Electro-conjugation between *Klebsiella pneumoniae* and *Proteus mirabilis* and between *Agrobacterium rhizogenes* R1601 and *Sinorhizobium meliloti*

الاقتران كهربائياً بين المسببات الجرثومية لذات الرئة و التهاب المجاري البولية وبين البكتريا المسببة للجذور الشعرية والمثبتة للنتروجين الجوي في النبات

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Abstract

Bacterial conjugations represent one of the possibilities to produce transformed bacteria. This study aimed to detect the occurrence of conjugation between the pathogenic bacteria *Klebsiella pneumoniae* and *Proteus mirabilis* and between *Agrobacterium rhizogenes* R1601 and *Sinorhizobium meliloti*. The results proved the conjugation in those bacterial parents through the sensitivity of bacterial species to specific types of antibiotics. The obtained data reported the stimulation of their growth when exposed to different electrical pulses. These electrotreatments include three voltages (200, 250, 300) volts of interval (0.5, 1.0, 2.0, 3.0, 4.0, 10.0, 20.0) msec. for each voltage .These treatments increase colonies numbers more than 50% which grown on the surface of the specific solid medium .The interesting results of this study are that pre-exposure of each type of bacteria and the exposure of the conjugation mixture to the above mentioned electrotreatments increase the transconjugant colonies at ratio (30-70)% and increased the conjugation frequency twice. This may be the key to enhance conjugation between those species of bacteria not enable or difficult for conjugation.

المستخلص

إن عملية الاقتران البكتيري تمثل إحدى إمكانيات التحول الوراثي في البكتريا . تناولت الدراسة التعرف على مدى نجاح الاقتران بين نوعي البكتريا iliالمرضية *Proteus mirabs* و *Klebsiella pneumoniae*وبين نوعين من البكتريا الممرضة للنبات *Sinorhizobium meliloti* و *Agrobacterium rhizogenes* R1601 وأكدت النتائج حدوث الاقتران في هذه الأنظمة البكتيرية بدلالة حساسية الأنواع البكتيرية لأنواع معينة من المضادات الحيوية . وأظهرت البيانات تحفيز نمو هذه الأنواع البكتيرية عند تعريضها لمعاملات كهربائية منتخبة شملت ثلاثة فولتيات (200 , 250 , 300) لأمد زمني (0.5 , 1.0 , 2.0 , 3.0 , 4.0 , 10.0 , 20.0) ملي ثانية ولكل فولتية وزيادة أعداد مستعمراتها النامية على أوساطها المناسبة إلى أكثر من 50% . ومن النتائج البارزة لهذه الدراسة لوحظ إن تعريض نوعي البكتريا قبل مزجها أو تعريض مزيجها لنفس المعاملات الكه ربائية أدى إلى زيادة أعداد المستعمرات البكتيرية المقترنة بنسبة (30 - 70)% وتردد الاقتران وفي كلا الحالتين .إن النتائج المتحققة في هذه الدراسة قد توفر إمكانات مناسبة لإحداث الاقتران بين الأنواع البكتيرية غير المستجيبة للاقتران أو التي تعد صعبة الاقتران .

Introduction

Klebsiella pneumoniae are G-negative bacteria has large mucoid and dome –like colonies, red-pink color on MacConkey, capsulated bacilli, non- motile, it cause

respiratory tract infection RTI, urinary tract infection UTI, Septicemia and liver abscess [1].

Proteus mirabilis gram-negative un-capsulated motile bacilli, urease positive. Swarm on solid media. It's one of the most important causes of UTI and bacteraemia [2].

Rhizobium is a soil living bacteria gram-negative motile bacilli not spores forming. Grow at 25-30 ^{o}C [3]. Each species of *Rhizobia* live symbiotically with one species of dicotyledonous forming nitrogen fixing nodules. *Sinorhizobium* posses symbiotic plasmid, nodulation and fixation genes, while *Agrobacterium rhizogenes* R 1601 is contain Ri- hairy root inducing plasmid producing unfamiliar amino acids named Opines, as a source for carbon and nitrogen [4].

Plasmids are a small circular double strand of extra chromosomal P-DNA replicates autonomously found in bacteria, yeast, fungi, algae and protozoa. Transformation, transduction and conjugation are three main methods for genetic exchange [5]. In conjugation, P-DNA transfer in one pathway from fertile (F^+) donor to Non fertile (F^-) recipient bacteria through conjugation bridge, usually happened in gram negative bacteria. This plasmid carry transfer genes, origin of transfer and mobilization protein for conjugation [6].

The aim of this work is to evaluate whether electrotreatment enhance bacterial conjugation between these two pathogenic bacteria and between the other two bacteria infected plants.

Materials and Methods

Bacterial cultures: The bacteria used in this study were *Klebsiella pneumonia* , *Proteus mirabilis* obtained from Dept. of Biology, College of Science, Mosul University. *Agrobacterium rhizogenes*R1601 was provided from Prof. E.W. Nester, Dept. of Microbiology and Biochemistry, Washington, Univ. U.S.A, and *Sinorhizobium meliloti* obtained from Plant Genetic manipulation group, Dept. of Biology, College of Education, Univ. of Mosul

Klebsiella pneumoniae and *Proteus mirabilis* grown on nutrient and MacConkey agar [7], while *Agrobacterium rhizogenes* was grown on Morgan APM medium [8] and *Sinorhizobium meliloti* on Yeast extract medium (YEM) medium [9].

Antibiotics used

Sixteen types of antibiotics were used in this study Table (1).

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

Antibiotics	Stock Conc.)(mg/ml	Final Conc. (µg/ml)	Antibiotics	Stock Conc.)(mg/ml	Final Conc. (µg/ml)
Trimethoprim (Tm)	20	**30,10	Gentamicin (Gm)	40	30
Chloramphenicol (Cm)	20	10	Ciprofloxacin (Cip)	10	30
Streptomycin (Sm)	25	25	Rifadine (Rif)	10	5
Ampicillin (Ap)	25	**10,50	Erythromycin (Er)	10	*15
Cefalixin (Cf)	20	30	Penicillin (Pn)	20	*50
Amoxcillin (Ax)	50	**25,50	Cefotaxime (Ctx)	20	*30
Nalidixic acid (Nal)	20	30	Carbenicillin (Carb)	50	*100
Tetracycline (Tc)	12.5	**30,15	Kanamycin (Kana)	50	*100

*: Selection of antibiotic according to ref.(1).

**: Conc. used with plant pathogenic bacteria.

Exposure of bacterial suspensions to electrotreatments

A volume 1.0ml of each of the four bacterial suspensions were exposed to electrotreatment using (200, 250, 300) volt for (0.5, 1.0, 2.0, 3.0, 4.0, 10.0, 20.0) msec [10] Each bacterial sample transferred to a glass chamber that connected with the two electrodes arising from electrostimulator apparatus. Then each electrotreatments were selected and the pulse was transit through the chamber. The electrotreated samples were cultured on the specific medium.

Bacterial conjugation

One volume (1ml) of *K. pneumoniae* suspension and one volume (1ml) of *P. mirabilis* suspension were mixed with a similar volume of nutrient broth .The mixtures were incubated for three hours in 37ºC, then 0.1 ml of each mixture were spread on the surface of nutrient agar supplemented with Streptomycin 25 µg/ml and Cefalixin 30 µg/ml .

In the same manner one volume (1 ml) of *A. rhizogenes* R 1601 suspension and similar volume of *S. meliloti* suspension were mixed with a same volume of liquid YEM. These mixtures were incubated in for three hours at 28 ºC, then 0.1 ml of bacterial mixture were spread on the surface of agar solidified YEM. This media was supplemented with Kanamycin 100 µg/ml and Rifadine 5 µg/ml [11].

Selection of these types of antibiotics depend on the bacterial sensitivity or resistance to those types of antibiotics. This was detected by adding the antibiotic to the agar solidified culture medium, then 0.1 ml of the bacterial suspension was streaked on the surface of the media.

Electrotreatment of conjugation mixtures

Each bacterial suspension was exposed to the selected electrotreatments then mixed together and incubated for three hours. In other case, the conjugation mixture directly exposed to the selected electrotreatments [10,12]. Then 0.1 ml of each mixture was spread on the suitable solid media.

Results

Bacterial responses towards antibiotics

The results proved that the different species of bacteria exhibit various responses to the tested antibiotics Table (2).

The interested results that *K. pneumoniae* was Cf $^{(R+)}$, Sm $^{(s+)}$ and Tc $^{(s+)}$ whereas *P. mirabilis* was Sm $^{(R+)}$, Tc $^{(R+)}$ and Cf $^{(S+)}$. Similarly, *A. rhizogenes* R1601 was Kana$^{(R+)}$, Carb $^{(R+)}$ and Rif $^{(S+)}$ while S. meliloti was Rif $^{(R+)}$,Kana$^{(S+)}$ and Carb $^{(S+)}$.

Table: (2) Detection the responses of bacterial species towards the used antibiotics .

Antibiotics	Bacterial responses			
	Enterobacteriaceae		Rhizobiaceae	
	K.pneumoniae	*P.mirabilis*	*A.rhizogenesR1601*	*S.meliloti*
Trimethoprim*	R	R	R	R
Chloramphenicol	R	R	R	R
Streptomycin	S	R	R	R
Ampicillin*	R	R	R	R
Cefalixin	R	S	R	R
Amoxcillin*	R	R	R	R
Nalidixic acid	S	S	S	S
Tetracycline*	S	R	S	S
Gentamicin	S	S	S	S
Ciprofloxacin	S	S	S	S
Rifadine	S	S	S	R
Erythromycin*	-	-	R	R
Penicillin*	-	-	R	R
Cefotaxime*	-	-	S	S
Carbenicillin*	-	-	R	S
Kanamycin*	-	-	R	S

R: Resistant, S: Sensitive, *: Con. Used with bacteria infected plants, -: Not tested.

Effect of electrotreatment on growth of bacterial species

Results of exposing bacterial suspensions to a group of electrotreatments indicate an increase in colonies numbers .The long term effect was clear with the two members of enterobacteria compared with the untreated samples. This effect was totally varied with the members of rhizobiaceae using the same electrotreatments Table (3).

Table (3): Effect of electrotreatment of bacterial suspensions of *K. pneumoniae* , *P.mirabilis* , *A. rhizogenes R1601 and S. meliloti* on their growth.

Electrotreatments	No. of colonies (±SD)			
(V/msec)	*K. pneumoniae*	*P.mirabilis*	*A.rhizogenesR1601*	*S. meliloti*
Control	63 ±1	36±1	25±3	16±2
200/0.5	87±2	49±1	29±2	21±1
200/1.0	32±2	36±2	0±0	23±1
200/2.0	9±1	47±4	0±0	23±3
200/3.0	93±3	65±1	54±1	26±2
200/4.0	156±1	50±3	36±4	40±1
200/10.0	152±2	32±1	16±3	33±4
200/20.0	115±1	46±2	65±1	36±4
250/0.5	82±3	40±3	29±2	43±1
250/1.0	98±3	47±2	47±4	31±2
250/2.0	120±4	55±3	56±1	38±2
250/3.0	132±2	63±1	35±3	39±1
250/4.0	119±2	38±2	44±1	26±2
250/10.0	141±1	27±3	49±3	17±1
250/20.0	136±3	47±1	39±2	22±5
300/0.5	89±3	38±2	19±1	18±4
300/1.0	48±4	94±1	26±3	21±2
300/2.0	63±1	38±3	66±1	36±1
300/3.0	123±2	47±2	41±2	36±1
300/4.0	148±1	47±4	36±1	32±2
300/10.0	104±3	48±1	65±2	21±4
300/20.0	112±4	63±2	21±4	24±3

***Control: Not electrostimulated.**

SD: Standard deviation.

Detection of the transconjugant

The results proved successful conjugation between *P.mirabilis* (as a donor) and *K. pneumoniae* (as a recipient) .The obtained transconjugant *K.pneumoniae* was Sm $^{(R+)}$, Tc $^{(R+)}$ and Cf $^{(R+)}$.

These results showed that genetic elements responsible for antibiotic resistance transferred from donor to recipient, gives strong evidence that plasmid had pass from donor to recipient.

Again the obtained results proved the occurrence of conjugation between *S. meliloti* (as a donor) and A. rhizogenes R1601 (as a recipient). The transconjugant *A. rhizogenes* R1601 became Kana $^{(R+)}$, Carb $^{(R+)}$ and Rif $^{(R+)}$.

In both cases the transconjugant bacteria have acquired new properties and exhibit new physiological activities .These including ability of transconjugant *K. pneumoniae* to producing H_2S, and transconjugant *A.rhizogenes* R1601 became able to develop root nodules on specific legume which normally doesn't happen.

Effect of electrotreatment on bacterial conjugation

The results demonstrated that electrotreatment encourages bacterial conjugation either by exposing the two parents of bacteria either before or after mixing .The exposure of bacterial mixture to electrotreatment had better effect than exposing each of them individually prior to mixing. Total numbers of transconjugant colonies and conjugation frequency reported high levels in the case of exposing bacterial suspension after mixing Table (4).

Additionally, these electrical pulses sustained the growth of the treated bacteria while the shape and morphological appearance of colonies were unaffected.

Table (4) Electrotreatment effect on conjugation between K. pneumoniae & P. mirabilis and between A. rhizogenes R1601 & S. meliloti on total account of colonies and conjugation frequency.

Electrotreatments (V/msec)	K. pneumoniae & P. mirabilis				A. rhizogenes R1601& S. meliloti			
	Exposure before mixing		Exposure after mixing		Exposure before mixing		Exposure after mixing	
	Total account of conjugation colonies	Conjugation frequency ×) 10(-6	Total account of conjugation colonies	Conjugation frequency ×) 10(-6	Total account of conjugation colonies	Conjugation frequency ×) 10(-6	Total account of conjugation colonies	Conjugation frequency ×) 10(-6
Control	5		0.09		7		0.28	
200/0.5	2	0.23	4	0.46	11	0.37	12	0.41
200/1.0	1	0.31	1	0.31	0	0	0	0
200/2.0	0	0	0	0	0	0	0	0
200/3.0	4	0.43	5	0.53	18	0.33	19	0.35
200/4.0	7	0.44	8	0.51	19	0.52	19	0.52
250/0.5	0	0	3	0.36	17	0.58	17	0.58
250/1.0	5	0.51	6	0.61	16	0.34	15	0.31
250/2.0	8	0.66	11	0.91	19	0.33	20	0.35
250/3.0	13	0.98	16	1.21	17	0.48	19	0.54
250/4.0	3	0.25	0	0	18	0.40	20	0.45
300/0.5	4	0.44	6	0.67	3	0.15	4	0.21
300/1.0	4	0.83	5	1.04	9	0.34	9	0.34
300/2.0	3	0.47	4	0.63	23	0.34	24	0.36
300/3.0	8	0.65	10	0.81	18	0.43	19	0.46
300/4.0	12	0.81	14	0.94	17	0.47	18	0.51

0: no growth

Discussion

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. Many authors expected that bacteria pump antibiotic before damage it [13] or produce enzymes that hydrolyze antibiotic like *B* –lactamase [14].

The available data mentioned that penicillin and cephalosporin affect bacterial cell wall [15].Whether Rifadine inhibit nucleic acid synthesis, but Streptomycin, Tetracycline, Gentamicin, Chloramphenicol and Erythromycin inhibit protein synthesis [16].

Resistance of *K. pneumoniae* to cephalixine is due to the presence of resistance genes while *P. mirabilis* harbor resistance genes to Streptomycin and Tetracycline [17].

A. rhizogenes R1601 helds on its plasmid-DNA(Ri) the genes responsible for resistance to Kana. $^{(R+)}$ and Carb.$^{(R+)}$ [8].While Rif $^{(R+)}$ gene is present in *S. meliloti* [18].

Bacterial conjugation occurrence depending on the growth scale of the transconjugant bacteria on media supplemented with suitable type of antibiotics. This case express the transfer of specific genetic element from F^+ to F^- [19].

The production of H_2S was the main expression and a strong marker to the success of conjugation between *K. pneumoniae* and *P. mirabilis*. This is due to the transfer of H_2S producing ability to the recipient. Many literatures stated this phenomenon in other bacteria such as *Clostridium* and *Salmonella typhimurium* [20].

The increase of bacterial conjugation (in both cases) by electrostimulation may due to the acceleration passage of genetic elements from donor to recipient cells .This could be explained by the pores formation on bacterial cells, or to high permeability of cell walls [12].

Many studies refers to transfer of symbiosis (sym-plasmid) from R. meliloti to other species of *Rhizobium* [21] and *Agrobacterium* to produce transconjugant Agrobacterium which can produce nodule like structures on roots of *Medicago sativa* legume host [22].

This finding introduce for the first time new possibility to carry out conjugation, as a method of transformation, and increase conjugation frequency in other bacterial system particularly those un-amenable to conjugation.

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