde and aldehyde dehydrogenase converting alkyl aldehyde to fatty acid which is ultimately degraded via the β-oxidation pathway. Pentadecane degradation may be initiated by monoterminal oxidation to produce 1-Pentadecanol by alkane monooxygenase and n-Pentadecanoic acid as suggested by previous investigators [27,37]. The strain showed increase in growth during three days of incubation in BTEX when used as sole carbon source. Growth in petroleum sludge was evaluated by increase in bacterial cell count from day zero to 14th day. TPH was reduced by more than 50% during this period. While growing in crude oil as sole carbon source, 10 fold increase in cell count was observed within the 7 days of inoculation. The bacterial count increased gradually till 28 days from 10^6 to 10^7 cells/mL, after which it started decreasing. Nearly 26% TPH was reduced during this period (Fig. S3b). Gallego et al. [13] in their study with *Pseudomonas* and *Acinetobacter*, isolated from oil tank bottom sludge, observed similar kind of growth pattern in which the cell numbers increased within 7 days and gradually declined after that. SEM analysis of our strain showed that the cells were scattered throughout the microscopic field when grown in absence of crude oil but they showed aggregation during their growth in crude oil (Fig. 2a and b).

3.2. Genome analysis

3.2.1. COG analysis

The draft genome of *Franconibacter pulveris* DJ34 is composed of 4,856,096 bp, with a GC content of 56.5% [35]. A total of 4382 protein coding sequences (CDSs) has been identified out of which 3478 was designated to cluster of orthologous groups (COGs). The list of protein products, annotated through NCBI PGAAP and assigned to COG by IMG was represented in Table S2 and S3. The genome encodes 23 rRNA, 76 tRNA, 60 pseudogenes and 14 noncoding RNA. The orthologous groups are assigned into 24 different categories, as presented in Table S4. The circular map representing the G + C content and GC skew is represented in Fig. S4.

The *t*-test analysis revealed higher abundances of genes for amino acid transport and metabolism (E, 9.84%), carbohydrate transport and metabolism (G, 10.22%), cell motility (3.14%), inorganic ion transport and metabolism (P, 5.72%), cell wall/membrane/envelope biogenesis (M, 6.43), intracellular trafficking, secretion, and vesicular transport (U, 2.48%) and transcription (K, 8.53%) in the DJ34 genome than the average values obtained for other hydrocarbon degrading bacteria. However the genes responsible for coenzyme transport and metabolism (H, 5.16%), defense mechanisms (V, 1.97%), energy production and conversion (C, 5.64%), extracellular structures (W, 0.66%), lipid transport and metabolism (I, 3.29%), nucleotide transport and metabolism (F, 2.4%), posttranslational modification, protein turnover, chaperones (O, 3.90%), replication, recombination and repair (L, 3.11%), secondary metabolites biosynthesis, transport and catabolism (Q, 2%), signal transduction mechanisms (T, 5.08%), translation, ribosomal structure and biogenesis (J, 6.02%) and cell cycle control, cell division, chromosome partitioning (D, 1.06%) were comparatively lower than the average levels (Table S5). Not much significant difference was observed when *t*-test analysis was performed between strain DJ34 and *Franconibacter* spp. as the values for the gene abundances in each COG categories were similar (Table S6).

3.2.2. Crude oil component degradation

Several genes which are known to be involved in oil component degradation were detected in the genome of *Franconibacter pulveris* DJ34 (Table S7). Among these, Alcohol dehydrogenase is responsible for chloroalkane and chloroalkene degradation. S-(hydroxymethyl) glutathione dehydrogenase (EC 1.1.1.284)/alcohol dehydrogenase (ACH50_RS05245) and salicylaldehyde dehydrogenase (EC 1.2.1.65) (ACH50_RS05500) is responsible for naphthalene degradation [10,26]. Five genes as found in our strain encode for catechol 2,3 dioxygenase or other lactoylglutathione lyase family enzyme in COG0346, which are known to be involved in aromatic biodegradation [31]. Catechol is an important intermediate in the aromatic compound degradation pathway. Salicylaldehyde dehydrogenase was reported to be involved in enhancing the mineralization of naphthalene by *Pseudomonas putida* by converting salicylaldehyde to salicylate [26]. Lanfranconi et al. [24] have also demonstrated that *P. stutzeri* AN10 having the gene for Salicylaldehyde dehydrogenase showed the capacity to degrade naphthalene and effectively convert the salicylate byproducts into tricarboxylic acid cycle intermediates. Strain DJ34 possessing Salicyldehyde dehydrogenase gene might be playing an important in aromatic hydrocarbon degradation in crude oil contaminated environment. One copy of aromatic ring-opening dioxygenase, of the LigB family (COG3384) and p-hydroxybenzoate 3-monooxygenase (EC 1.14.13.2) (ACH50_RS08280) which plays an essential role in the beta-ketioadipate pathway of aromatic compound degradation.

Protocatechuate 3,4-dioxygenase (EC 1.13.11.3) alpha subunit (ACH50_RS05270/COG3485) and beta subunit (ACH50_RS05275/COG3485) as found in the genome of our strain are known to be involved in the benzoate and xylene degradation. Strain DJ34 harbors various genes for aromatic hydrocarbon degradation via β-ketoadipate pathway, which included 3-carboxy *cis*, *cis* muconate cycloisomerase (EC 5.5.1.2) (ACH50_RS03975), 4-carboxymuconolactone decarboxylase (EC 4.1.1.44) (ACH50_RS03985), β-ketoadipate enol lactone hydrolase (EC 3.1.1.24), β-ketoadipate succinyl CoA transferase (EC 2.8.3.6) and β-keto adipyl CoA thiolase (EC 2.3.1.174) (ACH50_RS03970). Aerobic ring cleavage of protocatechuate is catalyzed by Protocatechuate 3,4 dioxygenase to generate 3-carboxy *cis*, *cis* muconate which is converted into 4-carboxymuconolactone by 3-carboxy *cis*, *cis* muconate cycloisomerase. 4-carboxymuconolactone is transformed into β-keto adipate enol lactone by 4-carboxymuconolactone decarboxylase which is further hydrolyzed to β-keto adipate by β-keto adipate enol lactone hydrolase. Conversion of β-keto adipate to β-keto adipyl CoA is governed by β-keto adipate succinyl CoA transferase to finally form succinyl CoA and acetyl CoA by β-keto adipyl CoA thiolase [42]. Benzoyl formate decarboxylase (BFDC) (EC 4.1.1.7), a thiamin diphosphate (ThDP) dependent enzyme was also found in DJ34 genome, which is reported to catalyze the decarboxylation of benzoyl formate forming benzaldehyde and carbon di oxide [43]. *Pseudomonas naphthalenivorans* CJ2T has the ability to grow on naphthalene suggest that the gene responsible for Benzoyl formate decarboxylase may have originated from an organism that shares the ability to grow on aromatic compounds [19]. Vanillin dehydrogenase (EC 1.2.1.67) belongs to the aldehyde dehydrogenase family. It is involved in converting xylene to methylbenzoate and toluene to benzoate respectively. Several other enzymes such as benzoylformate decarboxylase, 3 carboxy *cis*, *cis* muconate cycloisomerase (ACH50_RS03975) and 3-oxoadipate enol lactonase (ACH50_RS03980) which belong to the subpathway of the beta-ketoadipate pathway are actively involved in aromatic compound degradation. Two copies of gene encoding for 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (EC 3.7.1.8) (ACH50_RS05510) was also present in strain DJ34 genome, which may be responsible for the degradation of biphenyl or polychlorinated biphenyl [25].

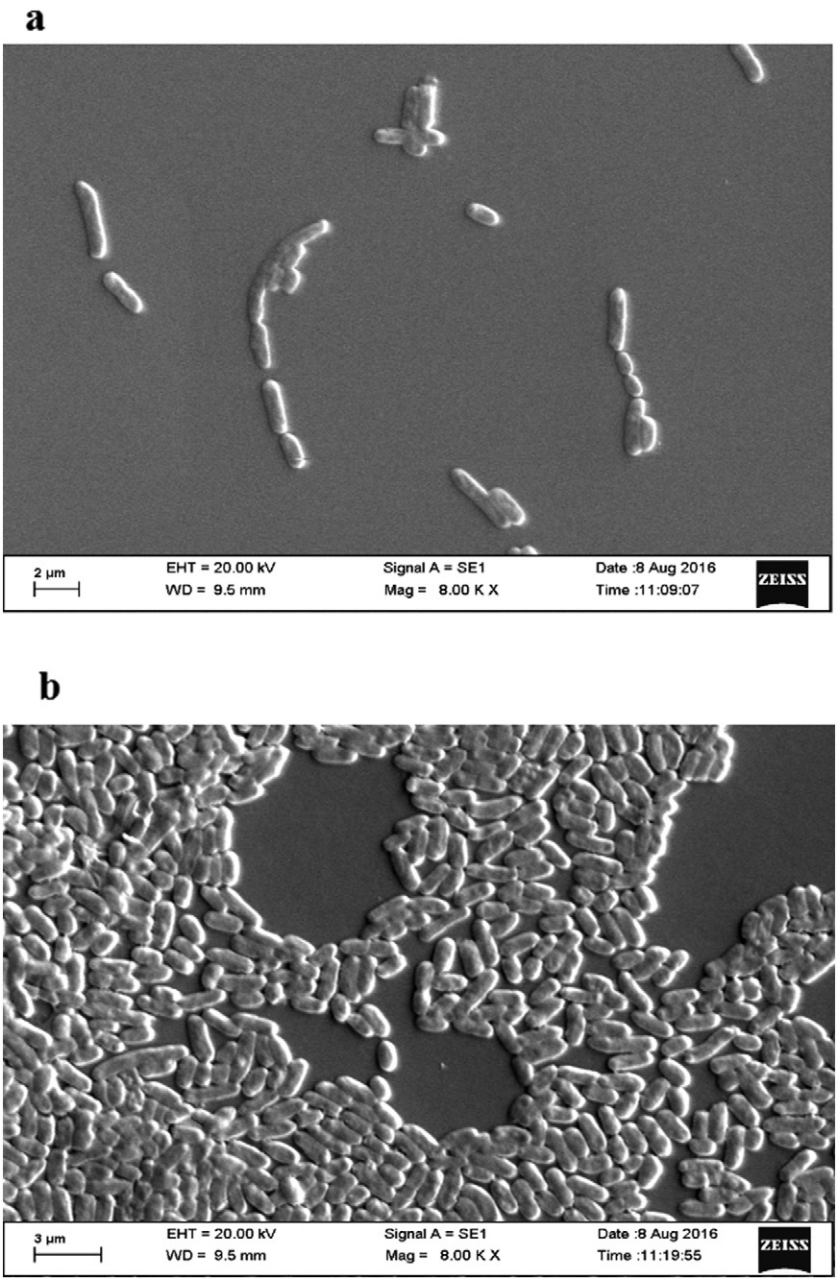

Fig. 2. (**a**): Scanning electron micrograph of *Franconibacter pulveris* DJ34 cells grown overnight in LB medium. The scale bar represents 2 µm; (**b**): Scanning electron micrograph of *Franconibacter pulveris* DJ34 cells grown overnight in crude oil containing medium. The scale bar represents 3 µm.

Two genes of alkane 1-monooxygenase (EC 1.14.15.3) (ACH50_RS05860 and ACH50_RS12560), one alkane sulfonate monooxygenase (EC 1.14.14.5) (ACH50_RS16705/COG2141), one alkane sulfonate transporter permease subunit (ACH50_RS16700), four genes for alcohol dehydrogenase (EC 1.1.1.1) (ACH50_RS04875/COG1028, ACH50_RS08610/COG1064, ACH50_RS12620/COG1454, ACH50_RS20910/COG1062) and four genes for aldehyde dehydrogenase (EC 1.2.1.3) (ACH50_RS05215, ACH50_RS13425, ACH50_RS14150, ACH50_RS20920 (COG1012)) was also present in the genome of DJ34. Zhang et al. [56] reported a strain of *Enterobacter mori*, isolated from crude oil well capable of degrading petroleum, possess a flavin dependent alkane degrading enzyme with which Alkane 1-monooxygenase from strain DJ34 showed 93% similarity in the amino acid sequence. Alkane once oxidized, genes encoding enzymes for oxidation of alcohols (Alcohol dehydrogenase) and aldehydes (Aldehyde dehydrogenase) complete the second and third step of mineralization, respectively. The substrate specificity of alcohol dehydrogenase is not only restricted to aliphatic alcohols, rather the physiological importance of this enzyme can be highlighted by its ability to metabolize xenobiotic aromatic and aliphatic hydroxyls through similar pathways. Aldehyde dehydrogenase plays an important role in detoxification of toxic aldehydes produced during various cellular metabolic pathways and is thus recognized as an essential enzyme during degradation of many hydrocarbon compounds. [47]. Involvement of alkane sulfonate monooxygenase in alkane and sulfonated alkane degradation is evident from the studies of [1,36], and are mostly carried out by the members of *Actinomycetales*, *Clostridiales*, *Burkholderiales*, *Pseudomonadales* and *Rhizobiales*. The overall structural similarity of alkane sulfonate monooxygenase with long chain alkane monooxygenase (LadA) may probably aid in alkane degradation [40]. Different pathways like monoterminal, diterminal, subterminal and terminal are being followed by the microorganisms during growth in alkane compounds [54].

Alkane monoxygenase or cytochrome P450 are reportedly involved in degradation of small, medium or long chain *n*-alkanes by oxidizing it into primary alcohols, monocarboxylic fatty acids, secondary alcohols and ketones [32]. Fatty acids are common degradation products of *n*-alkane [41]. ABC type long chain fatty acid transport system fused with permease and ATPase component (COG1133 and COG2067) was found in DJ34 genome along with four other putative transporter genes belonging to the FAT family(Ga0078663_11482, Ga0078663_114227, Ga0078663_12438, Ga0078663_125111 (COG0318)) for efficient transportation of fatty acids.

3.2.3. Biosurfactant synthesis

Crude oil constituents like alkanes and aromatics are hydrophobic in nature and therefore are less available to environmental microorganisms. Emulsification could enable microbes to contact hydrocarbon and solubilize it by lowering the interfacial surface tension thereby degrading it. The common biosurfactants synthesized by hydrocarbon degrading organisms are glycolipid and lipoprotein in nature [34]. The genome of DJ34 has genes, which encoded the key enzymes phosphomannomutase (ACH50_RS12800), glycosyl transferase (ACH50_RS12735 and ACH50_RS14490) and acyltransferase (ACH50_RS02725, ACH50_RS04605 and ACH50_RS12635) involved in glycolipid synthesis (Table S8).

3.2.4. Regulation pattern

Based on the COG analysis, a total of 337 CDSs in the DJ34 genome were classified to the transcription category (K) (Table S9), which was highest among all the *Franconibacter* spp. considered for this comparative study. Among these 62 proteins are LysR type regulators for transcription (LTTRs) (COG0583), the most abundant type of transcriptional regulators of prokaryotes. These regulators have been reported to play a pivotal role in regulating genes responsible for aromatic compound catabolism, motility of cell and quorum sensing [5]. Thirteen proteins belonged to the OmpR family of transcriptional regulators for two component system, 11 proteins belonged to MerR family regulators (COG0789), 21 proteins belonged to the multiple antibiotic resistance regulator (MarR) family (COG1846) [55]; 19 proteins were TetR family transcriptional regulators (COG1309 and COG3226); 22 proteins were GntR family transcriptional regulators (COG1167, COG1802, COG2186 and COG2188) known to be associated with aromatic compound degradation [14]; 27 proteins belonged to AraC family regulators (COG2207, COG4977 and COG1609) which are responsible for sugar metabolism [30]. In addition, 7 proteins belonged to IclR family transcriptional regulators (COG1414), 4 proteins AsnC family transcriptional regulator (COG1522) and only 1 for ArsR family transcriptional regulator (COG0640). The genes responsible for type IV pili and flagella assembly have been found in the strain DJ34which can function to emulsify the hydrocarbon for crude oil degradation. Flagella assembly plays an important role in cell motility and chemotaxis which could also help in bacterial movement to relatively close niches and attachment to oil water interface where the uptake and utilization of hydrocarbons can take place. A total of 124 genes encode for cell motility (Table S10) which include flagellar biosynthesis, flagellar assembly and methyl accepting chemotactic proteins. Presence of diverse and abundant genes involved in responding systems, regulation systems, metabolism and transportation might be suggesting the strong ability of the DJ34 strain to adapt to harsh environmental condition. 201 genes have been assigned to the signal transduction (Table S11) category which encodes for 33 kinases and 41 genes for two component system (TCS) (Table S12), which is known to be the basic stimulus-response coupling mechanism in bacteria for sensing and responding to changes in many different environmental conditions. Strain DJ34 encodes genes for TCS responding to phosphate limitation and regulation, nitrogen limitation, and chemotaxis. Phosphorous acts as major rate limiting element for biological activity in oil reservoirs [18]. PhoB (Ga0078663_103295) and PhoR (Ga0078663_103294) phosphate regulon response regulator, belonging to the OmpR family, is present in DJ34 which may play a vital role in phosphate/phosphonate transport system. Phosphate may also be taken up by Na^+/Phosphate symporter. DJ34 encodes two copies of this enzyme (COG1283).

3.2.5. Transporters

The abundance of toxic crude oil components, heavy metals and other antimicrobial compounds and relatively lower elemental nutrients such as nitrogen, phosphorous, sulfur etc. in crude oil contaminated environments affect the growth and survival of microorganisms [18]. Competent transporters for nutrient uptake and detoxification could play an important role in microbial survival in harsh environment. The genome of strain DJ34 codes for ABC type sulfate transport system and sulfate/molybdate transport system. Seventeen genes code for the Sulfate permease (SulP) family (TC: 2.A.53) and five for sulfur relay (sulfurtransferase) complex belonging to the DsrC/DsrE/DsrF/DsrH/TusC for sulfur transport. Nitrate is thermodynamically favorable as terminal electron acceptor which facilitates efficient oxidation of carbon substrates in oxygen deficient conditions, allowing bacterial growth as well as hydrocarbon catabolism [45]. Genes for both dissimilatory and assimilatory nitrate reductase is being encoded by the genome of strain DJ34 and it also codes for ABC-type nitrate/sulfonate/bicarbonate transport system (COG0600, COG0715, COG1116).

Transport system of strain DJ34 included, 2014 genes (57.9% of total CDS) (Table S13). Among them, 569 genes encoded ATP-binding Cassette (ABC) superfamily (TC: 3.A.1) related proteins that capable of transporting a broad range of compounds, including inorganic ions, metal ions, lipids and hydrocarbons. 157 genes encoded Major Facilitator Superfamily (MFS) (TC: 2.A.1) involved in resistance to multidrug and solute transport, 36 genes for Drug/Metabolite Transporter (DMT) superfamily (TC: 2.A.7) involved in transport of sugar and drug resistance. Mot-Exb superfamily belongs to TC: 1.A.30, which helps in bacterial flagellar rotation by translocating H^+ or Na^+. Twelve genes were related to K+ transporter (Trk) family (TC: 2.A.38) for concentrating K^+ during osmotic stress [34]. Strain DJ34 genome contained abundant copies of Na^+ ion dependent transporter (COG0385), a Na^+ ion efflux pump, encoded by gene NorM (COG0534) and a Na^+ ion/phosphate symporter expressed by gene NptA (COG1283). These transporters contribute to efficiency in mediating ion transport which is essential to the organism to survive in the oil-contaminated ecosystem. Heavy metals are ubiquitous in petroleum contaminated areas. Microbial cell possess various transporters to effectively export heavy metals outside the cell. DJ34 genome contained divalent metal cation transporters for Fe/Co/Zn/Cd (COG0053) and Co/Zn/Cd efflux system component (COG1230). Uptake of iron seems to be very important as plenty of Fe^{2+}/Fe^{3+} transport proteins are being coded by the genome of DJ34. Fe^{2+} transport is mediated by FeoA (COG1918) and FeoB (COG0370) along with high-affinity Fe^{2+}/Pb^{2+} permease (COG0672) and enterobactin specific ABC-type Fe^{2+} transport system (COG4592). Fe^{3+} transport is facilitated by ABC type siderophore and hydroxamate transport system (COG0609 and COG0614). Zn^{2+} transport was mediated by Zinc transporter ZupT (COG0428), ZnuA (ABC-type Zn-ion uptake transporter, COG0803), ZnuB (ABC-type Mn^{2+}/Zn^{2+} ion uptake and transport, COG 1108), ZnuC (COG1121) and an ABC-type periplasmic component of Zn^{2+} transport system. FbpB (ABC-type Fe^{3+} ion transporter, COG1178) and one set of Zn^{2+}/Fe^{2+} permease (ZIP) family (Ga0078663_114182) was also found. Other metal transporters include the CorABC, for Mg^{2+}/Co^{2+}, Mn^{2+}/Fe^{2+} (NRAMP/VIT1/CCC1 family), a putative Mn^{2+} efflux pump MntP and two Cu/Ag efflux pump encoded by CusA and CusF. Apart from ion transporters, DJ34 also possesses different metal-dependent hydrolases (COG0402) and a benzoate:H^+ symporter BenE (COG3134). The abundance of heavy metal transporting genes and other transporters in strain DJ34 reflected its capacity to persist in the nutritionally poor crude oil-contaminated environment.

3.2.6. Horizontal gene transfer (HGT) and genomic islands (GI)

Eighteen GIs have been detected in DJ34 genome using the integrated mode of Island Viewer 3 (Fig. S5). The total length comprised by genomic islands was 211,894 bp which encoded 217 genes and 75 hypothetical proteins (Table S14). Table S15 represents the list of source organisms from which the genes may have been acquired by DJ34 through HGT. The predicted functions involved capsule synthesis and active transport, transportation of sugar and carbon stress response, universal and oxidative stress response, redox reaction of azo dyes, synthesis, storage and secretion of membrane proteins, cell wall synthesis, sulfur transfer, pili and fimbri formation and certain regions coding for unknown functions. Horizontal gene transfer has been known as a universal microbial process to acquire properties that enable them to adapt to different selective pressures in their habitat. Genomic islands in microbial genomes are gene clusters known to be originate from HGT. Presence of transposes/integrases coding genes in the GIs of the strain could have supported potentially active HGT or genetic alterations via bacteriophage infection making it capable of surviving in oligotrophic environment [8].

3.2.7. Comparative genomics

Results of genomic comparisons between *Franconibacter* strains from IMG database revealed that major proteins of strain DJ34 were closely related to *Franconibacter pulveris* 24057^T, isolated from fruit powder. The abundance profile tool of IMG revealed the difference in number of proteins annotated based on functions and families. More number of proteins have been annotated for pfam and COG in the strain DJ34 as compared to *Franconibacter pulveris* 24057^T and *Franconibacter helveticus* 1159^T. Pairwise *t*-tests between the abundance profiles gave an idea about the average differences between two sets of abundance profiles of two different strains. For pfam, there is a significant difference between DJ34 and 1159^T ($t = 3.649710702$) > $t_{critical}$ ($= 1.960819224$)) with 99.9732623% confidence, as well as between DJ34 and 24057^T ($t = 3.175342791$) > $t_{critical}$ ($= 1.960819224$)) with 99.8487% confidence. For COGs, there is a significant difference between DJ34 and 1159^T ($t = 4.602197809$) > $t_{critical}$ ($= 1.961097518$)) with 99.99955692% confidence, as well as between DJ34 and 24057^T ($t = 8.006345173$) > $t_{critical}$ ($= 1.961097518$)) with 100% confidence. Strain DJ34 possess more number of LysR-type transcriptional regulators (LTTRs) and AraC family transcriptional regulator than *Franconibacter pulveris* 24057^T and *Franconibacter helveticus* 1159^T, important for regulating genes for hydrocarbon degradation, stress response and survival in crude oil contaminated habitats [34]. The detailed comparison is listed in Table S16. Comparison of strain DJ34 was also being performed with already reported hydrocarbon degrading bacteria from IMG database (as mentioned before) which is enlisted in Table S17. Analysis of the gene abundance in COG categories between different hydrocarbon degrading bacteria and the DJ34 strain revealed that the percentage of carbohydrate transport and metabolism gene was on the higher side in the DJ34 genome, whereas rest of the genes related to cell motility, amino acid transport and metabolism, inorganic ion transport and metabolism, secondary metabolite synthesis, signal transduction, transcription and other COG categories were similar in all these organisms.

4. Conclusion

Franconibacter pulveris DJ34, isolated from tank bottom sludge of Duliajan oil fields, could grow in wide array of environmental factors and utilize various hydrocarbons as growth substrates. Biochemical, chemotaxonomic and genome scale analyses revealed its phylogenetic closeness with *Franconibacter pulveris* LMG 24057^T. The genome of strain DJ34 possesses various genes involved in hydrocarbon degradation, signal transduction, environmental stress response, nutrient uptake and various transporters. Horizontally acquired genes provide the genetic basis for its survival and activity in oil contaminated environment. These results provide profound understanding for designing bioremediation strategy for the restoration of oil polluted environments.

Nucleotide sequence accession number

Genome sequence of *Franconibacter pulveris* strain DJ34 has been deposited at DDBJ/EMBL/GenBank under the accession no. LFEJ00000000.1.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygeno.2017.06.002.

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