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Original Research

Jet A-1 Bacterial Contamination: a Case Study of Cultivable Bacteria Diversity, Alkane Degradation and Biofilm Formation

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Abstract

Bacterial contamination during fuel storage can lead to fuel biodegradation (biodeterioration) or infrastructure problems (i.e., microbiologically influenced corrosion (MIC), biofouling, etc.). Jet A-1 storage tank samples were screened, and 23 morphologically distinct bacterial strains were isolated. 6 isolates revealed biofilm initiation capacity. For 12 isolates the alkane monooxygenase gene (*alkB*) was successfully detected. The 12 *alkB*-possessing isolates were 16S rDNA sequences identified as belonging to *Bradyrhizobium*, *Sphingomonas*, *Bacillus*, *Pseudomonas*, *Methylobacterium* and *Lelliottia* genera. In addition to possessing enzymatic equipment (AlkB) required for alkane oxidation, 10 out of 12 isolates were able to use Jet A-1 and *n*-tetradecane as their sole carbon source and energy. Biofilm initiation capacity and the ability to grow on different hydrocarbons highlights once again that fuel bacterial contamination can lead to serious fuel and storage system alterations. In contrast, their adaptation to high concentrations of hydrocarbons highlights the potential use of our isolates for bioremediation processes.

Keywords: Jet A-1, biofilm, alkane deterioration/degradation, *alkB* genes

Introduction

Microbial contamination during fuel storage is a chronic problem, especially for Jet A-1 kerosene or diesel fuels. Besides fuel deterioration, microorganism proliferation during storage can lead to system problems such as microbiologically influenced corrosion (MIC) and biofouling. Microbial biofilm formation is the main cause and the first step in biofouling. Through distribution facilities, from storage tanks, microorganisms are transported with fuel to aircraft and sometimes can block the aircraft tank-to-engine filters and have fatal consequences for flight operation. MIC is also associated with biofilm development due to anaerobic bacteria. Fuel deterioration is associated with aerobic biodegradation of fuel hydrocarbons and it is a cause of fuel failing specification tests that result in loss of commercial value [1].

As previously emphasized, fuel biodegradation has negative (biodeterioration) or positive (bioremediation) connotations only from a commercial point of view [1].

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Although microbial growth in Jet A-1 storage tanks can cause a variety of serious problems, it is reasonable to suppose that microbes that are able to proliferate at such high hydrocarbon concentrations might be powerful candidates for bioremediation (bioaugmentation) processes [2, 3].

The first and most important step in the aerobic bacterial degradation of alkanes (the major fractions of Jet A-1 hydrocarbons) is catalyzed by oxygenases. These enzymes play an important role in fuel biodeterioration or bioremediation. Up until the present, the best characterized alkane oxygenase is rubredoxin-dependent alkane monooxygenase AlkB, encoded by the *alkB* gene [4]. Specific detection of *alkB* biomarker genes by PCR using strain-specific oligonucleotide primers or degenerate oligonucleotides [4, 5] without any obvious strain-specific discrimination, is frequently used to determine the abundance and diversity of alkane-degrading bacteria in various ecosystems [6].

Besides fuel deterioration, some of the major problems associated with microbial growth in fuel storage systems are biofouling, sludge formation within pipes and valves, and filter blockage. Under these circumstances, it becomes very important to test Jet A-1 tank bacterial isolates for biofilm formation capacities [7, 8].

Thus, considering all the above-mentioned aspects regarding microbial contamination of Jet A-1, it becomes necessary to evaluate the extent of microbial implications on the quality of fuel and biofouling under storage conditions and to extrapolate those findings to a useful extent, such as bioremediation of fuel spills.

In this context, the current study focusses on (i) estimating the implications of Jet A-1 storage tank bacterial contaminants in system-related problems (biofouling) and fuel deterioration, (ii) revealing the taxonomic identity of potential hydrocarbon (alkanes)-degrading bacteria strains, and (iii) investigating their alkanes utilization ability for an eventual use as candidates for bioremediation or as experimental strains used to unfold important fuel degradation aspects.

Materials and Methods

Isolation and Characterization of Cultivable Microorganisms

Aqueous phase samples from the bottom of 7 Jet A-1 storage tanks were collected, serially diluted and spread onto R2 agar (R2A) and Luria-Bertani agar (LBA). After 2 days of incubation (28ºC), morphologically distinct colonies were picked, checked for purity and stored at -80ºC in 25% glycerol.

Phenotypic characterization of bacterial isolates was based on colony morphology, Gram stain reaction, oxidase and catalase tests and Biolog metabolic arrays (Biolog, Hayward, CA, USA). For Biolog biochemical tests, bacterial strains were grown according to the Biolog GN/GP microplates manufacturer recommendations. Strains were incubated at 28ºC until plate readings by a MicroLog MicroStation.

Biofilm Formation Asay

Biofilm formation assays were conducted according to a modified version of the O'Toole and Kolter method [9]. Microtiter plates containing 200 µl LB broth/well were inoculated with 10 µl cell suspension (OD_{600nm} = 1) and incubated at 28°C for 24h. Formed biofilms were stained with 0.1% w/v crystal violet solution for 15 min and then washed with distilled water. Biofilm-attached dye was extracted in 200 µl/well 95% ethanol and quantified by OD_{590nm} measurements (Biolog MicroLog MicroStation). All experiments were performed in triplicate with appropriate controls.

DNA Isolation

Genomic DNA extraction was performed using a Purelink Genomic DNA kit (Invitrogen) according to the manufacturer's recommendations. DNA purity and concentration were determined by spectrophotometry (GE Healthcare NanoVue Plus™) and 0.8% (w/v) agarose gel electrophoresis.

alkB Gene Screening

Detection of the alkane hydroxylase gene was performed by PCR amplification using two sets of oligonucleotide primers: alkB-1f/alkB-1r [4] and ALK-3F/ALK-3R [5]. Those primers were designed based on *alkB* sequences, including the *alkB* gene sequence of *Pseudomonas (P.) protegens* CHA0 (AJ009579). Type strain *P. protegens* CHA0 [10], used as *alkB*-PCR positive control, was obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. PCR reactions were carried out in a final volume of 25 µl, containing: 100 ng genomic DNA, 12.5 µl Dream Taq Green (2X) (Thermo Scientific), 10 µM for each primer and water up to the final volume. For alkB-1f/alkB-1r primers, the temperature cycling conditions were: 95ºC for 10 min, 15 cycles of 94ºC for 30s, 51ºC for 30s, 72ºC for 60s, followed by 20 cycles of 94ºC for 30s, 55ºC for 30s, 72ºC for 60s, and a final elongation at 72ºC for 5min. For primer pair ALK-3F/ALK-3R the temperature cycling conditions were: 95ºC for 5min, 35 cycles of 94ºC for 30s, 60ºC for 30s, 72ºC for 60s and a final elongation at 72ºC for 5min. PCR products were checked by 1% (w/v) agarose gel electrophoresis.

Microorganism Identification

16S rRNA gene from all positive *alkB* strains was amplified by PCR using universal primers 27F

(5'- AGA GTT TGA TCM TGG CTC AG – 3') and 1492R (5'- CGG TTA CCT TGT TAC GAC TT – 3'). PCR reactions were carried out in a final volume of 50 µl, containing: 100 ng genomic DNA, 25 µl Dream Taq Green (2X) (Thermo Scientific), 1 µl for each primer (10 µM) and nuclease-free water up to the final volume. Cycling parameters were 95ºC for 5 min, followed by 30 cycles of 95ºC for 1 min, 58ºC for 30s, and 72ºC for 2 min, with a final step at 72ºC for 10 min. Positive amplification products were purified using a Favor Prep PCR Clean-up Mini kit (Favorgene Biotech Corp., Taiwan) and sequenced by GENETIC LAB (Bucharest, Romania) using the primer sets 27F/1492R or 27F/533F (5'-GTG CCA GCA GCC GCG GTA A-3'). Strain identification was based on their rDNA sequences comparison with GenBank using the BLAST algorithm from EzTaxon.

Microbial Growth on Hydrocarbons

Bacterial growth on aliphatic hydrocarbons was evaluated using 30 ml screw cap sealed flasks containing 10 ml minimal salts medium (MSM) (per liter: $Na_2HPO_4 \bullet 12H_2O$ 1 g, KH_2PO_4 0.5 g, $(NH_4)_2SO_4$ 1 g, $MgSO_4 \bullet 7H_2O$ 0.2 g, NaCl 1 g, pH 7.0) supplemented with hydrocarbons 1% (v/v) (*n*-tetradecane (C14) or Jet A-1) as the sole carbon source, and inoculated with bacterial culture (to a final OD_{600nm} = 0.1). Flasks were incubated at 28ºC, 150 rpm for 30 days. Microbial multiplication was determined at the end of the incubation period by measurement of OD_{600nm}. All experiments were performed in triplicate with appropriate controls.

Results and Discussion

Microbiological Phenotypic Characterization and Biofilm Formation

Morphologically distinct bacterial colonies, designated D1-D23, were directly isolated from the fuel tank water layer on R2A and LBA plates. As expected for oligotrophic bacteria, the majority of isolates exhibited a better growth on R2A than on rich LBA medium. Basic microbiological characterization and Biolog biochemical tests were performed for all the isolates (Tables 1, 2).

In biofouling and system-related problems involving microbial colonization and deposition of biofoulants, microorganisms need to (i) have biofilm formation abilities and (ii) proliferate in fuel storage conditions using Jet A-1 hydrocarbons.

Biofilm formation capacity (OD_{590nm} values for adherent cells) was highlighted for 6 out of 23 bacterial isolates: an OD>1.4 was obtained for strains D4, D18, D19; for D2, D15, D16 strains was registered a moderate biofilm formation (OD<0.7) (Fig. 1). Three of those isolates (D2, D15, D19) were 16S rRNA identified as closely related to *Bacillus simplex*, *Pseudomonas synxantha* and *Sphingomonas zeae* commonly found in biofilms [11, 12]. Due to the short period of incubation used for this assay (24 h), those 6 biofilm-forming isolates could be considered the primary colonizers, responsible for initial adhesion step of biofilm formation and could be involved in biofouling, sludge formation, and pipes/valves/filters blockage events.

Table 1. Cell morphology, reaction to Gram staining, oxidase and catalase production of direct isolates.

Bacterial isolate	Cell morphology	Gram staining	Oxidase and catalase tests	Bacterial isolates	Cell morphology	Gram staining	Oxidase and catalase tests
D1	bacilli	(-)	**Oxi(+)**	D12	bacilli	(-)	**Oxi(-)**
D2	bacilli	(+)	**Cat(+)**	D13	bacilli	(-)	**Oxi(-)**
D3	bacilli	(-)	**Oxi(-)**	D14	bacilli	(-)	**Oxi(+)**
D4	cocci	(+)	**Cat(+)**	D15	bacilli	(-)	**Oxi(+)**
D5	bacilli	(+)	**Cat(-)**	D16	cocci	(+)	**Cat(-)**
D6	cocci	(+)	**Cat(+)**	D17	bacilli	(+)	**Cat(+)**
D7	bacilli	(-)	**Oxi(-)**	D18	cocci	(+)	**Cat(+)**
D8	cocci	(+)	**Cat(+)**	D19	bacilli	(-)	**Oxi(+)**
D9	bacilli	(+)	**Cat(-)**	D20	bacilli	(-)	**Oxi(-)**
D10	cocci	(+)	**Cat(+)**	D21	bacilli	(+)	**Cat(+)**
D11	bacilli	(+)	**Cat(+)**	D22	bacilli	(-)	**Oxi(+)**
				D23	bacilli	(-)	**Oxi(+)**

Table 2. BIOLOG taxonomic identification of bacterial isolates.

Bacterial isolates	Biolog identification	Similarity index*	Bacterial isolates	Biolog identification	Similarityindex*
D1	*Acidovorax sp.*	0.448	D13	*Kluyvera cryocrescens*	0.518
D2	*Bacillus anthracis*	0.492	D14	*Burkholderia glumae*	0.769
D3	*Ralstonia sp.*	0.411	D15	*Pseudomonas fluorescens*	0.708
D4	*Kocuria kristinae*	0.786	D16	*Staphylococcus sp.*	0.465
D5	*Curtobacterium pusillum*	0.626	D17	*Bacillus sp.*	0.471
D6	*Micrococcus luteus*	0.721	D18	*Micrococcus luteus*	0.528
D7	*Xanthomonas campestris*	0.741	D19	*Sphingomonas terrae*	0.816
D8	*Staphylococcus xylosus*	0.547	D20	*Acinetobacter sp.*	0.327
D9	*Arcanobacterium bernardiae*	0.610	D21	*Bacillus anthracis*	0.651
D10	*Staphylococcus xylosus*	0.531	D22	*Pseudomonas tolaasii*	0.642
D11	*Bacillus anthracis*	0.500	D23	*Pseudomonas synxantha*	0.734
D12	*Kluyvera cryocrescens*	0.620			

*First choice from the similarity list of species that most closely matches the unidentified strain.

Molecular Screening of Alkane Hydroxylase Genes

In order to estimate the ability of the 23 bacterial isolates to be involved in fuel hydrocarbon deterioration/degradation, alkane hydroxylase *alkB* genes presence was investigated by PCR-amplification with two sets of primers [4, 5]. The medium-chain-length alkanes (C10 to C16) hydroxylation enzyme AlkB (EC 1.14.15.3) gene, is the most common gene that has been used for investigating the composition of alkane-degrading communities and for abundance estimation of oil-degrading bacteria in various environments [10].

In this study, the appropriate size *alkB* PCR fragments were obtained for 12 out of 23 bacterial isolates, all showing the expected *alkB* gene fragments of 330 bp using ALK-3F/ALK-3R-specific primers (Fig. 2). For bacterial isolates D14, D19, D22, D23, where non-specific amplifications were observed, *alkB* gene fragments of 550bp were successfully amplified with the highly degenerated primers alkB-1f/alkB-1r for confirmation (Fig. 2). Non-*alkB* possessing strains can either use Jet A-1 aromatic fraction, use different enzymatic systems for alkane degradation or cannot metabolize Jet A-1 hydrocarbons [13].

The 12 *alkB*-possessing bacterial strains were 16S rRNA identified as belonging to 6 genera (Table 3), with the prevalence of *Bacillus* and *Pseudomonas* species. The dominant phyla for the 12 identified, *alkB*-possessing isolates was *Proteobacteria* (Table 4).

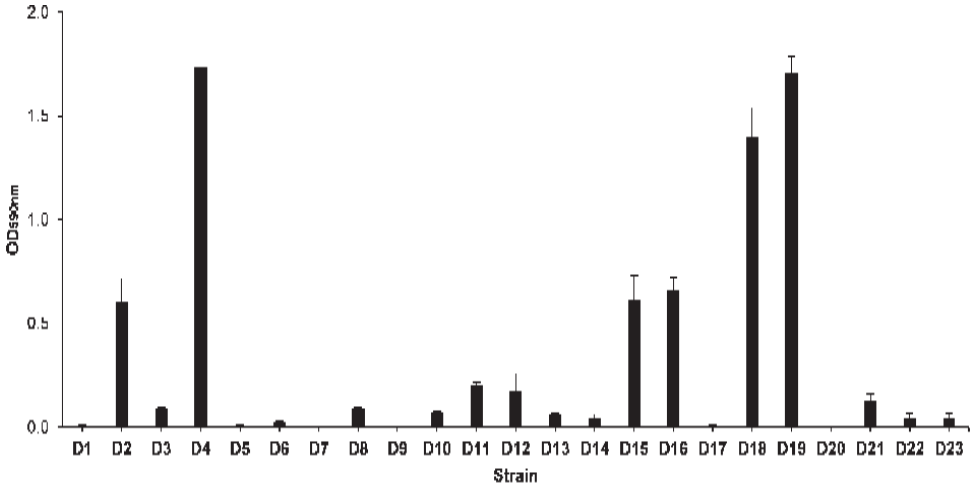

Fig. 1. Biofilm formation capacity of all Jet A-1 storage tanks isolates. The height of the bars represents adherent cells OD_{590nm} after 24 hours of incubation. Error bars display standard deviation based on four replicate wells.

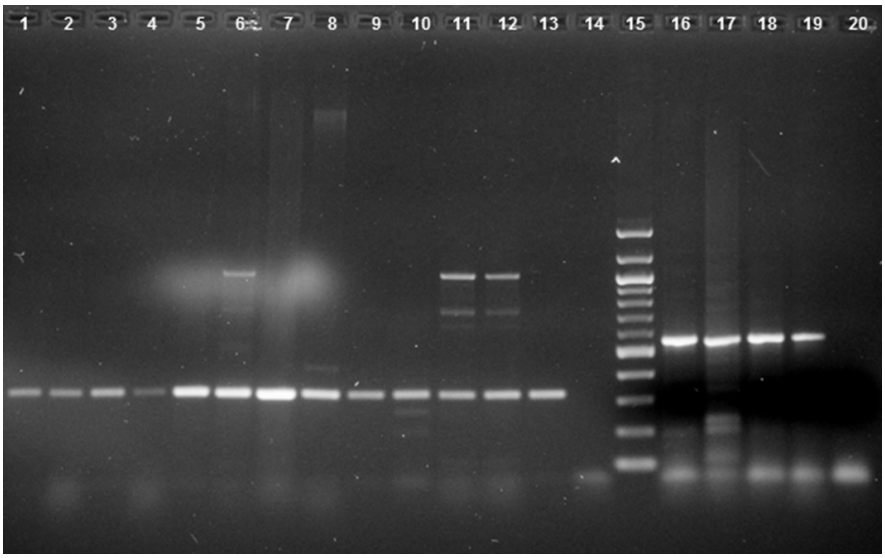

Fig. 2. Detection of *alkB* gene for direct isolates with ALK-B3F/ALK-B3R (lanes 1-14) and alkB-1f/alkB-1r (lanes 16-20). Lanes 1-14: D1, D2, D11, D12, D13, D14, D15, D19, D20, D21, D22, D23, *P. protegens* CHA0, no DNA control; lane 15: 100 bp DNA Ladder (New England Biolabs); lanes 16-20: D14, D19, D22, D23, no DNA control.

This it is in accordance with the previously reported dominance of *Proteobacteria* (*Gammaproteobacteria*) among fuel-isolated bacterial taxa by both cultivation-dependent and independent methods [14, 15].

Besides such frequent fuel-contaminating genera as *Bacillus*, *Pseudomonas* and *Sphingomonas* [14, 16], two bacterial isolates (D12, D13) were closely related to *Lelliottia amnigena* (former *Enterobacter amnigenus*). To the best of our knowledge, this is the first time the *Lelliottia* genus is mentioned among fuel-isolated bacteria or for possessing alkane monooxygenases codind genes. Isolates closely related with *Bradyrhizobium denitrificans* (D1) and *Methylobacterium plata*ni (D20) were previously mentioned as isolated from different fuel samples [14, 16-18] and associated with biofilms and MIC [19]. In the current study we found no correlation between biofilm formation and *B. denitrificans* D1 and *M. plata*ni D20 isolates (Fig. 1). Due to the iron-respiring (*Bradyrhizobium* sp.) and methane-oxidizing (*Methylobacterium* sp.) abilities, we consider that those genera could be biofilm inhabitants in the latest stages of biofilm development. The *Gammaproteobacteria* group found in our jet A-1 samples was mainly comprised of members of the *Pseudomonas* genus. The most abundant *Pseudomonas* species showed more than 99.7% 16S rRNA gene similarity to the *P. extremaustralis/veronii* lineage, species mentioned in relation with aromatic compounds degradation [20].

Table 3. 16S rDNA sequence taxonomic affiliation of bacterial isolates.

Strains	Molecular identification*	Class	Phylum
D1	*Bradyrhizobium denitrificans*	α-proteobacteria	*Proteobacteria*
D2	*Bacillus simplex, B. frigoritolerans*	*Bacilli*	*Firmicutes*
D11	*Bacillus cereus, B. anthracis*	*Bacilli*	*Firmicutes*
D12	*Lelliottia amnigena*	γ-proteobacteria	*Proteobacteria*
D13	*Lelliottia amnigena*	γ-proteobacteria	*Proteobacteria*
D14	*Pseudomonas extremaustralis*	γ-proteobacteria	*Proteobacteria*
D15	*Pseudomonas synxantha*	γ-aproteobacteria	*Proteobacteria*
D19	*Sphingomonas zeae, S.aeria*	α-proteobacteria	*Proteobacteria*
D20	*Methylobacterium aquaticum, M. variabile, M. tarhaniae*	α-proteobacteria	*Proteobacteria*
D21	*Bacillus cereus, B. anthracis*	*Bacilli*	*Firmicutes*
D22	*Pseudomonas extremaustralis*	γ-proteobacteria	*Proteobacteria*
D23	*Pseudomonas extremaustralis*	γ-proteobacteria	Proteobacteria

Table 4. 16S rDNA sequences taxonomic identification of isolates.

Strains	Accession no.	Molecular identification*	Similarity (%)
D1	*KY973665*	*Bradyrhizobium denitrificans*	99.82
D2	*KY973666*	*Bacillus simplex, B. frigoritolerans*	99.82
D11	*KY973667*	*Bacillus cereus, B. anthracis*	99.91
D12	*KY973668*	*Lelliottia amnigena*	98.88
D13	*KY973669*	*Lelliottia amnigena*	99.74
D14	*KY973670*	*Pseudomonas extremaustralis*	99.83
D15	*KY973671*	*Pseudomonas synxantha*	99.74
D19	*KY973672*	*Sphingomonas zeae, S. aeria*	98.88
D20	*MG000973*	*Methylobacterium platani, M. aquaticum, M. variabile*	98.05
D21	*KY973673*	*Bacillus cereus, B. anthracis*	99.91
D22	*KY973674*	*Pseudomonas extremaustralis*	99.73
D23	*KY973675*	*Pseudomonas extremaustralis*	99.91

* Identification based on comparison of their rDNA 16S sequences with GenBank by BLAST algorithm from EzTaxon. The closest relative type strain is detailed. Only hits with the same similarity percentage are included.

Bacillus species are frequently isolated from fuel storage tanks, one of the reasons being the high resistance to fuel additives due to their endospore-forming features. Species closely related to *B. cereus* and *B. anthracis* were previously described as contaminants for aviation fuels (i.e., JP-8) [15].

Even though the 16S rRNA gene is the basis for phylogenetic analysis and taxonomic identification of bacteria and new, improved techniques are continuously implemented for assessing diversity and metabolic profiles of microbes, methods like Biolog biochemical tests identification system it is still considered to offer relevant taxonomic information [21]. This identification method was employed in the past for environmental isolate taxonomic identification and found to be 68.3% accurate compared to the 90.6% identification accuracy given by 16S gene sequencing [22, 23]. Based on the degree of correlation between 16S taxonomic affiliations and Biolog MicroLog system identification of our 12 identified bacterial isolates (Table 2 and 3), we concluded that the Biolog system has a limited discriminatory power but was accurate to the genus level for 7 out of 12 bacterial isolates.

16S rRNA genes sequenced of the 12 *alkB*-possessing isolates in this study have the NCBI accession numbers KY973665-KY973675 and MG000973, and the Blast identification results are summarized in Table 4.

Hydrocarbon-Degrading Potential

Further, all the 12 *alkB* gene-possessing isolates were tested for their ability to grow on Jet A-1 and

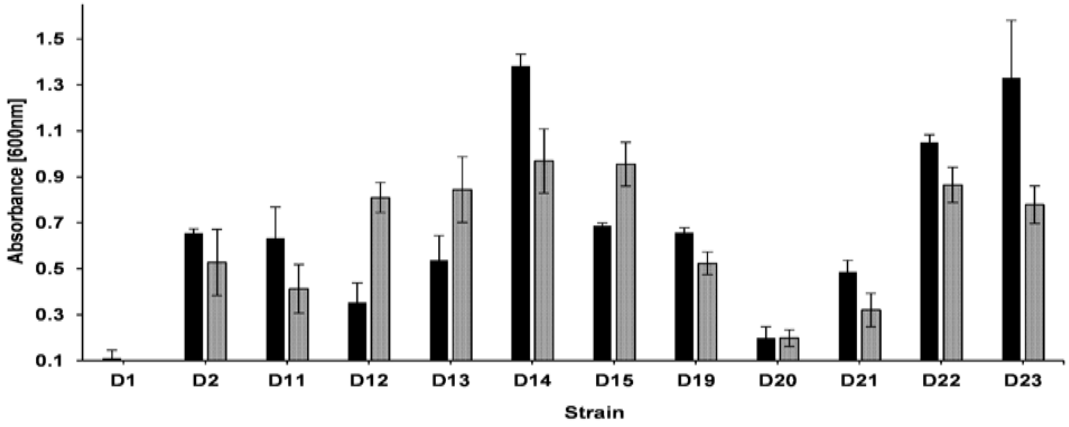

Fig. 3. Growth of *alkB*-possessing bacterial strain (OD_{600nm}) on minimal salts medium (MSM) supplemented with 1% Jet A-1 (black bars) or *n*-tetradecane (C14) (grey bars) as the sole carbon source after 30 days of incubation at 28ºC. Error bars display standard deviation based on three replicates.

one of the medium chain length Jet A-1 alkanes (C14) used as the sole carbon sources (Fig. 3). Except for *Bradyrhizobium* and *Methylobacterium* species (D1, D20), all other bacterial isolates were able to grow on tested hydrocarbons up to a final OD_{600}nm between 0.25 and 1.33.

As expected for *Pseudomonas* species, known as important hydrocarbon degraders and often mentioned as being implicated in Jet A-1 biodegradation [3,15], our *P. extremaustralis* strains (D14, D22, D23) highlighted the best growth values on both tested substrates. Biomass accumulation was higher using Jet A-1 mixture (OD_{600nm} >1), probably due to the *P. extremaustralis* strain ability to degrade different kerosene fractions [20, 24]. In contrast, for *P. synxantha* D15 strain bacterial growth was poorly supported by the Jet A-1 and the higher biomass accumulation was obtained using C14 alkanes as the sole carbon source. Interestingly, C14 also supported the growth of *Lelliottia amnigena* D12 and D13 strains. To the best of our knowledge, species of *Lelliottia* were never before described as hydrocarbon degraders. Jet A-1 mixture was preferred over C14 also by *Bacillus* sp. (D2, D11, D21) and *Sphingomonas* sp. (D19) bacterial isolates, but both tested substrates supported only a moderate grown for those strains. *Bradyrhizobium* and *Methylobacterium* species (D1, D20) were the only bacterial isolates that showed weak or no growth on tested hydrocarbons, although they possess the *alkB* gene encoding for alkane hydroxylase. This is either because their *alkB* gene is not a functional gene, or they are very slow growing bacteria since the cells were still viable by the end of the incubation period but exhibited no significant growth.

Conclusions

Jet A-1 is a middle distillate petroleum product – a mixture of: alkanes, cycloalkanes, aromatics and olefines from approximately C9–C20 lengths [3]. Bacterial contamination of such a complex hydrocarbon mixture is of interest in terms of fuel deterioration and system-related problems, as well as in terms of finding the best candidates for bioremediation processes. In this study the presumed connection between Jet A-1 bacterial contamination during storage, system-related problems, and contaminant bioremediation potential was confirmed by highlighting the bacteria isolates capacity to initiate biofilm, possess the genetic features needed for alkane degradation and by showing their ability to use hydrocarbons as the sole carbon source and energy.

The present study highlighted the capacity of different bacteria strains to colonize fuel storage tanks, be implicated in subsequent system problems associated with biofilm formation, and their involvement in fuel hydrocarbon deterioration/degradation, additionally underlining their potential use as experimental strains in revealing important fuel contamination and deterioration aspects or as candidates in bioremediation processes. The current study also revealed a new hydrocarbon-degrader *Lelliottia* species, represented by strains D12 and D13, which has not been previously mentioned among fuel-contaminating bacteria or for possessing the enzymatic equipment (*alkB*) required for alkane oxidation.

Conflict of Interest

The authors declare no conflict of interest.

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