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CLONING PROTEASE GENE FROM PSEUDOMONAS AERUGINOSA ISOLATED FROM HUMAN CORNEAL ULCERATION CASE

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ABSTRACT

In previous paper, clinical strain *Pseudomonas aeruginosa* was isolated from human corneal ulceration case. The bacterial cells secreted the extracellular alkaline protease that was partially purified and characterized. The aim of this paper is determined some antibiotics resistance and pyocyanin pigment production , as well as introduced protease encoding gene or genes into *Escherichia coli* HB101 to study and understand the role of protease enzymes in bacterial pathogenicity to develop therapeutics against *P. aeruginosa* future researches. Results: The isolate appeared pyocyanin pigment production and extracellular protease and has multiple drug resistances to twelve antibiotics including ampicillin, amikacin, amoxicillin, carbencillin, chloramphenicol, cefotaxime, erythromycin, lincomycin, penicillin, streptomycin, tetracycline, and trimethoprim and it is sensitive to ciprofloxacin, gentamycin, neomycin and rifampicin. The plasmid profile of the isolate revealed that the presence of single large plasmid on agarose gel electrophoresis. Plasmid transfer experiment including plasmid transformation and bacterial conjugation into the plasmidless competent cells of *E. coli* MM 294 (Rif^r), as the recipients strain at a frequency $5x\ 10^{-3}$ and$1.2x10^{-4}$ respectively, appeared that transformed *E. coli* cells acquired all antibiotic resistances but lincomycin and pyocyanin production. While the transconjugant cells acquired pigment production (pyocyanin) on King's medium and all studied antibiotic resistances including lincomycin that may be indicated multiple resistance genes related to the presence of the conjugative plasmid R68.45 or a derivative of R68 that available in *P. aeruginosa*. This plasmid is able to mobilize the bacterial chromosome genes from many origins and may be the *lin* resistance gene and pigment genes included in this transfer into *E.*

coli MM294 strain. Pseudomonad genes were introduced into *E. coli* HB101 Sm^r, after purified total DNA and cloning vector *pACYC184* were cleft by *EcoR* I restriction endonuclease at frequency 4.3 x 10^{-3}. Subsequently, these clones screened on skimmed milk agar to select protease – producing clones. Only 32 clones showed variable ability to produce protease enzyme out of 86 clones. These clones phenotypically divided into two groups, the first one appeared complete casein digestion (12 clones) on skimmed milk agar plate (clear zone) and the other group (20 clones) has low caseinolytic activity that may be indicated the DNA fragment encoding protease miss certain sequence caused less activity of protease enzyme or most enzyme molecules accumulate intracellular or in periplasmic space of bacterial cells and few enzyme molecules secreted from cells to the medium or may be these clones encoding different protease enzymes.

KEYWORD: *Pseudomonas aeruginosa*, protease gene, cloning, antibiotic resistance.

INTRODUCTION

The virulence of several bacteria is related to the secretion of several extracellular proteases. Gene cloning in these microbes was studied to understand the basis of their pathogenicity and to develop therapeutics against them. Proteases play an important role in cell physiology, and protease gene cloning, especially in *E. coli*, has been attempted to study the regulatory aspects of proteases [1].

P. aeruginosa is an opportunistic pathogen and can cause fatal infections in compromised hosts. This virulence is related to the secretion of several extracellular proteins. *P. aeruginosa* secretes two proteases, an alkaline protease and an elastase. The alkaline protease genes (*apr*) from *P. aeruginosa* IFO 3455 and PAO1 were cloned in *E. coli* [2, 3]. The DNA fragment (8.8 kbp) coding for the alkaline protease from strain PAO1 was expressed in *E. coli* under the control of a *lac* promoter. Active enzyme was found to be synthesized and secreted into the medium in the absence of cell lysis. The LasA protease (elastin degrading) of *P. aeruginosa* is also an important contributor to the pathogenesis of this bacterium. The enzyme shows a high level of staphylolytic activity. The *lasA* gene from strain FRD1 was overexpressed in *E. coli* that encodes a precursor, prepro-LasA, of about 45 kDa [4]. *P. aeruginosa* has a number of diverse proteases that have been purified and characterized from multiple strains: strain PST-01, strain PAO1 and strain DG1, strain PA103-29 and strain FRD2 , all these proteases are extracellular and directly associated with this species virulence [5-8]. *P. aeruginosa* secretes an elastase as a preproenzyme, with a weight of 53 kDa, which is then processed into

its mature form, with a molecular weight of 33 kDa. It is a neutral metalloprotease, requiring zinc for enzymatic reactivity and calcium for stability [6, 8]. Strain FRD2 secretes the LasA metalloendoprotease as a proenzyme with a molecular weight of 42 kDa. The protease is then processed extracellularly into its mature form with a molecular weight of 20 kDa [8]. Both elastase and LasA protease can degrade elastin, some collagens, and the LasA protease also has high stapholytic activity [6, 8]. Another extracellular protease, Protease IV, is as a serine protease. Produced by strain PA103-29, it has a molecular weight of 26 kDa, an isoelectric point of 8.7 and optimum enzymatic activity at pH 10 and 45° C [7]. *P. aeruginosa* strain PST-01 produces another type of protease, an organic solvent-stable protease with a molecular weight of 38 kDa, an optimum temperature of 55° C and a pH of 8.5. This protease is stable over a pH range of 8 – 12 and at temperatures below 50° C. Inhibitor assay experiments concluded the enzyme was a metalloprotease, inhibited by EDTA, 1,10-phenanthroline, and phosphoramidon [5].

N-terminal sequence analysis allowed the identification of a 31-aa signal peptide. pro-Las A (42 kDa) does not undergo autoproteolytic processing and possesses little anti-staphylococcal activity. The digestion of pro-LasA either by trypsin or by culture filtrate of the *P. aeruginosa lasA* deletion mutant yielded the active (20-kDa) staphylolytic protease [9].

In previous paper [10], clinical strain *P. aeruginosa* was isolated from human corneal ulceration case. The bacterial cells secreted the extracellular alkaline protease that was partially purified and characterized, the enzyme has an optimum activity at pH 9.5 and the activity is stable in the alkaline pH range (8- 10). The enzyme is stable at temperature under 30˚C and maximal activity at 45° C. Inhibitor assay experiments concluded the enzyme is regarding to a serine protease and its protein containing disulfide bonds that could be important in maintaining the molecular conformation required for activity. The aim of this paper is determined some antibiotics resistance and pyocyanin pigment production , as well as introduced protease encoding gene or genes into *Escherichia coli* HB101 to understand the role of protease enzymes in bacterial pathogenicity and develop therapeutics against bacteria in the next paper.

MATERIALS AND METHODS

1. Identification of Pseudomonas Aeruginosa

Clinical strain *Pseudomonas aeruginosa No.3* was isolated previously from human corneal ulceration. The bacterial cells secreted the extracellular protease in liquid culture and this

enzyme was produced, purified and characterized previously [10].The isolate was activated using brain heart infusion broth. After activation inoculated on the MacConKey agar, a single colonies were selected, for more purification inoculated on the selective medium cetrimide agar, oxidase test was done, and microscopically Gram negative rod shape, identified provisionally as *Pseudomonas aeruginosa*, subcultured on nutrient agar slants, after incubation at 37 °C for 24 hr., stored at 4 °C, till other bacteriological tests were done [11]. The api 20E Micro tube system (BioMerieux SA, Lyon, France) was used to re-identified. This system is a standardized, miniaturized version of conventional procedures for the identification of Enterobacteriaceae and other Gram negative bacteria.

2. Antibiotic Susceptibility Test

Antibiotic susceptibility test by disk-diffusion method was performed according Bauer et al. method (1966) that described in [12] and the results was compared with standard inhibition zone [13]. Antibiotic resistance test by pour method was used to screen the genetic transfer of antibiotic resistances in studied isolate was preformed according to Sambrook *et al*. and Baron and Finegold [12, 14].

3. Total DNA Extraction by Salting Out Method

Total DNA content of *P. aeruginosa* isolates was extracted according to the salting out method of Pospiech and Neuman [15] that modified by Omran [16]. Plasmid DNA content was extracted by using alkaline lyses [17].

The concentration of total DNA content was determined and calculated as according to Sambrook *et al*. as follows [14]:

Conc. of DNA µg/ml = optical density at 260_{nm} × dilution factor × 50 µg/ml.

4. Agarose Gel Electrophoresis Technique

Agarose Gel was prepared according to the method of Sambrook *et al*. [14]; agarose gel was prepared by using 0.7% agarose gel. The electrophoresis was run at 6volt/cm for 3hrs. The gels were illuminated with UV-transilluminator, and then photographed by digital camera.

5. Bacterial Conjugation

The transmissible ability of DNA plasmid of *P. aeruginosa* was tested according to Olsen *et al*. [6] method by *P. aeruginosa* isolates as donor and standard plasmidless strain of *E .coli* MM294 Rif^r (*hsd* R^-, *hsd* M^+, *edn AI*, *pro*$^-$, *thi*$^-$, Rif^r , its source from Biotechnology

department University of Nahrain) as recipient. The conjugation frequency was calculated according to the following equation:

$$\text{The frequency of conjugation} = \frac{\text{No. of transconjugant cells / ml}}{\text{Total No. of recipient cells / ml}}$$

6. Genetic Transformation

The genetic transformation was performed according the Sambrook *et al.* [14] method by using free plasmid that extracted from *P. aeruginosa* isolates (donors) and standard plasmidless strain of E .*coli* MM294 Rif^r as recipient. The genetic transformation frequency was calculated according to the following equation:

$$\text{The transformation frequency} = \frac{\text{No. of transforments (cells / ml) / DNA amount (µg)}}{\text{Total number of competent cells / ml}}$$

7. Cloning of protease genes of *p. Aeruginosa*

a). Cleavage by *EcoR* I Restriction Enzyme

Both the purified total DNA of *P. aeruginosa* and cloning vector *pACYC184* (*tra*$^-$,Tc^r, Cm^r, from CinnaGen company**)** were separately digested by *EcoR* I endonuclease enzyme (CinnaGen company) in sterile Eppendorf tube.

The concentration and purity of total DNA were 1µg/ml and 1.74 respectively. The reaction mixture consists of : 5 µl of 10x buffer, 33 µl of sterile D.W. was mixed very well , then 10 µl of *P. aeruginosa* total DNA and 2 µl of *EcoR* I enzyme (U/µl) were added. The final volume of the reaction mixture was 50 µl containing 10ng of DNA mixed gently. Cloning vector DNA (1 µg/ml) was digested in the same manner. The control reaction was the same contents but enzyme. All mixture reactions were incubated at 37°C for 1 hr, and then they were stopped by heating at 68 °C for 3 min. After that, equal volume of chloroform-Isoamyl alcohol was added to each reaction mixtures and mixed very well, then centrifuged at 12000 rpm for 10 min at 4°C. The aqueous phase containing digested pseudomonad DNA and cloning vector were transferred to sterile Eppendorf tubes separately. To check the digestion results, the restricted products and size marker Lambda DNA cut by *Hind* III & *EcoR* I (Fermentas) were migrated into 0.8% agarose gel at 3V/cm for 50 min. The restricted products were precipitate by adding 0.1 volume of 3 M sodium acetate and 2.5 volume of absolute ethyl alcohol, the mixtures were left to cool at -10 °C for 2 hrs. and then centrifuged

at 12000 rpm for 15min. After removing ethyl alcohol, the precipitates of restricted DNA were dried and dissolved by 20 µl of TE buffer [14].

b). Ligation with T_4 DNA Ligase

The restricted DNA fragments of both totals DNA of *P. aeruginosa* and cloning vector *pACYC184* were ligated together by *T_4* DNA ligase in sterile Eppendorf tubes. The reaction mixture consisted of ligated buffer (5X) and 18µl of sterile D.W. was mixed very well and then both of *EcoR* I restricted DNA fragments of *pACYC184* and *P. aeruginosa* were added in the ratio 1:3 by mixing 5µl of restricted cloning vector and 15 µl of pseudomonad restricted fragments. 2 µl of *T_4* DNA ligase (200 U/µl) was added to the mixture reaction. The final total volume of reaction mixture was 50 µl.The mixture reaction was mixed gently and incubated at 4°C for 18 hr. then 5 µl of ligated product was checked by gel electrophoresis into 0.8% agarose gel at 3V/cm for 90 min[14].

c). Transformation and Recombinant Colonies Selection.

To introduce the ligated mixture into plasmidless competent *E.coli* HB101 (*hsd R*$^-$,*hsd M*$^-$, *recA*$^-$, *leu*$^-$, *pro*$^-$, *lac*, *gal*$^-$, Sm^r , its source from University of Tehran Islamic Republic of Iran), 20 µl of ligated product was added to 200 µl of competent cells in a sterile Eppendorf tube as genetic transformation procedure mentioned above. Then 0.1 ml of the culture was spread on selective culture media containing 50µg/ml of chloramphenicol. All plates were incubated at 37°C for 24 to 48 hrs. Subsequently, the transformed and total recipients colonies were calculated. Then the transformed colonies were selected and grown by picking and pitching on LB agar plates containing ampicillin (150µl/ml), tetracycline (20µl/ml), and chloramphenicol (l50µl/ml) separately to select the clones. After that the clones were grown on skimmed milk agar to select protease encoding clones.

RESULTS AND DISCUSSION

The *P. aeruginosa* strain was isolated previously from patient suffering from corneal ulceration [10]. It was re-identified according to microbial, cultural and biochemical characteristics. The isolate produce pyocyanin and extracellular protease and has multiple drug resistances to twelve antibiotics including ampicillin, amikacin, amoxicillin, carbencillin, chloramphenicol, cefotaxime, erythromycin, lincomycin, penicillin, streptomycin, tetracycline, and trimethoprim and sensitive to ciprofloxacin, gentamycin, neomycin and rifampicin , this result agree with Shahid [17] that mentioned the pathogenic isolates of *P. aeruginosa* have several virulence factors including antibiotic resistances.

The plasmid profile of the isolate revealed that the presence of single large plasmid in agarose gel electrophoresis (Fig.1), this agree with Nordmann [20] who found the molecular weight of plasmid ranged between (1.9-45.0) MD or the size of plasmid in the bacteria ranged between (4-80) Kbp. Also it agree with previous our results[18,19] that revealed *P. aeruginosa* isolates which isolated from different human infections have single mega plasmid in addition to small plasmid encoding multiple drug resistances.

Genetic transformation was performed using *P. aeruginosa* isolate as plasmids donor and the plasmidless competent cells of *Escherichia coli* MM 294 (Rif^r) as the recipient strain at a frequency 5x 10^{-3}. The transformed *E. coli* cells acquired all antibiotic resistances but lincomycin and they showed inability to produce pyocyanin that may be indicated to the large plasmid (Fig.1) encoding multiple antibiotic resistances, while the lincomycin resistance gene and pigment production genes are encoded by chromosomal DNA genes and this agrees with that result obtained by Quadri *et al.* [21].

The results of bacterial conjugation between *P. aeruginosa* isolate and plasmidless *E. coli* MM294 standard strain appeared that the large plasmid has ability to transfer from donor to recipient cells at frequency $1.2x10^{-4}$ as indicated by DNA content profile of donor and recipient cells revealed that the presence large plasmid in agarose gel electrophoresis (Fig.1). The transconjugant cells acquired the resistance to all studied antibiotics as well as lincomycin resistance and pigment production (pyocyanin) on King's medium in comparison with transformed cells that appeared inability to resist lincomycin and non-producing pigment may be indicated the multiple resistance genes located on conjugative plasmid, but the interpretation for the foundation of lincomycin resistance and pigment production transfer from *P. aeruginosa* isolate into the transconjugant *E. coli* MM294 cells, the *P. aeruginosa* isolate may be harbor plasmid called R68.45 (a derivative of R68). This plasmid is able to mobilize the bacterial chromosome genes from many origins [20], and may be the Lin resistance gene and pigment genes included in this transfer. This results agree with the foundation of Hardy [23] how observed the production of pyocyanin in the transconjugant colonies could be related to the presence of the plasmid R68.45 carrying the genes encoding Amp, Gm and Tc resistances. This plasmid is available in the *P. aeruginosa* isolate which can be transmissible to the genes encoding pigment production together with Lin resistance gene which may be adjacent to the position of integration of this plasmid into the chromosomal DNA, and transferred into *E.coli* MM294 strain. Both transformed and transconjugant cells

showed inability to produce the proteases on skimmed milk plates that may be indicated the genes encoding proteases located on the chromosomal DNA; this agrees with the results demonstrated by Michael *et al*. [24], who observed the proteases encoded by chromosomal genes.

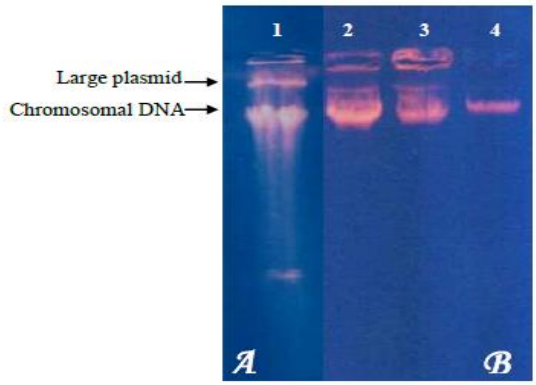

Fig-(1): The plasmid profile of *P. aeruginosa* and its transconjugant and transformed *E. coli* MM294.

The DNA plasmid extracted by salting out (Pospiech and Neuman, 1995) and migrated on agarose gel 0.7%, 50 volt, for 3hr.

Lane 1: DNA content of *P. aeruginosa*.
Lane 2: DNA content of transconjugant *E. coli* MM294.
Lane 3: DNA content of transformed *E. coli* MM294.
Lane 4: DNA content of standard plasmidless strain *E. coli* MM294.

Cloning of Proteases Gene from *P. Aeruginosa* Isolates into *E. coli* HB101

P. aeruginosa has multiple virulence factors including the production of extracellular alkaline proteases. Cloning of Pseudomonad proteases gene in *E. coli* HB101 was studied to understand the basis of their pathogenicity and to develop therapeutics against them. The skimmed milk agar plates were used to facilitate the detection of extracellular protease, the wild type of *P. aeruginosa* colonies produce clear zone on these plates due to alkaline proteases production. The total DNA content of *P. aeruginosa* was purified by modified salting out [16] and then both of total DNA and cloning vector *pACYC184* was cleft by (*EcoR* I) restriction endonuclease. *pACYC184* have a unique restriction target site for *EcoR* I located within the chloramphenicol resistance gene; therefore, there was only a single band which appeared in the gel electrophoresis after was digested by *EcoR* I . The presence of the *EcoR* I restriction target with chloramphenicol gene in *pACYC184* leads to the insertion and

inactivation of chloramphenicol gene; therefore, the clones that contains recombinant DNA plasmid will be sensitive to chloramphenicol and resist to tetracycline. The result of gene cloning experiment using *E.coli* HB101 Sm^r as recipient cells were obtained 95 clones that acquired tetracycline resistance and sensitive to chloramphenicol at frequency 4.3 x 10^{-3}. Subsequently, these clones screened on skimmed milk agar to select protease – producing clones. From this step were obtained 32 clones showed variable ability to produce protease enzyme and 63 clones were non-producing protease. The protease-producing protease clones divided into two groups , the first one (12 clones) appeared complete casein digestion on skimmed milk agar plate (clear zone) and the other group (20 clones) appeared low activity (turbid zone) on skimmed milk agar plate that may be indicated the partial digestion due to the DNA fragment encoding protease may be miss certain sequence caused less activity of protease enzyme or most enzyme molecules accumulated in periplasmic space of bacterial cells and few enzyme molecules secreted to the medium (Fig. 2), or may be these clones encoding different protease enzymes.

Fig-(2) Protease-Producing Protease Clones And The Wild Isolate *P. Aeruginosa*.

1: The wild isolate *P. aeruginosa*, 2: The clone 2 appeared complete skimmed milk digestion, 3: The clone 15 appeared partial or low caseinolytic activity.

Guzzo *et al.* [2] reported that the cloning of proteases structural gene on a cosmid with an insert of approximately 20 kbp, they succeeded in obtaining the expression of an 8.8 kb chromosomal DNA fragment, when expressed in *E. coli* HB101, active protease was synthesized and secreted in to the medium in the absent of cell lysis and without prior intracellular accumulation [2]. Furthermore, the same result was described by Pages *et al.* [25] who speculated cloning of the *apr* structural gene, showed that the APR determinant spans approximately (8.8 kb) of contiguous DNA which contains all of the information necessary for the synthesis and secretion of active protease when it is expressed in *E. coli*. Elsewhere,

Michael *et al*. [24] documented that a 6.5 kb chromosomal DNA fragment carrying the function required for specific secretion of the extracellular alkaline protease produced by *P. aeruginosa* was cloned to *E. coli* that allowed synthesis and secretion of the alkaline protease. Also, Ogino *et al.* [9] described that a gene of organic solvent-stable protease (PST-01 protease) secreted by *P. aeruginosa* PST-01 was cloned and its nucleotide was sequenced. The PST-01 protease produced in recombinant *E. coli* was secreted into the extracellular medium, but its proenzyme was released by the lysis of the cells and became a 33.1 kDa mature enzyme autoproteolytically, and also showed that the organic solvent stability characteristics were the same as those of the PST-01 protease secreted by *P. aeruginosa* PAT-01 [9].

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