# MOLECULAR AND GENETIC CHARACTERIZATION OF SOME *Rhizobium leguminosarum bv. vicieae isolates*.

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## ABSTRACT

*Rhizobium leguminosarum bv. vicieae* isolates which isolated and fully characterized in Agric. Microbiol. Dept., Agric. Fac., Zagazig Univ. were used in this work to study molecular and genetic diverisity.24 isolates were used to investigate lysogenicity ability. The results showed that 23 isolates from 24 were lysogen with lysogenecity percent 96%. 19 lysogenic isolates were contained more than one different prophage, since the phage released from them was able to lysis the same lysogenic isolate which released from it. The isolates RA21, RF12, RF13 and RR13 were contained one prophage only, since the phage released was not able to lysis the same lysogenic host.

Most bacterial isolates were sensitive to streptomycin, ampicillin and chloramphenicol at concentrations from 100 to 2000 µg/ ml.The RR23 isolate was resistance to ampicillin and chloramphenicol at the used concentrations. The RA11 and RR11 isolates were resistanc to ampicillin up to 1500 µg/ ml and chloramphenicol up to 500 µg/ ml. The RK11 and RF12 isolates were resistance to streptomycin up to 500 µg/ ml. Five rhizobiophages were used in this study. These phages were isolated from soil. The host range of these phages was studied by using the bacterial isolates as hosts. The phages A32, R11 and H21 were able to lysis all the used hosts. The phage K23 was lysis 10 from 11 host, while phage F13 was lysis 7 from 10 host. The plaque forming units (pfu/ ml) of these phages were varied, it ranged from $2.2\times 10^{6}$ to $9.67\times 10^{13}$. The ability of these phages to transduce some antibiotic resistance genes was assessed. The five phages were able to successfully transduce streptomycin and chloramphenicol resistance genes. Transduction frequency ranged from $0.39\times 10^{-8}$ to $4.5\times 10^{-5}$ for streptomycin and from $1.25\times 10^{-8}$ to $1.3\times 10^{-3}$ for chloramphenicol. Not all phages were able to transduce the ampicillin resistance gene. Also, transducing this marker was not success with all the recipients. Tranceduction frequency ranged from $4.3\times 10^{-9}$ to $9.4\times 10^{-8}$. The five phages were able to cotransduce streptomycin and chloramphenicol resistance gene together, tranceduction frequency ranged from $5.0\times 10^{-5}$ to $2.97\times 10^{-3}$.

Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) analyses were performed by genomic DNA extracted from 10 isolates of

*Rhizobium leguminosarum bv. Viciae.* Five out of 16 arbitrary decamer primers used in this study were informative and detected scoreable polymorphism in banding patterns of RAPD markers between these isolates. Each of primers used for analysis of individual isolates amplified different number of bands. Genetic similarity between isolates, calculated as the total number of band differences, were computed. The similarity coefficient value (1.000) was observed among RF31, RF31A, RS3, RZ11, RA21, RH31and RB2 isolates (group A). The similarity coefficient value between RR11 and (group A) was 0.919 and it was 0.973 between (group A) and both of RB2A and RK12 isolates. The similarity coefficient value between RR11 and RB2A was 0.946 while, it was 0.892 between RR11 and RK12.The dendrogram of genetic distances among isolates based on band polymorphisms generated by RAPD-PCR after using the primers clustered the 10 isolates into two main clusters, where RF31, RF31A, RS3, RZ11, RA21, RH31and RB2 isolates (group A) constituted one cluster correlated with RB2A and RK12 while, RR11 isolate formed the second cluster. RR11 isolate was distinguishable by 4 positive unique RAPD markers while, RB2A and RK12 isolates were identified by one positive unique RAPD markers.

**Key Word:** Rhizobium, transduction, bacteriophage, RAPD - PCR

## INTRODUCTION

In view of the numerous possible application of genetically engineered microorganisms(GEMs) in the environment (Tiedje *et al.,* 1989 Trevors *et a*l., 1990, Dergange and Bardin, 1995, Lynch *et al*., 2004, Zhou *et al,* 2004), research into the fate of these organisms and their genetic material has become increasingly important (Trevors *et la.,* 1987). Research has focused on the introduction and recovery of microorganisms in environmental samples, genetic interactions and analysis gane probing and on DNA amplification using polymerase chain reaction (Trevors *et al.,* 1989, Degrange and Bardin, 1995).

However, less in Known about environmental transport and dispersal of GEMs and factors that control this transport (Trevors *et al*.,1990, Zhou *et al*., 2004). Methods to assess the survival of microorganisms in soil have indicated that several bacterial genera such as *Pseudomanas, Rhizobium, Agrobacterium, Azospirllum, Bacillus , Azotobacter, Xanthomonan* and *Erwinia* have adapted to growth in rhizosphere. Rhizosphere bacteria of the genera *Rhizobium, Azorhizobium*, and *Bradyrhizobium* can interact with roots of legumes to form nodules, which function as sites for atmospheric nitrogen fixation.(Relic *et al*., 1994, Srinivasan *et al*., 1997). *Rhizobium* bacteria are used as microbial inoculants for agricultural purposes extends annually over 25x106 ha world wide, and is expected to increase due to land managerial practices such as sustainable agriculture, biological farming and land reclamation (Nuti *et al.*, 1994, Corich et al., 1996). So, the aim of this study was to investigate the genetic characterization of some *Rhizobium* isolates strairs which have been

isolated from different locatia is Sharkia area. The genetic diversity among these isolates has also been studied using RAPD-PCR analysis.

The randomly amplified polymorphic DNA (RAPD) assay, which detects nucleotide sequence polymorphisms by means of the polymerase chain reaction (PCR) and a single primer of arbitrary nucleotide sequence, is a useful method for generating molecular markers. RAPD assay was first described by two independent groups, Williams *et al.*, (1990) and Welsh and McClelland, (1990).The success of this method is due to the fact that no prior sequence information about the target is needed and a single short 10-mer oligonucleotide primer can be used in the reaction. The amplification happens at low stringency, allowing the primers to anneal to several locations on the two strands of the DNA. These primers detect polymorphisms in the absence of specific sequence information and the DNA sequence variations may work as genetic markers that can be used in genetic similarity and diversity studies. This technique has been used extensively to detect genetic diversity in Rhizobium spp. (Paffetti *et al.,* 1998, Willems *et al.,* 2001 and Zurdo-Piñeiro *et al.*, 2004).

## MATRIALS AND METHODS

This study was carried out in microbial and Molecular Genetic laboratories, Genetic Dept, Fuculty of Agric, Zagazig univ. Egypt.

**1- *Rhizobium leguminosarum bv. vicieae* bacterial isolates:**

The bacterial isolates that were used in this study were obtained from Howaida M. L., Agric. Microbiol. Dept., Fac. Agric. Zagazig Univ. These isolates were isolated from different location in Sharkia Gavernorate and full characterzed in Agric, Microbiol. Dept. Fac. Agric. Zagazig Univ. (Table 1) Also, the five rhizobiophages (K23, A32, F13, R11 and H21) were obtained from the same source.

**Table 1. *Rhizobium leguminosarum bv. vicieae* bacterial isolates.**

| Location | Nomination | Reference |
|---|---|---|
| Abo- Kibeer (K) | RK 11, 12, 13, 21, 32. | Salem, *et al*., (2006) |
| Abo- Hammad (A) | RA 11, 21. | Salem, *et al*., (2006) |
| Fakous (F) | RF 12, 13, 31, 32. | Salem, *et al*., (2006) |
| Zagazig (Z) | RZ 11, 22. | Salem, *et al*., (2006) |
| Kafer- Shaker (R) | RR 11, 13, 23, 31. | Salem, *et al*., (2006) |
| El- Hessenia (H) | RH 11, 21, 32. | Salem, *et al*., (2006) |
| Belbase (B) | RB 1, 2, 3. | Salem, *et al*., (2006) |
| Salhia (S) | RS 3. | Salem, *et al*., (2006) |

**2- Growth media:**

Yeast extract mannitol (YEM) agar and YEM broth media were used. The composition was (g / l) as follows: mannitol 10, yeast extract 0.5, MgSO4. 7H2O 0.2, K2HPO4 0.5, NaCL 0.1. To prepare YEM agar medium, 20 g agar has been added.

Soft agar (0.8 % w / v agar) was prepared in distilled water and kept at 45°C on waterbath. The antibiotics (streptomycin, chloramphenicol and ampicillin) were add as sterilized solution by filtration through 0.2 µm filter membrane to the media after autoclaving.

**3- Lysogenicity test:**

Each isolate cells were inoculated into 10 ml of YEM broth medium, then placed on a shaker incubator for two to three days at 28°C. Centrifugation was carried out on 10000 rpm for 30 min. The supernatant was removed and passed through a strile membrane (0.45 µm), spotted onto layer of baterial cells.

**4- Sensitivity to antibiotics:**

In this experiment, three antibioties were used (streptomycin, ampicillin and chyloramphenicol) at concentrations, 100, 500, 1000, 1500 and 2000 µg/ ml. Loop of liquid culture of each isolate was streaked onto YEM agar plates containing antibiotic and plates without antibiotic and incubated at 30°C for 2- 3 days.

**5- Host range of isolated phages from soil:**

Host range was carried out by using the spot test method (Barrangou, *et al*., 2002).Each individual phage lysate was spotted onto layer of bacterial host cells from the same location of isolated phage and other locations. The plates were incubated at 28°C for 48h.

**6- Phage titration:**

The phage activity was assayed using a double agar layer method (Sharma*, et al*., 2002 and payan *et al.,* 2005). Samples (0.2 ml) of exponentially growing culture of the bacterial isolates were suspended in 4 ml of melted soft YEM with 0.1 ml of diluted phage suspension and overlayed onto YEM agar plates. Plates were incubated at 28°C and observed after 48h. The number of plaques was counted and plaque forming units (pfu / ml) were calculated.

**7- Transducing antibiotic resistance genes:**

The five bacteriophages K23, A32, F13, R11 and H21 were propagated on donor RH11Al (resistant to streptomycin) to transduce streptomycin resistance gene. Equal volumes of phage lysates and recipient cells were mixed. The mixture was kept for 30 min at room temperature, serial dilutions were prepared and placed onto selective medium (Jensen *et al*., 1998 and Toth *et al*., 2003). This operation was repeated with transducing chloramphenicol

(donor was RH11A5), cotransduction (donor was RF12A8) and ampicillin (donor was RF12A17). Concentrations of antibiotics in this experiment were 4mg/ ml for streptomycin and 500 µg/ ml for chloramphenicol and ampicillin. Number of transductants were recorded and transduction frequency was calculated.

**RAPD fingerprinting**

**DNA isolation:** The assays were performed with the following: 15 µl from a single colony grown on nutrient agar (the colony was picked and resuspended in 150 µl of distilled water, the suspension was boiled for 5 min, cooled at room temperature, and the supernatant was collected after centrifugation at 10,000 rpm at 4˚C for 10 min).

**Primers:** A set of 16 primers was analyzed and based on the accurate amplified bands profiles and the produced polymorphic patterns of DNA fingerprinting we selected five different primers table ( 1 ).

**Table (1) Sequence and operon codes of the random primers used to detection of variation in *Rhizobium leguminosarum bv. Vicieae* isolates.**

| Primer codes | Sequence ($5^{-}$ to $3^{-}$) |
|---|---|
| OPB-11 | GTA GAC CCG T |
| OPD-03 | GTC GCC GTC A |
| OPC-08 | TGG ACC GGT G |
| OPA-11 | CAA TCG CCG T |
| OPB-17 | AGG GAA CGA G |

**Amplification reaction mixture:** The amplification conditions were based on Williams *et al*., (1990) with some modifications. The reaction was prepared using 25µl per tube, containing 2µl DNA of each strain(20 ng), 1 unit of *Taq* DNA polymerase enzyme, 2µl 10X buffer, 2 µl $MgCl_2$ (25 mM), 2µl $dNTP_s$ ( 2.5 mM of each), 2 µl primer (10 pmol ) and 14.8µl $H_2O$.

**DNA amplification cycles**: The temperature cycling program used with a Perkin-Elmer Gene Amp PCR system (model 2400) was as follows: one cycle at 94°C for 5 min followed by 30 cycles consisting of one step of denaturation (94°C) for 1 min, one step of annealing (35°C) for 1 min, followed by one step of synthesis (72°C) for 2 min and a final extension step consisting of 72°C for 7 min and finally 4°C infinitive.

**Band analysis:** The reaction products were analyzed by electrophoresis on 1.4% agarose gels, stained with ethidium bromide, and photographed under UV light. The synthetic DNA, ladder 100 bp (Pharmacia) was employed as molecular markers for bands weight. Each amplified band profile was defined by the presence or absence of bands at particular positions on the gel. Profiles were considered different when at least one polymorphic band was identified. Fragments were scored as 1 if present or 0 if absent based on standard marker. Pairwise combinations, genetic similarity and genetic distances were estimated

following Lynch (1990 and 1991). The computer package SPSS was used to generate distance matrices to determine isolates relationships using the unweighted pair-group method of analysis (UPGMA) (Sneath & Sokal1973).

## RESULTS ANSD DISCUSSION

**1-Lysogenicity ability:**

Data in Table 2 represent the lysogenicity ability of the 24 isolates of *Rhizobium leguminosarum bv. vicieae*, it seemed that 19 lysogenic isolates out of 24 contain more than one different prophage, since the phage released from them was able to make lysis on the same lysongenic isolate which released from it. These results come agree with the results of Ghanem, 2007. The rest of lysogens (RA21, RF12, RF13 and RR13 contained one prophage only, since the phage released was not able to lysis the same host lysogens because of lysogenic immunity.17 phage lysates prepared from lysogenes were able to lysis all the 24 host isolates. Whereas six phages (Ø RA21, Ø RF 12, RF13, RF31, RF 32, and RR13) were not able to lysis all the 24 host isolates Generally, this results clearly showed that 23 *Rhizobium* isolates from 24 were lysogen. So, the natural occurrence of lysogenicity among these isolates reached up 96%.

**2. Sensitivity to antibiotics:**

The sensitivity to antibiotics was presented in Table 3. The results showed that most bacterial isolates were sensitive to antibiotics that used in this study (streptomycin, ampicillin and chloramphenichol) at concentrations from 100 to 2000 µg/ml. The bacterial isolate RR23 was resistance to ampicillin and chloramphenicol at the used concentrations. The isolates RA11 and RR11 were resistance to ampicillin up to 1500 µg/ml and chloramphenicol up to 500 µg/ml. The two isolates RK11 and RF12 were resistance to streptomycin up to 500µg/ml). Schroder (1980) isolated *Rhizobium Japonicum* from soil, some of these isolates were naturally resistant to higher concentrations of four antibiotics, streptomycin, kanamycin, rifampicin and erythromycin. The resistance to antibiotics, streptomycin, kanamycin, rifampicin and erythromycin. The resistance to antibiotics may affect the symbiotic nitrogen fixation process. Sembiotic effectivness of 45 mutant strains of *R. leguminosarum* resisttance to streptomycin or kanamcin was determined on *Vicia faba*. Loss of effectiveness occurred in 20 of these mutants (Amarger, 1975). The spread of antibiotic resistance genes may due to the process of horizontal gene transfer which coupled with the selective pressures caused by the presence of increasing amounts of these substances in the environment (Davies, 1996 and Salyers and Shoemaker 1996). However, Cole and Elka, 1973 suggested that the resistance of *R.Japonicum* to a number of antibiotics was mediated to be plasmid-born genes.

**3.Host range of isolated phages from soil:**

The host range of five phages isolated from soil was studied using some bacterial isolates from the same location and different locations. The phages A32, R11 and H21 were able to lysis all the used hosts (7, 10, 8 respectively).The phage K23 was lysis 10 from 11 hosts. The phage F13 was lysis seven from nine. So, the phages A32, R11, H21 and K23 may have a wide host range, but the phage F13 has a moderate host range. The phages were titred using the same hostes that used in spot test. The pfu/m1 were varied depended on phage and host type (Tables 4, 5, 6, 7, 8).

**4.Transduction by Phage Lysates Isolated from Soil:**

The five phages that isolated from soil were used to transfer some antibiotic resistance genes to three different recipients (RK11, RF12 and RH11).

**4.1. Transducing streptomycin resistance gene:**

The five phages were able to successfully transduce the streptomycin resistance gene from donor RH11A1 to three recipients. Transduction frequency ranged from $2.5x10^{-7}$ to $2.9x10^{-5}$for phage K23, $1.4x10^{-7}$to$4.5x10^{-5}$ for phage A32, $2.2x\ 10^{-7}$ to $2.5x10^{-5}$ for phage F13, $1.2x10^{-8}$ to $2.8x10^{-5}$ for phage R11 and $0.39x10^{-8}$ to $2.9x10^{-5}$for phage H21(Table 9).

**4.2. Transducing chloramphenicol resistance gene:**

Also the five phages were able to successfully transducer chloramphenicol resistance gene from donor RH11A5 to the previous recipients. Transduction frequency was ranged from $2^{-3}x\ 10^{-7}$ to $1.5x10^{-4}$, $9.5x\ 10^{-8}$ to $2.6x10^{-4}$, $8.3x\ 10^{-7}$ to $2.4x10^{-4}$, $1.25x\ 10^{-8}$ to $2.1x10^{-4}$ and $1.3x\ 10^{-5}$ to $1.3x\ 10^{-3}$ for phages k23, A32, F13, R11and H21 respectively (Table 10).

**4.3.Transducing streptomycin and chloramphenichol resistance genes(cotransduction):**

The five phages were able to transduce streptomycin and chloramphenicol resistance genes together from donor RF12 A8 to reciplients RK11 and RF12 (Table 11).

**4.4. Transducing ampicillin resistance gene:**

Not all the five plages were able to transudce this marker. Three phges (K23 , R11 and H21)were able to transduce it to two reciplents (RK11 and RF12) only. Phages F13 and A32 were not able to transduce this marker. The recipient RH11 did not received this marker. In general, transduction frequency of ampicillin resistance gene was lower than streptomycin or chloramphenicol. It was ranged from $4.3x10^{-9}$ to $9.4x10^{-8}$ (Table 12). Transduction assay of *R. meliloti* and *R. leguminosarum* have been stablished and used to analyze *Rhizobium* genes (Martin and long, 1984, and Finan *et al*., 1984). The generalized transduction of *R. meliloti* by bacteriophage N3 will be useful in genetical and molecular studies including fine-structure genetic mapping, strain constructions and enhanced mutagenesis of specific regions of the megaplasid or chromosome such a transductional system will further advance the genetics of this bacterium (Martin and long, 1984).

**RAPD analysis performed for isolates:**

Single, random oligodeoxyribonucleotide primers were used to generate PCR-amplified fragments, termed RAPD (random amplified polymorphic DNA) markers. Five primers were chosen for this study (OPB-11, OPD-03, OPC-08, OPA-11 and OPB-17) and a fragment was considered polymorphic when absent in at least one isolate. These primers detected scoreable polymorphisms in banding patterns among the isolates. All Polymorphic PCR products were confirmed by repeating the reaction. Each of five primers used for analysis of individual isolate amplified different number of bands. The total number of amplified fragments from all primers for each isolate are summarized in Table (2). Figure 1 (a, b , c, d and e) represents the patterns of the five random primers with the 10 isolates. Each of five different random primers yielded from 6 to 9 DNA fragments whose molecular size ranged from approximately 255 to 1700 bp. The total number of amplified RAPD fragments after using all five primers was 37 bands with an average of 7.4 fragments / primer. The total number of polymorphic amplicons produced by the 5 primers was 4, thus, representing a level of polymorphism of 10.8% in all isolates. Primer OPB-17 produced the highest number of fragments among the primers used with an average of 9 fragments while, primer OPA-11 produced the lowest number of fragments with an average of 6 fragments. This indicated that the Primer OPB-17 was the highest among the tested five primers in their ability to flank the DNA sequences of 10 isolates tested in this work. Primers OPB-11 and OPD-03 produced the highest percentage of polymorphism (14.8%) in the isolates while, primer OPA-11 did not produce polymorphism. The highest number of amplified RAPD fragments (36) after using all five primers were detected in RR11 isolate with an average of 7.2 per primer.

The fragment size of the PCR products generated by primer OPB-11 (Fig. 1a) ranged from approximately 440 to 1500 bp. A band of size 440 bp was observed in RR11 isolate but was absent from all isolates, therefore it was positive unique RAPD marker. Primer OPD-03 (Fig. 1b) generated fragment size of the PCR products ranged from approximately 620 to 1370 bp. A band of size 855 bp was observed only in RR11 isolate while it was absent from all isolates, therefore it was positive unique RAPD marker. The fragment size of the PCR products generated by primer OPC-08 (Fig. 1c) ranged from approximately 270 to 1400 bp. A band of size 550 bp was observed in RR11 and RB2A isolates while it was absent from all isolates, therefore it was positive unique RAPD marker. Primer OPA-11 (Fig. 1d) generated fragment size of the PCR products ranged from approximately 305 to 1320 bp and produced six shared bands in all isolates. The fragment size of the PCR products generated by primer OPB-17 (Fig. 1e) ranged from approximately 255 to 1700 bp. A band of size 440 bp was observed in RK12 isolate but was absent from all isolates, therefore it was positive unique RAPD marker.

RR11 isolate was distinguishable by 4 positive unique RAPD markers while, RB2A and RK12 isolates were identified by one positive unique RAPD markers.

**Clusters analysis of isolates based on lysogen and sensitivity to antibiotics:**

Lysogen and sensitivity to antibiotics (Table ) were subjected to hierarchical Euclidean cluster analysis to determine the genetic divergence among the isolates. Considering the genetic distance among studied isolates (Table 3), the maximum distance (16.310) was recorded between RR11 and RF31A.The minimum Euclidean distance of (0.000) was observed among RF31A, RZ11, RA21, RH31, RB2 and RK12 isolates. Based on the extent of relative dissimilarity among isolates, the 10 isolates were grouped into two clusters ( Fig. 2). Cluster I consisted of all isolates except for RR11 isolate which formed cluster II.

**Phylogenetic Relationship among isolates based on amplified RAPD fragments (bands):**

The similarity coefficient values among isolates based on band polymorphisms generated by RAPD-PCR after using all primers are presented in Table (4). The similarity coefficient value (1.000) was observed among RF31, RF31A, RS3, RZ11, RA21, RH31and RB2 isolates (group A). The similarity coefficient value between RR11 and (group A) was 0.919 and it was 0.973 between (group A) and both of RB2A and RK12 isolates. The similarity coefficient value between RR11 and RB2A was 0.946 while, it was 0.892 between RR11 and RK12.

The dendrogram of genetic distances among isolates based on band polymorphisms generated by RAPD-PCR after using all primers is shown in Figure (3). This dendrogram clustered the 10 isolates into two main clusters, where RF31, RF31A, RS3, RZ11, RA21, RH31and RB2 isolates (group A) constituted one cluster correlated with RB2A and RK12 while, RR11 isolate formed the second cluster. In this regard, different authors reported the usefulness of RAPD-PCR technique for the identification of *Rhizobium leguminosarum bv. viciae* ( Corich *et al*., 2001, Rodriguez-Echeverria *et al*., 2004, Zurdo-Piñeiro *et a*l., 2004 and Moschetti *et al*. 2005).

**Table 2 : Lysogenesity ability.**

| Phage / Host | RK 11 | RK 12 | RK 13 | RK 21 | RK 23 | RA 11 | RA 21 | RF 12 | RF 13 | RF 31 | RF 32 | RZ 11 | RZ 22 | RR 11 | RR 13 | RR 23 | RR 31 | RH 11 | RH 21 | RH 32 | RB 1 | RB 2 | RB 3 | RS 3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **RK11** | + | + | - | + | + | + | - | - | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **RK12** | + | + | - | + | + | + | - | + | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **RK13** | + | + | - | + | + | + | - | - | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **RK21** | + | + | - | + | + | + | - | - | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **RK32** | + | + | - | + | + | + | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **RA11** | + | + | - | + | + | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **RA21** | + | + | - | + | + | + | - | - | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **RF12** | + | + | - | + | + | + | - | - | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **RF13** | + | + | - | + | + | + | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **RF31** | + | + | - | + | + | + | - | - | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **RF32** | + | + | - | + | + | + | - | - | - | + | - | + | + | + | - | + | + | + | + | + | + | + | + | + |
| **RZ11** | + | + | - | + | + | + | - | - | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **RZ22** | + | + | - | + | + | + | - | - | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **RR11** | + | + | - | + | + | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **RR13** | + | + | - | + | + | + | - | - | - | + | - | + | + | + | - | + | + | + | + | + | + | + | + | + |
| **RR23** | + | + | - | + | + | + | - | - | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **RR31** | + | + | - | + | + | + | - | - | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **RH11** | + | + | - | + | + | + | - | - | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **RH21** | + | + | - | + | + | + | - | - | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **RH31** | + | + | - | + | + | + | - | - | - | + | - | + | + | + | - | + | + | + | + | + | + | + | + | + |
| **RB1** | + | + | - | + | + | + | - | - | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **RB2** | + | + | - | + | + | + | + | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **RB3** | + | + | - | + | + | + | + | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **RS3** | + | + | - | + | + | + | - | - | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + |

+ = Lysis or lysogen.

- = No lysis or non lysogen.

**Table 3: Sensitivity to antibiotics.**

| Conc µg /ml / Strain | Str | | | | | Amp | | | | | Chl | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 100 | 500 | 1000 | 15000 | 2000 | 100 | 500 | 1000 | 15000 | 2000 | 100 | 500 | 1000 | 15000 | 2000 |
| RK11 | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| RK12 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| RK21 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| RK23 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| RK33 | - | - | - | - | - | + | - | - | - | - | + | - | - | - | - |
| RA11 | - | - | - | - | - | + | + | + | + | - | + | + | - | - | - |
| RA21 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| RF12 | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| RF13 | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - |
| RF31 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| RF32 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| RZ11 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| RZ22 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| RR11 | - | - | - | - | - | + | + | + | - | - | + | + | - | - | - |
| RR13 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| RR23 | - | - | - | - | - | + | + | + | + | + | + | + | + | + | + |
| RR31 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| RH11 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| RH21 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| RH31 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| RB1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| RB2 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| RB3 | + | - | - | - | - | - | - | - | - | - | + | - | - | - | - |
| RS3 | + | - | - | - | - | - | - | - | - | - | + | - | - | - | - |

+= Resistance

-= Sensitive

**Table 4: Host range of phage K 23.**

| Ø K23 / Host | Spot Test | Titration |
|---|---|---|
| RK11 | + | $2.36x10^{9}$ |
| RK12 | + | $6.0x10^{13}$ |
| RK13 | + | $1.69x10^{7}$ |
| RK21 | + | $7.0x10^{8}$ |
| RK23 | + | $1.90x10^{7}$ |
| RK33 | + | $2.17x10^{7}$ |
| RA11 | - | - |
| RF12 | + | $3.3x10^{8}$ |
| RZ11 | + | $4.0x10^{8}$ |
| RR11 | + | $5.7x10^{9}$ |
| RH11 | + | $2.9x10^{9}$ |

+ = Lysis

- = No Lysis

**Table 5: Host range of phage A32.**

| Ø A32 / Host | Spot Test | Titration |
|---|---|---|
| RA11 | + | $3.6x10^{13}$ |
| RA21 | + | $2.2x10^{6}$ |
| RK11 | + | $3.2x10^{8}$ |
| RF12 | + | $4.5x10^{8}$ |
| RZ11 | + | $3.9x10^{11}$ |
| RR11 | + | $4.8x10^{9}$ |
| RH11 | + | $3.2x10^{9}$ |

**Table 6: Host range of phage F13.**

| Ø F13 / Host | Spot Test | Titration |
|---|---|---|
| RF12 | - | - |
| RF13 | - | - |
| RF31 | + | $9.54x10^{9}$ |
| RF32 | + | $1.1x10^{9}$ |
| RK11 | + | $8.63x10^{9}$ |
| RA11 | + | $1.98x10^{9}$ |
| RZ11 | + | $5.30x10^{11}$ |
| RR11 | + | $1.8x10^{11}$ |
| RH11 | + | $2.50x10^{9}$ |

**Table 7: Host range of phage R11.**

| Ø R11 / Host | Spot Test | Titration |
|---|---|---|
| RR11 | + | $2.36x10^{9}$ |
| RR13 | + | $1.9x10^{9}$ |
| RR22 | + | $9.0x10^{11}$ |
| RR23 | + | $2.13x10^{7}$ |
| RR31 | + | $4.16x10^{8}$ |
| RK11 | + | $2.3x10^{8}$ |
| RA11 | + | $1.38x10^{11}$ |
| RF12 | + | $5.43x10^{7}$ |
| RZ11 | + | $2.15x10^{9}$ |
| RH11 | + | $3.5x10^{11}$ |

**Table 8: Host range of phage H21.**

| Ø H21 / Host | Spot Test | Titration |
|---|---|---|
| RH11 | + | $9.1x10^{9}$ |
| RH21 | + | $3.53x10^{12}$ |
| RH32 | + | $9.81x10^{9}$ |
| RK11 | + | $6.2x10^{8}$ |
| RA11 | + | $7.0x10^{9}$ |
| RF12 | + | $8.9x10^{9}$ |
| RZ11 | + | $9.67x10^{13}$ |
| RR11 | + | $2.11x10^{9}$ |

**Table 9: Transducing streptomycin resistance gene.**

| Recipient | Recipient (Cfu /ml) | Number of transductants | | | | | Transduction frequency | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Ø K23 | Ø A32 | Ø F13 | ØR11 | ØH21 | Ø K23 | Ø A32 | Ø F13 | ØR11 | ØH21 |
| **RK11** | $9.6x10^{9}$ | $1.79x10^{5}$ | $2.1x10^{5}$ | $1.87x10^{5}$ | $2.33x10^{5}$ | $1.68x10^{5}$ | $1.7x10^{-5}$ | $2.2x10^{-5}$ | $1.9x10^{-5}$ | $2.4x10^{-5}$ | $1.7x10^{-5}$ |
| **RF12** | $8.2x10^{9}$ | $2.35x10^{5}$ | $3.7x10^{5}$ | $2.07x10^{5}$ | $2.31x10^{5}$ | $2.4x10^{5}$ | $2.9x10^{-5}$ | $4.5x10^{-5}$ | $2.5x10^{-5}$ | $2.8x10^{-5}$ | $2.9x10^{-5}$ |
| **RH11** | $5.1\ x10^{9}$ | $1.3x10^{3}$ | $7.0x10^{2}$ | $1.1x10^{3}$ | $6x10^{1}$ | $2x10^{1}$ | $2.5x10^{-7}$ | $1.4x10^{-7}$ | $2.2x10^{-7}$ | $1.2x10^{-8}$ | $0.39x10^{-8}$ |

**Table 10: Transducing chloramphenicol resistance gene.**

| Recipient | Recipient (Cfu /ml) | Number of transductants | | | | | Transduction frequency | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Ø K23 | Ø A32 | Ø F13 | ØR11 | ØH21 | Ø K23 | Ø A32 | Ø F13 | ØR11 | ØH21 |
| **RK11** | $7.3x10^{9}$ | $1.7x10^{6}$ | $1.92x10^{6}$ | $1.78x10^{6}$ | $1.51x10^{6}$ | $9.8x10^{6}$ | $1.5x10^{-4}$ | $2.6x10^{-4}$ | $2.4x10^{-4}$ | $2.1x10^{-4}$ | $1.3x10^{-3}$ |
| **RF12** | $6.8x10^{9}$ | $6.14x10^{3}$ | $1.12x10^{6}$ | $1.51x10^{6}$ | $1.39x10^{5}$ | $1.23x10^{6}$ | $9.0x10^{-7}$ | $1.6x10^{-4}$ | $2.2x10^{-4}$ | $2.0x10^{-5}$ | $1.8x10^{-4}$ |
| **RH11** | $2.4\ x10^{9}$ | $5.6x10^{2}$ | $2.3x10^{2}$ | $2x10^{3}$ | $3x10^{1}$ | $3.2x10^{4}$ | $2.3x10^{-7}$ | $9.5x10^{-8}$ | $8.3x10^{-7}$ | $1.25x10^{-8}$ | $1.3x10^{-5}$ |

**Table 11: Cotransduction of streptomycin and chloramphenicol resistance genes.**

| Recipient | Recipient (Cfu /ml) | Number of transductants | | | | | Transduction frequency | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Ø K23 | Ø A32 | Ø F13 | ØR11 | ØH21 | Ø K23 | Ø A32 | Ø F13 | ØR11 | ØH21 |
| **RK11** | $2.11x10^{9}$ | $1.13x10^{5}$ | $1.14x10^{5}$ | $1.49x10^{6}$ | $1.88x10^{6}$ | $2.11x10^{6}$ | $5.3x10^{-5}$ | $5.4x10^{-5}$ | $7.1x10^{-4}$ | $8.9x10^{-4}$ | $1x10^{-3}$ |
| **RF12** | $2.31x10^{9}$ | $1.16x10^{5}$ | $6.87x10^{6}$ | $2.23x10^{6}$ | $5.09x10^{6}$ | $2.29x10^{6}$ | $5.0x10^{-5}$ | $2.97x10^{-3}$ | $9.6x10^{-4}$ | $2.2x10^{-3}$ | $9.9x10^{-4}$ |

**Table 12: Transducing ampicillin resistance gene.**

| Recipient | Recipient (Cfu /ml) | Number of transductants | | | | | Transduction frequency | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Ø K23 | Ø A32 | Ø F13 | ØR11 | ØH21 | Ø K23 | Ø A32 | Ø F13 | ØR11 | ØH21 |
| **RK11** | $2.11x10^{9}$ | $2x10^{1}$ | - | - | $1x10^{1}$ | $2x10^{2}$ | $9.4x10^{-9}$ | - | - | $4.7x10^{-8}$ | $9.4x10^{-8}$ |
| **RF12** | $2.31x10^{9}$ | $1x10^{1}$ | - | - | $2x10^{1}$ | $1x10^{1}$ | $4.3x10^{-9}$ | - | - | $8.6x10^{-9}$ | $4.3x10^{-9}$ |
| **RH11** | $9.24x10^{9}$ | - | - | - | - | - | - | - | - | - | - |

**Table 2 : Number of amplicons and the number of polymorphic bands produced by each RAPD primer for ever *Rhizobium leguminosarum bv. viciae* isolates.**

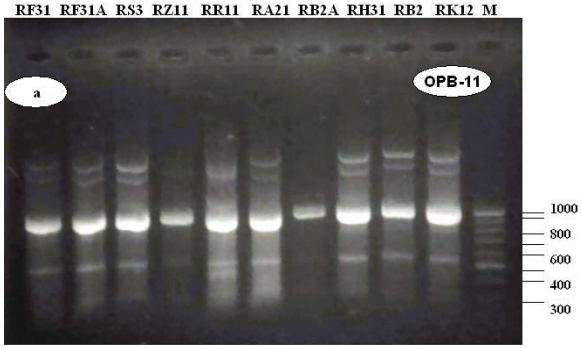
| Primers | RF31 | RF31A | RS3 | RZ11 | RR11 | RA21 | RB2A | RH31 | RB2 | RK12 | Total No. of amplicons | Polymorphic amplicons | Polymorphism % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **OPB-11** | 6 | 6 | 6 | 6 | 7 | 6 | 6 | 6 | 6 | 6 | 7 | 1 | 14.3 |
| **OPD-03** | 6 | 6 | 6 | 6 | 7 | 6 | 6 | 6 | 6 | 6 | 7 | 1 | 14.3 |
| **OPC-08** | 7 | 7 | 7 | 7 | 8 | 7 | 8 | 7 | 7 | 7 | 8 | 1 | 12.5 |
| **OPA-11** | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 0 | 0 |
| **OPB-17** | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 9 | 9 | 1 | 11.1 |
| **Total** | 33 | 33 | 33 | 33 | 36 | 33 | 34 | 33 | 33 | 34 | 37 | 4 | 10.8 |
| **Average** | 6.6 | 6.6 | 6.6 | 6.6 | 7.2 | 6.6 | 6.8 | 6.6 | 6.6 | 6.8 | 7.4 | 0.8 | |

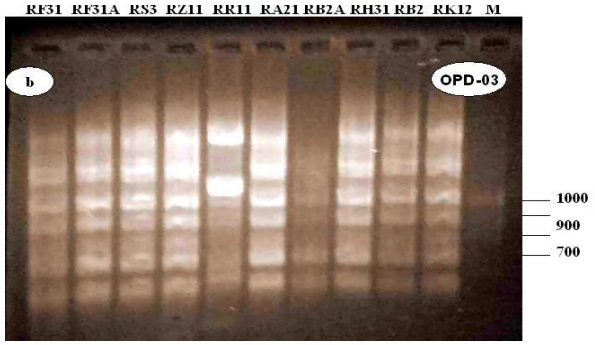
**Table 3: Euclidean genetic distances among studied isolates based on lysogen and Sensitivity to antibiotics.**

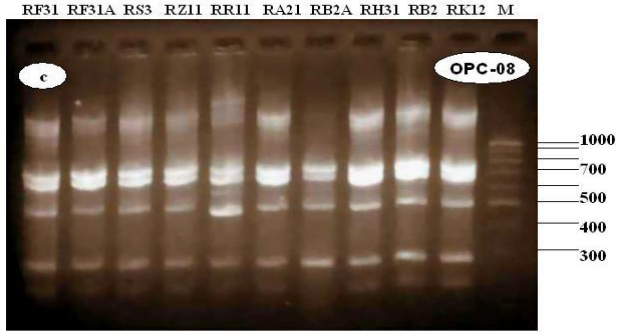
| | RF31A | RS3 | RZ11 | RR11 | RA21 | RB2A | RH31 | RB2 | RK12 |
|---|---|---|---|---|---|---|---|---|---|
| **RF31** | 4.000 | 1.000 | .000 | 15.811 | .000 | 4.000 | .000 | .000 | .000 |
| **RF31A** | | 4.123 | 4.000 | 16.310 | 4.000 | .000 | 4.000 | 4.000 | 4.000 |
| **RS3** | | | 1.000 | 15.524 | 1.000 | 4.123 | 1.000 | 1.000 | 1.000 |
| **RZ11** | | | | 15.811 | .000 | 4.000 | .000 | .000 | .000 |
| **RR11** | | | | | 15.811 | 16.310 | 15.811 | 15.811 | 15.811 |
| **RA21** | | | | | | 4.000 | .000 | .000 | .000 |
| **RB2A** | | | | | | | 4.000 | 4.000 | 4.000 |
| **RH31** | | | | | | | | .000 | .000 |
| **RB2** | | | | | | | | | .000 |

**Table 4: The similarity coefficient among *Rhizobium leguminosarum bv. vcieae* bacterial isolates based on combined analysis of amplified RAPD fragments after using all primers.**

| | RF31A | RS3 | RZ11 | RR11 | RA21 | RB2A | RH31 | RB2 | RK12 |
|---|---|---|---|---|---|---|---|---|---|
| **RF31** | 1.000 | 1.000 | 1.000 | .919 | 1.000 | .973 | 1.000 | 1.000 | .973 |
| **RF31A** | | 1.000 | 1.000 | .919 | 1.000 | .973 | 1.000 | 1.000 | .973 |
| **RS3** | | | 1.000 | .919 | 1.000 | .973 | 1.000 | 1.000 | .973 |
| **RZ11** | | | | .919 | 1.000 | .973 | 1.000 | 1.000 | .973 |
| **RR11** | | | | | .919 | .946 | .919 | .919 | .892 |
| **RA21** | | | | | | .973 | 1.000 | 1.000 | .973 |
| **RB2A** | | | | | | | .973 | .973 | .946 |
| **RH31** | | | | | | | | 1.000 | .973 |
| **RB2** | | | | | | | | | .973 |


RF31 RF31A RS3 RZ11 RR11 RA21 RB2A RH31 RB2 RK12 M
a
OPB-11
1000
800
600
400
300


RF31 RF31A RS3 RZ11 RR11 RA21 RB2A RH31 RB2 RK12 M
b
OPD-03
1000
900
700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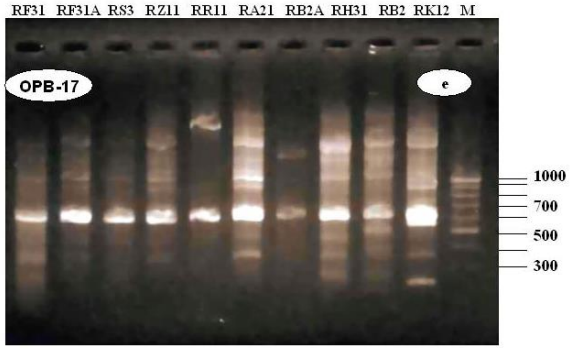
c
OPC-08
1000
700
500
400
300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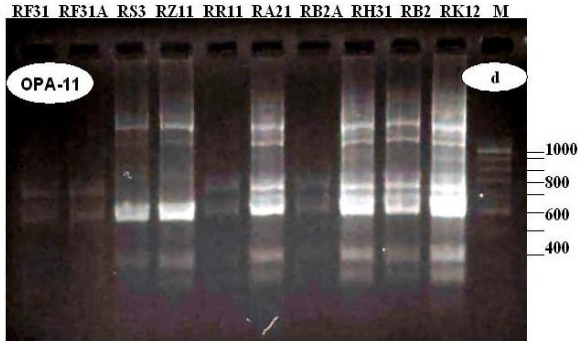
OPB-17
e
1000
700
500
300


RF31 RF31A RS3 RZ11 RR11 RA21 RB2A RH31 RB2 RK12 M
OPA-11
d
1000
800
600
400

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## التوصيف الوراثي والجزيئي لبعض عزلات *Rhizobium leguminosarum bv. vicieae*.

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استخدمت بكتريا الريزوبيوم (*Rhizobium leguminosarum bv. vicieae*) التي تم عزلها وتوصيفها في قسم الميكروبيولوجيا الزراعة- كلية الزراعة- جامعة الزقازيق لدراسة التنوع الوراثي والجزيئي.

تم استخدام ٢٤ عزلة من هذه البكتريا معزولة من أماكن مختلفة من محافظة الشرقية, تم اختبار lysogenicity ability لهذه العزلات وقد أوضحت النتائج أن ٢٣ عزلة من ٢٤ كانت ليسوجينية أى أن نسبة lysogenicity ٩٦%, وجد أن ١٩ عزلة من العزلات الليسوجينية كانت ليسوجينية بأكثر من بروفاج وذلك لأن الفاج المنطلق من كل عزلة استطاع أن يحلل نفس العزلة, العزلات RR13, RF13, RF12, RA21 كانت تحتوى على بروفاج واحد, وذلك لأن القاج المنطلق منها لم يستطيع أن يحلل نفس العزلة.

تم أختبار حساسية هذه العزلات لبعض المضادات الحيوية (استربتوميسين, أمبسلين, كلورامفينيكول بتركيزات من ١٠٠- ٢٠٠٠ ميكرو جرام/ ملل.وقد وجد أن معظم العزلات كانت حساسة إلا أن بعض العزلات كانت مقاومة.العزلة RR23 كانت مقاومة للأمبسلين والكلورامفينيكول على كل التركيزات المستخدمة, العزلات RA11, RR11 كانت مقاومة للأمبسلين حتى تركيز ١٥٠٠ ميكرو جرام/ ملل.والكلورامفينيكول حتى تركيز ٥٠٠ ميكروجرام/ ملل العزلات Rk11, RF12 كانت مقاومة للاستربتوميسين حتى تركيز ٥٠٠ ميكرو جرام/ ملل.

تم أيضاً استخدام خمس ريزوبيوفاج معزولة من تربة لنفس الأماكن التى عزلت منها البكتريا. تم دراسة المدى العوائلى لهذه القاجات بإستخدام العزلات البكتيرية كعوائل, كل فاج تم اختباره مع كل العزلات التى من منطقته مع عزلة واحدة من كل منطقة مختلفة.

الفاجات الثلاثة H21, R11, A32 استطاعت أن تحلل كل العوائل المستخدمة معها (٧, ١٠, ٨ على التوالى), الفاج K32 استطاع أن يحلل ١٠ من ١١ عائل, اما فاج F13 حلل ٧ من ١٠ عوائل. تم تقدير عدد الـ pfu/ ml لهذه الفاجات ووجد أنها تتراوح بين ٢,٢× ١٠^٦ إلى ٩,٦٧× ١٠^١٣. قدرة هذه الريزوبيوفاج على نقل بعض جينات المقاومة للمضادات الحيوية تم تقييمها.وجد أن الخمس فاجات استطاعت بنجاح نقل جينات المقاومة للأستربتوميسين والكلورامفينيكول وكان معدل النقل يتراوح بين ٠,٣٩ ×١٠^-٨ إلى ٤,٥ ×١٠^-٥ للأستربتويسين, ١,٢٥ ×١٠^-٨ إلى ١,٣ ×١٠^-٣ للكلورامفينيكول. إلا أنه لم تنجح كل الفاجات فى نقل جين المقاومة للأمبسلين, كما أن نقل هذا الجين لم ينجح مع كل العزلات المستقبلة إلا أن معدل النقل لهذا الجين كان من ٤.٣ ×١٠^-٩ إلى ٤.٩ ×١٠^-٨ استطاعت أيضاً الخمس فاجات أن تنجح فى النقل المصاحب cotransduction لجينات المقاومة للاستربتوميسين والكلورامفينيكول معاً بمعدل تراوح بين ٥ ×١٠^-٥ إلى ٢,٩٧ ×١٠^-٣.

أجريت تحليلات ال RAPD-PCR على ال DNA الجينومى المستخلص من عشرة عزلات من بكتريا ال *Rhizobium leguminosarum bv. viciae* وقد أعطت خمسة من البوادئ المستخدمة فى هذه الدراسة تعدد صور فى طرز حزم واسمات الرابيد بين هذه العزلات وقد أعطى كل بادئ عدد مختلف من الحزم. وقد تم تعيين التماثل الوراثى بين هذه العزلات على أساس العدد الكلى للفروق فى حزم الرابيد. وكانت قيمة التماثل الوراثى هى الواحد الصحيح بين عزلات البكتريا RF31 وRF31A و RS3 و RZ11 و RA21 و RH31 و RB2 (مجموعة أ) وكانت 0.919 بينRR11 و (مجموعة أ) وكانت 0.973 بين (مجموعة أ) وكلا من RB2A و RK12 وكانت 0.946 بين كلا من RR11 و RB2A بينما كانت 0.892 بين كلا من RR11 و RK12 . وقد قسم دندوجرام المسافات الوراثية بين العزلات على أساس تعدد صور حزم واسمات الرابيد بعد استخدام البوادئ العزلات العشرة الى مجموعتين رئيسيتين حيث أن المجموعة الأولى ضمت كلا من RF31 و RF31A و RS3 و RZ11 و RA21 و RH31 و RB2 (مجموعة أ) وهذه مرتبطة مع العزلتين RB2A و RK12 بينما مثلت العزلة RR11 فقط المجموعة الثانية. وقد تميزت

العزلةRR11 بأربعة واسمات رابيد موجبة بينما تميزت كلا من العزلتين RB2A و RK12 بواسم رابيد واحد موجب.