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RESEARCH ARTICLE

Ultrasound Stimulate Conjugation Between Wild Type and Genetically Engineered *Agrobacterium tumefaciens* and *Sinorhizobiummeliloti*

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Abstract

Bacterial conjugations represent one of possibilities that transform bacteria. This study aimed to detect the occurrence of conjugation between two genera of bacteria includes wild strain A.tumefaciens (Atmco1), standard strain *A. tumefaciens* (C58Cl) and *Sinorhizobiummeliloti*. The results proved the conjugation through the sensitivity of those bacteria to specific antibiotics. The obtained data reported the stimulation of bacteria growth when they exposed to different ultrasound treatments. These ultrasound treatments include five period (0.0, 240, 480, 720 and 900) second for (47.6) KHZ. The treatments (240, 480) s increase colonies numbers between 25-72% when bacteria grown on the surface of the specific solid medium. The interesting results of this study are that the exposures of the conjugation mixture to the ultrasound treatment increase the Trans conjugant colonies between (23-80) % and increase the conjugation frequency twice. This may be probably as akey to enhance conjugation between those of bacteria species which are not able to conjugation.

Introduction

Rhizobium is a soil living bacteria, Gram negative motile bacilli not spores forming grow at 25-30°C [1]. Each species of Rhizobia live symbiotically with one species of dicotyled forming nitrogen fixing nodules [2]. Most of genes required for nodule formation (*nod*) and nitrogen fixation (*nif* and *fix*) are carried on plasmid that is traditionally called the symbiotic plasmid or *pSym* [3, 4]. This plasmid is very large in size and number, the nodulation (*nodABC*) gene, the regulatory (*nodD*) gene and nitrogen (usually ≤ 100kb) symbiotic plasmids (*pSym*) [5]. In contrast *Agrobacterium tumefactions* strains harboring a tumor inducing (Ti) plasmid are the causative agent of grown gall tumor disease in dicotyledonous plant,

The transfer DNA (T-DNA) and virulence gene (*vir)* regions in the Ti plasmid are essential for tumor genesis [6].The *vir* gene products nick the T-DNA region at its left (LB) and right border (RB), and then transfer T-DNA in to the plant cell [7, 8]. Plasmids are a small circular double strand of extra chromosomal DNA replicates autonomously found in bacteria, yeast, fungi, algae and protozoa. Transformation, transduction and conjugation are three main methods for genetic exchange [9].In conjugation P-DNA transfers in one path way from fertile (F^+) donor to fertile (F^-) recipient bacteria through Conjugation Bridge that is usually in gram negative bacteria. This plasmid carries transfer genes, origin and mobilization protein for conjugation [10]. Ultrasound has been identified as a potential novel technique for affecting transformation, because it is recognized as a method responsible for casing biological effects [11]. Cavitation can effect the generation by ultrasound is considered the major mechanism responsible for causing the increase membraine permeability, and possibly bacteria during transformation [12, 13].

These changes are due to the stress induced via ultrasound [14]. That induces the formation of pores in the bacterial membrane that can permit transformation [13]. The pore sizes are 30-100nm, which the membrance recovery period being quite Rapid- a few seconds or at most a minute [12]. The aim of this work is to evaluate whether the ultrasound enhances bacterial conjugation between these two bacteria infected plants.

Materials and Methods

Bacterial Cultures

One local isolates of *A.tumefaciens* (Atmco1), which is isolated by from crown galls formed on stem of *Myrtuscommunis*, *A. tumefaciens* (C58Cl), obtained from (Ugen-VIB Research Belgium). *S. meliloti* which is isolated and curing of plasmid by using acridine orange from nodules of *Medicagostiva* plant [15]. *A. tumefaciens* (Atmcol) was grown on Agrobacterium mannitolmedium (AM) [15] while *A. tumefaciens* (C58Cl) was grown on yeast extract bef medium (YEB) [16] and *S. meliloti* was grown on yeast extract medium (YEM) medium [17].

Antibiotics Used

In this study, twelve types of antibiotics were used as shown in Table (1).

Table 1: Types of antibiotics, their stock and final concentrations

Antibiotics	Stock conc. mg/ml	Final conc. □g/ml	Antibiotics	Stock conc. mg/ml	Final conc. □g/ml
Ampicillin (AMP)	5	10	Erthromycin (Ery)	10	15
Amoxicillin (Amo)	5	25	Rifampicin (Rif)	30	100
Cefotaxime (Cef)	5	30	Streptomycin (Str)	50	25
Gentamycin (Gen)	40	40	Chloramphenicol (Chl)	35	35
Tetracycline (Tet)	5	30	Ciprodar (Cip)	20	30
Trimthprim (Tri)	5	30	Kanamycin (Kan)	30	30

Exposure of Bacterial Suspensions to the Ultrasound Treatment

A stock solution of two isolates *A. tumefaciens*and *S. melliloti* was prepared by removing a loop of bacteria from a mother dish. The loope of these bacteria then was used to inoculate (10) ml of (Am, YEB, YEM) broth. This was placed in shaking incubator at a temperature of (28±2) °C for 24 hours. After 24 hours.

A volume 1.0ml of the three bacterial suspensions, each was exposed to ultrasound treatment using (47.6) KHZ for (0.0, 240, 480, 720, 900) sec to study the effects of this treatment on growth and viability bacteria by measuring of the suspension optical density (OD) [18] and number of coloni which were cultured on the specific medium.

Bacterial Conjugation

One volume (1) ml of *A.tumefaciens* suspension and similar volume of *S.meliloti* suspension were mixed with a same volume of liquid YEM. These mixtures were incubated for three houres at (28)℃ , then (0.1) ml of bacterial mixture was spread on the surface of agar solidified YEM, this media then was supplemented with Cef (30)□g/ml and Rif (100)□g/ml [19] selection of these types of antibiotics depend on the bacteria sensitivity or the resistance to those types of antibiotics.

This was detected by adding the antibiotic to the agar solidified culture medium and then 0.1 ml of the bacteria suspension was streaked on the surface of the media.

Ultrasound Treatment of Conjugation Mixture

Each conjugation mixture was exposed to the selected ultrasound treatment [20] as diagram below, then 0.1 ml of each mixture was spread on the YEM medium.

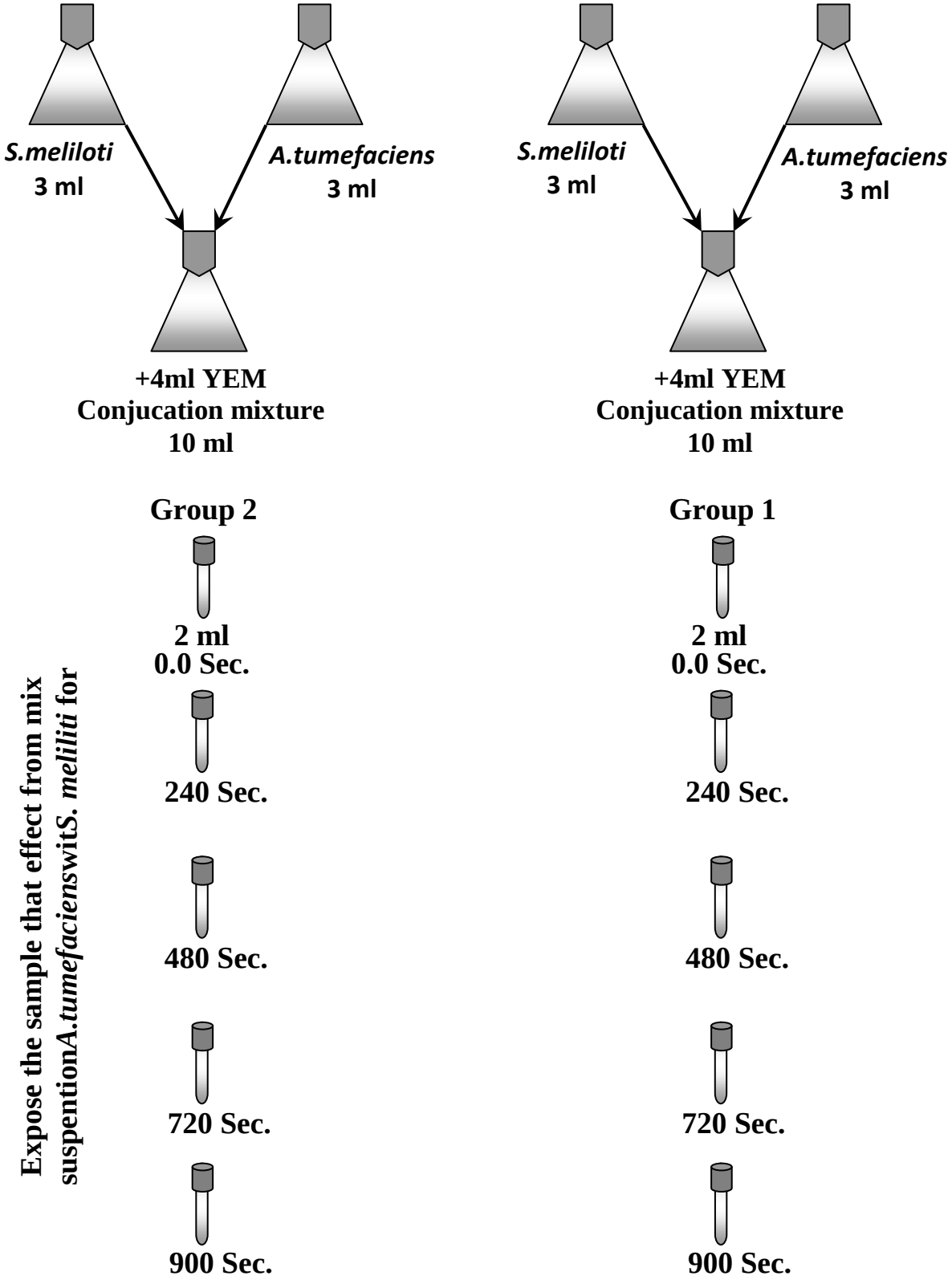

Fig.1: Ultrasound treatment of conjugation mixture

Isolation of DNA Plasmid and Screening

Ti plasmid of *A.tumefaciens* (C58 c1), *A.tumefaciens* (Atmco1) were isolated and extracted as previously described [21].

All cells were transformed (Transconjucat Sinorhizobium) and DNA then suspended in 0.1 X TE buffer. The plasmid content of bacterial strain was analyzed using gel electrophoresis. This method includes adding ethidium bromide at final concentration 0.5 □g/ml to the 0.7% melted agarose [22].

Preparation DNA Template

Template DNA for PCR either by total DNA extraction. To prepare total DNA from overnight broth cultures, I lysed cell in 50 mMtric (pH8). 20 mM EDTA-1 % sodium dodecyl sulfate-500 mMNaCl and performed phenol-chloroform extractions and an ethanol precipition as described by [23]. Next, I dissolved the nucleic acid in 10mM tric (pH 7.5)-0.1 mM EDTA, incubated the DNA-RNA mixture with RNases A and Ti, added ammonium acetate to 25□, and performed a second-ethanol precipitation.

PCR Amplification and DNA Analysis

I add 4□l of DNA templates to the reaction mixtures (25 □l) contained primer oligonucleotides at 10 picomole each, deoxynucleoside triphosphates at 200 mM each, 1U of thermo stable DNA polymerase, reaction cocktail supplied by the manufacturer (perkin-Elmer), 10mM tric [pH 8.3 at 25℃], 50mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin [sigma G 2500]; Epicenter, 50mM tris [pH 9.0 at 25℃], 20mM ammonium sulfate, 1.5 mM $MgCl_2$). PCR is performed by the denaturation at 94℃ for 1minutes, Then annealing at 50℃ for 1 minutes and extension at 72 for 5 minutes and final extension at 72 for 10 minutes. Then what cold to10 ℃.This was repeated for 35 cycles. The PCR products were separated on 2% agarose gel via electrophoresis, stained with ethedim bromide and observed under Uv light.

The ipt primer sequences come from some conserved region, are used as universal primers to detect Agro bacterium isolate and the *Transconjucant rhizobium* isolates the sequence of the sense strand primer, cyt was 5'GATCG (G/C) GTCCAATG(C/T) TGT3' and the sequence of the antisense-strand primer, cyt, was 5'GATATCCATCGATC (T/c) CTT3' this primer pair yields a 427-bP PCR product.

Results

Bacterial Responses towards Antibiotics

The results proved that the different species of bacteria exhibt various responses to the tested antibiotics Table (2);The interested results showed that *S.meliloti* was [Amp, Amo, Cef, Tet, Ery, Str, Chl, Cip]$^{(R)}$& [Gen, Tri, Rif, Kan]$^{(S)}$, whereas *A.tumefaciens* (Atmcol) was [Amp, Amo, Gen, Tet, Tri, Ery, Rif, Str, Chl, Cip]$^{(R)}$& [Cef, Kan]$^{(S)}$. But However, *A.tumefaciens* (C58Cl) was [Amp, Amo, Gen, Tri, Ery, Rif, Str]$^{(R)}$ and [Cef, Tet, Chl, Cip, Kan]$^{(S)}$.

Table 2: Detection the response treatment of bacterial species towards the used antibiotics

Antibiotics	Bacterial responses		
	S. meliloti	***A.tumefaciens* (Atmcol)**	***A.tumefaciens* (*C58Cl*)**
Amp	R	R	R
Amo	R	R	R
Cef	R	S	S
Gen	S	R	R
Tet	R	R	S
Tri	S	R	R
Ery	R	R	R
Rif	S	R	R
Str	R	R	R
Chl	R	R	S
Cip	R	R	S
Kan	S	S	S

S: sensitive, R: Risists

Effect of Ultrasound Treatment on the Growth of Bacterial Species

Results of exposing bacterial suspensions to a group of ultrasound treatment indicate an increase in colonies numbers and optical density. The long term of (240, 480) s effect was clean with *S.meliloti* using the same ultrasound treatment Table (3).

Table 3: Effect of ultrasound treatment of bacterial suspensions of *A.tumefaciens* (Atmcol), *A.tumefaciens* (S8Cl) and *S.meliloti* on their growth

Ultrasound treatment KHZ/Sec	*A.tumefaciens*Atmcol		*A.tumefaciens* Cs8Cl		*S.meliloti*	
	No. of colonies	Optical density	No. of colonies	Optical density	No. of colonies	Optical density
Control	26	1.60	24	1.43	18	1.13
47.6/240	43	2.77	41	2.52	31	1.95
47.6/480	33	2.16	30	1.86	23	1.44
47.6/720	17	1.14	19	1.25	13	0.96
47.6/900	13	0.85	15	0.87	0.0	0.42

Detection of the Transconjugant

The result showed successful conjugation between *A.tumefaciens* (C58C1) or *A.tumefaciens*(Atmcol) (as adonor) and *S.mlitoti* (as a recipient). The obtained transconjugent Rhizobium was $cef^{(R+)}$ and $Rif^{(R)}$ Table (4).These results showed that the genetic elements, responsible for antibiotics resistance transferred from doner to recipient, give strong evidence that plasmid can pass from donor to recipient. In both cases the transconjugant bacteria have acquired new properties and exhibit new physiological activities. These including ability of transconjant Rhizobium to fluorescent andpigmention in king B medium and become able to formation tumor like structure on root which normally formation nodules on specific legume.

Table 4: Conjugation between *A. tumefaciens* & *S. meliloti*

Bacterial Responses		Trasconjugantsinorhizobium	Total account of conjugation colonies	Conjugation frequency ($X10^{-6}$)
Donor isolate	Recipent isolate			
A.tumefaciens (Atmcol) $Cef^{S}Rif^{R}$	*S.meliloti* $Rif^{S}Cef^{R}$	*S.meliloti* $Rif^{R}Cef^{R}$	12	0.9
A.tumefaciens (C58C1) $Cef^{S}Rif^{R}$	*S.meliloti* $Rif^{S}Cef^{R}$	*S.meliloti* $Rif^{R}Cef^{R}$	10	0.87

Effect of Ultrasound Treatment on Bacterial Conjugation

The result demonstrated that ultrasound treatment encourages bacterial conjugation by exposing the two parents of bacteria, after mixing total numbers of transconjgant colonies and conjugation frequency reported on two case of exposing bacterial suspension mixture wich is above in (240, 480) sec respectively Table (5). Additionally, these ultrasound pulses substance the growth of the treated bacteria while the shape and morphological appearance of colonies were unaffected.

Table 5: Ultrasound treatment on conjugation between *S.meliloti* and *A.tumefaciens* on the total account of colonies and conjugation frequency

Ultrasound treatment KHZ/Sec	Exposure after mixing			
	S.meliloti&*A.tumefaciens* (Atmcol)		*S.meliloti*&*A.tumefaciens* (C58Cl)	
	Total account of conjugation colonies	Conjugation frequency $X(10^{-6})$	Total account of conjugation colonies	Conjugation frequency $X(10^{-6})$
Control	13	0.9	10	0.87
47.6/240	20	1.5	18	1.4
47.6/480	18	1.2	16	1.35
47.6/720	9	0.49	14	1.0
47.6/900	0.0	0.0	0.0	0.0

Plasmid Profile

By using Alkaline method for DNA extraction Figure (2) results revealed that the two transcojucants sinorhizobium have band of plasmid same to be with molecular size of Ti-plasmid of *A.tumefaciens*, which is transform from *A. tumefaciens* to *S. meliloti* through bacterial conjucaiton that is certain to successfully transfer plasmids to *S. meliloti*.

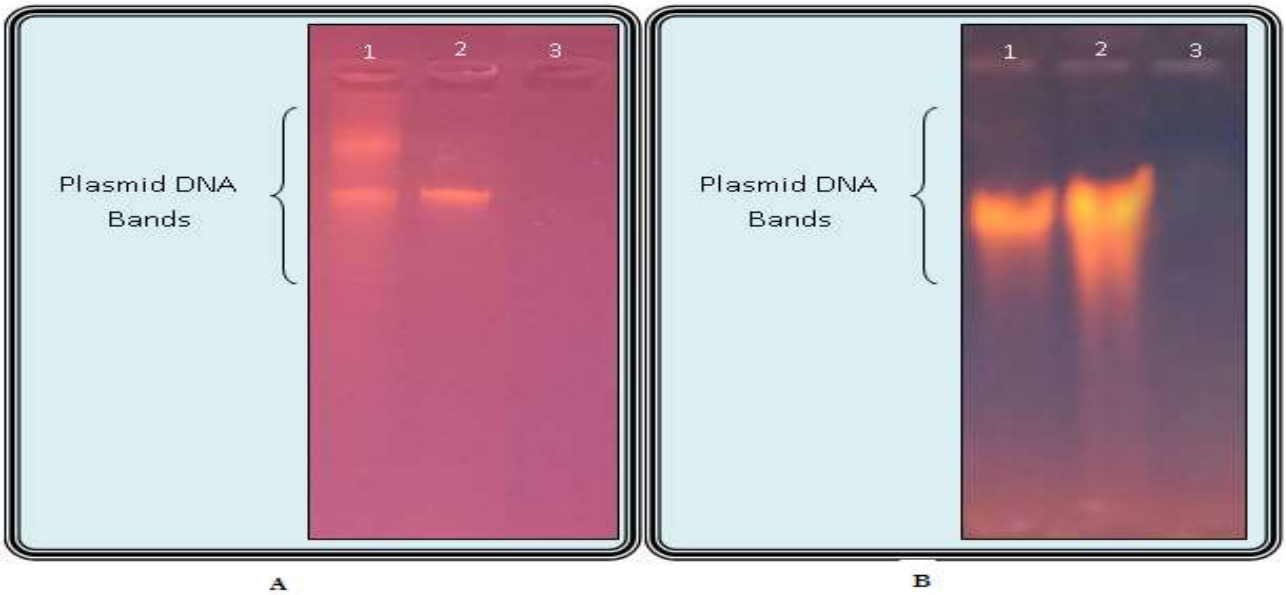

Fig. 2: Agarose gel electrophoresis of lysates

A: Plasmid profile of *A.tumefaciens*(C58c1) (1), Transconjugant Rhizobium (2) and *S.meliloti* (Cured)(3).
B: Plasmid profile of *A.tumefaciens* (Atmco1) (1), Transconjugant Rhizobium (2) and *S.meliloti* (Cured) (3).

PCR analysis of *A.tumfaciens* (C58Cl), *A.tumfaciens* (Atmco1) isolates and transconjucant Rhizobium gaves positive results. The presence of 427bp and represented the presence of *ipt* genefiger (3) in *A.tumfaciens* (Atmcol).

The same resulted were obtained by [24].And these results confirm success the process of conjugation between *A.tumfaciens* and *S. meliloti* terms of presence of *ipt* gene in transconjucant Rhizobium isolated

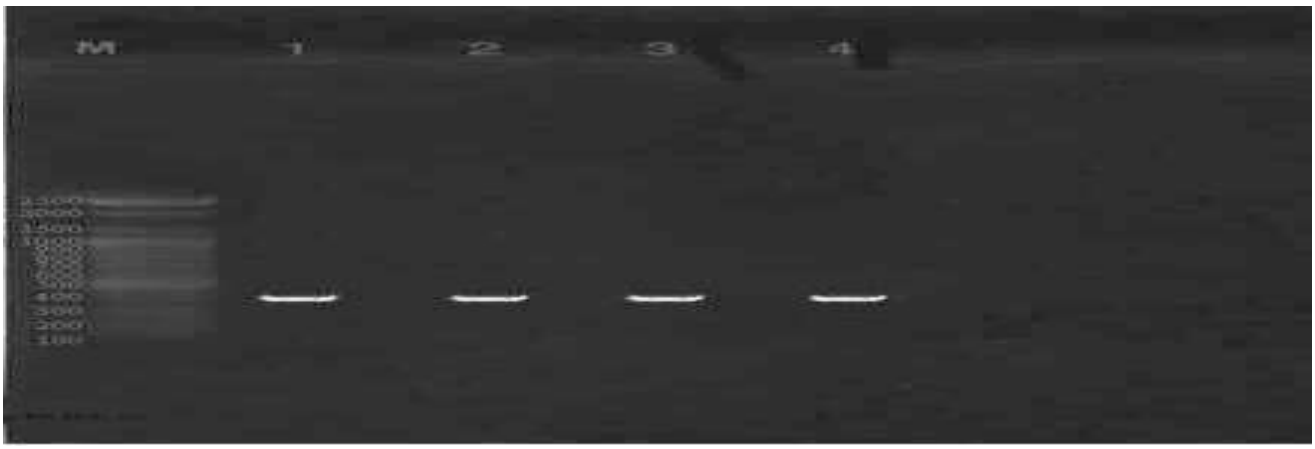

Figure 3: The presence of the *ipt* gene in the plasmid and implified plasmid DNA sample of TransconjugantSinorhizobium (1, 2) by electrophoresis

Path (M): volumetric guide (100 base pair)
Path (1): represents a positive result of plasmid DNA amplified and isolated from A.tumefaciens bacteria (C58C1 (positive comparison sample)
Path 2: represents a positive result of plasmid DNA amplified and isolated from A.tumefaciens (AtMcol) (positive comparison sample)
Path (3): Transconjugant Rhizobium(1)
Path (4): Transconjugant Rhizobium (2)

Discussions

The bacterial resistance to antibiotics could be due to the chromosomal mutation, which alter either the aim of antibiotics in the bacterial cell or cell membrane permeability as some studies many authors expected that bacteria pump antibiotic before damage it [25] or producing enzymes that hydrolyze antibiotic like □-lactamase [26]. The available data mentioned that penicillin and cephalosporin affect bacterial cell wall [27], whether rifadin inhibits nucleic acid synthesis.But streptomycin, tetracycline, gentamicin, chloramphenicol and erythromycin inhibit protein synthesis [28]. *A.tumefaciens*helds on its plasmid-DNA (Ti), the genes responsible for resistance to Rif $^{(R+)}$ and Gen [29] whil Cef$^{(R+)}$ gene is present in *S.meliloti* [15].

Bacterial conjugation occurrence depending on the growth scale of the transconjugant bacteria on media supplemented with suitable type of antibiotics. This case expresses the transfer of specific genetic element from F^+ to F^- [30]. The increase of bacterial conjugation (in both cases) by ultrasound stimulation may be due to the acceleration passage of genetic elements from donor to recipient cells.

This could be explained by the effects of ultrasound upon permeability on the cell walls of the gram-negative bacteria [31]. power ultrasound may disrupt the lipid membrane to have an impact on how grow and be transformed either by facilitating a higher degree of transformation in bacterial cell population [32] it is an ideal approach for plasmid or DNA fragments delivery by based upon acavitational effect, which physically generates reversible porosity in the cell membrane [20]. Many studies refer to the transfer of symbiosis (Sym-plasmid) from *R. melelioloti* on to other species of Rhizobium [33], and Agro bacterium to produce transconjugant Agro bacterium which can produce nodule like structures on roots of *M. sativa* legume host [15].

Our results have shown that ultrasound DNA delivery (UDD) is a viable technology for bacterial transformation, which is far less dependent on the ionic strength of the medium than electroporation allowing it to be used with bacteria growing in complex environments and under more natural condition. I believe UDD will open up nameros new opportunities for DNA and RNA delivery into a side vary of microorganisms.

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