Full Length Research Paper

Conjugational transfer and survival of plasmid encoding silver and antibiotic resistance genes of Acinetobacter baumannii BL54, E. coli K12 J53.2 transconjugants and pseudomonas transformants in different soil microcosms

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In this investigation we tried to transfer plasmid encoded silver and antibiotic resistance genes from Acinetobacter baumannii BL54 to E. coli k12 J53.2 and Pseudomonas (a soil microflora) by conjugation and transformation in different soil microcosms and study the survival of the isolated bacterium in each soil. Clay loam, fine clay, sandy and clay soils were collected from different area of maharashtra in India. Microcosm was developed for each type of soil in the glass tube (150 x 25 mm) with 2 g soil moistened with 1 ml 0.5% sterile saline. The conjugation frequency was lowest in sterile clay soil with frequency of $0.2 \times 10^{-6}$, while, it was maximum in clay loam soil with frequency of $0.6 \times 10^{-6}$. Similarly, in non-sterile soil microcosms, the rate of conjugation was highest in clay loam soil with frequency of $0.09 \times 10^{-6}$ while was lowest in sandy soil ($0.03 \times 10^{-6}$). Rapid death of the organisms was observed within 9 days of incubation in presence of selection in sandy soil, while in fine clay, survival of the organisms was extended beyond 11 days. The Pseudomonas transformant survived for more than 40 days in presence of selection. From above results it can be concluded that plasmid mediated silver and antibiotic resistant genes were transferred in different soil by conjugation process. However, the rate of conjugation was affected by soil type. Soil transformant considerably survived in fine clay containing high amount of organic carbon and neutral pH as compared to sandy soil.

Key words: Conjugation, soil type, survival, plasmid, Acinetobacter baumannii.

INTRODUCTION

Soil is a macrohabitat containing minute amounts of nutrients, and is subjected to temporal and spatial variation (Barkey et al., 1985). Plasmids have been found in many genera of soil bacteria (Chopade et al., 1985; Devanas et al., 1986) and play an important role in survival of soil organisms in presence of metals or antibiotics in the soil environment. Conjugation in the soil showed that plasmid encoding gene for degradation of 3-chlorobezoate was transferred to indigenous soil flora (Pseudomonas) with

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The above results are average of two independent experiments. Soils were collected from 15 cm depth above locations and subjected to analysis as described in text.

K = Potassium
P = Phosphorous

Table 1. Chemical analysis of soil samples used in this study.

<table>
<thead>
<tr>
<th>Soil type</th>
<th>Source</th>
<th>pH</th>
<th>Electric conductivity $\text{Ohm}^{-1}/\text{Cm}^{-1}$</th>
<th>Organic carbon (%)</th>
<th>P (gm %)</th>
<th>K (gm %)</th>
<th>Organic matter (%)</th>
<th>Total soluble salt (%)</th>
<th>CaCO3</th>
<th>Silt (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clay loam</td>
<td>Pune</td>
<td>8.1</td>
<td>0.2</td>
<td>0.65</td>
<td>0.0012</td>
<td>0.007</td>
<td>0.72</td>
<td>0.8</td>
<td>0.9</td>
<td>30</td>
</tr>
<tr>
<td>Fine clay (Black)</td>
<td>Pimperi</td>
<td>7.5</td>
<td>0.2</td>
<td>0.79</td>
<td>0.0014</td>
<td>0.001</td>
<td>1.3</td>
<td>0.8</td>
<td>0.96</td>
<td>18.75</td>
</tr>
<tr>
<td>Clay</td>
<td>Mahabale shwar</td>
<td>6.3</td>
<td>0.12</td>
<td>0.549</td>
<td>0.008</td>
<td>0.001</td>
<td>0.94</td>
<td>0.6</td>
<td>0.88</td>
<td>16</td>
</tr>
<tr>
<td>Sandy</td>
<td>mumbi beach</td>
<td>8.6</td>
<td>0.15</td>
<td>0.04</td>
<td>0.001</td>
<td>0.0006</td>
<td>0.04</td>
<td>-</td>
<td>-</td>
<td>50</td>
</tr>
</tbody>
</table>

P = Phosphorous
K = Potassium

Soils were collected from 15 cm depth above locations and subjected to analysis as described in text. The above results are average of two independent experiments.

MATERIALS AND METHODS

Collection of soil samples and developing soil microcosm

Clay loam, fine clay (forest, black), sandy and clay (red, latertic) soils were collected from different area of maharashtra provenience in India. Thirty gram of each soil was removed by spade from 15 cm depth and collected in polythene bag. The soils were dried over night at 30°C and subjected to grading with pestle followed by sieving through 0.242 mm pore size sieve. The processed soils were subjected to physicochemical analysis as shown in Table 1. Organic carbon was determined by Walkey Black potassium dichromate oxidation method and available phosphorus was determined by Olson method (Redford et al., 1981). The soils were then divided in two parts and one was sterilized by autoclaving at 121°C for one hour. Microcosm was developed for each type of soil in the glass tube (150 x 25 mm) with 2 g soil moistened with 1 ml 0.5% sterile normal saline and used for gene transfer and survival studies.

Conjugation in soil

Conjugation was carried out in each sterile and nonsterile soil as described by Naik et al. (1994). Briefly, 1 ml O/N growth of A. baumannii (10⁷/ml) in Luria-Bertani medium as donor and 1 ml of E. coli K12 J53.2 (Rif²) as recipient were added to each soil microcosm and incubated for 24 h at 30°C. With help of a sterile glass rod approximately 20 mg of soil was withdrawn and suspended in sterile saline and serially diluted. 0.2 ml aliquot of each dilution was spread in to Lactose electrolyte deficient medium (CLED) agar selective for transconjugant (Ag 200 µg/ml + Te 100 µg/ml + ampicillin 200 µg/ml and Rif 100 µg/ml). The frequency of conjugation was then calculated as number of colonies grown in this medium divided by number of recipient cells.

Transformation and plasmid stability

Transformation and stability of plasmid were determined by shakibaie et al. (1998) as previous described. Briefly, Plasmid DNA was prepared with the Qiagen plasmid purification kit (Qiagen, Hilden, Germany). Overnight culture of A. baumannii BL54 were grown in LB broth with 100 µg AgNO₃, washed twice in 0.9% NaCl and adjusted to a titer of $1 \times 10^{11}$ ml⁻¹. To 20 µl of this suspension 10 µl of plasmid DNA in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 50 mM ice cold CaCl₂ was added and the mixture was spotted onto LB
agar. Incubation was for 20 h at 37°C. Appropriate dilutions of the resuspended cells were plated on LB agar containing 100 µg AgNO\(_3\) recipients). Colonies were counted following incubation for 48 h at 37°C.

Survival of \textit{A. baumannii} BL54, \textit{E. coli} K12 transconjugant and \textit{Pseudomonas} transformant in different soil microcosms

2 ml of overnight growth of each isolate (10\(^8\) CFU/ml) was inoculated separately into 4 g of sterile and nonsterile (Fine clay, clay loam, and sandy) soil microcosms. All tubes were incubated up to 44 days. At two days interval 5 mg soil was taken from each soil microcosm and suspended in 10 ml 0.85% sterile saline and serially diluted. 0.1 ml of each dilution was spread onto CLED agar medium containing 25 µg/ml Tetracycline and 200 µg/ml AgNO\(_3\) and into CLED agar. After 24 h of incubation at 30°C, total viable count of bacteria in each soil then was calculated.

**RESULTS**

Chemical composition of different soil microcosms used in this study is shown in Table 1. Table 1 indicates, the organic matter as well as phosphorous and neutral pH while, sandy soil contain minimum amount of nutrient as well as alkaline pH (8.6). The results of conjugation in sterile and nonsterile soil microcosms are shown in Table 2a, b. The conjugation frequency was lowest in sterile Clay soil with frequency of 0.2 x 10\(^6\), while, it was maximum in clay loam soil with frequency of 0.6 x 10\(^6\). Similarly, in non sterile soil microcosms (Table 2b), the rate of conjugation was highest in clay loam soil with frequency of 0.09 x 10\(^6\) while was lowest in sandy soil (0.03 x 10\(^6\)). This indicate that transfer of metal and antibiotic resistance genes indeed occur in different soil microcosms and soil type influenced the frequency of conjugation. Concurrent testing of transconjugant in presence of ampicillin 50 µg/ml + Rif 100 µg/ml, Te 50 µg/ml + Rif and Ag 200 µg/ml + Rif showed simultaneous transfer of these genes to the recipient cells. Transfer of plasmid from \textit{A. baumannii} to \textit{Pseudomonas} a soil microflora had occurred by transformation process (data not shown) with frequency of 0.4 x 10\(^6\). Co-transfer study of transformant indicated simultaneous transfer of above antibiotic resistant markers to \textit{Pseudomonas}.

The survival of \textit{A. baumannii}, \textit{E. coli} K12 transconjugant and \textit{Pseudomonas} transformant was studied in different types of soil as shown in Figures 1 and 2. Rapid death of the organisms was observed within 9 days of incubation in presence of selection in sandy soil, while in fine clay soil survival of the organisms was extended beyond 11 days. This decrease in plasmid bearing cells was apparently the result of partitioning of the plasmid beyond 11 days. This decrease in plasmid bearing cells was apparently the result of partitioning of the plasmid beyond 11 days. The above conclusion was supported from comparatively high CFU of the organisms at the start of experiment as shown in Figure 1. This indicated that expression of resistant genes on plasmid was inhibited after long incubation under starvation condition. The above results were further supported by study of the viable count of pseudomonas transformant in presence and absence of selection as shown in Figure 2. The organism survived for more than 44 days in the absence of selection and for 40 days in presence of selection. This indicates that expression of these resistant genes was also depending on physiology of the organism as well. Since \textit{Pseudomonas} is as soil
isolate it better adapted in starvation condition, while, A. baumannii and E. coli transconjugant being zymogenous were not able to survive in starvation under pressure of antibiotic and metal containing medium.

**DISCUSSION**

The major concern about introduction of plasmid containing organisms in soil is not only their potential adverse ecological impacts on the homeostasis of soil but also on the bioremediation of pollutants like heavy metals from different type of soil. Krasovky and Stotzky, (1987) demonstrated that indigenous microflora of soil directly effects the survival of plasmid bearing bacteria in nonsterile soil.

In our study plasmid encoding Acinetobacter was quite stable in all soil tested. The stability was not depending on type of soil but it was depend on type of host. Chemical composition of different soil microcosms used in this study indicates that, the forest soil contain considerable amount of organic matter as well as phosphorous and neutral pH (7.5) while, sandy soil contain minimum amount of nutrient as well as alkaline pH (8.6). Therefore, it is appropriate to suggest that genetic markers could not be expressed up on long term in starvation condition in sandy soil. This was supported by study of population dynamics of Acinetobacter, E coli transconjugant and Pseudomonas transformant in different types of soil microcosms. This also indicated that plasmid have no role in long term survival of the organisms in soil. Infact, viable count declined rapidly when cells were plated in

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**Figure 1.** Survival of A. baumannii and E. coli transconjugant in sterile and nonsterile sandy, clay, clay loam and fine clay (forest) soil microcosms.

- *; A. baumannii plated on CLED agar, ●; E. coli plated on CLED agar, □; A. baumannii plated on CLED agar + 200µg/ml AgNO₃, ▲; E. coli transconjugant plated on CLED agar + 200µg/ml AgNO₃, ●; E. coli transconjugant plated on CLED agar + 50µg/ml Te.
Figure 2. Survival of Pseudomonas transformant (an indigenous soil microflora) in sandy, clay loam and fine clay (forest) soil microcosm

- Organism plated on CLED agar, ●; Organism plated on CLED agar + 50 µg/ml Te, ▲; Organism plated on CLED agar + 200 µg/ml AgNO₃

metal and antibiotic containing medium when plasmid was present in the cells. The viable count decreased rapidly in sandy soil, while in forest soil it was prolonged by few days.

Endogenous energy source is an important factor in microbial survival and it may be that autochthonous bacteria have a mechanism for reducing metabolic rate in order to enhance their survival under starvation condition (Trevors, and Oddie, 1986). Shakibaie et al. (1999) reported that accumulation of silver is an energy dependent process. It is quite possible nutrients were not available in soil and affect long term survival of the organisms. At present, research is carrying out by our group in this regard. Recently, horizontal transfer of antibiotic resistant genes was studied in sewage and lake water by Shakibaie et al. (2009). It was observed that the rate of conjugation was two fold high in sewage than in lake water. The physicochemical parameters of water also were contributed to gene transfer by conjugation.

Conclusion

From above results it can be concluded that metal and antibiotic resistant genes were transferred in different soil types by conjugation process. However, the rate of conjugation was depending on soil type as well as type of host. The plasmid containing soil microbiota transformant considerably survived under selective and non selective conditions and soil type also play role in the survival of isolated organisms as well.

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REFERENCES


Shakibaie MR, Dhakephalker PK, Kapadnis BP, Chopade BA (2003). Silver resistance in Acinetobacter baumannii BL54 occurs through