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Screening on Bacterial Strains Resistant to Tachyplesin-I Induced by Different Methods

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To obtain the strains resistant to tachyplesin-I, we explored whether tachyplesin-I could induce resistance in three strains by three different methods, which may provide certain theoretical support for research of antibacterial peptides resistant mechanism. *Escherichia coli* ATCC25922, JM109 and *Pseudomonas aeruginosa* CCTCC2620 were induced by continuous passages in sub-inhibitory concentration of drugs, microwave and UV mutagenesis techniques. The resistance to tachyplesin-I strains were selected through gradient concentration tachyplesin method, contrasted with the corresponding non-mutagenesis strains, and subsequently, the methods, of which was more available to obtain resistant strains to tachyplesin-I were compared. The experimental results were as follows: (1) No resistance strains to tachyplesin I in three strains were obtained for continuous forty-two generations in sub-inhibitory concentration of tachyplesin-I. (2) The results of microwave and UV mutagenesis indicated that *Escherichia coli* ATCC25922 was more sensitive to UV than microwave mutagenesis. One mutant resistant to tachyplesin I in *Escherichia coli* ATCC25922 and nine mutants in *Pseudomonas aeruginosa* CCTCC2620 were obtained by UV mutagenesis. The MIC values of tachyplesin I for induction strains were four times higher than those of the original strains. But tachyplesin I did not induce resistance in JM109 by UV mutagenesis. Microwave mutagenesis failed to obtain resistance strains in *Escherichia coli* ATCC25922 and JM109. The above studies have shown that under this research condition, the method of UV mutagenesis is easier to screen the drug-resistant strains. Resistance mechanism of tachyplesin-I-resistant strain induced by mutagenesis needs further research.

Key words: Tachyplesin I, resistance, Mutagenesis, Resistant strains selected.

The serious problem on antibiotic resistance in pathogenic bacteria and super-bacteria emergence has attracted much attention from health care workers of the world. Antimicrobial peptides (AMPs) are acknowledged to be promising candidates as one of novel alternative antibiotics. However, studies have indicated that some bacteria (especially human pathogens) are resistant to certain AMPs[1-4]. It is important to the research that the resistance mechanism of bacteria resistant to AMPs in the clinical application and new antimicrobial agents design aspects.

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At present, the study of resistant mechanism of bacteria resistant to cationic AMPs is mainly focusing on changing the structure and gene composition of bacterial cell membrane or getting resistant mutant strains by physical and chemical mutagenesis methods. For example, Peschel *et al*. studied the mechanism of defensins, protegrins and tachyplesin-I resistance through constructing *S. aureus* mutant which lost the mprF gene mutant, while the result showed that mprF gene was resistance gene[5]. And Bac7-resistant mutant in *E. coli* was obtained through mutagen MNNG mutagenesis method, which demonstrated that the resistance to Bac7 is related to the expression of the inner membrane protein sbmA[6].

Tachyplesin I, a cationic AMP with a disulfide-stabilized β-sheet conformation, was originally isolated from hemocytes of the marine horseshoe crabs, *Tachypleus tridentatus*[7]. It exhibited potent and broad-spectrum activities against both gram-positive and gram-negative bacteria[7-8]. With the increasing expression quantity of reorganization tachyplesin I in yeast strain, it would be widely used in the pharmaceutical industry, animal feed and food industry in the future[9-11].

Till now, it has not been reported that tachyplesin I could induce bacteria to produce resistance. Apart from the author's previous study indicating that neither non-successive induction nor successive induction (less than 20 serial passages) could induce resistance to tachyplesin I in *E. coli* ATCC25922, F41 or *S. aureus* ATCC25923, others have not been reported[12]. Resistance mechanism of bacteria to tachyplesin-I remain unclear. Reports indicated that the surface of cell membranes in gram-positive bacteria (*S. aureus*) exist in controlling tachyplesin-I resistance genes (dlt and mprF)[13-14].

To study the resistant mechanism of bacteria to some AMPs, how to rapidly screen resistant mutants is very important. At present, these methods including continuous drugs induction, physical and chemical mutagenesis-assist are widely used to screen bacterial strains resistant to drug.

Therefore, to obtain the mutant strains resistant to tachyplesin-I, three induction methods were used in the study. On the one hand, three original strains were selected for continuous passages in sub-inhibitory concentration of tachyplesin-I; on the other hand, microwave and UV mutagenesis techniques were used to perform tachyplesin-I-resistant mutation isolation. In the present study, we obtained tachyplesin-I-resistant mutants in *E. coli* ATCC25922 (one mutant) and *P. aeruginosa* CCTCC2620 (nine mutants) through UV mutagenesis. This study can provide certain reference for screening of AMPs–resistant mutants.

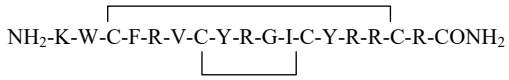
MATERIALS AND METHODS

Microorganisms, media and growth conditions

E. coli ATCC25922 was provided by the Microbial Culture Collection Center of Guangdong (GIMCC, China); JM109 and *P. aeruginosa* CCTCC2620 were provided by China Center for Type Culture Collection (CCTCC, China). Unless otherwise stated, the bacteria were cultured in nutrient broth media (1% peptone, 0.3% beef extract, and 0.5% NaCl) and nutrient agar plates. A portion of an overnight culture of strain was transferred into new nutrient broth and cultivated at 37°C with constant shaking. A log-phase culture was diluted to an appropriate concentration with new broth and used for the antibacterial assay.

Antibacterial agents

The agent used in this study was the AMP, tachyplesin I, which had a purity greater than 95%, synthesized by Shenzhen HYBIO (China). Its sequence was described as follows:



The peptide was solubilized in phosphate buffered saline (pH 7.2) yielding a 10 mg/mL stock solution which was filter-sterilized before use. Drug solutions were made fresh on the day of the assay or stored at -20°C for a short period.

The determination of growth curve

Growth curve was determined of *E. coli* ATCC25922, JM109 and *P. aeruginosa* CCTCC2620 according to the method described by Shen *et al* with some modification[15].

UV mutagenesis treatment

To make the light stability, UV lamp was first opened to preheat 30 min (irradiation distance 28 cm, 18 W) before strains were mutated. Then, logarithmic phase cells (concentration of 10^7 organisms /mL) were collected and added to sterile petri dish with a magnetic stirrer bar. Then, the dish cover opened, cell suspension was treated under different UV radiation doses (0-150 s). After UV radiation treatment, control and treatment cells (0.5 mL) were appropriately diluted and plated on nutrition agar plates. The samples were incubated at 37°C for 20 h in the dark, and subsequently the fatality rate was calculated[16].

Microwave irradiation treatment

The microwave irradiation was conducted in a household microwave oven (2450 MHz, 800W). i) The microwave mutagenic method was in accordance with the method reported with light modification[17]. *E. coli* ATCC25922 cells from the

logarithmic phase of growth (5mL, 10^6 organisms /mL) were added to each glass tube, respectively. In order to eliminate the heat effect caused by microwave irradiation, these tubes were put into the 200 mL beaker with 150 mL water before radiation, which was called the low-temperature-heat-diffusion method. Then, microwaves irradiated the sample under different radiation dose (0-150 s) at microwave oven (2450 MHz, 100W). ii) The suspension of JM109 (10^6 organisms / mL, 5mL) was added to each glass tubes, respectively. These tubes was radiated 10 s at microwave oven (2450 MHz, 100W) , taken out and cooled the using ice-water for 10 s, then radiated again for 10s, the proceeding cumulative radiation time for 0, 30, 60, 90, 120 and 90 s, respectively[18].

After microwave radiation treatment, the samples (0.5 mL) were appropriately diluted and plated on nutrition agar plates, respectively. The samples were incubated at 37°C for 20 h, and subsequently the fatality rate was calculated.

Isolation of mutagenized strain clones resistant to AMPs

After radiation treatment, the bacteria were plated on isolation nutrition agarose plates with gradient concentrations for tachyplesin I, respectively, and incubated at 37°C for 20 h. The single grown colony on high concentration of tachyplesin I plates was picked out, and cultured in nutrient broth overnight at 37°C. Then cultured cell was used to determine minimum inhibition concentration (MIC) of tachyplesin I for each selection strain.

Since Clinical Laboratory Standards Institute (CLSI) does not define standard breakpoints for isolates resistant to AMPs, we identified bacterial resistance to AMPs according to a method previously described[19] that it was a significant increase in MIC for mutant strains comparing to that of control strains. In this study, if the MIC value for mutation strain was more than or equal to fourfold that of control strain, it was identified to obtain resistance mutant.

MIC determination

MIC was determined using a broth microdilution method for AMPs as described previously[20]. Briefly, cultured cells in the logarithmic phase were diluted to 2×10^5-4×10^5 organisms/ml. The inoculum (100 µL) was added to each well of 96-well plates. The peptide samples diluted with fresh broth (100µL) were added to each well and the plates were incubated at 37°C for 20 h. Cell growth was assessed by measuring the OD_{490} on a Spectralmax M_2 model microplate reader. The MIC was considered the lowest drug concentration that reduced growth by more than 50% compared with growth in the control well. Experiments were performed in triplicate.

Sub-inhibitory concentrations of AMPs selection experiment

This procedure was performed according to a previously reported method[21]. The three stains were cultured in the nutrient broth medium with 1/2×MIC of tachyplesin I at 37°C for 20 h. This procedure was repeated forty-two times in sub-inhibitory concentration of tachyplesin I. In addition, each strain was grown in the absence of tachyplesin I for the duration of the experiment, which was referred to as the control selection experiment. Off-spring strains from each interval of every four passages were collected to determine the MIC values. Each experiment was designed in parallel. The breakpoint of resistance to tachyplesin I was in accordance with the above reported method.

Stability of resistance assay

To verify the stability of resistance, 10 µL of resistant strains were cultured in 2 mL nutrient broth medium without drug for five continuous passages with each passage of 20 h, and off-spring strains from each passage were stored in 15% (v/v) glycerol at -70°C. The MIC values for each passage strain were determined, respectively.

RESULTS AND DISCUSSION

Growth curve assay

The growth curve of three strains was shown in Figure 1. These strains grew slowly in initial 2 h. However, they grew rapidly from 2 to 12 h. This result showed that the bacteria started to enter the logarithmic phase. After 12 h, they grew slowly to enter the stationary phase. In this study, we selected the logarithmic phase of bacteria to screen the resistant mutants.

Isolation of Tachyplesin I-resistant mutant by UV mutagenesis

The lethality rate of three strains induced by different UV radiation doses was shown in figure

2. With the increasing of mutagenic dose, fatality rate for *E. coli* ATCC25922 and JM109 increased gradually, which indicated a certain proportional relationship between mutation time and the fatality rate. When mutagenesis time was beyond 60 s, the lethal ratio had exceeded 75%. Under the same mutagenic intensity and radiation distance, fatality rate for *E. coli* ATCC25922 and JM109 was proportional to mutagenesis time. But for *P. aeruginosa* CCTCC2620, with the increasing of mutagenic dose, the fatality rate of *P. aeruginosa* displayed the trend that it began to increase at first, then decrease and finally increase again. The reason why fatality rate for *P. aeruginos* sharply increased may relate to operation, different strains and bacteria liquid density. Therefore, UV irradiation time was selected for 60, 90, 120 s for further screening.

E. coli ATCC25922, *P. aeruginosa* CCTCC2620 and JM109 mutagenized colonies strains from different mutagenesis time were randomly selected to determine MIC of tachyplesin I, respectively. The results indicated that one strain for *E. coli* ATCC25922 (mutagenesis 60 s) and nine strains for *P. aeruginosa* CCTCC2620 (mutagenesis 90 s) resistance to tachyplesin-I were isolated by UV mutagenesis. The MIC value of these mutants was four-fold higher than that of their original strains (Table 1). However, no resistance to tachyplesin mutant in JM109 was obtained by UV mutagenesis under the same condition, which may be related to its own structure of JM109. JM109 possess resistance to Ampicillin properties, and its cell membrane was different from that of *E. coli* ATCC25922. Compared with the other two strains (*E. coli* ATCC25922, *P. aeruginosa* CCTCC2620), the outer membrane proteins of JM109 such as OmpA, OmpF and OmpC lost or changed maybe it was one of the reasons why it was not easily induced resistance to tachyplesin I in JM109 by UV mutagenesis.

As one kind of physical mutagens, UV mutagenesis has many advantages including high mutagenic efficiency, easy operation, etc. It was a very useful tool for obtaining microbial high yield mutations and resistance to drug strain. Several bacterial strains have been mutated successfully by UV mutagenesis[22].

Table 1. MIC comparison of Tachyplesin-I for bacteria UV induction before or after

Strain	MIC ($mg \cdot L^{-1}$)		
	Original stain	UV mutagenesis strain	Microwave mutagenesis strain
JM109	10	5 or 10	5 or 10
E. coli ATCC25922	5	5 or 10 or 20	5
P. aeruginosa CCTCC2620	20	equal to or more than 80	-

Note: MIC was measured using serial broth microdilution method. It was considered the lowest drug concentration that reduced growth by more than 50% compared with growth in the control well.

Table 2. MIC comparison of Tachyplesin-I for bacteria under forty-two successive passages sub-inhibitory concentration of drugs

Strain	MIC ($mg \cdot L^{-1}$) of tachyplesin-I		
	Original stain	(after forty-two successive passages unsupplemented group) control group	(after forty-two successive passages tachyplesin-I group) induction group
JM109	10	10	10
E. coli ATCC25922	5	10	10
P. aeruginosa CCTCC2620	20	20-40	20-40

Notes: Inducible strains were cultured in supplemented with tachyplesin I for forty-two successive passages, whereas control strains were cultured in unsupplemented nutrient broth medium under the same conditions. The method for MIC determination was the same as above.

Compared with the original strain, smaller colony morphology, slower growth and worse uniformity of morphology were observed in *E. coli* ATCC 25922, and *P. aeruginosa* CCTCC 2620 resistant to tachyplesin. And after five continuous transfers in nutrition broth medium, the MIC values of tachyplesin for ten mutants did not change, which showed resistance in ten mutants was stable after five transfers in nutrition broth medium.

Isolation of Tachyplesin I-resistant mutant by microwave irradiation

Microwave mutagenesis is a relatively new physical mutation breeding technology of non-ionizing electromagnetic radiation. It has been widely used in crops, plants and microbes breeding study[23-24]. Compared with the other induction methods, microwave irradiation has a lot of advantages. It is clean, effective, easy to operate, safe with high mutation rate and small radiation damage, and mutant strains are more stable etc.[23]. It also avoids the problem of photo-reactivation, and it is often seen in UV mutation[17].

Continuous irradiation and intermittent irradiation method was usually adopted in microwave mutagenesis. In this experiment, in order to observe the effects of two different methods to resistant mutation rate, low-temperature-heat-diffusion method with water as coolant radiating *E. coli* ATCC25922 and low temperature cooling intermittent irradiation method radiating JM109 were used.

The lethality rate of microwave radiation on *E. coli* ATCC25922 and JM109 was shown in Fig. 3, 4. With the increasing of microwave radiation dose, fatality rate for *E. coli* ATCC25922 displayed the trend that it began to increase at first, then decrease and finally increase again.

Combined with the feature of colony on the plate, we chose radiation time for 60, 80, 120 s for *E. coli* ATCC25922 to further screen mutant. The fatality rate for JM109 indicated a gradual increased trend, and then we chose radiation time

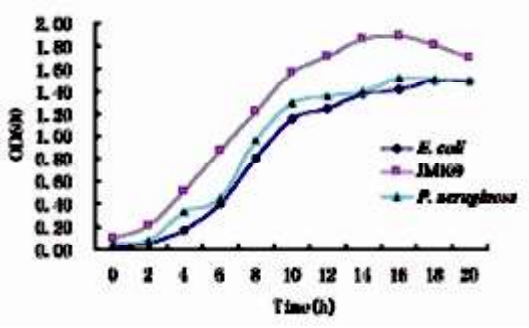


Fig. 1. Growth curve of three strains

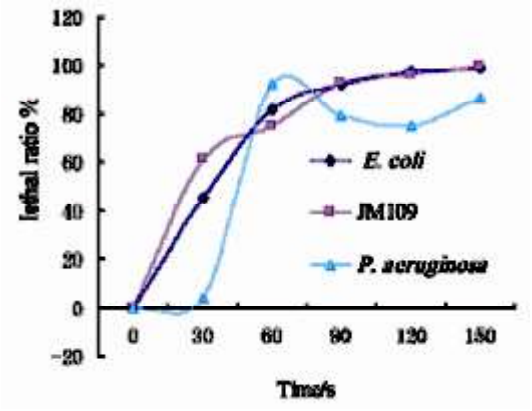


Fig. 2. Lethality rate of three strains induced by different UV radiation doses

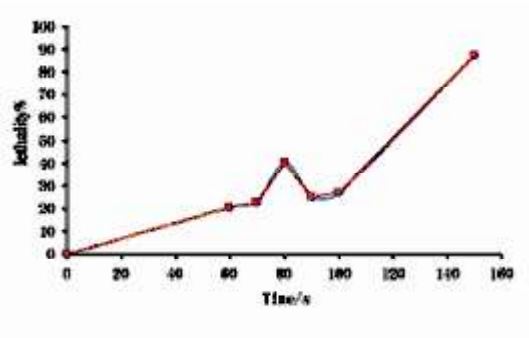


Fig. 3. Lethality rate of microwave radiation on *E. coli* ATCC25922

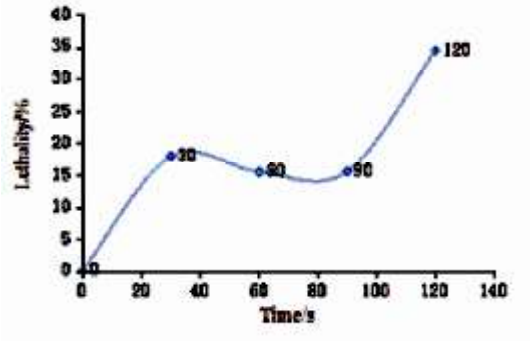


Fig. 4. Lethality rate of microwave radiation on JM109

for 90, 120 s to further screen mutant. The results showed that microwave mutagenesis failed to obtain strains resistant to tachyplesin I in *E. coli* ATCC25922 and JM109 (Table 1). The results indicated that original strain was more sensitive to UV than that of microwave in this study, so it is easier to obtain the mutants resistant to tachyplesin I by UV mutagenesis.

Isolation of Tachyplesin I-resistant mutant by sub-inhibitory concentrations selection

The strains (*E. coli* ATCC25922, JM109, *P. aeruginosa* CCTCC2620) were successively transferred in sub-inhibitory concentration of tachyplesin I for forty-two passages. As is shown in Table 2, there was no significant difference in the MIC between the control induction strains and the corresponding treatment induction strains. The result showed that no tachyplesin I-resistant mutants in *E. coli* ATCC25922, JM109 or *P. aeruginosa* CCTCC2620 were produced after forty-two continuous passages by this method. The result was also in agreement with the previous report that most cationic AMPs did not induce resistant mutants after as many as 20 passages at a concentration close to the MIC in the laboratory[25]. However, some evidence indicated that resistance to cationic AMPs can be evolved in bacteria through long and continual selection in the laboratory. *E. coli* and *P. fluorescens* independently evolved heritable mechanisms of resistance to pexiganan when propagating in medium supplemented with increasing concentrations of pexiganan for 600-700 generations (100 serial transfers) in the laboratory[26]; And resistance to porcine-rich PR-39 can rapidly develop in *Salmonella enterica*. Furthermore, some evidence demonstrated that bacteria could evolve resistance to AMPs when AMPs were used in the clinical application for a long time[27]. In present study why tachyplesin I was difficult to induce bacteria produce resistance may relate to the induction method and its antibacterial mechanism. Some studies show that tachyplesin I kill bacteria quickly by physical disruption of the cell membrane and causing damage to the key intracellular targets (DNA, RNA) in the short term[8,28-29], as well as causing inactivation of intracellular esterase and ultimate cell death. Moreover, cell membrane was mainly the target of killing bacteria (unpublished data).

Among the three methods, compared with others, sub-inhibitory concentration of drug selection method was accessible to clinical practice, which is more meaningful in application aspects. But this method needs to spend for longer periods of time with more drugs. So in the actual scientific research, we should choose the proper methods to screen the mutant strain according to our study aim.

To sum up, Tachyplesin I induced high resistance in *E. coli* ATCC25922 and *P. aeruginosa* CCTCC2620 by UV mutagenesis. The MIC values of tachyplesin I for mutant strains were four times higher than those of original strains. Microwave mutagenesis and sub-inhibitory concentration of drug selection failed to obtain resistance strains to tachyplesin I. Resistance mechanism of tachyplesin-I needs further research.

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