ISOLATION OF SPONTANEOUS BACTERIOPHAGE-RESISTANT MUTANTS OF SYMBIOTIC NITROGEN FIXING BACTERIA

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ABSTRACT

Bacteriophages specific for *Bradyrhizobium japonicum* and *Bradyrhizobium* spp. (arachis) were isolated from soils of the Experimental Farm of Faculty of Agriculture, Minia University. A spontaneous bacteriophage-resistant mutant of *Bradyrhizobium japonicum* and another one of *Bradyrhizobium* spp.(arachis) were successfully isolated.

Proteins of the wild types and their isolated mutants were electrophoretically analyzed. In case of *Bradyrhizobium japonicum*, seven polypeptides of 66, 60, 51, 47, 43, 29 and 24 kilodalton (KD) were found in the polypeptides pattern of the wild type and were undetectable in the isolated mutant. However, in case of *Bradyrhizobium* spp. (arachis) three bands of 66, 51, and 43 KD, were observed in the polypeptides pattern of the wild type and were absent in the isolated mutant. Moreover, one polypeptide of 39 KD was found in the two mutants of both strains (*Bradyrhizobium japonicum* and *Bradyrhizobium* spp.) and was not found in their wild types.

Presence of bacteriophages did not affect the efficiency of the isolated mutants of *Bradyrhizobium japonicum* and *Bradyrhizobium* spp. (arachis) in nodulating their host plants and in fixing nitrogen. On the other hand, in presence of bacteriophages values of number and dry weight of nodules/plant, plant dry weight and nitrogen contents of plants markedly decreased in soybean and groundnut inoculated with the wild types of their specific root nodule bacteria.

KEY WARDS: Bacteriophage, Mutants, *Bradyrhizobium* spp., *Bradyrhizobium japonicum*, Nitrogen fixation, Root nodules , Soybean, Groundnut.

INTRODUCTION

The use of root nodule bacteria as inocula for leguminous plants is of a great agricultural importance since they can be used as alternatives for nitrogenous-chemical fertilizers and hence the production costs of these plants and the environmental pollution can be reduced (Abdel-Ati *et al.* 1996). It is well known that successful nodulation is sufficient for supplying grown leguminous plants with their nitrogen requirements during the different growth stages (Goldsworthy and Heathoote, 1963). Abdel-Latif *et al.* (1992) reported that, in soil recently reclaimed , the effect of inoculation of ground nut with efficient strain of root nodule bacteria on yield was equivalent to the application of about 600 kg. $NaNO_3$ /ha . Therefore, knowledge of factors influencing the efficiency and maintenance of such desired bacteria in the soil is of particular interest. Bacteriophages of nodule bacteria are likely to have a significant role in the ecology of their economically important hosts. These bacteriophages are commonly found in soils especially when legumes are grown regularly. (Kowalski *et al.* 1974, Hammad 1993 , Hammad and Ali 1999).

This study aimed to isolate spontaneous mutants of *Bradyrhizobium japonicum* and *Bradyrhizobium spp.* (arachis) resistant to their specific bacteriophages. In addition, the ability of phage particles to lyse a bacterial strain depends on presence of certain micro-molecules on the surface of the cells, viz. surface receptors for bacteriophage adsorption. These receptors were found to be composed of protein (Barnet, 1972 and Kay, 1972). This study aimed also to compare protein of the wild types and the isolated phage resistant mutants to detect the changes in proteins of bacterial cells due to the mutation.

MATERIALS AND METHODS

1) Microorganisms:

Bradyrhizobium japonicum and *Bradyrhizobim spp.* (arachis) were obtained from the microbial collection of Dept. Agric. Microbiology, Fac. Agric., Minia Univ., Minia – Egypt.

2) Isolation of bacteriophages:

Four soil samples were collected from the Experimental Farm of Faculty of Agriculture, Minia University to be used as a source of bacteriophages. Liquid enrichment technique described by Barnet (1972) with minor modification, was used to isolate phages. The four collected soil samples were mixed in equal amounts together just before enrichment. To isolate the phages specific for *Bradyrhizobium japonicum* or *Bradyrhizobim spp.* twenty grams of the soil mixture were incubated overnight at 30-33°C with 40 ml of yeast extract mannitol broth "medium 79" (Allen, 1959). Five ml of chloroform were then added and the sample was shaken for10 min, followed by centrifugation at 4000 r.p.m, for 10 min. to remove soil and bacteria. The supernatant was added to 3 ml of 48h, old liquid culture of *Bradyrhizobium japonicum* or *Bradyrhizobium spp.* (arachis). After multiplication of phages (24-30 h. at 30-33°C), bacteria were killed by shaking with 5 ml chloroform for 10 min., then the sample was clarified by centrifugation at 4000 r.p.m. for 10 min. The supernatant (phage lysate) was subjected to phage detection .

Agar double layer plates method (Adams, 1966) was used for detection of phages. Plates were prepared by pouring a base layer of 20 ml of nutrient agar medium with 1.5% agar in Petri dishes 10 cm in diameter. The basal layer was allowed to solidify. A mixture of 3 ml nutrient agar melted medium containing 0.7% agar and 300 µl of the indicator bacteria was poured into each plate. The indicator bacteria were liquid cultures of 48h old of either *Bradyrhizobium japonicum* or *Bradyrhizobium spp.* (arachis). Each phage lysate was spotted with sterile micropipette on the upper layer after it had solidified. Plates were incubated at 30-33°C for 36-48h, and then examined for lysis of bacterial lawn at the sites where drops had been applied.

The lysed clear zones were picked and transferred separately into eppendorf tubes containing 1 ml. of SM medium (Maniatis *et al.*, 1982). Two hundreds µl chloroform were added to each tube, then maintained at 4°C.

3) Isolation of phage-resistant mutants:

The method described by Adams (1966) was used for isolation of spontaneous phage-resistant mutants of *Bradyrhizobium japonicum* or *Bradyrhizobium spp.* (arachis). One ml. of liquid bacterial culture containing

$2x10^8$ cells, was mixed with 1 ml. of phage lysate containing 10^{10} plaque forming unit (pfu.) in an eppendorf tube. The tube was incubated for 5 min. at 30°C, to ensure that all bacteria which can adsorb phages were infected. One hundred µl of the adsorption mixture was placed on the surface of a plate containing nutrient agar medium and spread uniformly with glass rod until all the liquid had been adsorbed by agar. After incubation for 24 –30 h, single colonies appeared. A single colony was picked from this plate, suspended in 1 ml. of nutrient broth and a loopful was streaked on another plate. Two repetitions of this procedure (streaking on agar plates) were carried out to obtain a pure strain of phage-resistant mutant free from contaminating phages.

4) The changes in total protein of the studied microorganisms after mutation:

The total proteins of *Bradyrhizobium japonicum* and *Brayrhizobium spp.* (arachis) as well as their induced mutants were extracted as described by Hames and Rickwood (1985) and fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Standard marker proteins of known molecular weight were run on the same gel.

Electrophoresis was carried out at 20 mA for 5 h. Gels were stained overnight in 100 ml of 0.1% coomassie brilliant blue in 5:5:2 water, methanol, glacial acetic acid and destained in 12.5 % isopropanol, 10 % acetic acid for three changes over a period of 24 h. (Hames and Rickwood, 1985).

5) Preparation of inocula:

a- Bacterial inocula.

The isolated mutants and the wild types of *Bradyrhizobium japonicum* and *Brayrhizobium spp.* (arachis) were grown separately in yeast extract mannitol broth "medium 79" (Allen, 1959) for four days at 30°C, (giving 12 to 23 $x10^8$ cell /ml). These liquid cultures were used as bacterial inocula.

b- High titre phage suspension (phage inocula .)

The confluent lysis technique of Maniatis *et al.* (1982) was used to prepare high titre phage suspensions . Titres of the prepared phage suspension were estimated using the method described by Kiraly *et al.* (1970).

6) Evaluation the efficiency of the isolated mutants:

Pots experiments were carried out to evaluate the efficiency of the isolated phage resistant mutants in nodulating their plant hosts and in fixing nitrogen. Fired clay pots containing 4kg soil/pot, were prepared. Pots containing soil were autoclaved at 121°C for 1 h. Six surface sterilized seeds of either soybean or groundnut were sown in each pot. Pots of each plant were subjected to the following inoculation treatments:

1-Inoculation with the wild types 2- Inoculation with the isolated mutants

3- Inoculation with the wild types and phage suspension. 4- Inoculation with the isolated mutants and phage suspension. Four replicates for each treatment were employed. Pots were inoculated twice with 5 ml of the prepared bacterial inocula. The first time was at the 7^{th} day after sowing and 7 days later was the second one. For the inoculation with phages, 10 ml of the high titre phage suspension were added to each pot just before sowing.

Sampling and determinations:

At age of 60 days, 10 plants from each treatment were carefully uprooted. The number and dry weight of nodules/plant and plant dry weight were estimated. The estimated values were the average of the ten plants. Nitrogen content of the plants of each treatment was determined by modified macro-kjeldahl's method (A.0.A.C., 1995).

RESULTS AND DISCUSSION

Bacteriophages of the studied microorganisms:

Widespread occurrence of bacteriophages specific for *Bradyrhizobium japonicum* and *Brayrhizobium spp.* (arachis) were detected in the collected soil samples. Similarly, Hammad (1993) and Hammad and Ali (1999) found a widespread occurrence of phages specific for *Bradyrhizobium japonicum* in the rhizosphere soil of soybean cultivated in the Experimental Farm of Faculty of Agriculture, Minia, Eygpt. In addition, Emam et al. (1983) stated that bacteriophages of root nodule bacteria were found to be common in the Nile Vally Soils cultivated with leguminous plants.

Titre of the prepared phage suspensions

The suspension titres which were estimated according to Kiraly *et al.* (1970) are shown in Table (1). The titre of phage suspensions specific for *Bradyrhizobium japonicum* and *Bradyrhizobium* spp. (arachis) were found to be 4.5×10^{10} and 3.3×10^{11}pfu/ml , respectively. These high concentrations of phages were not surprising, since a single plaque of 2 mm. in diameter may contain between 10^7 and 10^9 recoverable phage particles (Adams, 1966; Gunsalus and Stanier, 1960).

Table (1). Titre of phage suspensions prepared with twenty agar double layer plates showing confluent lysis.

Host strain	Total volume	Estimated titre
Bradyrhizobium japonicum	85 ml	4.5×10^{10}
Bradyrhizobium spp.	90 ml	3.3×10^{11}

1) Phage-resistant mutants:

As shown in Figure (1) the isolated mutants of *Bradyrhizobium japonicum* and *Brayrhizobium spp.* (arachis) exhibited resistance to phages of their wild types, since, no lyses were detected on plates seeded with the mutants and spotted with the isolated phages. On the other hand, lyses of the wild types can be clearly seen.

The changes in proteins of the tested bacteria due to mutation:

Bacterial cell walls were found to have multiple surface receptor sites for phage adsorption . (Kay, 1972). He reported that the cell wall of *E.coli* consists of three layers. The outer layer contains the receptors for phages T2 and T6 and is composed of protein. Therefore, it was of a particular interest to compare proteins of the wild types of *Bradyrhizobium japonicum* and *Bradyrhizobium spp.* (arachis) and their isolated phage resistant mutants by electrophoretical analysis.

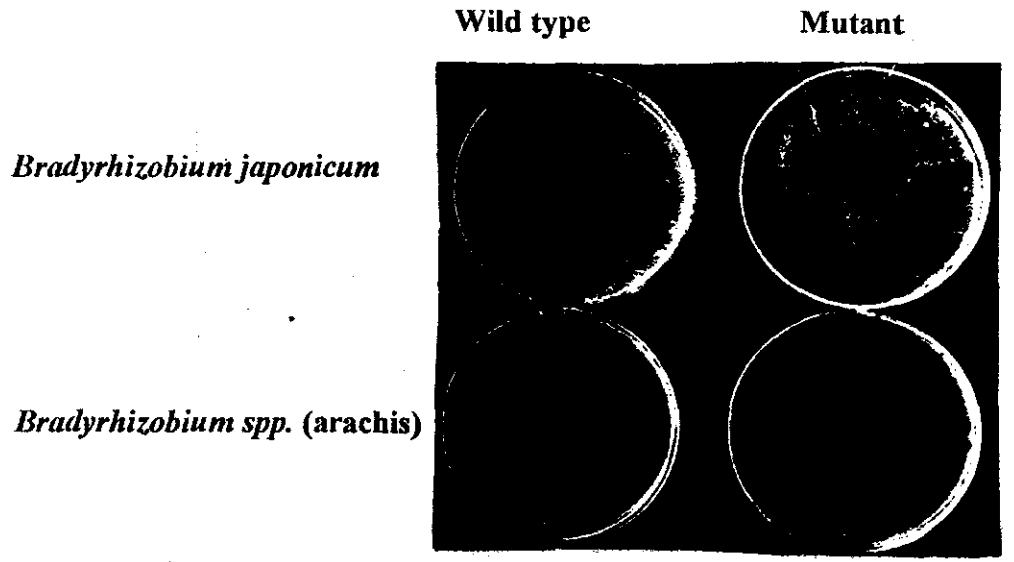

Fig. (1): Bacterial lawns of wild types and phage-resistant mutants of *Bradyrhizobium japonicum* and *Bradyrhizobium spp.* (arachis), spotted with their specific phage lysates. Susceptibility of the wild types and resistance of the isolated mutants can be clearly seen.

As shown in Figure (2) and (3) inspection of the polypeptide banding patterns of the wild type of *Bradyrhizobium japonicum* and *Bradyrhizobium spp.* and their resistant mutants indicate that although most of the detected polypeptides were found to be common in the wild type of each strain and its mutant and migrated with identical mobilities, few differences were apparent. As clearly shown in Fig. (2) there are seven bands having molecular weights of 66, 60, 51, 47, 43, 29, and 24 kilodalton (KD.) could be detected in the polypeptides pattern of the wild type of *Bradyrhizobium japonicum*. These seven polypeptides were absent in the isolated mutant. Moreover, one

polypeptide of 39 KD. was found in polypeptides pattern of the isolated mutant of *Bradyrhizobium japonicum* and was not detectable in the wild type.

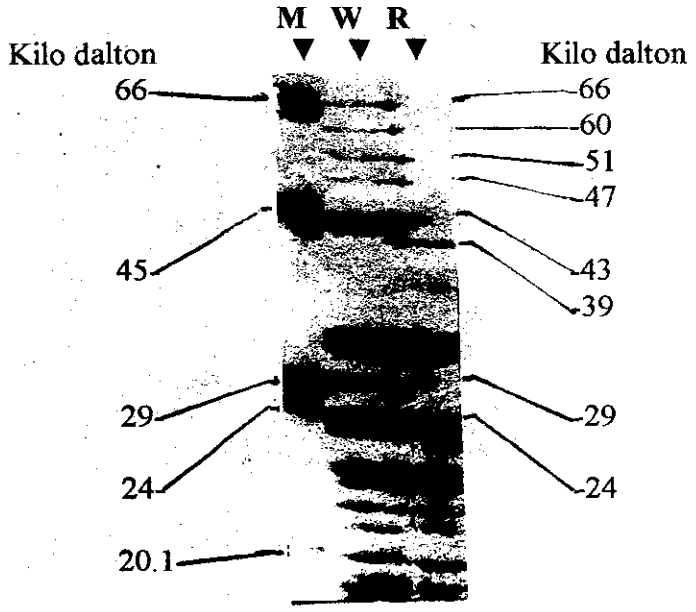

Fig. (2) :Electrophoresis on SDS-polyacrylamide gel of marker proteins (M), total proteins of wild type of *Bradyrhizobium japonicum*) W) and its phage resistant mutant (R).

In addition, as shown in Fig. (3) the electrophoretic analysis of the wild type and mutant-polypeptides of *Bradyrhizobium spp.* (arachis) indicate that three bands of 66, 51 and 43 KD, were detected in the polypeptides pattern of the wild type and were absent in the isolated mutant. However, one band of 39 KD. was observed in the polypeptides banding pattern of the isolated mutant and was not detectable in the wild type. Disappearance of some polypeptides as a result of the spontaneous mutations indicated that these polypeptides may be responsible for the formation of the receptor sites for phage adsorption on the wild types and their absence in the isolated mutants protect the bacterial cells from phage attack. These results are in agreement with those of Hammad and Ali (1999).

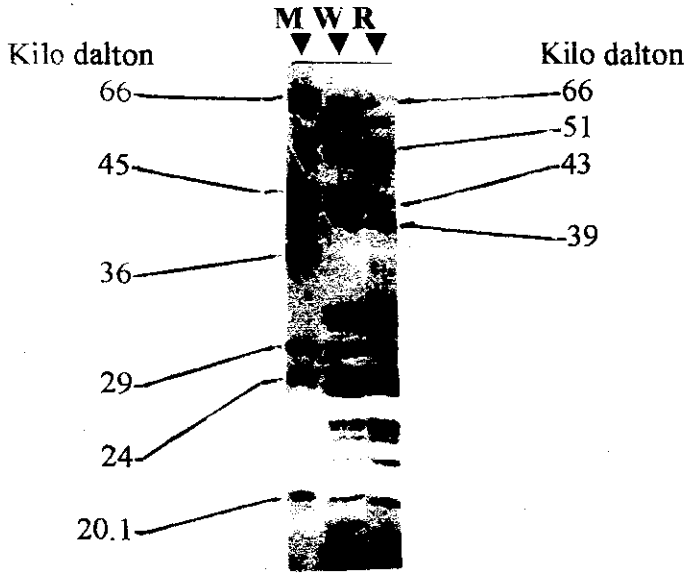

Fig. (3) :Electrophoresis on SDS-polyacrylamide gel of marker proteins (M), total proteins of wild type of *Bradyrhizobium spp*(W) and its phage resistant mutant (R).

Effect of phages on the activities of the wild types and mutants of root nodule bacteria

Data presented in Tables (2) and (3) indicate that in the absence of the bacteriophages; the number and dry weight of the formed nodules/plant as well as plant dry weight and nitrogen contents in either soybean or groundnut, which inoculated with the wild types of their nodule bacteria were similar to those inoculated with the isolated mutants (phage- resistant mutants). This may indicate that the mutation processes did not alter the efficiency of either *Bradyrhizobium japonicum* or *Bradyrhizobium spp.* (arachis) in nodulating their hosts and fixing nitrogen. Similar results were obtained by Hammad and Dora (1998). On the other hand in presence of bacteriophages values of the above

parameters were significantly lower in either soybean or groundnut inoculated with the wild types of their nodule bacteria as compared with those inoculated with the induced mutants. In addition, the recorded values in either soybean or groundnut inoculated with the wild types in the absence of phages were nearly equal to those inoculated with the isolated mutants in presence of phages. Such results may indicate that presence of phages has no effect on the efficiency of the isolated mutants. Similar results were obtained by Abebe *et al.* (1992).

Table (2) :Nodulation, dry weight and nitrogen content of soybean inoculated with the wild type of *Bradyrhizobium japonicum* and its isolated mutants and grown in phage treated soils or untreated ones

Inoculation treatment	No. nodules/ plant	Nodules D.W. (g./plant)	Plant D.W. (g./plant)	N-content (mg./plant)
Wild type	77.3	0.21	20.6	780.0
Mutant	76.8	0.22	21.2	782.1
Wild type+ phage	18.6	0.05	13.7	402.6
Mutant + phage	75.9	0.18	20.1	774.9
L.S.D	3.1	0.02	0.9	13.2

Generally, on the basis of the obtained results it can be concluded that presence of bacteriophages of root nodule bacteria in the soil considered one of the most important environmental factors which affect the efficiency of these bacteria in nodulating their host plants and fixing nitrogen.

Therefore, induction and /or isolation of phage-resistant mutants of such desired bacteria to be used as inocula is highly recommended to avoid the phage attack and to promote maintenance and efficiency of these microorganisms.

Table (3) :Nodulation, dry weight and nitrogen content of groundnut inoculated with the wild type of *Bradyrhizobium* spp. (arachis) and its induced mutants and grown in phage treated soils or untreated ones.

Inoculation treatment	No. nodules/ plant	nodules D.W. (g./plant)	Plant D.W. (g./plant)	N-content (mg./plant)
Wild type	80.2	0.13	35.6	325.4
Mutant	81.8	0.14	37.2	371.6
Wild type+ phage	30.5	0.05	20.8	157.1
Mutant + phage	78.9	0.11	34.3	302.0
L.S.D.	24.75	0.05	2.75	10.8

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عزل طفرات تلقائية مقاومة للبكتيريوفاج من البكتيريا المثبتة للنيتروجين تكافليا

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تم عزل الفاجات المتخصصة على بكتيريا العقد الجذرية لكل من فول الصويا والفول السودانى من تربة المزرعة التجريبية لكلية الزراعة – جامعة المنيا. كما تم بنجاح عزل طفرات تلقائية مقاومة للفاجات المتخصصة على هذه البكتيريا.
تم تحليل البروتين للطرز البرية والطفرات المستحدثة بالتفريد الكهربى . وقد تبين وجود سبعة أنواع من عديد الببتيدات ذات أوزان جزيئية ٦٦, ٦٠, ٥١, ٤٧, ٤٣, ٢٩ و ٢٤ كيلو دالتون فى الطراز البرى لبكتيريا العقد الجذرية لفول الصويا بينما لم تلاحظ فى الطفره المعزولة. أما فى حالة الطراز البرى لبكتيريا العقد الجذرية للفول السودانى فقد وجد به ثلاثة أنواع من عديد الببتيدات وهى ٦٦, ٥١ و ٤٣ كيلو دالتون بينما لم يتم ملاحظتهم فى الطفرة المعزولة . بالأضافة الى ذلك فإن عديد الببتيد ٣٩ كيلو دالتون قد وجد فى طفرتى كل من بكتيريا العقد الجذرية لفول الصويا و الفول السودانى ولم يلاحظ فى الطرز البرية . .
لم يؤثر وجود الفاجات على كفاءة الطفرات المعزولة فى تكوين العقد الجذرية و تثبيت النيتروجين . إلا أن وجود الفاجات أدى الى نقص ملحوظ فى أعداد و أوزان العقد الجذرية و الوزن الجاف للنباتات والمحتوى النيتروجينى للنباتات فى كل من فول الصويا و الفول السودانى الملقح بالطرز البرية .