Mutation of RNA polymerase β-subunit confers in both enhancement of environmental bioremediation and antibiotic production in a *Streptomyces* species

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Abstract

Research on environmental bioremediation has intensified in recent years because of a continuous increase in environmental pollution accidents. Scientists taking advantage of the microbial astonishing steps involved in metabolism and breakdown of toxic macromolecules to clean up our environment from pollutants. In this work some antibiotic producing *Streptomyces* inhabiting contaminated soil with crude petroleum "near Shiwashok oil field-Koya" were isolated, and characterized based on the morphological properties and sequence analysis of 16s rRNA coding region. The ability of the isolates was screened for antibiotic production by in *vitro* antagonism against Gram positive and Gram negative bacteria. Although the isolates were able to survive as free living organisms in the petroleum contaminated soil, only one isolate was able to grow and reduce the toxic hydrocarbons content in a synthetic media contains crude petroleum as a sole source of carbon. Mutation in the *rpoB* gene encoding RNA polymerase β-subunit (1280 A-T, and 1319 G-A) that confers bacterial resistance to rifampicin, resulted in significant increase of hydrocarbons degradation, and apparent increase in the antibiotic production by the *Streptomyces* isolate.
Keywords: Streptomyces, bioremediation, rpoB mutation, antibiotic production.

1 Introduction

Occurrence of ecosystems damages during the previous years have been significantly increased by accidental spills of crude oil during extraction, transportation, storage, refining and distribution. It has been reported that


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approximately five million tons of crude oil and refined oil enter the environment each year as a result of anthropogenic sources such as oil spills [1]. The damage can include pollution of water resources and contamination of the soil which negatively affects vegetation, livestock, and human health, because it contains hazardous hydrocarbon and its constituents are very much toxic and carcinogenic. As a result, many people have become aware of the need to protect ecosystems as well as to evaluate the environmental devastation caused by oil contamination. While most of the most of the developed physicochemical methods for remediation use chemical agents, they also produce additional source of contamination and also increase the oil recovery cost. Therefore it is necessary to search for alternative and appropriate clean-up methods. Biodegradation of hydrocarbons by microorganisms represents one of the primary mechanisms by which petroleum and other hydrocarbon pollutants are reduced or eliminated from the polluted ecosystem. In the past years, researchers have identified various microbial species that are effectively involved in metabolism and degradation of hydrocarbons in natural environments. It has been reported that petroleum hydrocarbons can be actively degraded by microorganisms such as bacteria, fungi, yeast and microalgae [2–5]. Biodegradation of pollutants (like crude petroleum or hydrocarbons) is a complex microbial metabolic process whose quantitative and qualitative aspects depend on the nature and amount of the oil or hydrocarbons present, the ambient and seasonal environmental conditions (e.g. temperature, humidity, pH) and the composition of the autochthonous microbial community [6]. Some microbes have regulatory systems that synthesize the enzymes which can lead the initial attack on these petroleum hydrocarbons only when ever required. Petroleum contains four classes of hydrocarbons: the substrates, the aromatics, the asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins), and the resins (pyridines, quinolines, carbazoles, sulfoxides, and amides) [7]. It is well known that these hydrocarbons may inhibit the growth of some microbial communities that are important in biogeochemical cycles of that ecosystem and this affects the efficiency of such ecosystems [8], and some organisms have ability to survive and enzymatically attack aliphatic or light aromatic fractions of the oil with high-molecular-weight aromatics, resins, and asphaltenes. The most rapid and complete degradation of the majority of organic pollutants is brought about under aerobic conditions. The initial intracellular attack of organic pollutants is an oxidative process and the activation as well as incorporation of oxygen is the enzymatic key reaction catalyzed by oxygenases and peroxidases. Peripheral degradation pathways convert organic pollutants step by step into intermediates of the central intermediary metabolism, for example, the tricarboxylic acid cycle [9, 10].

The species of the *Streptomyces* genus constitute 50% of the total population of soil actinomycetes and are well known for producing a variety of bioactive secondary metabolites including antibiotics, immunomodulators, anticancer drugs, antiviral drugs, herbicides, and insecticides [11]. However, nearly no report found about soil *Streptomyces* able to degrade petroleum or hydrocarbons [12]. Furthermore, no data available about antibiotic producing *Streptomyces*

able to degrade and use hydrocarbon as energy source, therefore, documentation of *Streptomyces* of a hydrocarbon contaminated site is important issue because it might result in isolation and identification of novel isolates with environmental and biotechnological importance. The current work included isolation and characterization of antibiotic producing streptomycetes isolated from petroleum contaminated soil samples based on 16s rDNA sequencing method. Improvement of the bacterial growth rate and antibiotic production in presence of hydrocarbons was done by induction of *rpoB* gene mutation in the bacterial genome.

2 Material and methods

2.1 Isolation of *streptomyces*

Crude-petroleum polluted soil samples inflicted by transportation trailer truck accidents were collected near the Shiwashok-Koya oil field. After removal of the above 3-5 cm surface layer, soil samples were pulled out from depth of 10 cm. The soil samples were stored inside sterile labelled polyethylene plastic bags at 4°C. 1 gm of the sample was suspended in 100 ml of sterile physiological solution (0.9% NaCl) with shaking (250 rpm) at 30°C for 30 minutes. The mixture was then allowed to settle, and serial dilutions up to 10^{-5} were prepared. For isolation of *Streptomyces*, 100 µl of each dilution was used to inoculate trypticase soya agar (TSA) plates supplemented with 25 µg/ml nalidixic acid, 50 µg/ml cyclohexamide to prevent growth of other bacteria and fungi, respectively. Plates were then incubated at 28°C for 7 days. Single colony isolation was performed by repeated streaking on Gauze synthetic agar containing (mg/ml): soluble starch 20, K_2HPO_4 0.5; $MgSO_4$ $7H_2O$ 0.5; KNO_3 1.0; NaCl 0.5; $FeSO_4$ $2H_2O$ 0.01 and agar 25 [13], for purification of bacterial colonies that showed a *Streptomyces*-like appearance. Putative *Streptomyces* colonies were recognized on the basis of colony morphology, culture odor, and microscopical examinations. The taxonomic properties of isolate SJE177 were evaluated following the methods given in the international *Streptomyces* project (ISP) [14]. The sporophores were studied under a light microscope. The morphology of the mature culture and color was observed on yeast extract-malt extract agar (ISP2), as described in Bergey's Manual [15]. To study the ability of the isolates to grow in a medium contains crude petroleum as sole source of carbon. 0.5 ml of well grown culture was used to inoculate 50 ml of gauze medium contains 0.2% crude oil instead of soluble starch in Erlynmeyer flask, and grown at 28°C in rotary shaking incubator (150-200 rpm).

2.2 Bioassay determination assay and antibiotic extraction

Bioassay determination was carried out by agar diffusion method [16]. After incubation of the selected strains on Gauze plates for 7 days at 28°C, agar disk (10 mm in diameter) from well grown *Streptomyces* colony mass was prepared by using sterile cork borers. For antibacterial activity, discs were aseptically placed on top of Lauria Bertani agar plates freshly covered by 3 ml of top agar

containing 50 µL of a 5 hr old culture of *Escherichia coli* (ATCC25922, and *Bacillus subtilis* (ATCC21332). Antifungal test activity of the strains was tested against *Candida albicans* (ATCC10231) on potato dextrose agar plates (PDA). Plates were first kept in a refrigerator (4°C) for 2 h to allow the diffusion of any antibiotics produced, then incubated overnight at 37°C for *E. coli* and *B. subtilis*, or at 28°C for *C. albicans*. The antimicrobial activity was observed after 24 hr for bacteria and 48 hr for yeast based on appearance of inhibition zone. To test antibacterial activity of *Streptomyces* culture extract, bacterial cells were grown in 50 ml Gauze medium in Erlenmeyer flasks at 28°C in rotary shaker for 7-10 days. After culture centrifugation at 4000 rpm at 4°C, cells and supernatant was extracted using acidified ethyl acetate using 0.1% glacial acetic acid. The ethyl acetate layer was separated and evaporated by rotary evaporator at 45°C. The residue was dissolved in 0.5 ml methanol (90%). Whatman filter paper discs (6 mm) were impregnated with 25 µl of the cultural extract and dried under aseptic condition. Discs were aseptically placed on top of agar plates pre-inoculated by *E. coli*, *B. subtilis*, or *C. albicans* and grown at appropriate temperature. Alternatively, 2-5 µl of the extract was added into 150 µl (1:200) diluted overnight culture of *E. coli*, *B. subtilis*, or *C. albicans* in a microtire plates and grown for 24 h at 37°C for bacteria growth, or 48 hr at 28°C for yeast growth, (90% methanol was used as control). Antimicrobial activity and growth rate of test organisms were measured by reading optical density OD_{600nm}.

2.3 Analysis of petroleum biodegradation by the Streptomyces isolates

To test ability of the *Streptomyces* isolates for usage and biodegradation of crude petroleum or hydrocarbons, cells were grown in 50 ml Gauze medium prepared with starch, 0.2% crude petroleum, or 2% n-hexadecane (Sigma) as sole source of carbon. To monitor bacterial growth, optical density (OD_{600}) of the culture was measured every 24 hr for 10 days. 20 µl of *p*-iodonitrotetrazolium violet dye (7.5 mg/ml) was added to 1.5 ml grown cultures after 10 days incubation. Mixture was incubated for 2 hr at room temperature. Change of the indicator's colour from colourless to red confirms presence of actively respiring bacteria and ability to growth in presence of crude petroleum or hydrocarbons. To test antibiotic production by the *Streptomyces* isolates in presence of crude petroleum or *n*-hexadecane as sole source of carbon, bacterial cells were inoculated in Gauze medium which contained starch, or 0.2% crude petroleum, or 2% *n*-hexadecane and incubated at 28°C in rotary shaker for 10 days. Culture extraction was performed as described formerly, and *B. subtils* culture was used to test antimicrobial activity.

2.4 Genomic DNA isolation

Streptomycete isolates were grown to the late exponential phase in TSB at 28°C and 2 ml of culture was centrifuged at 10.000 rpm for 5 minutes. Cells were washed twice with Tris/HCl-EDTA buffer, and re-suspended in 0.4 ml SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.5). Lysosome was added to concentration of 1 mg/ml and incubated at 37°C for 1 h. Then 40 µl 10% SDS and 10 µl of 10 mg/ml proteinase K were added and incubated at 42 °C for 2 hr

or until the suspension cleared. 1/10 volume 5M NaCl and gently mixed thoroughly. The mixture was extracted twice each with one volume phenol/chloroform/isoamyl alcohol (25:24:1 v/v), and once with isoamyl alcohol (24:1 v/v). The mixture was centrifuged at 4500 g for 15 min and the aqueous phase was transferred to a new tube using a blunt-ended pipette tip. Chromosomal DNA was precipitated by addition of 1/10 volume 3M sodium acetate pH 4.8, 2.5 volumes cold ethanol (100%), and centrifugation at 13000 rpm for 10 minutes. The pellet was washed with 70% cold ethanol, dried, dissolved in TE (10 mM Tris/HCl-1 mM EDTA, pH 8.0) buffer and stored at 4°C.

2.5 PCR amplification and sequencing of 16s rDNA

Polymerase chain reaction of *Streptomyces* 16s rDNA was performed in a thermal cycler (Corbett life science). The degenerated *Streptomyces* oligonucleotides AM47 (5'-GTG GGC AAT CTG CCC T(TG)C ACT C-3'), and AM45 (5'-GTG AGT CCC CA(GAT) CAC CCC GAA G-3') [17] were used to generate an internal fragment (~1100 bp) of the bacterial 16s rDNA. PCR reaction was performed in a total in a total volume of 50 µl containing 30–50 ng genomic DNA, 100 µM each primer, 10 µM dNTP, 1x buffer (100 mM Tris/HCl, pH 8·0, 500 mM KCl, 20 mM MgCl2, 0·1 % gelatin), 1 µl DMSO, and 1·5 U Taq DNA polymerase (Fermentas, Germany). The reaction was performed under the following conditions: 2 min at 96°C, followed by 30 cycles of 1 min at 96°C, 30 sec min at 52-56 °C, 1 min 72°C followed by one cycle of 10 minutes at 72°C. 5 µl of the resulted PCR was then analyzed by gel electrophoresis using 1.0 % agarose with ethidium bromide at 8V/cm and the reaction product was visualized under Gel doc/UV trans-illuminator (Syngene). The PCR product was purified by gel extraction kit (Fermnetas) according to the manufacturer's protocol. BigDye® terminator v3.1 cycle *sequencing* kit *and ABI 3130 Applied Biosystems 3130* Genetic Analyzer were used for sequencing of the PCR fragment. Sequencing of both strands of the 16s rDNA PCR was succeeded by using the AM47 or AM45 primers which provided 951 bp sequence information.

2.6 Data analysis

The resulted 16s rDNA sequence was used to blast against NCBI GenBank database and to find similarities with previously published sequences. The phylogenetic tree was constructed from the *Streptomyces* Koy7216s rDNA nucleotide sequence with that of 27 *Streptomyces* species in the nucleotide database using the Neighbor-Joining method as implemented in the Geneious Pro 5.0.3 software.

2.7 Induction of rifampicin resistance mutations

10, 20, 30, 40, and 50 µl from 2 day old culture of *Streptomyces* koy72 isolate were plated out onto TSA plates supplemented with 10, 25, 50 and 100 µg/ml rifampicin. After incubation at 28 °C for 10 days, a number of rifampicin resistant colonies (Koy72-*rif*) were appeared predominantly on plates with the

lowest rifampicin concentration. 30 colonies were then screened for enhanced growth on crude petroleum and antibiotic production in comparison to the wild type isolate. Cultivation was carried out in 50 ml Gauze medium (with starch, crude petroleum, or n-hexadecane (Sigma)). After extraction with equal amounts of ethylacetate, the organic phase was evaporated and redissolved in 500 µl methanol (90%). Analysis antibiotic production was carried out by addition of 5 µl of the cultural extract of the resulted mutants to 150 µl freshly inoculated culture of *B. subtilis* in 96 well sterile tissue culture microtiter plate. Plates were incubated at 37°C for 24 hr and bacterial growth was measured by reading optical density OD_{600nm}.

2.8 Molecular analysis of *rpoB* gene mutation

An internal fragment (~1kb) *rpoB* genes of the wild type *Streptomyces* Koya-72, and the dervative rifampicin resistance strain Koy72-*rif*12 were amplified by PCR using appropriate genomic DNA as templates, and the oligonucleotides rpoBPF (5'-GAG CGC ATG ACC ACC CAG GAC GTC GAG GC-3'), and rpoBPR (5'-CCT CGT AGT TGT GAC CCT CCC ACG GCA TGA-3'), as described formerly [18]. PCR was performed by using Taq polymerase (Fermnetas) according to the manufacturer's recommendations, and the PCR conditions were as follows: 2 min of preincubation at 94°C; followed by 30 cycles of 96°C for 30 sec., 55°C for 30 sec, and 72°C for 45 sec, and final step at 72°C for 10 min. PCR products were purified and directly sequenced by the dideoxy chain termination procedure by using the BigDye terminator cycle sequencing kit as described previously. Data analysis was performed using the Geneious Pro 5.0.3 software for sequence alignment and finding *rpoB* gene mutations.

3 Results and discussion

Samples of soil contaminated with crude petroleum from a strongly polluted environment near an oil-field were analyzed to isolate streptomycetes that are able to grow and produce antibiotics and in a media contains crude petroleum as sole source of carbon. The putative Streptomycetes isolates were characterized according to Bergy's Manual of Determinative Bacteriology [19]. The occurrence of total bacterial isolates in the petroleum contaminated soil samples was $1.6*10^2 - 3.6*10^2$ cfu/gram of dry weight soil, while for streptomycetes was 7-12 cfu/gram. This was apparently much lower than those isolated from non contaminated soil reported previously [20–22]. This might resulted from presence of high level of toxic pollutants which often kill or inhibiting large sectors of soil microbial population [23]. The grown *Streptomyces* were characterized by small colony (3-12 mm in diameter), dry or chalky in consistency, folded, lichenoid, leathery with wavy edges, and the colonies color was ranged from white to beige or gray as shown in table 1. They are noted for their distinct "earthy" odor. Gram staining of bacterial cells showed gram positive cells and appearance of mycelia filaments and spores.

Five out 81 isolates of *Streptomyces* isolates (Koy9, Koy21, Koy47, Koy72, Koy86) showed antimicrobial activity against Gram-positive, and Gram negative test bacteria, and only two strains (Koy34, and Koy70) inhibited growth of *C. albicans*. Previously published data showed that some soil microorganisms are able to utilize crude petroleum or hydrocarbons for their growth. The driving force behind usage of these materials is because they are equipped with metabolic machinery to utilize petroleum and hydrocarbons as alternative carbon source to satisfy their cell growth and energy needs, which is very important for environmental bioremediation [24–26].

Here, we aimed to test the ability of the isolated *Streptomyces* for growth in a carbon source free synthetic medium supplemented with either crude petroleum or *n*-hexadecane hydrocarbon. The results showed that four *Streptomyces* isolates (Koy53, Koy67, Koy72, and Koy 76) were able to grow in presence of crude petroleum as sole source of carbon. However, only one isolate (Koy72) was able to grow in the presence of crude petroleum or *n*-hexadecane as sole source of carbon and also produce antibiotics (table 1). To confirm resistance and survival of this isolate in presence of crude-petroleum or hydrocarbons, *p*-iodonitrotetrazolium indicator was added to the bacterial culture which showed change of the indicator color from colorless to red color. The result might indicate growth and presence of actively respiring cells in the culture, and presence of hydrocarbon or petroleum-degrading activity [27]. We further confirmed the characterized the Koy72 isolate by computational analysis of the bacterial genetic information. Genomic DNA of the isolate was extracted, followed by PCR amplification of 16s rDNA, sequencing and comparison with nucleotide database of NCBI webserver through blast tool. The result showed alignment with a number of *Streptomyces* species sequences. The query sequence was best pairwise aligned with the 16s rDNA sequence of *Streptomyces roseoviolaceus* (accession nr. AB_184298) with similar sequence similarity and identity of 99.1%, followed by the sequence of *Streptomyces violatus* (accession nr. AJ_399480), and *Streptomyces violaceus* (accession nr. NR_041115). For phylogenetic analysis, a new data set of alignment was

Table 1: Represents colony morphology of the 81 *Streptomyces* isolates from polluted soil, and ability for antibiotic production, and growth in presence of hydrocarbons as sole carbon source.

Bacterial Isolates (81)	Colony morphology				Antibiotic production		Growth on hydrocarbon	Growth on hydrocarbons and antibiotic production
	White	Gray	Beige	Grayish pink	Anti-Bacteria	Anti-fungi		
Number	41	33	5	2	5	2	4	1
%	50.7	40.7	6.2	2.4	6.2	2.4	5	1.2

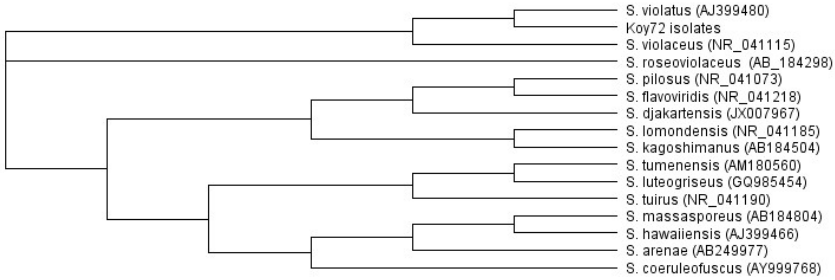

Figure 1: Neighbor-joining tree of selected 16S rDNA gene sequence of the genus *Streptomyces* obtained from BLAST search of the *Streptomyces* isolate Koy72 sequence for phylogenetic inference.

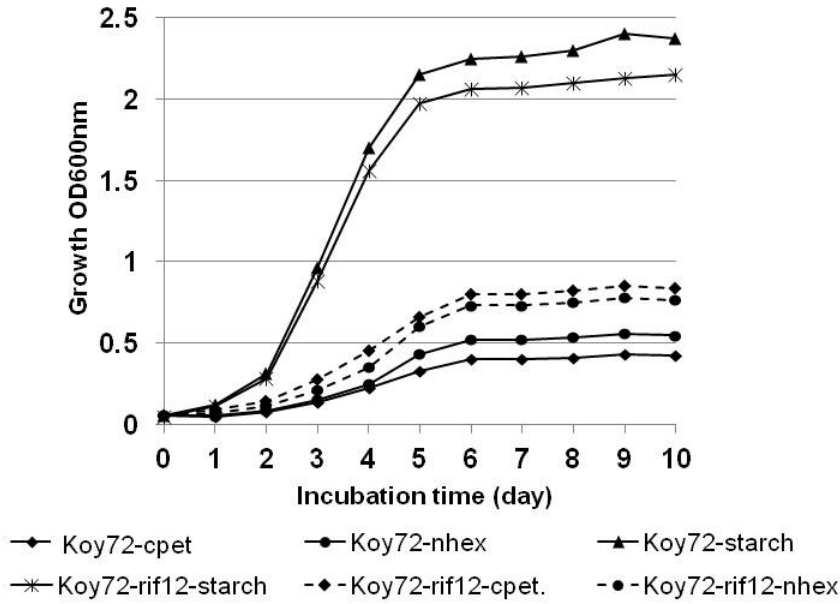

Figure 2: Represent growth kinetics of the *Streptomyces* Koy72 and the *rif* mutant (Koy72-rif12) isolate in gauze medium supplemented with either starch, crude petroleum (cpet), or n-hexadecane (nhex) as sole source of carbon. Growth was measured by optical density during cultivation for 10 days.

prepared after removing the sequences of non-cultured and sequences of repeating strains from the BLAST results. The phylogenetic tree was then drawn using Neighbour joining method [28]. The data revealed that Koy72 isolate formed a distinct lineage with species *Streptomyces violatus* (Figure 1). As the molecular analysis of the bacterial 16s rDNA sequence showed no 100% sequence identity, we can classify the isolate as novel genomic species of the

genus *Streptomyces*. It has been noticed that the growth of the *Streptomyces* isolate in presence of 0.2% crude petroleum and 2% hexadecane was lowered 4.5 fold and 2.7 fold, respectively in comparison to the presence of starch (Figure 2). Bioassay for antibacterial activity of the isolate against *B. subtilis* in presence of either crude petroleum or n-hexadecane showed nearly 75% and 67% reductions in comparison to starch (Figure 3). Rifampin is a major drug used in the treatment of tuberculosis infections, and increasing rifampin resistance represents a worldwide clinical problem. Resistance to rifampin is caused by mutations in the *rpoB* gene, encoding the *β*-subunit of RNA polymerase [29, 30]. It has been investigated that mutation in *rpoB* mutation plays role in regulation of many microbial pathways like production of actinorhodin, salinomycin, and bottromycin in *Streptomyces lividans* [31], *Streptomyces albus* [32], and *Streptomyces bottropensis* [33], respectively. In the present work, rifampicin resistance was induced by growing the bacterium on agar plates supplemented

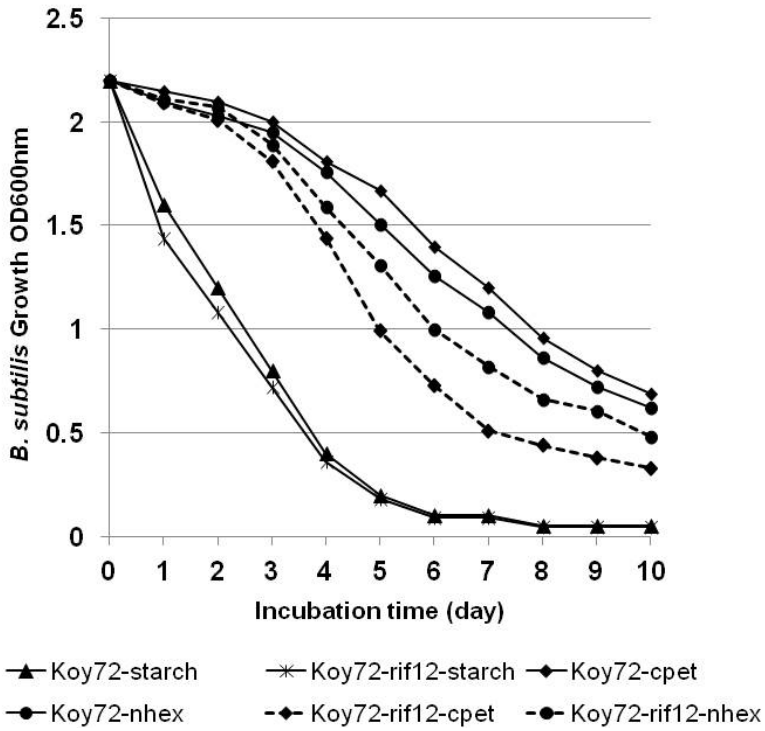

Figure 3: Shows growth inhibition of *B. subtilis* by the cultural extracts of the *Streptomyces* Koy72 wild type and the rifampicin mutant variant, *Streptomyces* Koy72-*rif*12. Methanolic extract of 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 day old *Streptomyces* cultures grown in the presence of either starch, crude-petroleum (cpet), or n-hexadecane (nhex), was used to test antibiotic production and inhibition of *B. subtilis* growth.

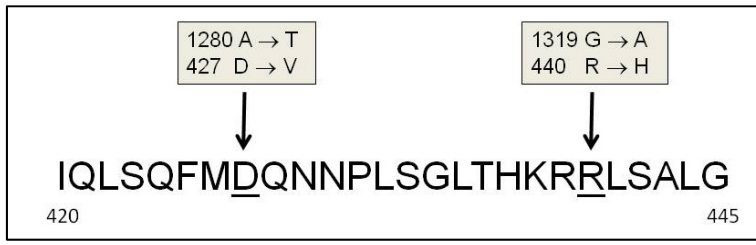

Figure 4: Represent nucleotide mutations detected in *rpoB* gene and the corresponding amino acid alterations in the *β*-subunit of RNA polymerase in the Koy72-*rif*-12 mutant. The mutation positions are underlined and indicated by arrows. Nucleotide change and amino acid substitution are denoted in boxes.

with different concentrations of the antibiotic. Later, the resistant isolates (Koy72-*rif*) were tested for growth and antibiotic production in comparison to the wild type. One of the rifampicin mutant strains (Koy72-*rif*-12) showed an increase in growth rate (2 fold, and 1.4 fold) in the presence of crude petroleum or n-hexadecane, respectively. It has been also noticed that antibacterial activity of the resulted mutant was also increased 1.8 fold and 1.2 fold when tested against *B. subtilis growth*. Interestingly no significant change was found in the growth rate and antibiotic production in *rif*-mutant when grown in presence of starch in comparison to the wild type. This might indicate that the induction of growth and antimicrobial activity of the *rif*-mutant strain is related to increase in metabolism of the crude petroleum and activation of antibiotic biosynthesis pathway. Molecular analysis of the *rif*-mutant strain, Koy72-*rif*-12 was done by amplification and sequencing of a fragment of nearly 1 kbp of the *rpoB* gene. The resulted sequence covers an internal region of the gene starting from the nucleotide 1162 to the nucleotide 2155 corresponding to the sequence of the *S. coelicolor* and *S. lividans rpoB* genes. Sequence alignment of the *rpoB* fragment in Koy72-*rif*-21 mutant with that of the wild type showed mutations in the nucleotides A1280T, and G1319A. The point mutations result in amino acids substitution D427V, R440H as shown in figure 4. This finding might serve as promising step forward toward approaches for finding new alternative carbon source for pharmaceutical and biotechnological fermentations, as well as enhancing the technology of bioremediation using genetically modified microorganisms.

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References

[1] Hinchee, E.R., Kitte, A.J. and H.J., *Applied Bioremediation of Petroleum Hydrocarbons*, Columbus [OH): Battelle Press, 1995.

[2] Riser-Roberts, E., Bioremediation of Petroleum Contaminated Sites, NCEL, Port Hueneme, CA: C. K. Smoley Publishers, CRC Press, Inc, pp. 197-198. 1992.

[3] Bundy, J.G., Paton, G.I. and Campbell, C,D., Combined microbial community level and single species biosensor responses to monitor recovery of oil polluted soil. Soil Biology and Biochemistry, 36, pp. 1149-1159, 2004.

[4] Nikolopoulou, M., Pasadakis, N. and Kalogerakis, N., Enhanced bioremediation of crude oil utilizing lipophilic fertilizers, *Desalination*, 211, pp. 286–295, 2007.

[5] McGenity, T.J., Folwell, B.D., McKew, B.A. and Sanni, G.O., Marine crude-oil biodegradation: a central role for interspecies interactions. *Aquat Biosyst*, 8(1), pp. 1-19, 2012.

[6] Leahy, J.G. and Colwell, R.R., Microbial Degradation of Hydrocarbons in the Environment. *Microbiol Re,*154 (3), 305-315, 1990.

[7] Colwell, R.R., Walker, J.D. and Cooney, J.J., Ecological aspects of microbial degradation of petroleum in the marine environment. *Critical Reviews in Microbiology*, 5 (4), pp. 423–445, 1977.

[8] Rhodes, A.N. and Hendricks, C.W., A continuous-flow method for measuring effects of chemicals on soil nitrification. *Toxicity Assessment*, 5, pp 77–89, 1990.

[9] Okoh, A.I., Biodegradation alternative in the cleanup of petroleum hydrocarbon pollutants. *Biotechnol. Mol. Biol. Rev*, 1 (2), pp. 38-50, 2006.

[10] Das, N. and Chandran, P., Microbial Degradation of Petroleum Hydrocarbon Contaminants. *Biotechnol Res Int*, 2011, pp. 1-13, 2010.

[11] Mohammad, S.J., Malakawi, H.I., Al-Momani, F. and Meqdam, M., Diversity of soil Streptomycetes in northern Jordan. *Actinomycetes*, 9 (3), pp. 52-60, 1998.

[12] Barabas, G.V., Szabo, I.M., Penyige, A., Damjanovich, S., Szollosi, J., Matko, J., Hirano, T., Matyus, A. and Szabo, I., N-Alkane uptake and utilisation by Streptomyces strains. *Antonie Van Leeuwenhoek*. 79(3-4), pp. 269-276, 2001.

[13] Gauze, G.F., Kochetkova, G.V., Preobrazhenskaia, T.P., Kubrina, E.C., Sveshnikova, M.A. and Popova, O.L., Investigation on inhibiting effect of actinomycetes on actinophages. *Mikrobiologiia*, 26(6), pp. 729-735. 1957.

[14] Shirling, E.B. and Gottlieb, D., Method for characterization of *Streptomyces* sp. *Int J Syst Bacteriol*, 16, pp. 313-340, 1966.

[15] Holt, J.G., Krieg, N.R., Sheath, P.H.A., Sterley, J.T. and Williams S.T. (1994). *Bergey's Manual of Determinative Bacteriology*. 9th Ed. Williams and Wilkins. Maryland, USA. 1994.

[16] Bauer, A.W., Kirby W.M., Sherris J.C. and Turck, M. Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol*, 36, pp. 493-496, 1966.
[17] Mehling, A., Wehmeier, U. F. and Piepersberg, W., Nucleotide sequences of streptomycete 16S ribosomal DNA: towards a specific identification system for streptomycetes using PCR. Microbiol,141, pp. 2139–2147, 1995.
[18] Guo, Y., Zheng, W., Rong, X. and Huang, Y., A multilocus phylogeny of the *Streptomyces griseus* 16 S rRNA gene clade .Use of multilocus sequence analysis for streptomycetes systematics. *Int J Syst Evol Microbiol*, 58, pp. 149-159, 2008.
[19] Buchanan, R.E. and Gibbons, N.E., *Bergy's manual of determinative bacteriology*. 8th ed. Williams and Wilkin Co., Baltimore, pp. 599-881, 1974.
[20] Mohammad, I.S., Hameed, K.M. and Shawaqfah, M., Microbial populations of crude oil spill polluted soils at the Jordan-Iraq desert (The Badia Reion). *Braz J Microbiol*, 39, pp. 453-456, 2008.
[21] Lee, J.Y. and Hwang, B.K., Diversity of antifungal actinomycetes in various vegetative soils of Korea. *Can J Microbiol*, 48, pp. 407-417, 2008.
[22] Lee, G.C., Kim, Y.S., Kim, M.J., Oh, S.A., Choi, I., Choi, J., Park, J.G., Chong, C.K., Kim, Y.Y., Lee, K. and Lee, C.H., Presence, molecular characteristics and geosmin producing ability of actinomycetes isolated from South Korean terrestrial and aquatic environments. *Water Sci Technol*, 63, pp. 2745-2751, 2011.
[23] Zhang, Q.R., Zhou, Q.X., Ren, L.P., Zhu, Y.G. and Sun, S.L., Ecological effects of crude oil residues on the functional diversity of soil microorganisms in three weed rhizospheres. *J Environ Sci* [China], 18(6), pp. 1101-1106, 2006.
[24] Ghazali, M.F., Zaliha, N.R., Abdul, R.N., Salleh, A.B. and Basri, M., Biodegradation of hydrocarbons in soil by microbial consortium. *Int Biodet and Biodeg*, 54, pp. 61-67, 2004.
[25] Sun, Y., Chen, Z., Xu, S. and Cai, P., Stable carbon and hydrocarbon isotopic fractionation of individual n-alkanes accompanying biodegradation: Evidence from a group of progressively biodegraded oils. *Org Geochem*, 36, pp. 225-238, 2005.
[26] Gerdes, B., Brinkmeyer, R., Dieckmann, G. and Helmke, E., Influence of crude oil on changes of bacterial communities in Arctic sea-ice. *FEMS Microbiol Ecol*, 53, pp. 129-139, 2005.
[27] Sherr, B.F., Del -Giorgio, P. and Sherr, E.B., Estimating abundance and single-cell characteristics of respiring bacteria via the redox dye CTC. *Aquat Micro Ecol,* 18, pp. 117-131, 1999.
[28] Saitou, N. and Nei, M., The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol*, 4, pp. 406-425, 1987.
[29] Mariam, D.H., Mengistu, Y., Hoffner, S.E. and Andersson, D.I., Effect of *rpoB* mutations conferring rifampin resistance on fitness of *Mycobacterium tuberculosis.* Antimicrob Agents Chemother, 48, pp. 1289–1294, 2004.

[30] Kim, H., Kim, S.H., Ying, Y.H., Kim, H.J., Koh, Y.H., Kim, C.J., Lee, S.H., Cha, C.Y., Kook, Y.H. and Kim, B.J., Mechanism of natural rifampin resistance of *Streptomyces* spp. *Syst Appl Microbiol,* 28, pp. 398–404, 2005.
[31] Hu, H., Zhang, Q. and Ochi, K., Activation of antibiotic biosynthesis by specified mutations in the *rpoB* gene [encoding the RNA polymerase beta subunit] of *Streptomyces lividans*. *J Bacteriol*, 184, pp. 3984-91, 2002.
[32] Tamehiro, N., Hosaka T., Xu J., Hu H., Otake N. and Ochi K., Innovative approach for improvement of an antibiotic-overproducing industrial strain of *Streptomyces albus*. *Appl Environ Microbiol*, 69, 6412-7. 2003.
[33] Huo, L., Rachid, S., Stadler, M., Wenzel, SC. and Müller, R., Synthetic biotechnology to study and engineer ribosomal bottromycin biosynthesis. *Chem Biol*, 27, pp. 1-10, 2012.