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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., Fac. Agric., Zagazig Univ. were used in this investigation to study molecular and genetic diverisity.24 isolates were used to investigate their ability of lysogen. The results showed that 23 isolates from 24 were lysogen with 96% of lysogenecity. 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 with concentrations used. The RA11 and RR11 isolates were resistance 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 that isolated from soil were used in this study. The host range of these phages was studied using the bacterial isolates as hosts. The phages A32, R11 and H21 were able to lysis all 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 2.2×10^{6} to 9.67×10^{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 0.39×10^{-8} to 4.5×10^{-5} for streptomycin and from 1.25×10^{-8} to 1.3×10^{-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, and the transduction frequency ranged from 4.3×10^{-9} to 9.4×10^{-8}. The five phages were able to cotransduce streptomycin and chloramphenicol resistance gene together, transduction frequency ranged from 5.0×10^{-5} to 2.97×10^{-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, was 0.892 between RR11 and RK12.The dendogram 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.
keyword: *Rhizobium*, transduction, bacteriophage, RAPD – PCR, dendogram.

INTRODUCTION

In view of the numerous possible application of genetically engineered microorganisms(GEMs) in the environment (Tiedje *et al.,* 1989 Trevors *et* al., 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 of gene probing and on DNA amplification using polymerase chain reaction (Trevors *et al.,* 1989, Degrange and Bardin, 1995).

However, less is 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).

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 with several locations on the two strands of the DNA molecule. 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).

The aim of this study was to investigate the genetic characterization of some *Rhizobium* isolates which have been isolated from different locations in Sharkia Governorate. The genetic diversity among these isolates has also been studied using RAPD-PCR analysis.

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 locations in Sharkia Governorate and fully characterized 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 (salem *et al.*, 2006).

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

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 water bath. 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 bacterial cells.

4- Sensitivity to antibiotics:

In this experiment, three antibiotics 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 a 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 .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 profile and the produced polymorphic patterns of DNA fingerprinting ,five different primers were selected(Table 2).

Table 2: 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 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 molecular 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. Pair wise 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 relationship using the un weighted pair-group method of analysis (UPGMA) (Sneath & Sokal1973).

RESULTS ANSD DISCUSSION

1-Lysogenicity ability:

Data in Table 3 represent the ability of lysogenicity for 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 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 4. 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 high concentrations of four antibiotics, streptomycin, kanamycin, rifampicin and erythromycin. The resistance to antibiotics may affect the symbiotic nitrogen fixation process. Symbiotic 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, Schroder (1980) suggested that the resistance of *R.Japonicum* to a number of antibiotics was mediated to be plasmid-born genes.

Table 3 : Lysogenecity 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 4: Sensitivity to antibiotics.

Conc µg /ml / Strain	Str 100	Str 500	Str 1000	Str 15000	Str 2000	Amp 100	Amp 500	Amp 1000	Amp 15000	Amp 2000	Chl 100	Chl 500	Chl 1000	Chl 15000	Chl 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

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 as well as different locations. The phages A32, R11 and H21 were able to lysis all 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 hosts that used in spot test. The pfu/m1 were varied depended on phage and host type (Tables 5, 6, 7, 8 and 9).

Table 5: 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 6: 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 7: 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 8: 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 9: 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	+	$2.11x10^{9}$

4.Transduction by Phage Lysates Isolated from Soil:

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 10).

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 11).

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

Five phages were able to transduce streptomycin and chloramphenicol resistance genes together from donor RF12 A8 to recipients RK11 and RF12 (Table 12).

4.4. Transducing ampicillin resistance gene:

Not all the five phages were able to transudce this marker gene. Three phages (K23 , R11 and H21)were able to transduce it for two recipients (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 13). Transduction assay of *R. meliloti* and *R. leguminosarum* have been established 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).

5.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 14. 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.

Table 10: 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 11: 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 12: 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 13: 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	$9.24x10^{9}$	-	-	-	-	-	-	-	-	-	-

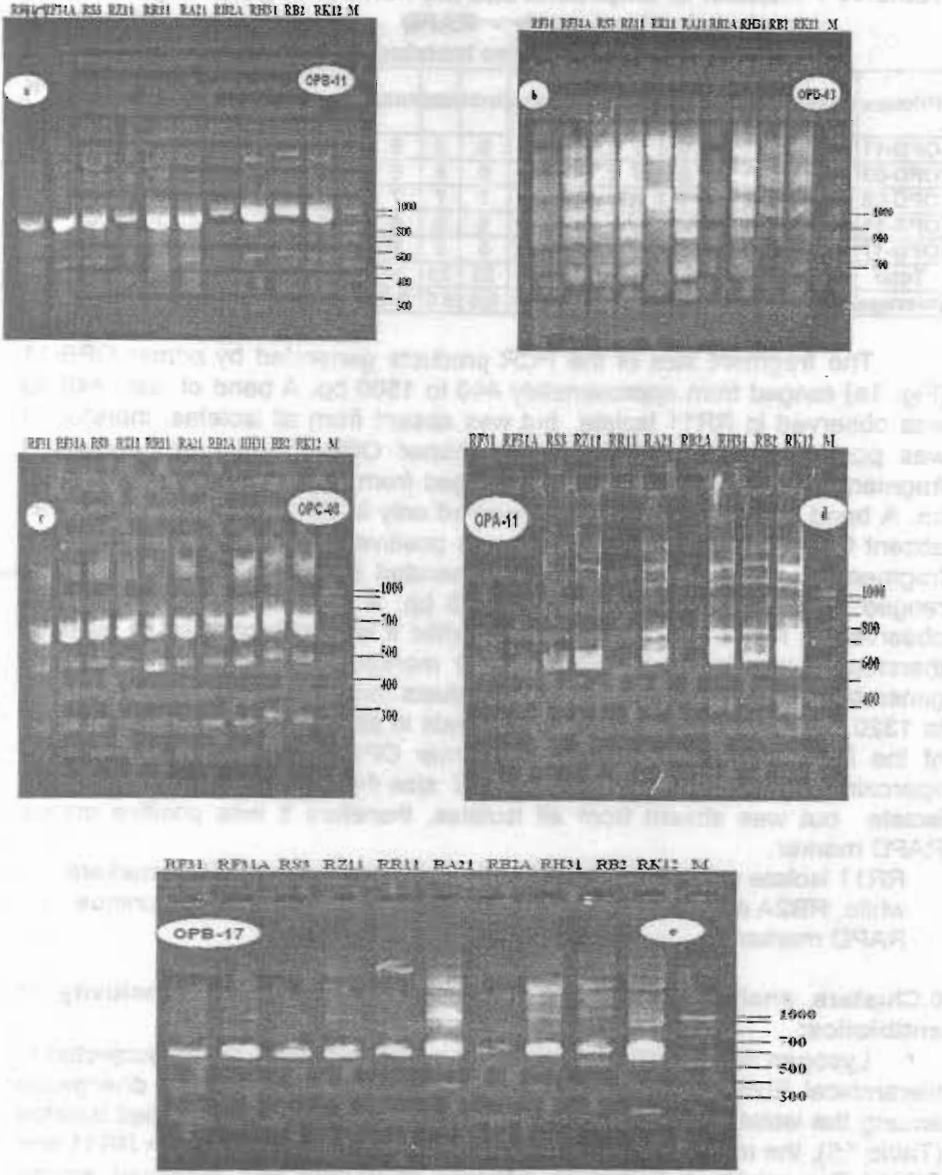

Figure 1: Amplified band profiles generated with primers OPB-11, OPD-03, OPC-08, OPA-11 and OPB-17.

Table 14 : Number of amplicons and the number of polymorphic bands produced by each RAPD primer for *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	

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 distinguished by four positive unique RAPD markers while, RB2A and RK12 isolates were identified by one positive unique RAPD markers.

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

Lysogen and sensitivity to antibiotics (Table 3 and 4) were subjected to hierarchical Euclidean cluster analysis to determine the genetic divergence among the isolates. Considering the genetic distance among studied isolates (Table 15), 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.

Table 15: 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

7.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 16. 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 dendogram of genetic distances among isolates based on band polymorphisms generated by RAPD-PCR after using all primers is shown in Figure 3. This dendogram 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 al.*, 2004 and Moschetti *et al.* 2005).

Table 16: 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

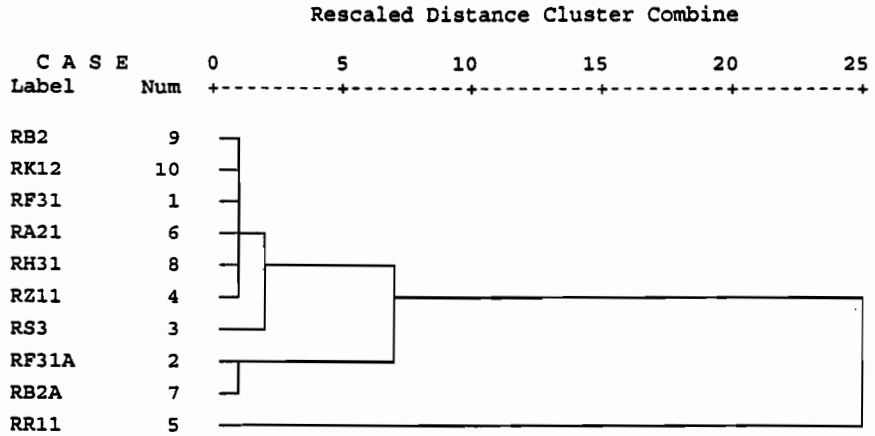

Fig. 2: Linkage dendogram of studied isolates based on lysogen and sensitivity to antibiotics.

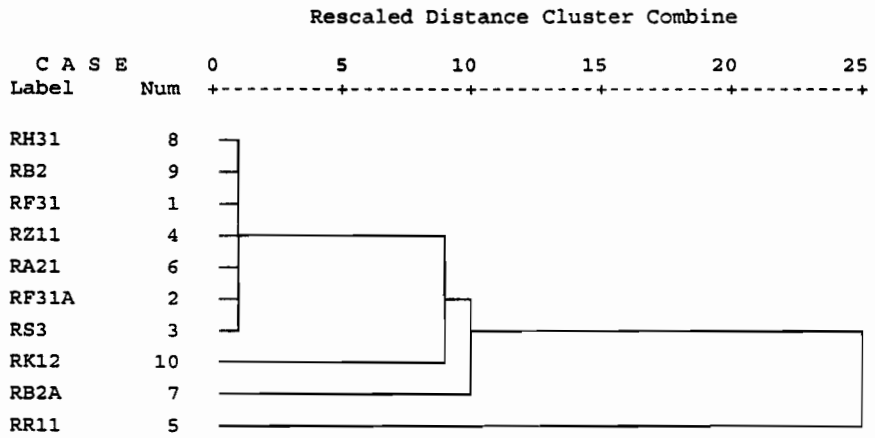

Fig. 3 : Linkage dendogram for *Rhizobium leguminosarum bv. vicieae* bacterial isolates based on combined analysis of amplified RAPD fragments after using all primers.

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

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

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 أيضا 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 الثلاثةH21، 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 ٩,٤ ×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 RZ11و 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 RB2Aبينما 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.