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Streptomyces flora in chronically fuel oil-polluted soils and analysis of their alkane hydroxylase (*alkB*) gene by PCR

Ismail Saadoun[1,*], Mohammad Alawawdeh[2], Ziad Jaradat[3], and Qotaiba Ababneh[3]

[1]Department of Applied Biology, College of Sciences, University of Sharjah, Sharjah, P.O. Box 27272, UAE, [2]Department of Applied Biological Sciences, Faculty of Science and Arts, Jordan University of Science and Technology, P. O Box 3030 Irbid 22110 Jordan, [3]Department of Biotechnology and Genetic Engineering, Faculty of Science and Arts, Jordan University of Science and Technology, P. O Box 3030 Irbid 22110 Jordan

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Running Title: alkB gene in hydrocarbon-degrading Streptomyces

Abstract

This study reports the examination of the chronically fuel oil-polluted soil *Streptomyces* flora and their analysis for harboring the alkane hydroxylase gene (*alkB*) by PCR. Twenty *Streptomyces* isolates were recovered and the PCR analysis for the occurrence of *alkB* gene in the isolates 1d, 1e, 1f, 6a, 9a, and 9k showed three groups with different band size products; group 1 (G1) (215-227 bp), group 2 (G2) (334 bp), and group 3 (G3) (460-550 pb). Variation of the PCR size product of G1 from previous reports probably represents another form of *alkB* gene in these isolates.

Keywords: *alkB* gene; Biodegradation; Hydrocarbon; PCR; *Streptomyces*

1. Introduction

Attention to isolate and identify microbes prevailing in oil-polluted soils has long been reported by several investigators (Beilen *et al*., 2003; Jonathan 2003; Radwan 1998; Saadoun 2002; Saadoun 2004) and lists of hydrocarbon-degrading bacteria, actinomycetes, yeasts, fungi, and algae are therefore available. Some studies showed that *Streptomyces* flora could play a very important role in degradation of hydrocarbons (Barabas 2001; Radwan 1998; Saadoun *et al*., 2008; Saadoun and Alawawdeh 2019). For example, Barabas *et al*. (2001) have isolated 3 *Streptomyces* strains (*S. griseoflavus*, *S. parvus*, and *S plicatus*) from Burgan oil field in Kuwait with the capability to utilize *n*-hexadecane, *n*-octadecane, kerosene, and crude oil as sole carbon and energy sources. Later, Saadoun *et al*. (2008) have recovered 3 *Streptomyces* strains from soils in Jordan that are heavily polluted with crude petroleum as a result of oil spills' transportation accidents with the ability to grow on diesel as sole carbon and energy sources.

Alkane monooxygenase (*alkB*) gene is one of the key genes involved in the catabolism of alkanes and has been reported in several bacteria linked to hydrocarbon degradation including *Alcanivorax borkumensis*, *Pseudomonas aeruginosa*, *Mycobacteria* and *Rhodococcus* genera (Hamamura *et al*., 2001; Jonathan *et al*., 2003; Rojo 2013), as well as *Streptomyces* spp. (Saadoun *et al*., 2008). However, Saadoun and Alawawdeh (2019) reported the isolation of *Streptomyces* isolates from hydrocarbon-contaminated soils lacking the *alkB* gene but with a capability to degrade diesel, which implies that genes other than *alkB* gene might code for alkanes-degrading enzymes.

Saadoun (2002; 2004) has conducted studies in Jordan on isolation of different bacteria other than actinomycetes from soils polluted with oil and showed their ability to degrade diesel and short chain alkanes. Saadoun *et al.* (2008) and more recently Saadoun and Alawawdeh (2019) have shown that streptomycetes prevail in chronically oil-polluted soils of Jordan Refinery that has been historically exposed to crude or fuel oil spills for tens of years. However, these studies did not include the occurrence and recovery of streptomycetes in chronically fuel oil (refined)-polluted soils of gas stations. Therefore, the work presented here aimed to enumerate and isolate streptomycetes from soils polluted with refined fuel oil spills and to test the presence of the alkane hydroxylase gene (*alkB*) in the recovered isolates by PCR.

2. Materials and Methods

2.1. Location, sampling and sample processing.

Six gas stations in the City of Irbid/Northern Jordan with chronically fuel oil-polluted soils were selected in this study to describe the occurrence and recovery of streptomycetes in soils historically exposed to fuel spills for more than 10 years.

One fuel oil-polluted soil sample of approximately 300-400 g was collected from each station. Soil samples were taken from the 10 cm top soil, after removing the top 3 cm of the soil surface. A control soil sample was collected from a site far away from all gas stations included in this study. Samples were placed in polyethylene bags, closed

* Corresponding author e-mail: isaadoun@sharjah.ac.ae.

tightly and immediately transported to the laboratory to be stored in a refrigerator at 4 ±1 °C until processed.

2.2. Processing and characterization of the sample.

Prior to analysis, collected soil samples were crushed thoroughly to remove the large debris, and then mixed and sieved through a 2 mm pore size mesh (Retsch, Haan, Germany). The soil color, pH, moisture, and total petroleum hydrocarbons were determined for all sieved samples as prescribed below.

2.3. Bacterial count, isolation and characterization of Streptomyces isolates.

Standard serial dilutional and plating techniques were used to calculate the total bacterial count (CFU/gm soil) and total *Streptomyces* count (CFU/gm soil) in all sieved soil samples.

Enumeration and isolation of *Streptomyces* spp. that were able to grow on starch casein nitrate agar (SCNA) plates were performed as described by Saadoun *et al*. (2008). Morphological and physiological characterization of all recovered *Streptomyces* isolates was performed as described by Saadoun *et al*. (2008).

2.4. Soil pH, moisture and total petroleum hydrocarbon (TPH) measurements.

To measure the soil pH, soil suspension was prepared in distilled water (1:2 w/v), and the pH was determined at 25° C (Hanna, Italy). Soil moisture was determined by drying 1 g of each soil sample at 65 °C in the oven (WTB Binder, Germany), and the difference in the weight of the sample before and after drying was considered as the moisture content. For total petroleum hydrocarbon (TPH) determination, 10 g of each soil sample were constitutively extracted with 100 ml each of hexane, dichloromethane and chloroform. The three extracted portions were all pooled and then evaporated to dryness in a fume hood (CMS, Spain) at room temperature. After solvent evaporation, the amount of residual TPH was determined gravimetrically (Saadoun *et al*., 2008; Williams *et al*., 1972).

2.5. PCR amplification of alkB gene.

2.5.1. Growth conditions.

The recovered *Streptomyces* isolates were cultured in Oxoid tryptic soy broth (TSB) (Oxoid, UK) as per Hopowood et al. (1985) and incubated at 28 °C with shaking at 140 rpm for 48 h. To check for purity of the cultures, 0.1 ml of broth was plated on starch casein nitrate agar (SCNA) plates and then incubated at 28 °C for 72 h (Küster and Williams 1964).

2.5.2. Extraction of genomic DNA from pure Streptomyces isolates.

Wizard Genomic DNA Purification Kit (Promega, USA) was used for genomic DNA extraction from approximately 40 mg (wet weight) of *Streptomyces* mycelia. DNase, RNase-Free barrier tips (Promega, USA) were used for all DNA manipulation, handling and PCR work.

2.5.3. Quantitation of the extracted DNA and estimation of its purity.

The isolated DNA was quantitated spectrophotometrically and checked for purity as per Sambrook *et al*. (1989). The extracted DNA samples were diluted in Tris EDTA (TE) buffer and measured at 260 and 280 nm wavelengths (Genesys 2, Milton Roy, USA). DNA concentration in the original sample was calculated by taking the readings at 260 nm, where 1 OD corresponds to 50 µg/ml of double-stranded DNA. However, purity of the DNA was estimated by calculating the ratio between the readings at 260 nm and 280 nm (OD_{260} /OD_{280}).

2.5.4. PCR amplification.

The following two primers; ALK-F: 5´-TCGAGCACAACCGCGGCCACCA-3´ and ALK-R: 5´ CCGTAGTGCTCGACGTAGTT-3´; (Operon Technologies, USA) described before by Saadoun *et al.* (2008) and Saadoun and Alawawdeh (2019) were used to detect the presence of *alkB* gene in all isolates. Amplification reactions were performed as described before by Saadoun *et al*. (2008) and Saadoun and Alawawdeh (2019).

2.5.5. Electrophoresis and photography.

PCR products were separated by electrophoresis on 2% agarose (Promega, USA) gels in 1X TBE buffer at 100 Volts for 1.5 h. A 100 bp DNA ladder (Promega, USA) was used to estimate the size of the PCR product. DNA bands were detected on the agarose gel by staining with ethidium bromide (EB) (Acros Organic, USA) at 0.5 µl/ml concentration. Gels were visualized and documented using Gel Doc (Bio-Rad, USA).

3. Results and Discussion

3.1. Characterization of soil samples and their Streptomyces flora.

Color, pH and moisture of the collected soil samples were determined. Results showed that the colors of the samples were black, brown or dark brown, while pH ranged from 6.89 to 7.66, and the moisture content ranged from 2.4 to 6.5% (Table 1). Total petroleum hydrocarbons (TPH) were found between 44 and 116 mg/gm of soil (Table 1). Hydrocarbons spilled on soil increase the levels of organic carbon, and may either serve as substrates for microbial growth or be toxic to microbial growth and activity (Bossert and Bartha 1984). Variation between soil-polluted samples in color, pH or the moisture content could be due to differences in levels and types of organic matter as well as differences in precipitation rates and exposure to sun. All measured values characterizing the polluted-soil samples might reflect the best conditions for microbial activities.

A control unpolluted soil sample was also evaluated for color, TPH and moisture. The sample showed a light brown color, contained 0.00 mg/g TPH and a moisture content of 4.0%. The low TPH content in the control sample is explained by lower or no exposure to fuel oil spells. This also explains the higher total bacterial and *Streptomyces* count and diversity in this sample. This observation indeed points to the inhibition effect of hydrocarbons on the growth of *Streptomyces.*

Table 1. Hydrocarbon-contaminated soil samples collected from different gas stations and their characters and *Streptomyces* content.

Sample	Gas Station	Soil sample characters					Bacterial count			*Streptomyces* color diversity	
No.	Name	Color	Weight (gm)	Moisture %	pH	TPH mg/gm	Total count CFU X10^6/ gm soil	*Streptomyces* count CFU X10^4 /gm soil	% of *Streptomyces*/ gm soil	White	Grey
1	Modern	Brown	580	4.2	7.10	56	0.87	6	6.90	3	2
2	New Irbid	Brown	335	5.5	7.32	92	0.20	2	10.0	1	1
3	Aldejany	Dark brown	682	6.5	7.66	45	0.49	6	12.24	3	3
4	Industrial City	Brown	114	4.3	7.42	50	0.20	2	10.00	1	1
5	Kuforyoba 1	Black	116	2.4	7.35	116	0.244	2	8.20	1	1
6	Boshra	Brown	220	4.1	6.89	44	0.305	3	9.83	2	1
Control	Irbid City	Dark brown	670	4.0	6.81	0.00	7.00	16	2.28	9	7

The average *Streptomyces* count ranged between 2 x 10^4 and 6 x 10^4 colony forming unit (CFU)/ gm dry soil (Table 1). However, *Streptomyces* count in the un-polluted soil was 16 ×10^4 CFU/g (Table 1). In this study, the percentage of *Streptomyces* in the polluted soils was higher than that in the control soil. Such observation might reflect how the indigenous streptomycetes get acclimated to the hydrocarbons spilled in the gas stations and might be due to the refined types of oil (benzene, diesel and kerosene), thus containing less toxic substances as compared to the crude oil that usually contains more toxic substances. On the other hand, the lower counts of *Streptomyces* in soils from New Irbid, Industrial city, Kuforyoba 1, and Boshra gas stations suggest how difficult it is for these indigenous microorganisms to prevail as a result of accumulation of TPH, which could reach toxic levels. The non-polluted control soil sample showed slightly more counts with an average of 16 ×10^4 CFU/g dry soil (Table 1). The variation in the microbial count might reflect the location of sampling as well as the amount of spilled refined-oil or concentration of pollutant. Moreover, the presence of *Streptomyces* in various habitats is affected by several factors such as pH, temperatures, nutrients and moisture (Saadoun *et al*., 2008).

The *Streptomyces* colonies observed on starch casein nitrate agar plates (SCNA) after 10 days of incubation at 27 ˚C were small (1-10 mm diameter), discrete and leathery, initially exhibited smooth surface but later developed a weft of aerial mycelium that appeared granular, powdery and velvety (data not shown). Twenty different *Streptomyces*-like isolates were recovered from the polluted soils (Table 1). However, more diverse isolates (16) were recovered from the control soil sample. The recovered *Streptomyces* isolates were phenotypically characterized into two color series only (Table 1). The occurrence of the white and the grey series was obviously more than other color series, which is in agreement with other studies by Saadoun and Al-Momani (1997) and Saadoun *et al*. (1999) who also found that these two color series occur more frequently in Jordanian soils.

3.2. Detection of alkB gene sequence in Streptomyces isolates by PCR.

The presence of *alkB* gene in the isolated *Streptomyces* was tested by designing a pair of primers by multiple alignments of several sequences from all alkane hydroxylase genes deposited in the Gene Bank (Saadoun *et al*., 2008). There are currently over 250 *alkB* gene homologues found in diverse bacterial species in which a large portion of these genes was detected in oil-contaminated environments (Wang *et al*., 2010).

PCR analysis revealed 3 groups of PCR products; group 1 (G1) isolates gave a product size of 215-227 bp, group 2 (G2) isolates gave product sizes corresponding to 334 bp, while group 3 (G3) consisting of isolates gave product sizes corresponding to 460-550 bp (Figure 1). G1 isolate 6a gave a band of 227 bp, isolate 1d gave a band of 220, while isolate 9a gave a band of 215 bp. (Fig. 1). G2 isolates (isolate 9k) gave a product with a band size of 334 bp (Fig. 1). However, G3 isolates gave a product with a higher size (460-550 bp). The isolates 1e and 1f gave a band of 544 bp each (Fig. 1). The product size of those in G2 clearly matches product size observed by Kohno *et al*. (2009) and Saadoun *et al*. (2008). Either the PCR size product of G1 did not match those observed by Kohno *et al*. (2009) or Saadoun *et al*. (2008), thus indicating another form of *alkB* gene in these isolates. This variation in product size encourages us to conduct additional molecular tests, especially for the 6a, 1d, and 9a isolates that showed a fragment size of 227, 220, and 215 bp, respectively.

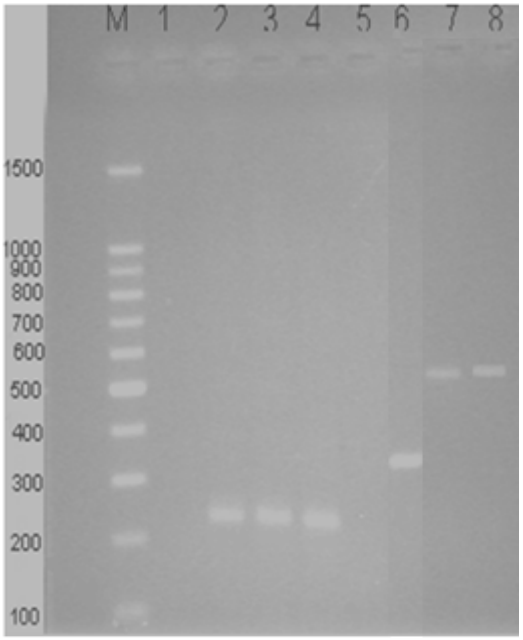

Figure 1: 2% Agarose gel electrophoresis of PCR amplification of alkane hydroxylase gene (*alkB*) from *Streptomyces* isolates with Alk primer, lane M: a 100 bp ladder, lanes 1 and 5: negative control (no DNA template), lane 2: 6a (227 bp), lane 3: 1d (220 bp), lane 4: 9a (215 bp), lane 6: 9k (334 bp), lane 7: 1e (544 bp), lane 8: 1f (544 bp).

4. Conclusion

This study highlights the power of molecular biology techniques as PCR analysis for studying the existence of alkanes-degrading *Streptomyces* flora that prevails in hydrocarbon-polluted environments. Variation of the PCR size product reported here in this study probably represents another form of *alkB* gene in *Streptomyces* isolates.

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