Enhancement of the Chitinolytic Properties of *Azospirillum* Strain against Plant Pathogens via Transformation.

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Abstract: Antagonistic rhizobacteria were identified using biochemical profiling as *Pseudomonas aeroginosa* (D), *Azospirillum sp.* in our previous study. In this study, three *Azospirillum sp.* and *Pseudomonas aeroginosa* (D) strains were described for their plasmid content. Different plasmid profiles were found. Plasmid numbers were ranged from one to five per tested strains. Five plasmids have been found in *P. aeruginosa* (D) strain, four plasmids were found in *A. lipoferum* (N) strain and one plasmid with the same size was found in each of *A. brasilense* (B) and (T) isolates. Plasmid curing was performed to locate the antagonistic properties on plasmids or bacterial chromosomes. The results of antagonistic test against *Fusarium solani* indicated that seven cured colonies of *A. brasilense* (T) strain were still showing antagonistic activity as the original strain, only one cured colony showed greater antagonistic activity. The *A. lipoferum* (N) colonies lacking plasmid showed a stronger antagonistic activity against tested pathogen than the original ones. Bacterial transformation technique was performed to transfer the *chi* -gene responsible for chitinase enzyme activity from *Bacillus cereus* Bc Nv-29 to *Azospirillum lipoferum* (N) and *Pseudomonas aeruginosa* (D). Transformants harboring chitinase gene were produced by about 2% of treated bacterial cells in both cases. [Journal of American Science 2010;6(9):169-176]. (ISSN: 1545-1003).

Keywords: Plasmid profile, plasmid curing, antagonism test and transformation.

1. Introduction

Biological control is considered as an alternative or a supplemental way of reducing the chemicals in agriculture [40]. Phylogenetically diverse microorganisms can act as natural antagonists of varies plant pathogens [9].

Plant growth-promoting bacteria (PGPB) control the plant damage by phytopathogens, through different mechanisms including: outcompeting the phytopathogen, physical displacement of the phytopathogen, secretion of siderophores to prevent pathogens in the immediate vicinity from proliferating, synthesis of antibiotics, synthesis of a variety of small molecules that can inhibit phytopathogen growth, production of enzymes that inhibit the phytopathogen and stimulation of the systemic resistance of the plant. Catecholate types of siderophore are produced by *Azospirillium lipoferum* [31, 32] and by *Pseudomonas* sp. [42].

Azospirillum spp. are not known as typical biocontrol PGPBs; *A. brasilense* lacks the capacity to produce significant amounts of antibacterial substances (apart from some bacteriocins and siderophores and cyanide (HCN) [25, 33, 31,16]. This bacterium is unable to induce systemic resistance in plants [6]. Most biological control effects reported earlier [5], *Azospirillum* spp., indirectly suppressed some pathogens, suggesting potential for *Azospirillum* inoculation in prevention programs. The possible mechanisms used by *Azospirillum* to reduce damage inflicted by pathogens that have been demonstrated so far are related to environmental competition and displacement of pathogens, inhibition of seed germination of parasitic weeds, general enhancement of plants to resist pathogen infection and possible inhibition of fungal growth via the production, at least *in vitro*, of microbial toxic substances.

Chitinases are antifungal proteins their expression in *Azospirillum* would help to develop strains with potential antifungal activities [19].

P. aeroginosa (D) and *Azospirillum sp* were isolated from tomato and bean fields and characterized as antagonistic against different plant pathogens as *Alternaria* and *Fusarium* species under *in vitro* conditions [12].

In this study the role of the indigenous plasmids of *P.aeroginosa* (D) and *Azospirillum sp* isolates in fungal antagonistic property will be clarified. This study aimed also to produce more efficient bacterial transformants in controlling the plant pathogen *F. solani* by chitinase gene transfer.

2. Material and Methods

Microbial strains, media and culture conditions:

A. brasilense (B), *A. brasilense* (T), *A. lipoferum* (N) and *P. aeruginosa* (D) isolates were obtained from previous study (El-Gebally *et al.* [12]).

Chitinolytic *Bacillus cereus* BcNv-29 strain was obtained from El-Hamshary and Khattab [13]). *Fusarium solani* was kindly obtained from El-Hussini, Soil Microbiology Unit., Desert Research Center, El-Mataria, Cairo, Egypt. Luria- Bertani medium (LB) (Davis *et al.* [11]) was used for bacterial growth. Potato Dextrose agar (PDA) medium (Burr *et al.* [8]) was used for fungal growth. M9 medium was used for detection of chitinase activity (Armentraut and Brown, [3]). The following concentrations of antibiotics were used when required: Ampicilin (Amp) 100 µg/ml and chloramphenicol (Cm) 35 µg/ml.

Detection of antagonistic activity:

Antagonistic properties of tested bacterial strains against *Fusarium solani* was done according to the method of Agarry *et al.* [2]. Bacteria were grown in LB broth at 30˚C for 24 h, they were then centrifuged at 3900 rpm for 30 min. The supernatant was used immediately. A volume of 25 ml PDA medium was inoculated with 1.0 ml of fungal growth and poured into Petri dish. Wells were made in the solidified agar using cork borer and 0.3 ml of culture supernatant of the tested bacterium was poured into each well at the center. The plates were incubated at 28˚C from 2 to 4 days and examined for clear inhibition zone around the well. The assay was carried out in duplicate for all the tested organisms.

Plasmid isolation:

Indigenous plasmids of *A. brasilense* (B), *A. brasilense* (T), *A. lipoferum* (N) and *P. aeruginosa* (D) were isolated using mini prep method of Rodriguez and Tait[5]. They were then analyzed by agarose gel electrophoresis (Maniatis *et al.* [21]).

Plasmid curing:

A. brasilense (B) Amp^r, *A. brasilense* (T) Cm^r, *A. lipoferum* (N) Amp^r Cm^r and *P. aeruginosa* (D) Amp^r Cm^r isolates were cured from their own plasmids using elevated temperature method (36˚C) (Toama *et al.* [34]). Then, appropriate dilution was spread on LB plates and incubated at 30˚C. Random single colonies were picked up and tested for their sensitivity against ampicilin or chloramphinicol.

Preparation of colloidal chitin:

Colloidal chitin was prepared from commercial chitin by the method of Roberts and Selitrennikoff [27] with a few modifications described as follows: Five gram of chitin powder was added slowly in to 60 ml of concentrated HCl and left at 4˚C overnight with vigorous stirring. The mixture was added to 2 liters of ice-cold 95% ethanol with rapid stirring and kept overnight at 25˚C. The precipitate was collected by centrifugation at 5000 rpm. for 20 min. at 4˚C and was washed with sterile distilled water until the colloidal chitin became neutral (PH 7.0). Colloidal chitin solution (5%) was prepared and stored at 4˚C until further application.

Bacterial transformation for chitinolytic activity:

Induction of competent cells and transformation of *A. lipoferum* (N) or *P. aeruginosa* (D) was performed according to Sambrook *et al.* [30]. Bacterial strain was inoculated in 100 ml LB broth medium and grown with shaking at 150 rpm over night. Ten ml of the culture was then transferred to 50 ml of LB broth medium, incubated at 30˚C with vigorous shaking until $O.D_{650}$ = 0.35 (~ 90 min.) was obtained. The culture was placed on ice for 10 min. and centrifuged at 5000 rpm for 10 min. at 4˚C. The pellet was suspended gently in 25 ml of ice cold 50 mM $CaCl_2$ and placed on ice for 20 min. centrifuged at 5000 rpm for 10 min. The pellet was resuspended very gently in 5 ml of 50 mM $CaCl_2$.

One hundred µl of competent cells and 40 µl of purified fragmented donor DNA solution were mixed in sterile Eppendorf tube. The content was incubated on ice for 1h., subjected to heat shock at 42ºC for 1.5-2.0 min. and kept on ice for 5 min. The content was completed to 1 ml with sterile LB broth medium and incubated at 30ºC for 1h. Distilled water was used instead of DNA as a control.

Detection of chitinolytic activity:

Cells of the parental strain and their transformants were inoculated on a chitinase detection agar plate which was prepared by mixing 1 g of colloidal chitin and 2 g agar in M9 medium. The plates were incubated at 30º C and clearing zones were detected around the colonies.

DNA Isolation, Manipulation and PCR Amplification:

Genomic DNA was isolated from the chitinolytic *Bacillus cereus* Bc Nv-29 strain using DNeasy genomic DNA isolation kit (Qiagen). Agarose gel electrophoresis was performed as described by (Sambrook *et al.* [31]) for DNA analysis. Polymerase Chain Reaction (PCR) technique was performed with Taq polymerase (Fermentas) according to (El-Hamshary *et al.* [14]).

Primer Design and PCR:

Polymerase chain reaction (PCR) technique was performed for amplification of a conserved region of chitinase gene. A set of primers were designed based on sequencing alignment of chitinase producing Bacilli (ac: D10594; D63702; AB04193; EF103273; AF275724; AF510723; EF040226;

M57601; AF265220 and AB110081). The conserved region of aligned nucleotide sequences were used to design degenerate 20-mer oligonucleotides forward and reverse PCR primers F: 5 ' TTCA (T/C) GTTCAACACTACAA3 '; R: 5'CATTAGGCCGCGGA (A/G) TG3', respectively. PCR was performed in 50 µl reactions containing 50 ng of template DNA, 10 pmoles of each primer, 200 µM of each dNTP, standard buffer (supplied with the *Taq*) containing 1.5 mM MgCl2, and 1 U of *Taq* DNA polymerase, in thermal cycler, as follows: initial denaturing step 94 ºC for 3 min., then (30 cycles) comprised a denaturing step at 94ºC for 1 min, annealing at 50 ºC for 1 min and extension at 72 ºC for 1.5 min. followed by final extension 72 ºC for 7 min..

3. Results

Plasmid patterns of *Azospirillum* isolates and *P. aeruginosa* (D)

The plasmids number of *Azospirillum* and *P. aeruginosa* D were determined using the method described by Rodryguez and Tait [5]. The plasmid patterns of the four isolates are presented in Fig. (1). the results showed different plasmid patterns among the four isolates, the plasmid numbers were ranged from one to five plasmids. Five plasmids has been found in *P. aeruginosa* (D) isolate, four plasmids were found in *A. lipoferum* (N) isolate and one plasmid with the same size was found in each of *A. brasilense* (B) and (T) isolates.

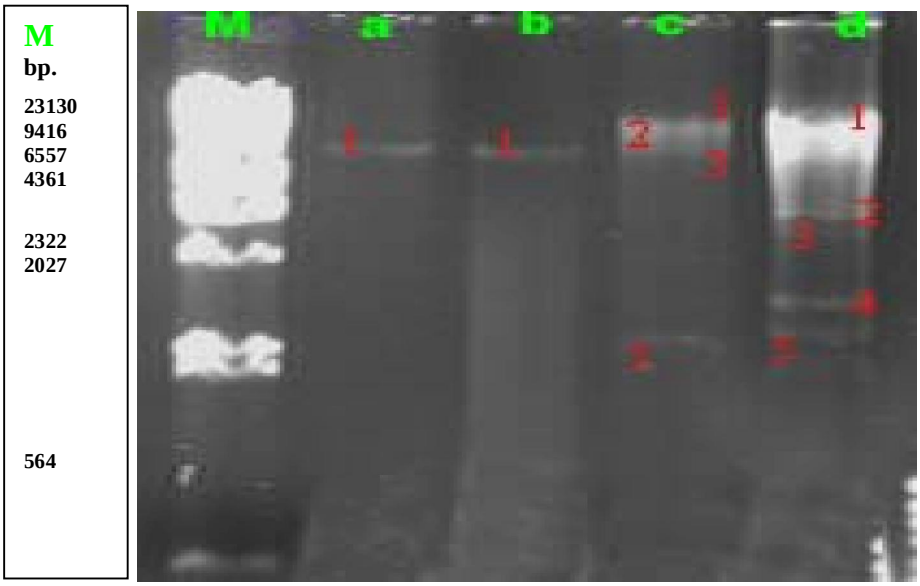

Fig. 1: Plasmid patterns of A. *brasilense* (B) (lane a); *A. brasilense* (T) (*lane* b); *A. lipoferum* (N) (lane c); *P. aeruginosa* (D) (lane d) and lane M; Lambda DNA *Hind III* marker.

Investigation of plasmid coded function (plasmid curing test)

Elevated temperature (36˚C) procedure was used to cure each of the four bacterial isolates from their plasmid (s). After treatment single colonies were isolated from each strain and checked for their antibiotic resistance patterns comparing with their original strains. A total of 24 colonies were isolated from *P. aeruginosa* (D), of which 9 colonies were sensitive to each of Amp^s and Cm^s. Among 14 colonies isolated from *A. lipoferum* (N), 7 colonies were sensitive to Amp^s and Cm^s, Among 14 colonies isolated from *A. brasilense* (B) of which 5 colonies were sensitive to ampicilin (Amp^s). Among 14 colonies isolated from *A. brasilense* (T) of which 7 colonies were sensitive to chloramphinicol (Cm^s) (Table 1). The plasmid curing efficiency was 38%, 50%, 50% and 36 %, respectively (Table 1). These isolates were confirmed as cured of their plasmid by the absence of one plasmid from *A. lipoferum* (N), one plasmid from *A. brasilense* (T) and three plasmids of *P. aeruginosa* (D) derivative.

Table 1: Antibiotic sensitivity of single colonies obtained after elevated temperature treatment of *A. brasilense* (B), *A. brasilense* (T), *A. lipoferum* (N) and *P. aeruginosa* (D) strains.

Bacterial strains	NO. **of tested colonies obtained after curing**	NO. **of** Amp^s	NO. **of** Cm^s	NO. **of** Amp^s and Cm^s	**plasmid curing %**
***A. brasilense* B**	**14**	**5**	**-**	**-**	**36 %**
***A. brasilense* T**	**14**	**-**	**7**	**-**	**50%**
***A. lipoferum* N**	**14**	**-**	**-**	**7**	**50%**
***P. aeruginosa* D**	**24**	**-**	**-**	**9**	**38%**

A. brasilense (B) Amp^r; A. brasilense (T) Cm^r; A. lipoferum (N) Amp^r, Cm^r and P. aeruginosa (D) Amp^r, Cm^r

Antagonistic test of *A. brasilense* (T) and *A. lipoferum* (N) after plasmid curing:

Antifungal activity of the bacterial isolates and their cured strains, against *Fusarium solani* is shown in Table (2). The results indicated that each cured colony of *A. brasilense* (T) exhibited antagonistic activity as the original strain except the cured strain number 2 which showed greater antagonistic activity. Moreover, all cured strains of *A. lipoferum* (N) showed a stronger antagonistic activity than the original strain.

Table 2: Antagonistic test for the four isolates against *Fusarium solani* after plasmid curing**.**

Bacterial isolates	**Inhibition zone**	**Note**
***A. brasilense* T**	+	**Original strain**
Cured 1	+	**Cured**
Cured 2	++	
Cured 3	+	
Cured 4	+	
Cured 5	+	
Cured 6	+	
Cured 7	+	
***A. lipoferum* N**	+	**Original strain**
Cured 1	++	**Cured**
Cured 2	++	
Cured 3	++	
Cured 4	++	
Cured 5	++	
Cured 6	++	
Cured 7	++	

(+) inhibition zone dimeters from 0.5 to 0.7 cm.
(++) inhibition zone dimeters from 0.8 to 1.0 cm.

A. lipoferum (N) and *P. aeruginosa* (D) transformation:

DNA of *Bacillus cereus* Bc Nv-29 was mechanically fragmented to the appropriate fragment lengths for transformation, i.e., from 1 to 3 kb by passing through a 27 gauge needle, using a syringe several times up to 100 times. The length of DNA-fragments produced was then detected by agarose–gel electrophoresis, DNA was stained with ethidium bromide and visualed under UV-transilluminator using a λ-DNA-*hind III* digested as standard molecular weight (Abdel Aal, [1]. This DNA was used to transform each of *A. lipoferum* (N) and *P. aeruginosa* (D) according to Sambrook *et al.* [30]. Results showed that passing the DNA sample 60 times through the needle produced the appropriate fragment length for transformation, where most of the DNA fragments were between 1 to 3 kb in length. The DNA concentration was adjusted to one hundred microgram of fragmented DNA in 40 µl sterilized distilled water. *A. lipoferum* (N) and *P. aeruginosa* (D) competent cells were mixed with DNA.

Transformants acquiring *chi*-gene were selected using M9 agar plates supplemented with 1% chitin where transformants showed clear zone around the colonies (Fig.2).

The results showed that three colonies out of one hundred forty colonies of *A. lipoferum* N and three colonies from *P. aeruginosa* D hydrolyzed colloidal chitin after two weeks (Fig. 2). The three *A. lipoferum* N transformants and the three *P. aeruginosa* D transformants had less chitinase activity than *B. cereus* Bc Nv-29.

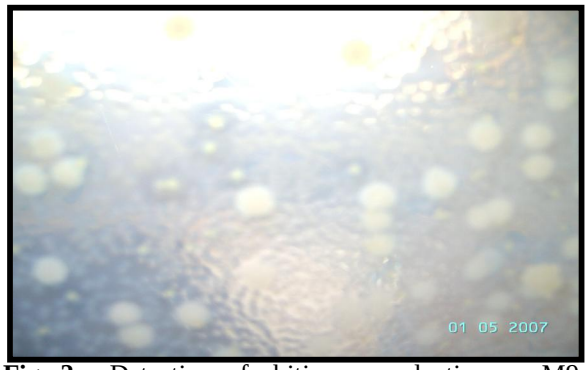

Fig. 2: Detection of chitinase production on M9 medium containing 1 % colloidal chitin.

In a program for molecular screening of chitinase producing strain, PCR primers were deigned according to alignment data of chitinase nucleotide sequences of different species of Bacilli as described previously. The primers were used to amplify a conserved region of the gene (310 bp). The PCR reaction was carried out on genomic DNA of two transformants and local *Bacillus cereus* Bc Nv-29 strain as positive control**,** where transformants strains as well the local isolate showed the amplification fragment with an expected molecular size (310 nucleotides) (Fig. 3).

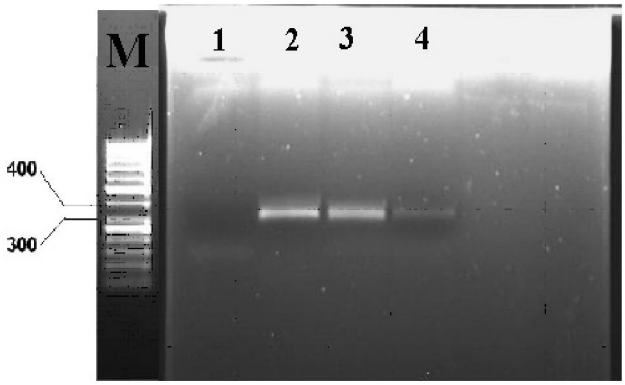

Fig.3: Agarose gel electrophoresis analysis of PCR products. M: 100 bp DNA marker; Lane 1: Negative control; Lane 2: local isolate *Bacillus cereus* Bc Nv-29 strain; *Lane* 3 and 4: *A. lipoferum* (N) and *P.aeruginosa* (D) transformants respectively.

4. Discussion

Research on the mechanisms of biocontrol employed by effective bacterial strains has revealed a variety of natural products that can be exploited for the development of chemical control measures.

Azospirillum spp. have moderate biocontrol capabilies against crown gall disease, bacterial leaf blight of mulberry and bacterial leaf and/or vascular diseases of tomato [29] . In addition, *A. brasilense* can restrict the proliferation of other non pathogenic rhizospher bacteria [17]. Some reports indicated that the protective effect of *Azospirillum* may be indirectly explained by plant growth promotion effect as by outcompeting other bacterial hosted by the same plant [4].

The plasmids number of *Azospirillum* and *P. aeruginosa* (D) were determined. Molecular plasmid profile studies of *Azospirillum* and *P. aeruginosa* (D) strains indicated different plasmid patterns among the four strains, the plasmid numbers were ranged from one to five plasmids. *A. brasilense* (B) and *A. brasilense* (T) showed only one plasmid with the same size; *A. lipoferum* (N) showed four plasmids and *P. aeruginosa* (D) showed five plasmids. *A. brasilense* and *A. lipoferum* contain several plasmids with size ranging from 40 kbp to 550 kpb [41].

Curing of the plasmids of *A. brasilense* (B), *A. brasilense* (T), *A. lipoferum* (N) and *P. aeruginosa* (D) was done using elevated temperature (36˚C). It was obvious that *Azospirillum brasilense* (B) showed resistance to ampicilin, *A. brasilense* (T) showed resistance to chloramphinicol. On the other hand, *A. lipoferum* (N) and *P. aeruginosa* (D) exhibited resistance to ampicilin and chloramphinicol.

The results indicated that Among 24 colonies were isolated from *P. aeruginosa* (D), 9 colonies were sensitive to each of Amp^s and Cm^s. Among 14 colonies isolated from *A. lipoferum* (N), 7 colonies were sensitive to Amp^s and Cm^s, Among 14 colonies isolated from *A. brasilense* (B), 5 colonies were sensitive to ampicilin (Amp^s). Among 14 colonies isolated from *A. brasilense* (T) of which 7 colonies were sensitive to chloramphinicol (Cm^s).

The plasmid curing efficiency was 38%, 50%, 50% and 36 %, respectively. These isolates were confirmed as cured of their plasmid by the absence of one plasmid from *A.lipoferum* (N) derivative, one plasmid from *A. brasilense* (T) derivative and three plasmids of *P. aeruginosa* (D) derivative. *A. lipoferum* (N) and *P. aeruginosa* (D) strains are resistant to antibiotic ampicilin (Amp^r) and chloramphenicol (Cm^r) and it was observed that ampicilin and chloramphenicol resistance genes are associated with the plasmids since curing the isolates of its plasmids made it Amp^s or Cm^s sensitive.

The different curing efficiencies obtained reflect the difficulty of curing plasmid where plasmids of high molecular weight are more difficult to be eliminated [18].

All *A. brasilense* and some *A. lipoferum* strains have 90-MDa plasmids (p 90), that share conserved regions and carry several genes involved in the *A. brasilense*-plant root interaction [27, 10]. Croes *et al*. [10] demonstrated that P90, of *A. brasilense* and *A. lipoferum*, carries genes involved in motility, adsorption to roots, colony morphology and growth on minimal media. Such plasmid contains two different loci conferring resistance to ampicillin [26] and two loci involved in chemotaxis [37].

Plasmid patterns of cured strains showed the loss of a large plasmid in derivatives from *A. brasilense* UAP 02 strain, whereas in clones from *A. brasilense* UAP 29 strain significant deletions in the plasmids were observed. The large bacteriocinogenic plasmid harbored by UAP 02 strain was designated pBc02. The gene (s) coding for the production of inhibitory substance(s) became derepressed [39].

Antifungal activity of the bacterial and their cured strains, against *Fusarium solani* was carried out. The results indicated that each cured colony of *A. brasilense* (T) exhibited antagonistic activity as the original strain except the cured strain number 2 which showed greater antagonistic activity, this may be on to the effect of heating treatments on production of mutation concerning the regulatory gene of the antagonism.

Moreover, all cured strains of *A. lipoferum* (N) showed a stronger antagonistic activity than the original strain. This may be indicating the existence of a regulatory gene (s) on one or more of indigenous plasmids. These genes repressing antifungal activity, by curing this plasmid, the antifungal activity is enhanced.

In the present investigation, there was variation in the ability of bacterial isolates, after plasmid curing, to form inhibition zone, plasmid-less cells displayed different antagonistic properties toward *Fusarium solani*. The regulation of biocontrol traits (production of antifungal metabolites, chitinases and biosurfactants) by phase variation was reported for *Pseudomonas* spp. strains [36]. Phase variation and DNA rearrangement phenomena in *Azospirillum* strains were investigated by Vial *et al*. 2006[38].

They showed large-scale genomic rearrangements in *Azospirillum* strains and correlates them with phase variation. The molecular origin for lipopolysaccharide phase variation in *Legionella pneumophila* relies on a 30-kb unstable element located on the chromosome in the wild type, whereas excision from the chromosome and replication as a high-copy plasmid result in the avirulent phenotype variant [20]. Moreover, major DNA rearrangements between the chromosome and plasmids that are not involved in phase variation were also well documented for many bacteria, including the plant-associated bacterium *Rhizobium*. These genomic rearrangements include deletion, amplification, inversion, and cointegration [23, 43, 24, 16, 7]. Some of these rearrangements have biological consequences [22, 16].

Bacillus cereus Bc Nv-29 was tested for the production of chitinase on M9 agar medium supplemented with colloidal chitin as the sole carbon source. *B. cereus* Bc Nv-29 hydrolyzed colloidal chitin after 72-168 h [13].

To enhance the antagonistic effect of *A. lipoferum* (N) and *P. aeruginosa* (D), bacterial transformation technique was performed to transfer the *chi* -gene responsible for chitinase enzyme activity from chitinolytic *B. cereus* Bc Nv-29 strain to *A. lipoferum* (N) and *P. aeruginosa* (D) antagonistic isolates.

Transformants acquiring *chi*-gene were selected using M9 agar plates supplemented with 1% chitin where transformants showed clear zone around the colonies.

The results showed that three colonies out of one hundred forty colonies of *A. lipoferum* (N) and three colonies from *P. aeruginosa* D hydrolyzed colloidal chitin after two weeks. In a program for molecular screening of chitinase producing strain, PCR primers were deigned. The primers were used to amplify a conserved region of the gene (310 bp). The PCR reaction was carried out on genomic DNA of *P.aeruginosa* (D) and *A. lipoferum* (N) transformants and local *B. cereus* Bc Nv-29 strain as positive control, where transformants strains as well the local isolate showed the amplification fragment with an expected molecular size (310 nucleotides). Transformants harboring chitinase gene were produced by about 2% of treated bacterial cells in both cases.

5. Conclusions

The present study addressed the ability of the A. *lipoferum* (N) and *P. aeruginosa* (D) transformants to produce the chitinase enzyme. This transformants will be tested for inhibition of growth of deferent plant pathogens.

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References

1. Abd EL-Aal, S. Kh. (1992). The effect of nucleotide sequences repetition of a dicotyledonous plant on microbial genetic repair. Ph. D. Thesis. Genetics, Agric. Botany Dept., Fac. of Agric., AL-Azhar University.
2. Agarry, O.O.; Akinyosoye, F.A. and Adetuyi, F. C. (2005). Antagonistic properties of microorganisms associated with cassava (Manihot esculenta Crantz) prouts. African J. Biochnol., 4 (7):627-632
3. Armentraut, R. W. and Brown, R. D. (1981). Molecular cloning of genes for cellobiose utilization and their expression in *Escherichia coli*. Appl. Environ. Microbiol., 41: 1355-1362.
4. Bashan, Y. and de-Bashan, L. E. (2002). Protection of tomato seedlings against Infection by *Pseudomonas syringae* pv. Tomato by using the plant growth-promoting bacterium *Azospirillum brasilense*. Appl. Environ. Microbiol., 68(6):2637–2643.
5. Bashan,Y.and Holguin, G. (1997). *Azospirillum*/plant relationships: environmental and physiological advances (1990 -1996). Can. J. Microbiol., 43:103-121.
6. Bashan, Y. (2001). Reduction of bacterial leaf speck disease (*Pseudomonas syringae* pv. tomato) in tomatoes treated with a combination of *Azospirillum brasilense*, bactericides and mild heat, p. 296–300. *In* S. H. De Boer (ed.), Plant pathogenic bacteria. Kluwer Academic Publishers, Dordrecht, the Netherlands.
7. Brom, S.; Girard, L.; Tun-Garrido, C.; Garcia-de los Santos, A.; Bustos, P.; Gonzalez, V. and Romero, D. (2004). Transfer of the symbiotic plasmid of *Rhizobium etli* CFN42 requires cointegration with p42a, which may be mediated by site-specific recombination. J. Bacteriol. 186:7538–7548.
8. Burr, I. J.; Schroth, M. N. and Suslow, T. (1978). Increased potato yields by treatment of seed pieces with specific strain of *Pseudomonas fluorescens* and *Pseudomonas putida*. Phytopathol., 68:1377-1383.
9. Cook, R. J. (2000). Advances in plant health management in the 20th century. Annu. Rev. Phytopathol., 38:95-116.
10. Croes, C. L.; Moens, S.; Van Bastelaere, E.; Vanderleyden, J. and Michiels, K. W. (1993). The polar flagellum mediates *Azospirillum brasilense* adsorption to wheat roots. J. Gen. Microbiol., 139:2261-2269.
11. Davis, R.W.; Botstein, D. and Roth, J.R. (1980). A Manual for Genetic Engineering Advanced Bacterial Genetics, Cold Spring Harbor Lab. Cold spring habor, New York. pp.
12. El-Gebally, O.; Abou-El-Khier, Z.A.; El-Hamshary, O.I.M.; Arafa, R. A.; Mousa, Sh. A. and Awad, N.S. (2008). Enhancement of the antagonistic properties of *Azospirillum* strains against plant pathogens via cell manipulation. Egyptian J. of Applied Science, 23: (10B).
13. El-Hamshary, O.I.M. and Khattab, A.A. (2008). Evaluation of Antimicrobial Activity of *Bacillus subtilis* and *Bacillus cereus* and Their Fusants against *Fusarium solani*. Research Journal of Cell and Molecular Biology, 2(2): 24-29.
14. El-Hamshary, O. I. M.; Halima H. Salem and Nadia A. Soliman (2008). Molecular screening of chitinase gene in *Bacillus spp*. Journal of Applied Sciences Research, 4 (9): 1118-1123.
15. Gonçalves, A.F.S. and de Oliveira, R.G.B. (1998). Cyanide production by Brazilian strains of *Azospirillum*. Rev. Microbiol. 29: 36–39.
16. Guo, X.; Flores, M. ; Mavingui, P.; Fuentes, S. I. ;Herna´ndez, G.; Da´vila, G. and Palacios, R. (2003). Natural genomic design in *Sinorhizobium meliloti* novel genomic architectures. Genome Res. 13:1810–1817.
17. Holguin, G. and Bashan, Y. (1996). Nitrogen-fixation by *Azospirillum brasilense* CD is promoted when co-cultured with a mangrove rhizosphere bacterium (*Staphylococcus* sp.). Soi. Biol. Biochem., 28:1651–1660.
18. Hynes, M.F. and McGregor, N.F. (1990) Two plasmids other than the nodulation plasmid are necessary for formation of nitrogen-fixing nodules by *Rhizobium leguminosarum*. Molecular Microbiology 4: 567-574.
19. Jayara, J., Muthukrishnan, S. and Liang, G. H. (2004). Transfer of a plant chitinase gene into a nitrogen-fixing *Azospirillum* and study of its expression. Microbiol., 50 (7): 509–513.
20. Lu¨neberg, E.;Mayer, B. ;Daryab, N. ;Kooistra, O. ;Zahringer, U.; Rohde, M. ;Swanson, J. and Frosch, M. (2001). Chromosomal insertion and excision of a 30 kb unstable genetic element is responsible for phase variation of lipopolysaccharide and other virulence determinants in *Legionella pneumophila*. Mol. Microbiol. 39:1259–1271
21. Maniatis, T.; Fritsch, E. F. and Sambrock, J. (1982). Molecular Cloning, a Laboratory Manual. Cold Spring Harbor Lab. Cold Spring Habor, New York.

22. Mavingui, P.; Flores, M.; Romero, D.; Martinez-Romero, E. and Palacios, R. (1997). Generation of *Rhizobium* strains with improved symbiotic properties by random DNA amplification (RDA). Nat. Biotechnol. 15:564–569.
23. Mavingui, P.; Laeremans, T.; Flores, M.; Romero, D.; Martinez-Romero, E. and Palacios, R. (1998). Gene's essential for Nod factor production and nodulation are located on a symbiotic amplicon (AMP*Rtr*CFN299pc60) in *Rhizobium tropici*. J. Bacteriol. 180:2866–2874.
24. Mavingui, P.;Flores, M. Guo, X.; Davila, G.; Perret, X.; Broughton, W. J. and Palacios, R. (2002). Dynamics of genome architecture in *Rhizobium* sp. Strain NGR234. J. Bacteriol. 184:171–176.
25. Oliveira, R. G. and Drozdowicz, A. (1987). Inhibition of bacteriocin producing strains of *Azospirillum lipoferum* by their own bacteriocin. Zentbl. Mikrobiol. 142:387–391
26. Onyeocha, I.; Vieille, C.; Zimmer, W.; Baca, B. E.; Flores, M.; Palacios, R. and Elmerich, C. (1990). Physical map arid properties of A 90, MDa plasmid of *Azospirillum brasilense* Sp7. Plasm., 23:169-182.
27. Roberts, W. K. and Selitrennikoff, C.P. (1988). Plant and bacterial chitinases differ in antifungal activity J. Gen. Microbiol., 134:169-176.
28. Rodriguez, R. L. and Tait, R.C. (1983). Recombinant DNA Techniques: An Introduction. The Benjamin/ Cummings Publishing Company, Inc.,Menlo Park, California, Massachusetts, London, Amstedam,Don Mills, Ontrio, Sydney.
29. Romero, A. M.; Correa, O. ; Moccia, S. and Rivas, J. G. (2003). Effect of *Azospirillum*-mediated plant growth promotion on the development of bacterial diseases on fresh-market and cherry tomato. J. Appl. Microbiol., 95:832–838.
30. Sambrook, J.; Fritsch, E. F. Maniatis, and T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. Cold Spring Harbor Laboratory press., pp. 1-82.
31. Shah, S.; Karkhanis, V. and Desai, A. (1992). Isolation and characterization of siderophore, with antimicrobial activity, from *Azospirillum lipoferum* M. Curr. Microbiol. 25:34–35.
32. Shah, S., Karkhanis, V. and Desai, A. (1993). Production of catecholate type of siderophore by Azospirillum *lipoferum*. Indian J. of experim. Biol. 31(1): 41-44.
33. Tapia-Hernandez, A., M. A. Mascarua-Esparza, and J. Caballero-Mellado (1990). Production of bacteriocins and siderophore-like activity in *Azospirillum brasilense*. Microbios. 64:73–83.
34. Toama, M.A.; El-Kersh,T.A. and Ahmady, A.M. (1983). Antibiotic resistance pattern and resistance curing of *E.coli* locally isolated. Bull.Fac.Pharm.,Cairo Univ. 22 (1): 129.
35. van den Broek, D.;Chin-A-Woeng, T. F.; Eijkemans, K. ;Mulders, I. H.; Bloemberg, G. V. and Lugtenberg, B. J. (2003). Biocontrol traits of *Pseudomonas* spp. are regulated by phase variation. Mol. Plant-Microbe Interact. 16:1003–1012.
36. Vande-Brock, A.; van Gool, A. and Vanderleyden, J. (1989). Electroporation of *Azospirillum brasilense* with plasmid DNA. FEMS Microbiol. Lett., 61:177 -182.
37. Van Rhijn, P.; Vanstockem, M.; Vanderleyden, J. and De Mot, R. (1990). Isolation of behavioral mutants of *Azospirillum brasilense* by using Tn5-*lacZ*. Appl. Environ. Microbiol., l4: 990-996.
38. Vial, L.; Lavire, C.; Mavingui, P.; Blaha, D.; Haurat, J.; Moe¨nne -Loccoz, Y.; Bally, R. and Wisniewski-Dye, F. (2006). Phase Variation and Genomic Architecture Changes in *Azospirillum*. Bacteriol, 188:5364-5373.
39. Velazquez-Cruz, C.; Tapia-Hernandez, A.; Mascarua-Esparza, M.A.; Caballero-Mellado, J. and Baca, B.E. (1992). Plasmid profile modification after elimination of bacteriocin activity in Azospirillum brasilense strains. Microbios. 69(280-281) 195-204.
40. Welbaum, G., Sturz, A. V. Dong, Z. and Nowak. J. (2004). Fertilizing soil microorganisms to improve productivity of agroecosystems. Crit. Rev. Plant Sci. 23:175–193.
41. Wood, A. G.; Menezes, E. M.; Dykstra, C. and Duggan, D. E. (1982). Methods to demonstrate the megaplasmids (or mini chromosomes) in *Azospirillum*. In W. Klingmu¨ller (ed.), *Azospirillum* I: genetics, physiology, ecology. Burkha¨user Verlag, Basel, Switzerland, P. 18.
42. Yeole, R.D.; Dave, B.P.; Dube, H.C. (2001). Siderophore production by fluroscent *Pseudomonas* colonizing roots of certain crop plants. Indian Journal of Experimental biology 39: 464-468.
43. Zhang, X. X., Kosier, B. and Priefer, U. B. (2001). Symbiotic plasmid rearrangement in *Rhizobium leguminosarum* bv. viciae VF39SM. J. Bacteriol. 183:2141–2144.

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