Transfer of nif genes from Nitrogen fixer Azotobacter chroococcum to phosphors solubilizing Bacillus megaterium var. phosphaticum by conjugation

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ABSTRACT

The probability of transferring the nitrogen fixation genes (nif genes) from Azotobacter chroococcum to Bacillus megaterium Var phosphaticum were explored in this research. A total of 50 isolated of Azotobacter sp. from many soil samples of Erbil city-Iraq, were subjected to cultural, morphological and biochemical characterization. The nitrogen fixing genes (nifH1, nifH2, nifU, nifV and FV genes) which located in chromosomal DNA from Azotobacter chroococcum were transferred through the conjugation process to the Bacillus megaterium Var phosphaticum as recipient cell. The presences of (nif genes) was confirmed by gene detection tests to the donor and recipient genomes and transconjugants cell by PCR amplification of nifH1, nifH2, nifU, nifV and FV genes. The transconjugant cells were showed the ability and the effectively action on nitrogen fixation when they growth on solid N2 free medium, and solubilizing phosphorus.

1. INTRODUCTION

Bacillus megaterium is one of the most important Gram positive free living bacteria successfully growing in water, air and soil in several locations (Banerjee et al., 2007).

The role of Bacillus spp. in agricultural fields has been reported to enhance many plants growth phenomenon directly and or indirectly (Kumar et al., 2011). The growth Increases in the crop yield is reported on various plants crops including sugar beet (Çakmakçı et al., 1999), barley (Salantur et al., 2005), alfalfa (Dasci and Omakli, 2009), clover, wheatgrass, perennial ryegrass (Holl et al., 1988) and cicer (Elkoca et al., 2007), while the production of amino acids, vitamins, indole acetic acid (IAA), gibberellic acids (GA3) where confirmed to be more valuable by (Bottini et al., 2004), (Bloemberg and Lugtenberg, 2001). Meanwhile the activities against antibiotics were reported by (Weller, 1988), as well as the induction of systemic resistance to plant pathogens, production of siderophore and inhibition of plant ethylene synthesis mentioned by (Richardson et al., 2009, Idris et
al., 2007, Gutiérrez-Mañero et al., 2001, Whipps, 2001) these reports confirm the possibility mechanism for crop yield increases in addition to nutrient solubilization.

The role of *Bacillus megaterium* var. *phosphaticum* in the promoting of (organic Phosphors) solubilization is well documented by (Lach et al., 1990) and (Vazquez et al., 2000). Several mechanisms have been proposed to explain the phosphorus solubilization by *Bacillus megaterium* var. *phosphaticum* due to the association with the release of organic phosphorus in soil.

*Azotobacter* is an N₂-fixing soil bacterium, free-living and aerobic bacteria. Genetics studies in this genus are supposed to take into consideration their *nif* genes which are responsible for fixing nitrogen (Betancourt et al., 2008). The two-component nitrogenase enzyme complex requires 3 genes *nifH*, *nifD* and *nifK* that encode respectively, the Fe-protein, and the alpha and beta-subunits of Mo-Fe protein. While both components of nitrogenase require extra genes products for activity. The *nifM* is required to give an activity for Fe-protein (Raina et al., 1993). The *nifE*, *nifN* and *nifB* genes are essential for the synthesis or insertion of the Fe and Mo containing cofactor (FeMo-Co). The *nifV* gene involved in stability of dinitrogenase and proposed to protect dinitrogenase from O₂ inactivation (Klipp et al., 2004). The chromosomal transferred *nif* genes among strains of *A. chroococcum* has been reported by Jones et al. (1984), and to *Bacillus subtilis* by Lotareva and Prosorov (2006) and Hewson et al. (2007). Copper resistance genes found on the chromosome of *A. chroococcum* were transferred to *Xanthomonas citri*. (Khider, 2011) transferred chromosomal *nif* genes (*nifH1, nifH2, nifH3, nifU* and *nifV*) by conjugation from *Azotobacter chroococcum* to *Lactobacillus planetarium*.

The current research aims at finding integrated solutions to several soil disadvantages in Erbil province, because this soil is calcareous and total phosphorus is more than (200) ppm; however, the available rate does not exceed (3) ppm. Phosphate is a major element needed by the plant and is vital to processes that occur in plant cell. In this study we attempted to incorporate the genes necessary for increasing the efficiency of nitrogen fixation and phosphate solubilization by one single bacterial species instead of two.

2. MATERIALS AND METHODS

2.1. Bacterial strains:

The bacterial strains, *Azotobacter* spp. and *Bacillus megaterium* var. *phosphaticum* were isolated from the soil: *A. chroococcum* was isolated from the soil of Erbil city, Iraq from November 2013 to March 2014 using Ashby and Sperper media. Morphological and biochemical tests (Gelatense, Catalase, Indol, Oxidase and Carbohydrate integration) were performed by stranded model (Atlas et al., 1995, Jackson, 1973, Forbes et al., 2002). *Bacillus megaterium* var. *phosphaticum* was isolated from rhizosphere soil in Erbil city using Sperber’s medium followed the enrichment culture techniques (Niemenen et al., 2007). Identification of the isolates were performed by a series of microbiological and biochemical tests. The serial diluted soil samples were pasteurized at 80 degrees for 15 minutes to isolate the spore forming bacteria, then plates onto nutrient agar and incubated at 37°C for 24 hr. Then, the plates were examined for typical colonies identified as catalase-positive, Gram-positive, endospore-forming rods(Reddy et al., 2010).

2.2. Preservation of bacterial isolates

The bacterial isolates were preserved at -20°C after suspension in 20% (W/V) glycerol (Ausubel et al., 1987).
2.3. Nitrogenase Activity Assay by Acetylene Reduction.

Eighteen ml serum bottles containing 6 ml of modified Ashby’s broth medium were inoculated with 1ml (35x10⁷ cfu/ml) of previously activated *Azotobacter* spp. (Mollica et al., 1985). After the incubation period, the cotton plugs were replaced under aseptic conditions by rubber stoppers, and 10% of the air was poured off with a syringe and replaced with acetylene to represent 10% of the gas phase. Then, the serum flasks were incubated for 1h at 30°C. From each bottle, 0.3 ml samples from the gas phase were injected into Gas Chromatography (PYE unicam). The temperature of the column was adjusted at 100°C and N₂ was used as a carrier gas at a flux rate of 120 ml/min. The produced ethylene was calculated according to the equation adopted by Hardy et al. (1973):

\[
\mu \text{ Mole } \text{C}_2\text{H}_4/\text{ml}/\text{h} = \frac{\text{CLC reading} \times 10^8 \times \text{volume of air}}{\text{volume of media} \times 22.4 \times \text{incubation period (1h)}}
\]

2.4. Phosphatase Activity Test

Ten milliliters of bacterial culture containing 35x10⁷ cfu/ml of each activated tested isolates were transferred separately to sperber’s medium, and then incubated with shaking at 30°C and 100 rpm for 7 days. After incubation, the culture were centrifuged at 7500 rpm for 10 minutes; the supernatant was examined for Phosphatase activity according to the procedure indicated by Jackson (1973).

2.5. Antibiotic Resistance

The following concentrations (µg/mL) of antibiotics such as Trimethoprim (20), Rifampicin (5), Cefotaxim (30), Erythromycin (10), Strptomycin (25), Chloramphenicol (30) Ampicillin (50), and Tetracycllin (15) were used in this experiment.

*A. chroococcum* isolates and *Bacillus megaterium var phosphaticum* were grown at 30°C on Ashby's and Sperber’s media respectively with added antibiotics when compulsory.

2.6. Isolation of bacterial genomes

The extraction and purification of Genomic DNA from bacterial cells have been performed via the AccuPrep Genomic DNA Extraction Kit Bioneer (Korea).

After the sample preparation from the bacterial isolates which was a gram negative bacteria *Azotobacter chroococcum* then the first step started with Lysis bacterial cells, DNA Binding, wash the extraction and elution which achieved depending on dried GD column was transferred to a clean 1.5 ml microcentrifuge tube. 100 µl of pre-heated elution buffer was added TE buffer into the center of the column matrix and wait for 3 minutes to allow elution buffer, TE Buffer completely absorbed then centrifuged at 14,000 rpm for 30 seconds to elute the purified DNA.

2.7. Amplification of nitrogenase genes

Various genes including *nifH1, nifH2, nifN, nifU* and FV sequence were taken from NCBI site and designed in Cinnaclon co. As shown in Table (1).

The melting and annealing temperature are computed followed (Womble, 1999) and primers amplification (Setubal et al., 2009) was completed using the protocol and reagents suggested by Rajeswari and Mangai (2009). The temperature sequence (TS) follows the flowing steps :a) 95°C for 3 min as preheating; b) 96°C followed by 55°C for one min; c) 72°C for 1 min. The TS is run for 30 cycles followed by the final product extension step which is carried out at 72°C for 6 min followed by 4°C temperature hold.
2.8. Agarose Gel Electrophoresis

The PCR product was run on agarose gel 2% electrophoresis for 45 minutes following the protocol discussed by Bürgmann et al. (2004).

2.9. Conjugation process

In order to transfer the genes responsible for nitrogen fixation, which occur on chromosomal DNA in A. Chroococcum (Evans et al., 1988) to non-nitrogen fixer bacterium Bacillus megaterium var phosphaticum, bacterial conjugation was carried out between them. These two bacteria must differ at least in two genetic markers. The method of (Olsen et al., 1992) was-used, 10ml of Ashpy's broth was inoculated with single colony of A. chroococcum (donor cell), and 10 ml Sparer's broth was inoculated with single colony of Bacillus megaterium var phosphaticum (recipient cell), then incubated at 30°C for 48 hours with shaking 100 rpm. After incubation, 0.8ml of the donor cells mixed with 0.2ml of the recipient cells and 1 ml of fresh Sparer's broth, the mixture incubated at 30°C for 3 hours under aerobic condition. Then 0.1 ml of conjugated mixture was spread on to Sparer's agar plates containing two antibiotics which were used as genetic markers, and control plates were prepared, by spreading 0.1 ml for each donor and recipient suspensions on agar plates containing the same markers, then all plates were incubated at 30°C till the colonies appeared, then the number of transconjugant colonies were screened after purification several times on same plates. The conjugation frequency was calculated according to the following equation (Puhler and Timis, 1984):

\[
\text{conjugation frequency} = \frac{\text{Number of transconjugant colonies in 1 ml of conjugation mixture}}{\text{Total number of recipient cell in 1 ml of bacterial culture}}
\]

3. RESULTS AND DISCUSSION

Total of (50) non-symbiotic nitrogen-fixing bacteria (Azotobacter spp.) were isolated from the soil using Ashby's medium. Ten isolate were selected according to the growth on Ashby's medium. These selective properties were gram- negative and motile rods, positive for oxidase, catalase, and water soluble pigments as shown in table (2), and they were negative for gelatinase, treptofinase and ethanol soluble pigments.

The isolates utilized mannitol, rafinose, sucrose and maltose, while some isolates failed to utilize starch, lactose, xylose and inositol (table 3).

Morphological studies on the isolates revealed strong similarity with the genus Azotobacter. Moreover, the utilization test of different sugars showed that out of the ten isolated bacteria, nine isolates of Azotobacter chroococcum, one of Azotobacter vinelandii. Bacillus megaterium var phosphaticum was isolated from the soil based on cultural characteristics, morphological characterization and biochemical tests. The isolates appeared to be gram-positive swollen, rod shape (bacilli), positively react with catalase and oxidase tests (table 4). The isolates grew when cultured on Sperber’s medium for phosphorus solubilization. Furthermore, the bacteria failed to grow on the same medium containing lysozyme (0.001%) and under anaerobic condition. One isolate from A. chroococcum and Bacillus megaterium Var. phosphaticum was selected based on antibiotic sensitivity test used as marker for the conjugation process. All transconjugant colonies were culturally and biochemically characterized.

The results indicate that minor colonies, insoluble brown color, anaerobically grow weak. The cells were long, rod shapes; appear individually or in chain, gram-positive, non-capsulated cells, spore forming, non-motile. The bacteria were positive to oxidase and catalase, and liquefied gelatin. Isolates have grown at temperatures 5 °C and 20°C but not at 50°C. They did not ferment glucose, and they grew on general-purpose medium (nutrient agar). The isolates appeared resistant
to erythromycin, ampicillin and rifampicin. These results are considered similar to the original donor cell *A. chroococcum*. The previous results confirmed that the resulting colonies (transconjugant) share all characteristics of the receiving cell *Bacillus megaterium* Var. *phosphaticum*. The transconjugant cells can grow on free nitrogen in Ashby’s agar medium and followed the usual pattern as *A. chroococcum*. The transconjugant cells were tested for *nif* genes transfer.

The PCR product is shown figures (1) and (2). *N*$_2$-fixing genes were detected with the same size (lane 5 and 6) in figure (1) and (lane 5, 6 and 7) in figure (2). These results were confirmed when transconjugant cells grown on *N*$_2$-free medium were able to utilize its nitrogenase enzyme and fixed atmospheric nitrogen.

4. DISCUSSION

Purified *Azotobacter* isolates were tested for the utilization of different sugars according to Bergey’s Manual (1984). *Azotobacter chroococcum* used mannitol, glucose and sorbitol as a carbon source, grew on *N*$_2$-free medium. They were gram negative and produced pigments when cultured on agar medium. All *B. megaterium* isolates grow well on Sperber’s medium and solubilized phosphorus where clear zone developed around the colonies and but failed to grow on Ashby’s medium. *A. chroococcum* was resistant to tetracycline and sensitive to cefixime, while *Bacillus megaterium* Var *phosphaticum* was sensitive to tetracycline and resistant to cefixime. Transconjugant colonies grew on Sperber’s agar containing tetracycline and cefixime. The success of obtaining transconjugant colonies may be due to the formation of the conjugation bridge between the donor and the recipient bacteria and transmission of oriT genes across this bridge (Snyder and Champness, 1997).

The transferred chromosomal *nif* markers has been performed previously and reported by chromosomal DNA transfer between *Azotobacter* (Khider, 2011) and (Rhon-Calderon et al., 2016), or *Bacillus subtilis* (Lotareva and Prosorov, 2006) has been reported as well.

Bacterial conjugation is a promiscuous DNA transport mechanism. Conjugal plasmids transfer themselves between most bacteria, thus being one of the main causal agents of the spread of antibiotic resistance among pathogenic bacteria. Moreover, DNA can be transferred conjugatively into eukaryotic host cells in this review. Conjugation can be visualized as a DNA rolling-circle replication (RCR) system linked to a type IV secretion system (T4SS), the latter being macromolecular transporters widely involved in pathogenic mechanisms. The scheme ‘replication + secretion’ suggests how the mechanism would work on the DNA substrate and at the bacterial membrane (Llosa et al., 2002).

Saitia et al. (1989) pointed out that the genetically modified cell showed resistance to antibiotics such ampicillin and erythromycin due to the success of the process of transferring genes located on R-plasmid of the donor cell. Meanwhile, the transconjugant cells were able to grow on nitrogen-free media means that they can fix atmospheric nitrogen. Colonies secreted pigments like the donor cells which indicate that the genes responsible for catalase enzyme, and pigment formation genes were transferred since they are located on the same chromosome (Evans et al., 1991). It was noted that many genes such as *nifV, nifB, nifE, nifN*, and *nifH* play a role in the formation of the enzyme cofactors such as Fe- and Mo compound which are important in the composition of a complex enzyme (FeMo-co) (Klipp et al., 2004). Additionally, to prove the transfer of *nif* genes from *A. chroococcum* to nitrogen non-fixing bacteria such as *B. megaterium* var. *phosphaticum*, genes *nifH, nifU* and *nifV* were selected. *NifH* genes (nifH1 and nifH2) are required for biosynthesis of Fe-protein (component 2) and S-subunits of MoFe-protein (component 1). The nifH2 gene was 246 bp, while, nifH1 gene was 1102 bp. These two...
genes can be separated by electrophoris. Genes involved in nitrogen fixation are shown in figure (1).

The protein involved in FeMo-co biosynthesis may be divided into three classes: molecular scaffolds (nifEN, nifU and nifB) where FeMo-co can gather, metallocluster carrier proteins (NifY and NifX) that carry FeMo-co precursors between assembly sites in the pathway (Li et al., 2016). The region of the chromosome, in which this gene located are nifH1, nifH2, nifH3. There are other nif genes like nifK,D,M,A,N,B,Q,Z,P,F,W,L and nifY (Hamilton et al., 2011).

Due to the importance of this chromosomal fragment, which has nifH1, nifH2 and nifFv, nifV and nifU it has been selected for the current study. This was revealed by PCR technique in both A. chroococcum and B. megaterium var phosphaticum transconjugant cells (figure 1). The results in (figure 1) show that transconjugant B. megaterium var phosphaticum cells as recipient cell and A. chroococcum as donor cells containing nifH1 primers display positive amplicon on the gel lane 2 and lane 5, with (1102bp), also there is a match between the donor and the recipient cell with nifH2 and have the same size (246bp) shown in lane 3 and lane6 while the lane 4 was a negative control. While the result in (figure 2) show the Conformity between the genes of the donor cell and recipient cell in each of the nifU (9302bp) , nifV(1146 bp) and Fv (550bp). Although Bacillus megaterium var phosphaticum show a -ve PCR product in (figure 3) lane2,3,4,5 and 6 that is Reflecting the success of the process of gene transfer and is consistent with (Khider, 2011) and (Hogg, 2013). The conjugation process between non-fixing nitrogen bacteria such as B. megaterium var phosphaticum which can't growth in free nitrogen media and Azotobacter chroococcum which can't solubilize phosphorus and combining characterizes between them also contributed to nitrogen fixation and solubilizing phosphate at same time. We assuming that the transformation of the Azotobacter hall genome to the Bacillus megaterium enhancing that ability to growth under anaerobic condition because one of the most important factor of the Azotobacter genome the existence of the nitrogenase enzyme (Raina et al., 1993). These transconjugate bacteria may be exploited as biofertilizer and to improve the nitrogen and phosphorus ratio in the soil.

5. CONCLUSION
The main results of the conjugation process between selected Azotobacter chroococcum as a donor and Bacillus megaterium var. phosphaticum as a recipient successfully obtained in new TB cell. The new TB cell poses the nif gene after the confirmation with the stander identification techniques for that gene using PCR and agarosgel the new bacteria carryout the both functions at the same time nitrogen fixation non-symbiotically and phosphate solubilization activities.
Table (1): The primers used in this study.

| Genes   | Sequences                                           | Size     |
|---------|-----------------------------------------------------|----------|
| $nifH1$-F* | 5'-cagacacgaagaagccgggc-3'                          | (1102) bp|
| $nifH1$-R* | 5'gaccacagcttgtgtga3'                              |          |
| $nifH2$-F  | 5'cgccggegcagttgtgcgg-3'                           | (246) bp |
| $nifH2$-R  | 5'cactcgctgtgegcgtgcgc3'                           |          |
| $nifU$-F   | 5'atgtgggattatcggaaaa3'                           | (930) bp |
| $nifU$-R   | 5'tcacgctccatgcccgtgg-3'                           |          |
| $nifV$-F   | 5'gatggctagggtcatcgcagga3'                         | (1146) bp|
| $nifV$-R   | 5'gcattctctgtgcgcagttgc3'                          |          |
| FV-F       | 5'tacagtaggaggttaggt3'                            | (550) bp |
| FV-R       | 5'tcagccgccgacctgtgctg-3'                          |          |

$F^*$ is the forward primer and $R^*$ is the reverse primer sequences.

Table (2): Biochemical and Morphological characteristics of *Azotobacter* spp.

| Isolate | Pigment    | Water solub. | Ethanol solub. | oxidase | catalase | gelatinase | Trepotofinase | Cell shape | flagella |
|---------|------------|--------------|----------------|---------|----------|------------|---------------|------------|----------|
| 1       | Yellow     | +            | -              | +       | +        | -          | +             | Ovoid      | Peritrichous |
| 2       | brown      | +            | -              | +       | +        | -          | +             | Ovoid      | Peritrichous |
| 3       | Gray       | +            | -              | +       | +        | -          | +             | Ovoid      | Peritrichous |
| 4       | white      | +            | -              | +       | +        | -          | +             | Ovoid      | Peritrichous |
| 5       | brown      | +            | -              | +       | +        | -          | +             | rod        | Peritrichous |
| 6       | Yellow/brown | +            | -              | +       | +        | -          | +             | Ovoid      | Peritrichous |
| 7       | brown      | +            | -              | +       | +        | -          | +             | Ovoid      | Peritrichous |
| 8       | white      | +            | -              | +       | +        | -          | +             | Ovoid      | Peritrichous |
| isolates | Starch | Mannitol | Rhafinose | Sucrose | Lactose | Maltose | Xylose | Inositol |
|----------|--------|----------|-----------|---------|---------|---------|--------|----------|
| 1        | -      | +++      | ++        | ++      | +++     | +       | ++     |
| 2        | ++     | +++      | ++        | +       | +++     | +       | +      |
| 3        | +++    | +++      | +         | +       | ++      | +       | +++    | ++       |
| 4        | ++     | +++      | +         | +       | ++      | ++      | +      | ++       |
| 5        | +      | +++      | +         | +       | +       | +++     | ++     | +        |
| 6        | +      | +++      | ++        | +       | ++      | ++      | +      | ++       |
| 7        | +      | +++      | +         | +       | +       | +       | -      | +        |
| 8        | -      | +++      | ++        | ++      | +       | +++     | -      | +        |
| 9        | -      | +++      | +++       | +       | -       | ++      | -      | -        |
| 10       | -      | +++      | +++       | +       | +++     | -       | +      | +        |

Table(3): Substrate utilization isolates of *Azotobacter* spp.
Table (4): Biochemical and Morphological characteristics of *B. megaterium var phosphaticum*.

| Isolate | oxidase | catalase | Cell shape | Lysozyme .001% | Anaerobic growth |
|---------|---------|----------|------------|-----------------|-----------------|
| 1       | +       | +        | Swollen Cell Rod | -              | -               |
| 2       | +       | +        | Swollen Cell Rod | -              | -               |
| 3       | +       | +        | Swollen Cell Rod | -              | -               |
| 4       | +       | +        | Swollen Cell Rod | -              | -               |
| 5       | +       | +        | Swollen Cell Rod | -              | -               |
| 6       | +       | +        | Swollen Cell Rod | -              | -               |
| 7       | +       | +        | Swollen Cell Rod | -              | -               |
| 8       | +       | +        | Swollen Cell Rod | -              | -               |
| 9       | +       | +        | Swollen Cell Rod | -              | -               |
| 10      | +       | +        | Swollen Cell Rod | -              | -               |

Table (5): Characterizes of Donor, Recipient and Transconjugant strain.

|                  | Donor          | Recipient      | Transconjugant |
|------------------|---------------|----------------|----------------|
| **Media**        | *Azotobacter* | *Bacillus megaterium var phosphaticum* | *Bacillus megaterium var phosphaticum* |
|                  | chroococcum   |                 |                |
|                  | *Ashby's media* | *Sperber's media* | *Ashby's media or Sperber's media* |
Figure 1. Agarose gel electrophoresis. Electrophoresis was performed on (2%) agarose gel showing the PCR amplicon of the \textit{nif}H1 (1102bp) and \textit{nif}H2 (246 bp).

- Lane 1: DNA ladder 100bp
- Lane 2: \textit{A. chroococcum} (+ve PCR product)
- Lane 3: \textit{A. chroococcum} (+ve PCR product)
- Lane 4: Negative control
- Lane 5: transconjugant \textit{B. megaterium var phosphaticum} (+ve PCR Product)
- Lane 6: \textit{B. megaterium var phosphaticum} (+ve PCR product)
Figure 2. Agarose gel electrophoresis. Electrophoresis was performed on (2%) agarose gel showing the PCR amplicon of the *nifU* (9302bp), *nifV*(1146 bp) and Fv (550bp).

Lane 1: DNA Ladder 100bp,
lane 2: *A. chroococcum* (+ve PCR product),
lane 3: *A. chroococcum* (+ve PCR product),
lane 4: *A. chroococcum* (+ve PCR product),
lane 5: transconjugant *B. megaterium var phosphaticum* (+ve PCR product),
lane 6: transconjugant *B. megaterium var phosphaticum* (+ve PCR product) and
lane 7: transconjugant *B. megaterium var phosphaticum* (+ve PCR product).

Figure 3. Agarose gel electrophoresis. Electrophoresis was performed on (2%) agarose gel showing the PCR amplified products of the *nifH1* (1102bp), *nifH2*(246 bp), *nifU* (9302bp), *nifV*(1146 bp) and FV (500bp).

lane 1: DNA marker 100bp,
lane 2: *B. megaterium var phosphaticum* (-ve PCR product),
lane 3: *B. megaterium var phosphaticum* (-ve PCR product),
lane 4: *B. megaterium var phosphaticum* (-ve PCR product),
lane 5: *B. megaterium var phosphaticum* (-ve PCR product) and
lane 6: *B. megaterium var phosphaticum* (-ve PCR product)
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