Isolation and characterization of *Staphylococcus aureus* mutants sensitive to Lysozyme by UV radiation

Farah Tarik Abdulkareem[+] and Hameed Majeed[++]

[+] Ministry of Sciences and Technology
[++] Al-Nahrain University

Abstract

Staphylococcus aureus, an opportunistic pathogen commonly found on human skin, were exposed to 1, 2, 3, 4 and 5 J/m^2 UV radiation; UV radiation was highly lethal and mutagenic. We detect the mutational effects of irradiation by culturing the isolates on lysozyme containing medium of isolate that was unable to grow in lysozyme and was affected by radiation. Four mutants were obtained and they were sufficiently effective for the isolation of macromolecules such as plasmid and deoxyribonucleic acids, from the cells after lysozyme-induced cell lysis.

Keywords: *Staphylococcus aureus*, UV radiation, lysozyme, mutation.

الحساسة لأنزيم اللايسوزايم عزل وتشخيص بكتريا المكورات العنقودية المطفرة

باستخدام الأشعة فوق البنفسجية

فرح طارق عبد الكريم و حميد مجيد

الخلاصة

تعد بكتريا المكورات العنقودية من الممرضات الانتهازية التي تتواجد عادة في جلد الإنسان. لقد تم تعريض هذه البكتريا الى 1 و 2 و 3 و 4 و5 جول/م^2 من الأشعة الفوق البنفسجية ووجد أن لها تأثير قاتل ومطفر على البكتريا وقد تم الكشف عن البكتريا المطفرة عن طريق زرع البكتريا على أوساط زرعيه تحتوي لايسوزايم. تم الحصول على أربع عزلات مطفرة واستخلاص الكروموسومات والبلازميدات بعد معاملتها بإنزيم اللايسوزايم وتشخيص هذي الطفرات ومقارنتها بالعزلة الأصلية.

الكلمات المفتاحية: بكتريا المكورات العنقودية، الأشعة الفوق البنفسجية، اللايسوزايم، الطفرة الوراثية.

Introduction

Staphylococci are Gram positive spherical bacteria that occur in microscopic clusters resembling grapes, it is a normal inhabitant of the skin and mucous membrane in the nose of healthy human [1].

Staphylococcus aureus, literally "Golden Cluster Seed" and also known as golden staph, is the most common cause of staph infections. It is a spherical bacterium, frequently living on the skin or in the nose of a person. Approximately 20–30% of the general populations are "staph carriers" [2].

S. aureus is, golden yellow colonies, often with β-hemolysis, when grown on blood agar plates , *S. aureus* is a facultative anaerobe and opportunistic pathogen [3].

Staphylococci cell walls are composed of teichoic acids, and wall-associated surface proteins. Stress-bearing murein represents continuous macromolecular units covering the whole cell [4].

The ability to evade host immune surveillance is a critical virulence determinant for any pathogen along with the capacity to defend against the immune defenses, and both of these aspects can be provided by the peptidoglycan ,a large polymer that provide much of the strength and rigidity to the bacterial cell wall. It consists a long glycan chains of alternating N-acetyleglucosamine (NAG) ,and N-actylmuramic acid (NAM) subunits [5].

The antibacterial nature of lysozyme was first witnessed by Sir Alexander Fleming (6). Because lysozyme is cationic, it closely adheres to bacteria through electrostatic interactions with negatively charged tiechoic and lipotiechoic acids and phospholipids on the bacterial surface this interaction can result in bacterial lysis by hydrolyzing the bond between NAG and NAM [7].

Although, lysozyme is known to be bactericidal to certain bacteria but its antimicrobial function is limited to certain gram positive bacteria owing to the differences found in their membrane structure [8].

S. aureus showed a remarkable ability to survive antibiotics treatment by developing new resistance mechanisms against them within a short time of its introduction. Some strains are now resisting most conventional antibiotics; it is worrisome that it seems to be there are no new antibiotics on the horizon and any recent antibiotic developments are merely a modification to existing drugs; *S. aureus* is known to be

notorious in their acquisition of resistance to new drugs [9].

Mutagenesis plays a central role in our lives. A low level of mutagenesis is advantageous, and ensures the survival of species by promoting evolution. Programmed mutagenesis of immunoglobulin genes promotes diversity and provides a dynamic defense against invading pathogens. However, many human diseases, including most cancers, arise as a consequence of mutations that occur either spontaneously, or are induced by copying errors in the genetic material during cell division, by exposure to ultraviolet or ionizing radiation [10].

Materials and methods

Bacterial Isolate

Staphylococcus aureus isolate used in this study was obtained from the Department of Biotechnology/College of Science/Baghdad University; it was isolated locally in 2007.

Media

Ready to use media

The following media were prepared according to the instruction of the manufacturing company; pH was adjusted to 7.0 and sterilized by autoclaving at 121ºC.

- Nutrient agar
- Nutrient broth
- Brain heart agar
- Brain heart broth

Laboratory prepared media [11].

- Luria – Bertani agar
- Luria broth

Antibiotic discs

The following antibiotic discs were used for antibiotic sensitivity test, Results of inhibition zone of *S. aureus* were then compared with those indicated in NCCLS (2002) [12] for *S.aureus*.

Buffers and solutions

Buffers and solutions used in this study were 12:

- Normal saline solution 0.85%
- Phosphate buffer solution (0.2M)
- Lysozyme Stock solution (500µg/ml)
- DNA extraction solutions
- Tris-EDTA buffer solution (TE)
- Sodium chloride Tris-EDTA buffer solution (SET)
- Sodium dodecyle sulphate solution (SDS) 10%
- Tris-borate Buffer solution TBE(1X)
- Agarose gel (0.7%)
- Ethidium bromide
- Loading buffer

Methods

Physical mutagenesis

Mutagenesis by UV irradiation was done (14) by subjecting fresh culture of *Staphylococcus aureus* suspended in phosphate buffer solution (pH7.0) to UV radiation in a dark place using the UV- transilluminator. The tray of the irradiation approximately 15X25 cm exposes sample in glass Petri dish and the distance between the UV source and irradiated suspension was 11 cm.

The suspension of *S. aureus* was prepared by inoculating 5 ml of L-broth with single colony of *S. aureus* overnight at 37ºC , cells were then precipitated at 3000 rpm for 15 min, and washed twice with normal saline and resuspended in 5 ml of phosphate buffer (pH 7), the cell suspension were poured in sterilized Petri dishes and subjected to 1, 2, 3, 4, and 5 J/m² UV of irradiation, then 0.1ml of cell suspension was taken after each treatment diluted to appropriate dilution and spread on brain-heart infusion agar plates, plates were then incubated over night at 37ºC to determine the viable count and survivals of *Staphylococcus aureus*. Lysozyme sensitive mutants were screened by replica plating on brain heart infusion agar plates containing 3.4µg/ml and 12.5µg/ml of lysozyme.

Plasmid profile

Total DNA of *S. aureus* was extracted alkaline lysis method and as follows [15]:

- Hundred ml of fresh culture of *S. aureus* in BHI broth was centrifuged at room temperature for b 5min.
- Cell pellets were resuspended in the same volume of SET buffer solution (pH 8), the cells were recenterifuged, suspended in 0.5ml ice-cold acetone, and kept on ice for 5 minutes.
- The cells were centrifuged again and residual acetone was removed with gentle stream of air.
- Bacterial pellets were resuspended in lysis buffer. Lysozyme was then added at final concentration 200µg/ml and incubated at 37°C for 30 minutes.
- Lysis was achieved by adding 0.4 ml of SDS (10%). Cellular debris was removed by centrifugation 12000rpm for 15min.
- The supernatant was then extracted twice with an equal volume of a mixture of chlorophorm- isoamyl alcohol (24:1 v/v).
- The DNA was precipitated with (0.6 v/v) isopropanol alcohol; the mixture was incubated at -20°C overnight and recentirfuged 12000rpm for 15 minutes.
- The supernatant was decanted and precipitate was washed with 70% ethyl alcohol, centrifuged again and

the precipitate was dried with gentle stream air.

- TE buffer (0.025 ml) was added for gel electrophoresis analysis.

Agarose gel electrophoresis

Agarose gels (0.7%) were run horizontally in Tris Borate- EDTA buffer (TBE 1 X), samples of extracted DNA were mixed with loading buffer in 1:10 ratio and added to the wells on the gel. Generally, gel was run for 2-3 h at 5 $v.cm^{-1}$ and the agarose gel was stained with ethidium bromide by immersing them in ethidium bromide solution (0.5 µg/ml) for 30-45 min, DNA bands were visualized by U.V in transilluminator cabinet, and photographed.

Characterization of lysozyme sensitive mutants

Antibiotic sensitivity test [11]

The disc diffusion method was used to test the antibiotic sensitivity of the bacterial isolate .A sterile cotton swap was applied in to the inocula (fresh culture for 18 hour) and the entire surface of the brain heart infusion agar plates was swabbed three times by rotating the plate approximately 60º after each streaking to ensure even distribution. Then the disc of antibiotics were applied on streaked plates and incubated at 37ºC. Inhibition zone was measured after incubation for 16 hour.

Protoplasts formation of *S. aureus* mutants by lysozyme

Protoplast formation of *S. aureus* involves the conversion of selected mutant cells into protoplast using lysozyme alone was achieved [16].

lysozyme sensitive mutant of *S. aureus* was obtained. Mutagenesis by UV radiation (S2) were selected by replica plating on brain heart infusion agar. Mutants were inoculated into 50ml of brain heart infusion broth and incubated over night at 37˚C with shaking till 0.50 optical density of the growth was achieved, then five ml of each growth culture were centrifuged at 3000rpm for 10min and resuspended in phosphate buffer (pH 7). Lysozyme was added to the cell suspension at final concentration of 50µg/ml and incubated at 37˚C with shaking 150rpm for 2 hours. The formation of protoplasts was observed by light microscope.

Temperature sensitive growth

Temperature sensitive growth of mutants *S. aureus* were tested and compared with the wild type [17].

Single colony of *S. aureus* (wild type) and the mutants cells (S2, S3, S4, S5) which were described previously

by replica plating, were inoculated into 50ml brain heart infusion broth over night at 37˚C, then 0.1ml of each culture spread on brain heart infusion agar and incubated over night at 30, 37, 40, 43 and 45˚C. Growth was observed and survivals were counted after each incubation.

Results and Discussion

Physical mutagenesis

DNA is one of the key targets of UV-induced damage; therefore, organisms have developed a number of repair mechanisms to counteract the DNA damage caused by UV. These repair mechanisms will however be unable to cope if the UV dose applied is higher than the repair capacity [18].

Results of subjecting fresh cultures of *S. aureus* to different doses of UV ray presented in Figure 1 showed lethal effect on bacterial cell; this can be noticed from reduction in the viable count of bacterial cells from $46x10^7$ CFU/ml at zero time to $13x10^7$ CFU/ml after exposure to UV ray decreases to 28.2%,6.9, 2.6 and 1.3% respectively as exposed to 1, 2,3and 4 $J/m^{2,}$ while the survivals was decreased to zero% after subjection to the final dose ($5J/m^2$) of UV ray. Results of treated cells with UV radiation showed that all of the selected colonies were able to grow on brain heart infusion agar containing 3.4µg/ml lysozyme. While results in Figure 2 showed that four of the total colonies were unable to grow on the medium containing 12.5µg/ml lysozyme. These colonies were probably affected by the mutagenic activity of UV radiation that may cause alterations in deoxyribonucleic acid (DNA) (19).

These results are due to pigmentation, since pigmented isolates were found to be more resistant to the effect of UV radiation (20).

In a previous study [21] it was found that with the proper mutation, *S. aureus* cells were rendered to lysozyme hydrolysis.

As shown in Figure 3, lysozyme sensitive mutants (S2, S3, S4, and S5) showed clear chromosomal and plasmid DNA bands on agarose gel. Which indicates lysis of these mutants; the wild type, on the other hand, was resistant to lysis by lysozyme. These results confirmed that lysozyme sensitive mutants isolated by chemical and physical mutagenesis have altered bacterial cell wall.

It appears that lysozyme sensitive mutants provide obvious economical advantages over the wild type strain for the preparation and study of macromolecules.

Characterization of lysozyme sensitive mutants

Antibiotic sensitivity test

Antibiotics sensitivity test was performed on wild type and mutants of *S. aureus* in order to reveal that the changes resulted from the effect of UV treatment on the antibiotics sensitivity pattern of the wild type, for this purpose twelve antibiotic discs were used in this test.

Results in Table 1 showed that the wild type of *S. aureus* was resistant to gentamycin, cephalothin, cefotaxime, erythromycin, penicillin, streptomysin, vancomycin, pipracillin and fucidic acid, while sensitive to impenem, amoxicillin and tetracycline. These results were constitute with other findings [22] which stated that *S. aureus* has become resistant to many commonly used antibiotics, only 2% of all *S. aureus* isolates were sensitive to penicillin, due to the penicillinase (a form of β-lactamase).

However, lysozyme sensitive mutants obtained after mutagenesis with UV radiation were variable in their resistance to these antibiotics; *S. aureus* mutants (S2, S3, S4, and S5) lost their ability to resist 4, 3, 3 and 3 different antibiotics respectively, these results may be due to the genetic alteration in different genes that express antibiotic resistance to *S. aureus*, occurred after mutagenesis with UV radiation [23].

Protoplast formation of *S. aureus* mutants by lysozyme treatment

Bacterial protoplasts and spheroplasts are osmotically fragile forms that lack rigid or partially rigid cell walls. A conversion of bacterial cells to protoplasts or spheroplasts achieved normally by the addition of lysozyme growth medium. Removing cell wall by lysozyme has been used with success on a variety of bacteria. However, protoplasts of *Mycobacterium smegmatis*, *S. aureus*, and *Clostridium pasteurianum* cells were not readily prepared by lysozyme alone [16].

lysozyme sensitive mutant of *S. aureus* isolated induced by UV radiation (S2) were treated with lysozyme at concentration of 50µg/ml, and incubated for 2 hours. The formation of protoplasts was observed by light microscope. Cells appeared as spherical bodies of various sizes and density. They were transparent and swollen in comparison with wild type. Figure 4 showed the morphological changes of *S. aureus* cells.

However, prolonged incubation may be necessary to cell division and loss of cell wall, which result in protoplast formation. As a result of this treatment, the cell contents would be enclosed only by a cell membrane.

Temperature-sensitive growth

Lysozyme sensitive mutants isolated by MNNG and UV mutagenesis might show temperature sensitive growth when incubated at elevated temperatures. *S. aureus* mutants were investigated for temperature sensitive growth and compared with the wild type(24).

Results in Table2 show the temperature sensitive growth of *S. aureus*. The wild type and mutants obtained after UV radiation (S4 and S5) showed growth at 30, 37, 40, 43 and 45˚C. While two of the mutants obtained after UV radiation (S2, and S3) were unable to grow at high temperature (above 40˚C), which suggested defects in cell surface structure and the membrane integrity as results of mutagenesis [25].

UV mutagenesis causes point mutation that produce defect in RNase. Temperature sensitive growth considered to be caused by simultaneous defect of RNase which is related to the synthesis and maintaining of the cell surface of this bacterium [26].

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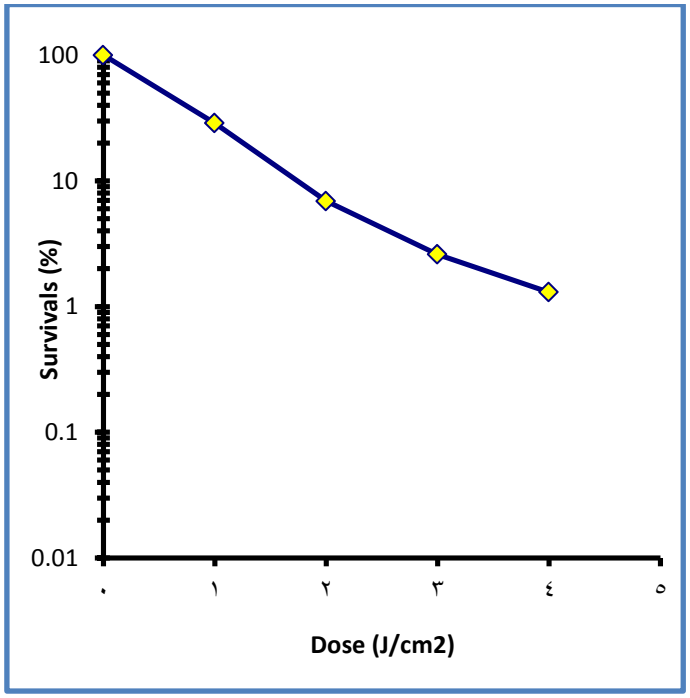


Figure 1. Survival curve for *S. aureus* after subjection to different doses of UV radiation (J/m^2).

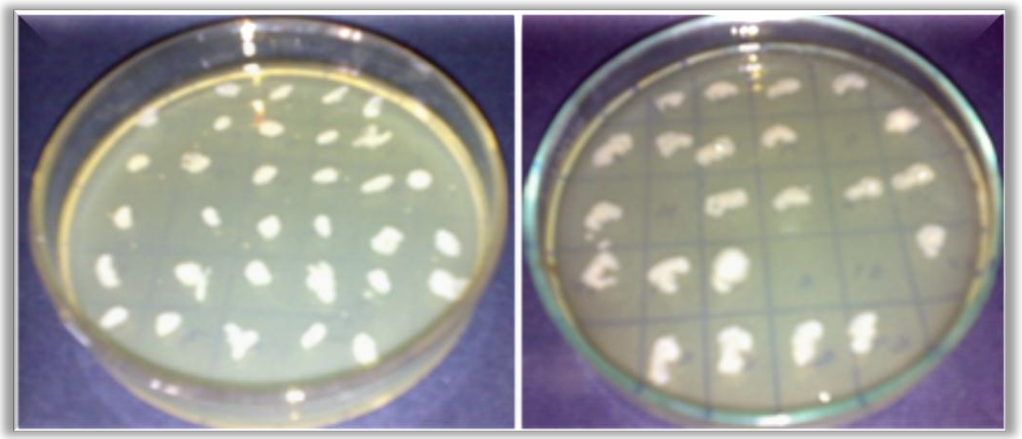


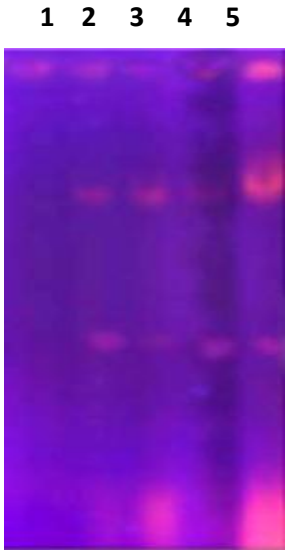
Figure 2. Screening lysozyme sensitive mutants of *S. aureus* after subjection to physical mutagenesis by UV radiation.

A: colonies plated on BHI agar (master plate).
B: colonies plated on BHI on agar containing 12.5μg/ml lysozyme.

Table 1: Antibiotic sensitivity of the wild type and mutants of *S. aureus* after subjection to UV mutagenesis and MNNG mutagenesis

`Antibiotic		Wild type	UV induced mutants			
Type	Conc. μg/ml		S2	S3	S4	S5
Gentamycin	10	R	S	R	R	R
Cephalothin	30	R	S	R	S	S
Cefotaxime	30	R	S	S	S	S
Erythromycin	15	R	S	S	S	S
Penicillin	10	R	R	R	R	R
Amoxicillin	25	S	S	S	S	S
Fucidic acid	10	R	R	R	R	R
Imipenem	10	S	S	S	S	S
Tetracycline	30	S	S	S	S	S
Pipracillin	100	R	R	R	R	R
Streptomycin	10	R	R	R	R	R
Vancomycin	30	R	R	S	R	R

R: Resistance S: Sensitive



Lane 1: DNA extracted from wild type.
Lane 2: DNA extracted from lysozyme sensitive mutants S2.
Lane 3: DNA extracted from lysozyme sensitive mutants S3.
Lane 4: DNA extracted from lysozyme sensitive mutants S4.
Lane 5: DNA extracted from lysozyme sensitive mutants S5.

Figure 3. Genomic DNA extracted from *S. aureus* after subjection to mutagenesis by UV and MNNG on agarose gel (0.7%) electrophoresed for 2hours at 5v/cm.

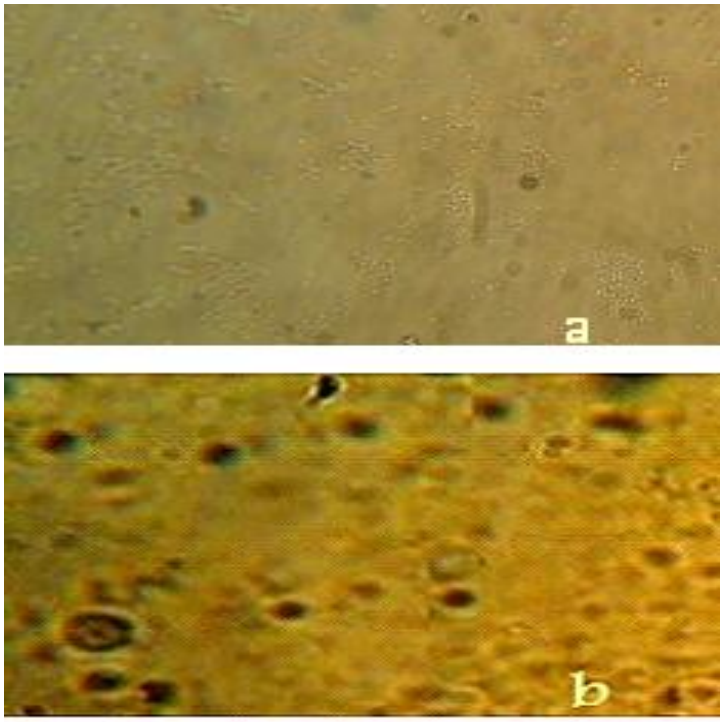


Figure 4: *Staphylococcus aureus* cells under light microscope.

a: wild type
b: lysozyme sensitive mutant S2 obtained after physical mutagenesis by UV radiation.

Table 2. Lysozyme sensitive mutants of *S. aureus* incubated at different temperatures.

Bacterial Isolate	Growth Temperature (°C)				
	30	37	40	43	45
WT	++	+++	++	+	+
S2	++	+++	++	-	-
S3	++	+++	++	-	-
S4	++	+++	++	+	+
S5	++	+++	++	+	+

+++ Very good growth ++ Good growth + Low growth - No growth
WT Wild type of *S. aureus*
S2, S3, S4, S5 Lysozyme sensitive mutants