Protection of phosphate dissolving bacteria against bacteriophage attack

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ABSTRACT

Bacteriophages specific for *Bacillus megaterium* were isolated and were found to be common in soil of the Experimental Farm of Fac. Agric., Minia Univ., Egypt.

A bacteriophage resistant mutant of *B. megaterium* was successfully isolated. The efficiency of the isolated mutant, free and immobilized cells of *B. megaterium* in dissolving insoluble phosphate and their susceptibility to phages were studied in pure liquid cultures (*in vitro*) and under cultivated soil conditions (*in vivo*).

Presence of phages did not affect the efficiency of both the phage resistant mutant and the immobilized cells of *B. megaterium* in dissolving insoluble phosphate in pure liquid cultures. Whereas, bacteriophages completely inhibited the free cells of *B. megaterium* in the pure liquid cultures.

Under cultivated soil conditions inoculation of wheat plants with either immobilized cells or phage resistant mutant of *B. megaterium* markedly increased numbers of phosphate dissolving bacteria in the rhizosphere soil and significantly increased plant P% as well as fresh and dry weight/plant, as compared to those inoculated with the free cells. In free cells inoculated plants bacteriophages had a marked depressive effect on numbers of P-dissolving bacteria in the rhizosphere soil and significantly reduced plant P% as well as fresh and dry weight/plant, as compared to those inoculated with the free cells in absence of phages. In plants inoculated with either immobilized cells or phage resistant mutant, no significant effect for the presence of phages was detected.

Key words: Bacteriophage, Rhigobium, Immobilize, Phage, Resistant mutant.

حماية البكتيريا المحللة للفوسفات من الإصابة بملتهمات البكتيريا

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الملخص

عزلت الفيروسات البكتيرية التي تصيب البكتيريا المحللة للفوسفات، وقد وجد أنها منتشرة بصورة عامة في تربة مزرعة تجارب كلية الزراعة في جامعة المنيا، جمهورية مصر العربية، وكما عزلت بنجاح أيضاً طفرات من البكتيريا المحللة للفوسفات *Bacillus-megaterium* المقاومة لملتهمات البكتيريا، وقد درست فعالية السلالات البكتيرية المعزولة سواء كانت حرة أو مفيدة بألجينات الصوديوم وكذلك الطفرات المقاومة لملتهمات البكتيريا في إذابتها للفوسفات في مزارع سائلة في الزجاج in vitro وفي الحقل in vivo.

لوحظ أن وجود الملتهمات البكتيرية لم تؤثر في فعالية كل من الخلايا الطافرة والمقاومة لملتهمات البكتيريا و كذلك الخلايا المفيدة بالجينات الصوديوم في إذابة الفوسفات في المزارع السائلة in vitro في حين وجد أن هذه الملتهمات البكتيرية قد ثبطت الخلايا الحرة للبكتيريا المدروسة في المزارع السائلة.

كما ظهر أن تلقيح نباتات القمح المزروعة في أصص التجارب بالبكتيريا المحللة للفوسفات *Bacillus-megaterium* المفيدة بالجينات الصوديوم أو ببكتيريا المقاومة للملتهمات أدى إلى زيادة معنوية في عدد البكتيريا المحللة للفوسفات في المنطقة المحيطة بالجذور Rhizosphere، كما أدى إلى زيادة نسبة الفوسفور في النبات وكذلك زيادة الوزن الرطب و الجاف للنبات مقارنة بالنباتات الملحقة بالخلايا الحرة.

وجد أن التلقيح بالخلايا الحرة للبكتيريا المحللة للفوسفات المدروسة بوجود الفيروس أدى إلى انخفاض ملحوظ في عدد البكتيريا المحللة للفوسفات في المنطقة المحيطة بالجذور وكذلك انخفاض نسبة الفوسفور في النبات، وكذلك الوزن الرطب والجاف للنبات مقارنة مع الخلايا الحرة بغياب الفيروسات، ولم تظهر النباتات الملحقة بالخلايا المفيدة بالجينات الصوديوم أو بالخلايا المقاومة للملتهمات البكتيرية تأثيراً معنوياً في حال وجود الملتهمات البكتيرية أو في غيابها مقارنة بالمعاملة المماثلة بوجود هذه الفيروسات.

الكلمات المفتاحية: فيروسات بكتيرية، بكتريا محللة للفوسفات، بكتريا مفيدة، بكتيرية مقاومة للفيروسات.

Introduction

Due to alkalinity of some soils, the soluble forms of phosphate fertilizer applied to such soils, is rapidly converted to a complex of precipitated form of $Ca_3 (PO_4)_2$ (Rajan *et al*., 1996; Zayed, 1998 Hammad, 1999. Some soil microorganisms play an important role in supplying the grown plants with available forms of phosphorus by producing organic acids and CO_2 which increase the soil acidity and convert the insoluble forms of phosphorus into soluble ones. The use of these bacteria as a biofertilizer in the alkaline soils is required to increase the avilability of soil phosphorus.

Zayed (1998) and Hammad, (1999) stated that presence of bacteriophages specific for *Bacillus megaterium* had a depressive effect on the efficiency of this bacteria in dissolving phosphate. Hammad (1998) and Fathy (2004) found that immobilization system offered high protection to *Bacillus megaterium* against their specific phages and increased their efficiency in dissolving phosphate.

Hammad (1999) isolated phage resistant mutant of *Bacillus megaterium*. He reported that presence of bacteriophages did not affect the efficiency of the spontaneous mutant of *B. megaterium* in dissolving insoluble phosphate in pure liquid cultures and under cultivated soil conditions.

Upon the above mentioned information, the presence of bacteriophages may affect the density and activity of such important P-dissolving bacteria (*Bacillus megaterium*) in the soil. Therefore, this investigation was carried out as an attempt to protect such desired bacteria against phage attack via the immobilization system and isolation of phage resistant mutant of *B. megaterium.*

Materials and Methods

The used soil: A clay loam soil was collected from the surface 15 cm layer of the Experimental Farm of Faculty of Agric. Minia University, Minia, Egypt. The mechanical and chemical analysis of the used soil are presented in Table 1. The collected soil was used for isolation of bacteriophages and cultivation of wheat plants.

Table (1) Mechanical and chemical properties of the used soil.

Coarse Sand%	Fine sand %	Silt %	Clay %	Texture grade	Total N%	$CaCO_3$ %	Available P, ppm	Organic matter %	pH
2.5	26.0	31.0	40.5	Clay loam	0.14	2.14	18.4	1.51	8.07

P-dissolving bacteria: An efficient isolate of phosphate dissolving bacteria (*B. megaterium*) was obtained from the microbial collection of Dept. Agric. Microbiology, Fac. Agric. Minia University.

Isolation of rhizobiophages: The liquid enrichment technique was used to isolate the phages of phosphate dissolving bacteria (*B. megaterium*) as described by Barnet (1972). Twenty grams of the collected soil were incubated overnight with 40 ml of nutrient broth (Allen 1959) at 30-33°C. Five ml of chloroform were then added and the sample was shaken for 10 min followed by centrifugation at 4000 rpm to remove soil and bacteria. The supernatant was added to 10 ml of 24h old liquid culture of *B.megaterium.* After multiplication of phages incubation for (24-30 h at 30-33°C), bacteria were killed by shaking with 5 ml of chloroform for 10 min, then the sample was clarified by centrifugation at 4000 rpm. The supernatant (phage lysate) was subjected to phage detection.

Detection of phages: Double layer agar plates method (Adams, 1966) was used for phage detection as described by Hammad (1993). 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 of melted yeast nutrient agar medium containing 0.7% agar and 300 µl of liquid culture of the indicator bacteria (*B. megaterium*) was poured in each plate. The phage lysate was spotted with a sterile micropipette on the upper layer after it had solidified. Plates were incubated at 30-33°C for 24-30 h and then examined for lysis of bacterial lawn at 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), which contains per litre 5.8 g NaCl, 2 g $MgSO_4.7H_2O$, 50 ml of 1M Tris-HCl (pH 7.5) and 5 ml of 2% gelatin. Two hundreds µl chloroform were added to each tube, then maintained at 4°C.

Preparation of high titre phage suspension: The agar double layer plates method of Maniatis, *et al.* (1982) was used as described by Hammad and Dora (1993) for preparing the high titre phage suspension. The phage suspensions prepared from the formed clear zones in the spot test were diluted to (10^{-4}) in SM medium. Double layer plates were prepared as described above but the top layer contained a mixture of 3 ml of semi-solid nutrient agar medium, 300 µl of liquid culture of indicator bacteria (*B. megaterium*) and 50 µl of the diluted phage suspension. After incubation at 30-33°C for 24-30h plates showed almost complete lysis. Five ml of SM medium (Maniatis, *et al.*, 1982) were added to the surface of each plate. The top agar layer of each plate was scraped off and combined in a flask together with the added SM. After 30 min of occasional shaking, agar and bacterial debris were sedimented by centrifugation at 4000 rpm. for 30 min. The supernatant containing the phages was stored at 4°C over 3ml of chloroform.

Titre estimation: Titre of the prepared phage suspension was estimated using the method described by Kiraly, *et al.* (1970). From the phage suspension a series of tenfold dilutions was prepared in sterile eppendorf tubes. Ninety µl of SM medium were placed in each tube. Ten µl of phage suspension were added to the first vial and mixed, then 10 µl from the first vial were transferred into the second one and so on until the last tube. After dilution, 300 µl of indicator bacterial liquid culture (*B. megaterium*) were placed in each tube. The contents of each tube were shaken and transferred to a sterile test tube containing 3 ml of semi-solid medium (nutrient agar) which had been prepared beforehand and kept at 50-55°C. Each tube was shaken separately and the contents were poured onto previously prepared solid media plates. Plates were then incubated at 30-33°C for 24-33 h. The formed plaques were counted and the titre was calculated and expressed as plaque forming unit (pfu)/ml.

Isolation of phage-resistant mutant: The method described by Adams (1966) was used. One ml of liquid bacterial culture containing 10^8 cells of (*B. megaterium*) was mixed with 1 ml of phage suspension containing 10^{10} plaque forming unite in an eppendorf tube. The tube was incubated for 5 min at 30-33°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 a 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 from this suspension a loopful was streaked on another plate. Two repetitions of this procedure (streaking on agar plates) were carried out to obtain a pure isolate of phage-resistant mutant free from contaminating phages. A liquid culture of the obtained pure isolate of phage-resistant mutant was prepared to be used as inoculum.

Preparation of free cells inocula: The used phosphate dissolving bacterial isolate (wild type of *B. megaterium*) and the isolated phage resistant mutant were grown in Erlenmayer flasks containing 100ml of nutrient broth medium (Allen, 1959)/flask and incubated in a shaker at 30°C for 96 h. (giving 16-19 x 10^8 cell/ml). These liquid cultures were used as inocula.

Sodium alginate-immobilized cells inoculum: One hundred ml of a sterile solution of sodium alginate (5% w/v) was mixed with an equal volume of the prepared liquid culture of *B. megaterium* (the wild type). The mixture was added dropwise into 200 ml of 2% $CaCl_2$ sterile solution using a sterile Pasteur pipette. Beads of approximately 2 mm in diameter were formed and were hardened in 2% $CaCl_2$ solution for 2 h before washing. The beads were then washed with sterilized water and stored at 4°C. All steps were carried out under aseptic conditions.

Experimental design and treatments

In vitro study:

Efficiencies of free, immobilized cells and the phage resistant mutant of *B. megaterium* in releasing phosphorus as well as their susceptibility to phage attack were evaluated. The evaluation was carried out in Erlenmeyer flasks containing 90 ml of Bunt and Rovira liquid medium (1955). The flasks which contained Bunt and Rovira liquid medium were supplemented with 0.25 gm tricalcium phosphate/flask and the pH was adjusted to 6.8. The prepared flasks were subjected to the following treatments:

1-Inoculation with free cells. 2- Inoculation with immobilized cells. 3- Inoculation with phage resistant mutant. 4- Inoculation with free cells and phage suspension. 5 - Inoculation with immobilized cells and phage

suspension. 6- Inoculation with phage resistant mutant and phage suspension.

In treatments inoculated with free cells and phage resistant mutant, 5 ml of the prepared liquid cultures inocula were added to each flask. In case of inoculation with the immobilized cells, a calculated weight of beads containing the same number of bacterial cells (in the 5 ml of free cells inoculum) was added to each flask. For inoculation with phages, 5 ml of the high titre phage suspension were added to each flask. Four replicates for each treatment were employed. All flasks were incubated at 30°C for 7 days. Changes in pH and amount of soluble phosphorus in each treatment were measured at intervals of 24 h up to 7 days. The soluble phosphorus was determined colorimetrically according to Wilde *et al.* (1979).

***In vivo* study:**

A pots experiment was carried out to evaluate the efficiency of free, immobilized cells and phage resistant mutants of *B. megaterium* in dissolving precipitated form of phosphate and their susceptibility to phages. Fired clay pots containing 3 kg soil/pot were prepared. The pots were planted with wheat and were subjected to the same inoculation treatments used in the *in vitro* study. Uninoculated plants as a control were involved. Four replicates for each treatment were employed.

In the treatments inoculated with free cells of either the wild type or mutant of *B. megaterium*, 5 ml of the prepared liquid cultures inocula were added to each pot. In case of inoculation with the immobilized cells, a calculated weight of beads containing the same number of bacterial cells (in the 5 ml of free cells inoculum) was added to each pot. For inoculation with phages, 5 ml of the high titre phage suspension were added to each pot.

Number of P-dissolving bacteria was determined in rhizosphere soil of wheat plants at intervals of 15 days up to 75 days. The standard plate method was used for determining number of P-dissolving bacteria as described by Abdel-Moniem et al (1988). Fresh and dry weight/plant as well as phosphorus percent in plants were determined when plants were 60 days old.

Results and Discussion

1- Bacteriophages of *Bacillus megaterium*

As shown in Figure (1), the spot test indicated that phages of *B. megaterium* are of widespread occurrence and were found to be common in the collected soil sample. Similarly, Hegazi *et al*. (1980) isolated phages of *B. mycoides, B. subtilis* and *B. cereus* from several soil samples collected from the Nile Valley. Zayed (1998) and Hammad (1999) isolated phages of *B. megaterium* from soils of Minia Governorate. Moreover, Abo-Sinna (2004) isolated phages of *B. subtilis* from soils of different Egyptian Governorates.

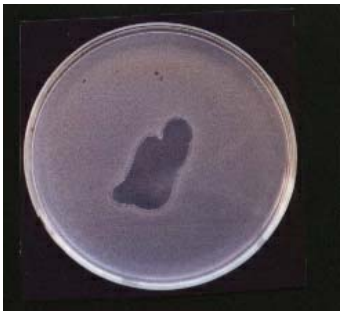

Figure (1) A bacterial lawn of *B. megaterium* spotted with a drop of the prepared phage lysate and incubated for 24-30 h, at 30-33°C.

2- Titre of the prepared phage suspension

Two hundred ml of high titre phage suspension were prepared as described by Maniatis *et al.* (1982) to be used in this study. Titre of the prepared suspension was estimated and was found to be 9.2×10^{11} pfu/ml. Such high concentration of phages was not surprising, since a single plaque of 2mm in diameter may contain between 10^7 and 10^8 recoverable phage particles (Gunsalus and Stanier, 1960; Adams, 1966; Hammad, 1998).

3- Phage-resistant mutant of *B. megaterium*

As shown in figure (2), the isolated mutant of *B. megaterium* exhibited resistance to the phages of the wild type. *I.e*. no lyses was detected on plates seeded with the mutant and spotted with the isolated phages whereas lyses of the wild type can be clearly seen. Defives *et al.* (1996); Coakley *et al.* (1997) and Hammad (1999) successfully isolated phage resistant mutants of *B. megaterium* and *Azospirillum* sp.

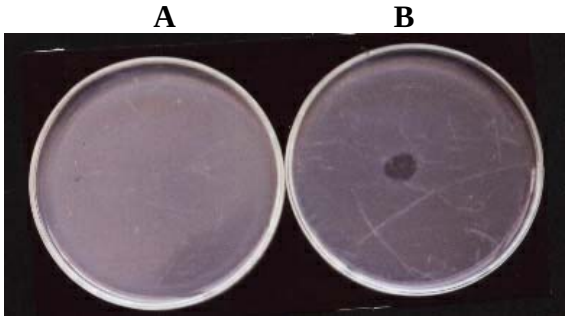

Figure (2) Bacterial lawns of the phage-resistant mutant (A) and the wild type (B) of *B. megaterium*, spotted with the phage lysate. Susceptibility of the wild type and resistance of the mutant can be clearly seen.

4- Effect of Bacteriophages on activity of B. megaterium in dissolving insoluble phosphate

i- *In vitro* study:

The reduction in pH values and the increase of the soluble phosphorus in the media inoculated with the different forms of *B. megaterium* inocula were used as indicators for their efficiency in dissolving insoluble phosphate.

Data presented in Table (2) indicate that in absence of phages the initial pH (6.8) was brought down due to inoculation with any form of P-dissolving bacteria (free, immobilized cells or phage resistant mutant). The lowest values were recorded at the 6^{th} day after inoculation and hence the highest concentrations of soluble phosphate were detected.

On the other hand, in the presence of phages no pronounced change in pH values was observed in media inoculated with the free cells. However, phages had no pronounced effect on the efficiency of the immobilized cells. Such observation may indicate that the immobilization system protects the cells against phage attack. This may be due to the presence of the host cells inside alginate beads, which may prevent the direct adsorption of phage particles on the bacterial surface and hence no infection can occurr. Similar results were obtained by Hammad (1998) Zayed (1998) and Fathy (2004).

Moreover, the presence of phages had no effect on the efficiency of the phage resistant mutant in dissolving insoluble phosphate. Such results may indicate that the mutation process did not alter the efficiency of this bacteria in dissolving phosphate. These results are in agreement with those obtained by Hammad (1999) and Fathy (2004).

Table (2) pH values and concentrations of soluble-phosphate in media inoculated with different forms of *B. megaterium* inocula in the presence and in the absence of phages.

Treatments	Days after incubation at 30°C							
	2		4		6		8	
	pH	P. ppm	pH	P. ppm	pH	P. ppm	pH	P. ppm
Free cells	4.21	196	4.10	218	3.86	251	4.20	246
Free cells + phages	6.70	23	6.81	26	6.78	29	6.81	27
Immobilized cells	4.08	228	3.81	230	3.53	336	3.86	321
Immobilized cells + phages	4.13	223	3.92	226	3.71	325	3.91	317
Mutant	4.23	212	4.00	219	3.81	261	4.00	245
Mutant + phages	4.22	207	4.13	211	3.96	252	4.11	238

ii- *In vivo* study:

Since the immobilized cells of *B. megaterium* and the isolated phage resistant mutant exhibited high resistance to bacteriophages and high efficiency in dissolving insoluble phosphate, under aseptic condition, it was of a particular interest to evaluate their efficiencies under the cultivated soil conditions.

Densities of phosphate dissolving bacteria in rhizosphere soil of wheat plants

Data presented in Table (3) indicate that under any inoculation treatment numbers of phosphate dissolving bacteria in rhizosphere soil of wheat plants tended to increase progressively and reached their maxima when plants were 60 days old , then decreased. This may be due to the changes in multiplication rate of these bacteria as a result of qualitative changes in the nature of root exudates of the plants during the different growth stages (Fayez *et al*., 1985; Abdel-Ati *et al*., 1996; Hammad, 1999; Fathy, 2004).

At any sampling time, the rhizosphere soil of wheat plants which inoculated with the immobilized cells of *B. megaterium* contained much higher numbers of P-dissolving bacteria than those inoculated with the free cells. Such results may indicate that the immobilization system provides suitable condition for growth and multiplication of the immobilized cells inside the beads and hence high number of these bacteria can be liberated from the beads to colonize the rhizosphere zone. Similar results were obtained by Van Elsas *et al.* (1991); Saad and El-Mohandes (1998) and Fathy (2004).

The presence of phages markedly reduced the number of P-dissolving bacteria in the rhizosphere soil of wheat which inoculated with free cells of *B. megaterium* as compared to those inoculated with free cells in the absence of phages. On the other hand, the presence of phages had no pronounced effect on densities of P-dissolving bacteria in rhizosphere soil of wheat which inoculated with either immobilized cells of *B. megaterium* or the phage resistant mutant. These results are in agreement with those obtained by Hammad (1999) and Fathy (2004).

Table (3) Densities of phosphate dissolving bacteria in rhizosphere soil of wheat inoculated with free and immobilized cells of *B. megaterium* and its phage resistant mutant, in the presence and in the absence of phages.

Treatments	Days after inoculation					
	0	15	30	45	60	75
	Number of P-dissolving bacteria x10^4					
Free cells	10.3	36.8	42.6	51.0	62.7	47.3
Free cells + phages	10.3	19.6	22.1	32.0	38.9	27.1
Immobilized cells	10.3	47.6	51.9	63.2	70.1	55.8
Immobilized cells + phages	10.3	45.2	50.8	63.6	68.1	61.1
Mutant	10.3	38.4	40.3	56.8	61.0	45.8
Mutant + phages	10.3	36.0	42.3	53.9	60.8	41.0
Control	10.3	16.3	22.8	28.0	32.6	23.3

Growth of wheat plants and their phosphorus content

As shown in Table (4), plant height, fresh and dry weight/plant as well as phosphorus percent in plants inoculated with the free cells of *B. megaterium* plus phages were lower than in the other treatments. This may indicate that bacteriophages had a depressive effect on their bacterial host. Therefore, densities of this host decreased due to the presence of phages in the rhizosphere soil and consequently the growth and phosphorus content of the plants reduced as well. Moreover inoculation of wheat plants with the immobilized cells of *B. megaterium*, significantly increased the studied measurements as compared to the uninoculated plants (control), even in the presence of phages. Such results may indicate that the immobilization system provides these bacteria with high resistance against phages. Similar results were obtained by Hammad (1998) and Fathy (2004).

The presence of phages in the soil did not affect neither growth nor P% in plants inoculated with phage resistant mutant. This may indicate that the mutation process does not alter the efficiency of *B. megaterium* in dissolving insoluble phosphate. These results are in agreement with those obtained by Hammad (1999) Zayed (1998) and Fathy (2004).

Table (4) The growth and phosphorus percent in wheat plants inoculated with *B. megaterium* in free, immobilized form or phage resistant mutant in the presence or absence of phages.

Treatments	Plant height	Fresh weight g./plant	Dry weight g./plant	P%
Free cells	41.1	14.31	3.20	0.43
Free cells + phages	32.8	11.62	2.69	0.28
Immobilized cells	47.6	16.94	4.11	0.58
Immobilized cells + phages	45.9	15.40	3.90	0.54
Mutant	43.0	13.82	3.16	0.47
Mutant + phages	42.6	13.51	2.98	0.45
Control	28.8	8.96	2.61	0.26
L.S.D. 5%	3.9	2.52	0.40	0.13

Generally, on the basis of the obtained results it can be concluded that, the presence of phages specific for *B. megaterium* in the soil is one of the most important environmental factors affecting the activity and maintenance of such desired bacteria. Whereas, application of such bacteria as a biofertilizer in alginate immobilized form may provide these bacteria with high resistance against phages.

Moreover, the phage attack can be avoided by isolation of phage-resistant mutant of these bacteria to be used as biofertilizer.

Therefore, preparation of *B. megaterium* in alginate immobilized form or isolation of phage-resistant mutant of these bacteria to be used as phosphate dissolving biofertilizer is highly recommended to avoid phage attack.

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