Detection the influence of plasmid involvement in hydrocarbon utilization by a *Streptomyces* sp. Isolated from contaminated soil surrounding local generators

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Abstract

A *Streptomyces* sp. isolated from hydrocarbon contaminated soil surrounding local generators was investigated to determine the involvement of plasmid DNA in hydrocarbon utilization. This was made by cultivating the bacterium on a complete medium for 10 sub – cultures without the stress factor (diesel) in a high temperature (45^0C) of incubation as a physical way to cure plasmid DNA and avoiding DNA alteration if chemical method is used. Surface tension calculations for control (media only), media + diesel, and media + diesel + *Streptomyces* from 10^{th} sub – culture showed significant change when compared to each other and was a match to that measured for original culture of the bacterium indicating that there is no plasmid DNA involvement in hydrocarbon utilization. This was confirmed by plasmid isolation from both the 10^{th} sub – culture and original one which showed the loss of a band when compared to each other.

Introduction

Streptomycetes are one of the most abundant microbial genera in soil and are well known for their ability to produce biologically active small molecules, such as antibiotics. The biosynthesis of secondary metabolites has been investigated thoroughly by biochemical and genetic analysis, and many different pathways have been characterized. More than 5% of the genes of streptomycetes appear to be involved in the production of small molecules (Bentley *et al.* 2002).

In contrast, although streptomycetes are saprophytic and known to play important roles in biotransformation and biodegradation in nature, the metabolic pathways have been little studied (Omura *et al.*, 2001).

Relatively little is known regarding the environmental determinants of microbial population selection in soil environments contaminated with complex hydrocarbon mixtures. The predominant factors influencing microbial community structure after contamination likely include (i) contaminant mixture type, (ii) soil type (i.e., physical, chemical, and biological characteristics of soils), and (iii) time (Polman,, and Clair. 1995).

Other cyclic hydrocarbons enter the environment as a result of human activities, e.g., transport and application of mineral oils and products derived thereof. Aromatics are especially abundant because of applications such as fuels, industrial solvents (benzene, toluene), polymer synthesis (styrene), and starting materials for chemical syntheses. The cycloalkanes are less abundant although biologically more-persistent compounds. In particular, cyclohexane is becoming increasingly important as an industrial solvent replacing benzene, which is known to be carcinogenic (Gibson, and Subramanian. 1984, Smith, 1990, Smith, 1993, Gibson and Harwood, 2002).

Bacterial biodegradation has been extensively characterized in gram-negative bacteria; *Pseudomonas* species are capable of degrading many organic compounds, including chlorinated polyaromatics. Among the gram-positive bacteria, *Rhodococcus* spp. are effective in the biodegradation of a wide range of xenobiotics Comparative studies of salicylate degradation in gram-positive bacteria, including *Streptomyces* spp., have revealed the presence of both pathways degradation processes in gram-positive and -negative bacteria . However, no molecular studies of these processes have been reported. (Dı´az *et al.* 2001).

In *Streptomyces*, two types of these elements were identified; the first is cccDNA form with various copy numbers, ranging from 6 – 100 Kb, whereas the second type is the giant linear form that may range 50 – 350 Kb. The importance of these plasmids may come from their involvement in conjugation, antibiotic production, and some evidences in biotransformation (Chater and Hopwood, 1993).

Molecular characterization of plasmids carrying the genes for catabolism of aromatics has revealed the modular structure of these plasmids: the catabolic genes are usually parts of composite transposons or they are found to be flanked by genes having similarity to transposase genes of IS elements. This indicates that IS elements could play an important role in the evolution of catabolic pathways in soil bacteria and in the regulation of gene expression. However, little is known about the mechanism of transposition of these DNA elements in soil bacteria. It was shown that introduction of a plasmid carrying the *phe*BA genes encoding catechol 1, 2-dioxygenase and phenol monooxygenase, respectively; into *Pseudomonas putida* PaW85 enables the bacterium to use the hybrid plasmid- chromosome-encoded pathway for phenol degradation (Lee *et al.*, 1995).

However, during our research we try in a simple procedure to investigat if plasmid DNA in our local *Streptomyces* play a role in diesel assimilation by an attempt to neutralize plasmid DNA in physical methods without exposing the chromosomal DNA to any alteration.

Materials and methods

Media

Streptomyces sp. was cultivated on gauza medium (Komagata, K. 1986)

(Soluble starch, 20 g, KNO_3, 1 g, NaCl, 0.5 g, $MgSO_4$. $7H_2O$, 0.5 g, $FeSO_4$. $7H_2O$, 0.01 g, K_2HPO_4, 0.5 g, Agar, 20 g, Distilled water, 1000 ml) for preservation of the bacterium, S – medium (Okanishi, and Umezawa 1974) (Glucose, 10 g, Pepton, 4 g, Yeast extract, 4 g, $MgSO_4$. $7H_2O$, 0.5 g, KH_2PO_4, 2 g K_2HPO_4, 4 g, Distilled water, 1000 ml) was used for preparing the bacterium for plasmid isolation, complete medium (Agar, 10g, K_2HPO_4, 5 g, NaCl, 0.5 g, $MgSO_4$. $7H_2O$, 0.5 g, Bacto – pepton, 2 g, Yeast extract, 1 g, Casaminoacids, 1.5 g L – Histidine, 50 mg, L – Proline, 50 mg, Yeast nucleic acid

hydrolysate, 5 ml, Vitamin solution, 1 ml, Glucose, 25 g, Distilled water, 1000 ml) was used for cultivation and growing of the bacterium during plasmid neutralization with high temperature cultivation period up to 50^0C (Hopwood *et al.*, 1985).

Plasmid isolation

Plasmid isolation was done using salt precipitation method according to (Schremph *et al.*, 1975).

Electrophoresis

Plasmid DNA was loaded into 1% agrarose gel electrophoresis mixed with loading buffer and a field of 10 v/cm was applied for 3 hours. After electrophoresis was completed, the gel was submerged in electrophoresis buffer containing 2µg/ml ethedium bromide for 1 hour. After staining was completed, the gel was visualized under U.V light.

Surface tension measurement.

Surface tension was done using a Beckman tensiometer with control, medium inoculated with the bacterium and medium inoculated with the bacterium and diesel.

Results

In a former study (unpublished data) we managed to isolate a *Streptomyces* sp. able to assimilate diesel fuel within its medium as a source of carbon and we found that the bacterium flourished in a shorter time in the medium with the stress factor (diesel) when compared of its growth on a diesel free medium.

The question is if genes responsible for hydrocarbon utilization are located on a plasmid DNA or on the chromosome. Thus, we try to neutralize the plasmid DNA using physical and nutritional method to avoid any DNA alteration if chemical methods are used.

Cultivating *Streptomyces* sp. on a rich medium (complete medium) at high temperature may help expulsion of plasmid DNA out of the cell (Bibb and Hopwood, 1981) helping to test the microorganism on diesel containing medium.

Therefore, we cultivated the *Streptomyces* sp. on complete medium for 10 generation without stress factor to facilitate the elimination of extra – chromosomal DNA molecule(s). We found that the bacterium retained its ability to grow on diesel containing even after repeated sub-culturing without the stress factor suggesting that plasmid DNA does not have any role in affecting hydrocarbon utilization in our *Streptomyces*.

Further confirmation was performed in two stages. The first stage is the isolation of plasmid DNA from original isolate and the one from the 10^{th} sub – culture to be compared with each other. The out come showed that the 10^{th} sub – culture lost a plasmid band when gel electrophoresis viewed under U. V. transilluminator that may indicate that repeated subculture and high cultivation temperature helped elimination of an extra – chromosomal molecule from the cell.

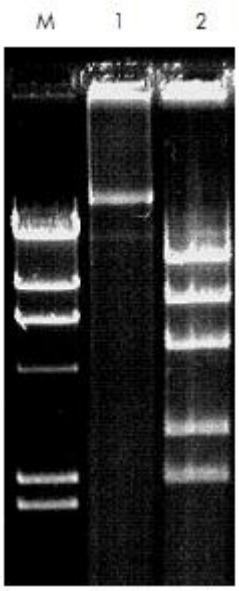

Figure 1 Agarose gel analysis of plasmid DNA isolated from *Streptomyces*. The marker is ١µg of undigested lambda–-HindIII. Lane no. 1 is *Streptomyces* sp. after the 10^{th} sub – culture where as lane no. 2 is *Streptomyces* sp. from stock culture.

Stage 2 included the use of measurement by tensiometer for the broth of the original culture, the 10^{th} sub – culture cultivate both of them containing diesel, with control of growth medium and the same as before but without diesel.

Results showed a drop in surface tension for both original and the 10^{th} sub culture growth in comparison with the control medium (bacterium free) and cultivate without diesel. This may suggest that genes responsible hydrocarbon utilization are located on the chromosome. Table (1) shows results obtained for tensiometer measurements.

Table (1). Tensiometer measurements for *Streptomyces* sp. under study.

	Sample	Surface tension mN/m
1	Media only (control)	50
2	Media + diesel	47
3	Media + *Streptomyces* sp.+ diesel	42

It is worth to say that the *Streptomyces* sp. under study showed a genetic stability when compared with the original culture suggesting that the hypervariable region on the chromosome has low frequency of alteration (Kieser and Hopwood, 2000). This genetic stability may make the *Streptomyces* sp. studied a good candidate to be used for biological remediation and elimination of contaminants of oil from the soil.

Discussion

Streptomyces is a widely spread microorganism and can be isolated from different part of world and occupy a wide range of habitats because of its ability to adapt and production of enzymes and secondary metabolites that enabling it to utilize resources of this habitat. Crude oil and oil derivatives contaminated environments were one

of these habitats that we were able to isolate *Streptomyces* sp. that actually able to grow and utilize diesel. During our research we determined that that this isolate was genetically stable and only a small change was observed in morphology of the colonies when cultured on different media. This may be due to the low changes that occur in the hypervarialble region of the chromosome. This may sustain this trait of diesel assimilation.

The ability of *Streptomyces* sp. to grow in a wide range of temperature (27 – 45 ^{0}C) was determined during our experiments to cure plasmid DNA may be due to the habitat from which the bacterium was isolated. This habitat is near locale generators that operate on diesel that produce heat during its operation that spread around it that when measured was found to be 40 – 50 ^{0}C. This heat is reduced when these generators are shut down which may last for long hours. Such rise and drop of heat had induced the criteria of growth within such range of temperature.

Many literatures (Chater and Hopwood, 1993, Floriano and Bibb, 1996, Hopwood, 1999) showed that plasmid DNA may be a burden on microorganisms and may lie toward expulsion of these plasmids unless it interferes with viable process. Using this fact and avoiding alteration of DNA if chemical curing is used our attempt to cure plasmid(s) of our *Streptomyces* sp. by physical stress for showed no effect on this isolate to utilize gas oil. This may be explained in tow ways: (i) the plasmid DNA may play no rule in hydrocarbon utilization and genes responsible for such trait may be located on chromosomal DNA; (ii) giant plasmids which are known to be found in streptomycetes (Kinashi *et al.*, 1987) may play a part in this process was not cured because of it size that reach 700 KB and / or is could be integrated with chromosomal DNA. The repeated subculture did not affect the criteria of growth and maturation on gas oil containing medium. Our conclusion that the trait of utilizing gas oil is stable within the *Streptomyces* sp. under study and not affected by small plasmids that may be found within the bacterial cells. However, more investigation is needed to study the rule of large plasmids with this process and if such trait is transmissible to other types of bacteria.

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الملخص

تم التحري عن العوامل الوراثية المسؤولة عن تحليل الهيدروكربون في بكتريا ستربتومايسيس المعزولة من التربة الملوثة المحيطة بالمولدات المحلية لمعرفة دور البلازميدات في تحلل الهيدروكربون. تم هذا عن طريق اعادة تنمية البكتريا على الوسط الكامل لعشر مرات بدون عامل الضغط(زيت الغاز) وفي درجة حرارة عالية وصلت الى 45^0 م كنوع من التاثير الفيزياوي لتحييد البلازميد بدون التسبب بطفرة وراثية قد تنتج عن استخدام المواد الكيمياوية. تم قياس الشد السطحي لوسط السيطرة (الوسط فقط بدون زيت الغاز) و الوسط+ زيت الغاز و الوسط+ زيت الغاز + البكتريا من اخر زرعة وهي العاشرة. اظهرت النتائج تغيرا واضحا عند مقارنة النتائج من الوسط المزروع بالبكتريا والمضاف اليه زيت الغاز عند مقارنته بوسط السيطرة والوسط المضاف اليه زيت الغاز مما يدلل على عدم ضلوع البلازميد في تحليل الهيدروكربون في هذه العزلة. تم التاكد من ذلك عند عزل البلازميد من البكتريا المأخوذة من الزرع الاخير والتي اظهرت تحييدا للبلازميد.