# Antibiotic resistance in *Staphylococcus aureus* and the effects of methylation and epigenetic inheritance within bacterial DNA

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## Abstract

The role of epigenetics in antibiotic resistance is an important factor in understanding how bacteria can obtain resistance without changing the genome. The purpose of this study was to show if DNA methylation would cause antibiotic resistance within two strains of *Staphylococcus aureus* (ATCC 43300 and ATCC 29213) using restriction endonuclease DNA digestion and pulsed-field gel electrophoresis (PFGE) analysis. A minimum inhibitory concentration (MIC) of the antibiotic oxacillin was determined for both *Staphylococcus* strains and both strains were plated on agar media containing various concentrations of the antibiotic to force a resistance/mutation. Resistant colonies were tested for the methicillin resistance gene (mecA) using polymerase chain reaction (PCR) and then subjected to restriction endonuclease digests using the enzymes SmaI and XmaI for the subsequent PFGE analysis. The result of this study shows that one strain (ATCC 43300) carried the mecA gene yet displayed susceptibility to oxacillin in the MIC test. The other strain (ATCC 29213) did not carry the mecA gene, but was able to grow on media containing a concentration of oxacillin three times the value of its MIC. The restriction digests and PFGE analysis detected no DNA methylation on the 5'-CCCGGG-3' sites for both strains indicating that the resistance observed may be a result of DNA methylation located on a different site or by some other mechanism.

## Introduction

Epigenetics in prokaryotes and eukaryotes, is a mechanism of gene transcription that does not permanently change the DNA and can usually be reversible (2, 21). Epigenetics maintains an important role within bacterial species by allowing these bacteria to change given their environment and not have these changes alter their DNA. These epigenetic regulations enable these organisms to respond rapidly to any environmental stresses or signals by multiple different mechanisms (21). One epigenetic mechanism bacteria use is DNA methylation. The methylation of DNA allows another way for epigenetic inheritance to occur (5).

The role of DNA methylation in bacteria is thought to protect DNA from restriction endonucleases and any cleaving of DNA (15). Another role of DNA methylation in bacteria is the activation of genes. Methylation of the DNA can happen when bacteria are exposed to environmental conditions that are not suitable for its function, and these environmental agents can alter gene expression which can evolve into a heritable epigenetic change (2, 5). These changes can lead to antibiotic resistance within bacterial species. For example, *Staphylococcus aureus* has been found to become antibiotic resistant by DNA methylation after being exposed to an antibiotic agent (2). These epigenetic mechanisms, like DNA methylation, can increase the virulence of a bacterial species (5).

Antibiotic resistant bacteria, like Methicillin-Resistant *Staphylococcus aureus* (MRSA), has been a threat to public safety especially for patients in the hospital. Understanding the mechanisms behind antibiotic resistant pathogens is essential to fight against these infectious agents. Bacteria have been known to become persister cells, which means that a few bacteria can remain dormant until the environment they are in is more suitable for growth. These persister cells can occur without any genetic alterations and can therefore become resistant or persistent by epigenetic mechanisms, like DNA methylation (18). Since these mechanisms are reversible it allows gene expressions to alternate between active or ON phase and inactive of OFF phase (14, 18). This means that a bacteria, like *Staphylococcus aureus*, can become antibiotic resistant without changing any DNA sequence. The purpose of this study is to determine if DNA methylation plays a role in antibiotic resistance through a forced mutation within two different strains of methicillin-susceptible *Staphylococcus aureus*.

## Materials and Methods

**Forcing the mutation.**

A minimum inhibitory concentration (MIC) of the antibiotic oxacillin was determined for two methicillin-susceptible strains of *Staphylococcus aureus*: ATCC 43300 and ATCC 29213. Oxacillin is routinely used to test for methicillin resistance because it is in the same class of drugs as methicillin but is more stable and readily available (6). Using the 0.5 McFarland Standard, 3mL sterile DI water was added to a test tube and put into a spectrophotometer set at 625nm. Suspended bacteria in a sterile water tube were added drop by drop until the spectrophotometer had an absorbance reading between 0.08-0.13. The absorbance recorded for each of the two *Staphylococcus aureus* strains were 0.12 for ATCC 43300 and 0.12 for ATCC 29213. Using a sterile swab, the diluted bacteria were transferred onto a normal Mueller-Hinton plate and a MIC oxacillin strip was added to the center. The plates were placed face-up in a 37℃ incubator overnight. The MIC was found to be around 2.0mg for ATCC 43300 and 0.25mg for ATCC 29213 (Figures 1 & 2).

Antibiotic plates were made using 500mL of sterilized Difco Mueller-Hinton agar along with increasing concentrations of the antibiotic oxacillin. A 0.1M solution of oxacillin was made by dissolving 0.05g of oxacillin powder into 11.8mL of DI water. This solution was then added into the liquid Mueller-Hinton agar (cooled to 60℃) to produce 5 plates for each of the following antibiotic concentrations: 0.0 (no antibiotic added), 0.75, 1.5, 2.0, and 5.0mg/L. Each plate was poured using a volume of 20mL.

Using the 0.5 McFarland Standard described above, both *Staphylococcus* strains were plated onto one of each of the antibiotic concentrations using a sterile swab. Plates were incubated face-up at 37℃ overnight.

**Determining the rate of mutation.**

After incubation of the antibiotic plates, colonies were found on the 0.75mg/mL plate for ATCC 29213 and on the 2.0mg/mL for ATCC 43300. These mutated colonies were then transferred to their own tryptic soy agar (TSA) plate with no antibiotic concentration to amplify their own growth using the basic isolation streak method. These plates were incubated overnight at 37℃ and were used later for a DNA extraction.

To estimate the number of cells that mutated, ten colonies were taken from each stock (nonmutated) *Staphylococcus* strain grown on a TSA plate and were suspended in 1mL of tryptic soy broth (TSB) inside of a 1.5mL microcentrifuge tube. One tube was prepared for each of the *Staphylococcus* samples. This 1mL suspension was then diluted by pouring it into a tube containing 9mL of sterile water. This suspension was then diluted again by taking 1mL and transferring it to another tube containing 9mL of sterile water. This process, known as serial dilution, was repeated for a total of five tubes giving a dilution factor of $10^{-5}$, or 1:100,000. This procedure was performed for both *Staphylococcus* samples.

From the final dilution tube of each *Staphylococcus* sample, $100\mu$L was transferred to new TSA plates containing concentrations of 0.0, 0.75, and 2.0mg/mL oxacillin. The $100\mu$L of liquid was spread over the entire agar surface using a glass spreader. These plates were incubated face-up overnight at 37℃.

After incubation, the number of colonies was counted on each of the four plates with the following results: ATCC 43300 had 564 colonies on the 0.0mg/mL plate, 83 colonies on the 0.75mg/mL plate, and 12 colonies on the 2.0mg/mL plate. ATCC 29213 had 550 colonies on the 0.0mg/mL plate, 2 colonies on the 0.75mg/mL plate, and zero colonies on the 2.0mg/mL plate. The number of colonies counted was then calculated to represent the estimated number of cells contained in the initial 1mL TSB suspension. The calculation was as follows:

**# of colonies counted x 100,000** (the dilution factor) **x10** (since only $100\mu$L of each sample was plated).

The final resulting numbers indicate that the original 1mL sample of ATCC 43300 contained 564,000,000 nonmutated cells (the number of colonies counted on the 0.0mg/mL plate). The number of mutants produced were 83,000,000 cells on the 0.75mg/mL plate and 12,000,000 cells on the 2.0mg/mL plate. The original 1mL sample of ATTC 29213 contained 550,000,000 nonmutated cells (the number of colonies counted on the 0.0mg/mL plate). The number of mutants produced were 2,000,000 cells on the 0.75mg/mL plate and zero cells on the 2.0mg/mL plate.

To get the rate of mutation, the number of mutated cells was divided by the number of nonmutated cells giving the final results of 14.2% on 0.75mg/mL and 2.13% on 2.0mg/mL for ATCC 43300, and 0.369% on 0.75mg/mL for ATCC 29213.

**Determining presence of the mecA gene.**

Determining the presence of the mecA gene is vital in determining if antibiotic resistance is due to inherent genomic resistance or by some other means. The mecA gene encodes an altered penicillin-binding protein, which is not inhibited by existing β-lactam antibiotics, such as methicillin and oxacillin, making bacteria resistant to this class of antibiotic (16). DNA was extracted from the two original stock *Staphylococcus* strains, from the 2.0mg/mL mutant of ATCC 43300, and from the 0.75mg/mL mutant of ATCC 29213. The extraction was performed using the Purelink Microbiome DNA purification kit protocol (12). To amplify the 533 base pair (bp) fragment of the mecA gene, polymerase chain reaction (PCR) tubes were made in $20\mu$L volumes containing $10\mu$L G2 colorless MasterMix, $2\mu$L of mecA forward primer with sequence 5' – AAAATCGATGGTAAAGGTTGGC – 3' (16), $2\mu$L of mecA reverse primer with sequence 5' – AGTTCTGGAGTACCGGATTTGC – 3' (16), $4\mu$L of nuclease-free water, and $2\mu$L of

DNA. One tube was prepared for each of the four samples. Amplification of the mecA gene was done by performing the PCR on a thermocycler. The four tubes were placed on the temperature block in the thermocycler with the following temperature settings: set pre-run at 95℃ for 3 minutes and then for 35 cycles at 94℃ for 1 minute, 53℃ for 30 seconds, 72℃ for 1 minute. After the PCR was finished, the samples were mixed with $5\mu$L of 5x Orange G loading dye. Samples were then loaded onto a 2% agarose gel (1.2g of agarose dissolved in 60mL of 1x TAE buffer) in $20\mu$L volumes along with a 100 base pair ladder. The gel was run using horizontal gel electrophoresis at 75 volts for one hour and 150 volts for another half-hour. After the electrophoresis, the gel was stained in a solution of SYBR safe DNA stain (60mL DI water and $6\mu$L SYBR safe stain) for 15 minutes and then visualized using a blue-light transilluminator.

**Determining DNA methylation.**

Pulsed-field gel electrophoresis (PFGE) is a process used to separate large DNA fragments (greater than 10 kilobases) from an organism's entire genome. The genomic DNA must be extracted inside of agarose plugs and then cut into fragments using a restriction endonuclease, which is an enzyme that can cleave double-stranded DNA. The enzymes used for this procedure were SmaI and its isoschizomer XmaI. Both enzymes cut the DNA at the specific sequence of nucleotides 5' - CCCGGG - 3', but SmaI activity is inhibited in the presence of methylated DNA whereas XmaI is not (3, 19). The protocol for agarose plug preparation and subsequent PFGE analysis was provided by the CDC (7).

*Pre-lysis Preparation*
The bacterial samples used for the plugs were from ATCC 29213 (one sample of the original nonmutated bacteria and one sample of the 0.75mg/mL oxacillin resistant mutant) and ATCC 43300 (one sample of the original nonmutated bacteria and one sample of the 2.0mg/mL oxacillin resistant mutant). Colonies from each of the four bacteria were resuspended in 1X TE buffer and diluted with TE buffer until each sample recorded a 25% transmittance in a spectrophotometer set at 600nm. $400\mu$L of this suspension was transferred to a 1.5mL microcentrifuge tube along with $20\mu$L of a 25mg/mL lysozyme solution and $5\mu$L of recombinant lysostaphin solution. Recombinant lysostaphin is used because it effectively breaks down the peptidoglycan of the cell wall without damaging the DNA (11). The samples were then incubated on a heat block at 55℃ for 45 minutes to complete this pre-lysis step.

*Agarose Plug Preparation*
A 1.0% low melt agarose (LMA) solution was prepared by dissolving 0.2g low melt agarose and 20mL of TE buffer in a flask and microwaving in 15 second intervals until the agarose was completely dissolved. 15mL of the LMA solution was added into a conical centrifuge tube and stored at 60℃ to be used later in the gel preparation. Approximately $425\mu$L of 1% LMA solution was added to each of the microcentrifuge tubes on the heat block after the incubation period was finished. Samples were mixed by gently pipetting up and down and then approximately $800\mu$L from each sample was transferred to its own 1mL syringe (the end of each syringe was previously cut off), capped off with parafilm, and stored in a fridge to solidify (Figure 3).

*Lysis of Agarose Embedded Bacteria*

A 15mm section of agar from each of the syringe molds were cut and added to their own 15mL conical tube along with 5mL of lysis buffer from the PureLink Microbiome DNA Purification Kit (12) and 25$\mu$L proteinase K (20mg/mL). The tubes were then placed on a rack and into a reciprocal shaking water bath set at 165rpm and at a temperature of 54℃ for two hours. After incubation, the plugs were transferred from the conical tubes into a flask with 10mL of sterile DI water and washed for ten minutes. This process was repeated a second time using fresh DI water. The plugs were then washed three times with 10mL of 1X TE buffer for 10 minutes each using fresh TE buffer for each wash. All washes were performed at room temperature with constant 150-170rpm agitation using an orbital shaker. Plugs were stored in fresh TE buffer and placed in the refrigerator to be used later.

*Cutting the DNA by Restriction Endonuclease*

Using a metal spatula, each plug was removed from the TE buffer and sliced into 1-2mm slices using a straight razor blade (Figure 4). Two slices were cut and dispensed into sterile 1.5mL microcentrifuge tubes for each sample. In each tube, 175$\mu$L of molecular-grade water, 22$\mu$L 10x restriction buffer containing Bovine serum albumin, and 3$\mu$L of a specific restriction endonuclease (four tubes with SmaI and four tubes with XmaI) were all added. Each of the four bacterial samples genomes were cut using both enzyme treatments. SmaI samples were incubated for two hours at 25℃ and XmaI samples were incubated for eight hours at 37℃. After incubation, the liquid from each tube was aspirated using a 200$\mu$L pipette and discarded. The restricted plug slices were then immersed in fresh TE buffer until they were ready to be loaded into the gel.

*Performing the Pulsed-field Gel Electrophoresis (PFGE)*

Assembly of our PFGE apparatus was done following the design and using firmware created by Lagos-Susaeta et al (13). A 12V power supply along with parts to build the circuitry, buffer cooling system, and gel rotational mechanism were purchased, and 3-D printed parts were provided by the Lewis and Clark Library. A horizontal gel electrophoresis chamber that was already in the lab was modified to accommodate the PFGE procedure (Figure 5).

A 1% agarose gel was prepared by dissolving 0.6g of low melt agarose into 60mL of 0.5X TBE buffer in a flask. The flask was heated in a microwave in 15 second intervals until the agarose was completely dissolved. After cooling to about 60℃, the gel was poured into a mold with a 6-tooth comb and left to solidify. One plug slice from each SmaI digest was placed in a gel well. The order of the samples was: 1) Blank (no sample added), 2) ATCC 43300 nonmutant, 3) ATCC 43300 2.0mg/mL mutant, 4) ATCC 29213 nonmutant, 5) ATCC 29213 0.75mg/mL mutant, and 6) Blank (no sample added). Each plug was sealed in the wells with the extra 1% LMA solution stored in the conical tube using a micropipette and left to solidify.

The equipment for the PFGE was setup and parameters for the run were input on the OpenPFGE app, also created by Lagos-Susaeta et al (13), using an Android tablet (provided by Mary Ann George) and are listed as follows: gel angle at 120°, initial switch time at 5.0s, final switch time at 40.0s, run time at 20hrs, and buffer temperature at 12℃. The horizontal gel electrophoresis chamber was connected to a high voltage power supply set at 6.0V/cm.

After the 20hr run, the gel was removed from the electrophoresis chamber and was set in a solution of SYBR safe DNA stain for 20 minutes. After staining, the gel was visualized using a blue light transilluminator. This process was repeated for the XmaI restricted samples.

# Results

The results for the antibiotic plates showed that ATCC 43300 had distinct colony growth on the 0.75mg, 1.5mg, and 2.0mg oxacillin concentrated plates. The antibiotic plates with ATCC 29213 showed no distinct colonies but as a precaution, the plates were scraped using a sterile loop and then plated on a nonantibiotic plate to see if any growth would occur. The results showed that for the 0.75mg plate colonies were in fact produced (Figure 6).

Results of the PCR and gel electrophoresis for the mecA gene showed that *Staphylococcus* strain ATCC 43300 was mecA positive for both the nonmutant and mutant samples. This positive result was distinguished by the presence of a DNA fragment at around the 550bp mark on the gel (Figure 7). For the strain ATCC 29213, both the nonmutant and mutant samples were mecA negative (Figure 7).

The results for the pulsed-field gel electrophoresis (PFGE) showed that each sample had DNA cut by both the SmaI and XmaI restriction endonucleases (Figures 8 and 9). There were 9 identifiable fragments for each *Staphylococcus* strain using SmaI, but there was a slight banding pattern difference between the two strains (Figure 8). There were 8 identifiable fragments for each *Staphylococcus* strain using XmaI, but there was a slight banding pattern difference between the two again (Figure 9).

# Discussion and Future Research

The results of the PFGE analysis show that no evidence of methylation was detected from the methods used. The restriction endonucleases SmaI and XmaI were both able to cut each *Staphyloccus* strain at the genomic site 5'- CCCGGG - 3'. The SmaI restriction enzyme is blocked from cutting methylated DNA at that specific site and the XmaI restriction enzyme will cut any methylated DNA at that same site (3, 19). Since the results of the PFGE showed that SmaI cut every DNA sample, it concludes that no DNA methylation occurred on that site. This could mean that other epigenetic mechanisms occurred, or other non-epigenetic factors played a role in causing the two strains of susceptible *Staphylococcus aureus* to form antibiotic resistant colonies.

The results of the PFGE gels also showed a difference in fragment patterns between the two strains, but there are at least 3-4 fragments of the same size shared by both. While both are *Staphylococcus aureus* species, they aren't genetically identical. ATCC 43300 was shown to carry the mecA gene, whereas ATCC 29213 did not. The different physical characteristics between the two, ATCC 43300 produces white colored colonies and ATCC 29213 produces gold colored colonies (Figures 1 and 2). It makes sense that the banding of genomic DNA fragments would be different between the two strains, and variations in banding patterns have been seen in many *Staph* isolates, particularly with MRSA (4).

The *Staphylococcus* strain ATCC 43300 showed susceptibility in the MIC test and within the antibiotic concentrated plates (results not shown). However, in both tests there were colonies that appeared on the oxacillin plates 0.75mg, 1.5mg, and 2.0mg. Further analysis of this strain using PCR, revealed that ATCC 43300 carried the mecA gene. The mecA gene could have been silent until introduced to increasing concentrations of oxacillin, which could have activated the mecA gene allowing some bacteria to resist the effects of the antibiotic (22). The mecA gene also could have caused this strain to form a few resistant colonies by a phenomenon called heteroresistance. Heteroresistance is a resistance to antibiotics that is expressed by a subset of a

bacterial population which would generally be considered to be susceptible to those antibiotics (10). This heteroresistance can be a precursor stage to actual full-blown resistance which has been observed to take place in *Staphylococcus aureus* (10). Essentially, this means that the bacteria can be both susceptible and resistant to an antibiotic, and could be an explanation as to why ATCC 43300 showed an initial susceptibility to oxacillin but was then able to grow on plates containing the antibiotic at high concentrations (up to 12mg/mL, results not shown).

The *Staphylococcus* strain ATCC 29213 also showed resistance when grown on oxacillin plates. The PCR analysis showed that this strain was mecA negative (Figure 7), but the absence of the mecA gene did not stop this strain from displaying resistance to oxacillin. Heteroresistance could be a factor of the resistance observed in this strain as well, or it could be due to the presence of other genes or amino acids present in the bacteria that contribute to β-lactam resistance (9).

These two strains, ATCC 43300 and ATCC 29213, could have also gained resistance from other epigenetic mechanisms other than DNA methylation. One other potential epigenetic mechanism is non-coding RNA (ncRNA)-associated gene silencing. This mechanism is the most recent epigenetic factor and suggests that the ncRNA molecules harbor a crucial role in epigenetic gene expression and are likely to account for the great difference in phenotype between species (1). These ncRNAs or riboregulators can control gene regulation and cause antibiotic resistance. Many of these riboregulators sense ribosome-inhibiting antibiotics by directly measuring ribosome activity (8). Any of these epigenetic mechanisms could have caused the antibiotic resistance observed in this study. DNA methylation could still have played a role, however on a different genomic site other than 5' – CCCGGG - 3', which is the only site that SmaI and XmaI can cut.

Future research could involve using other methods to identify DNA methylation, such as bisulfate conversion, or using different restriction endonucleases that cut at different genomic sites. Another way could be using a DNA methylation extraction kit (20), or possibly extracting DNA directly from cells grown on the antibiotic plates rather than streaking them onto antibiotic-free plates first. Other genetic primers could be used for detection of different methylation sites (17), or for other genes that may contribute to resistance (9). However, all of these suggestions would be time consuming and expensive. Sending out the bacterial DNA and sequencing the whole genome would be more efficient, and could potentially show exactly where methylation occurred in the genome, but ultimately would also be quite costly.

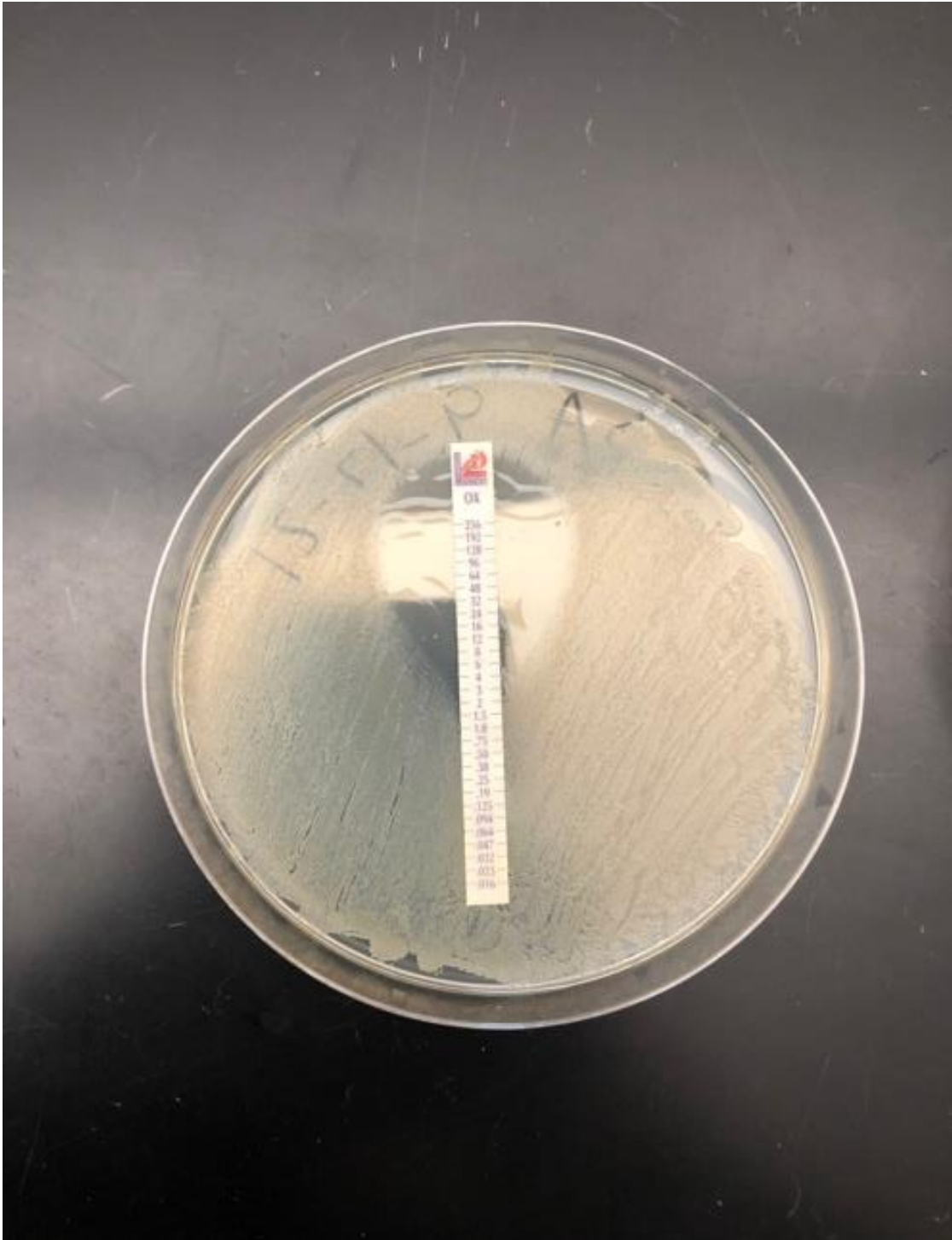## Acknowledgements

Figures



**Figure 1**: MIC test for *Staphylococcus aureus* strain ATCC 43300. Results are read at the intersection of growth with the oxacillin test strip (the bottom of the tear drop shape) and was determined to be around 2mg/mL.
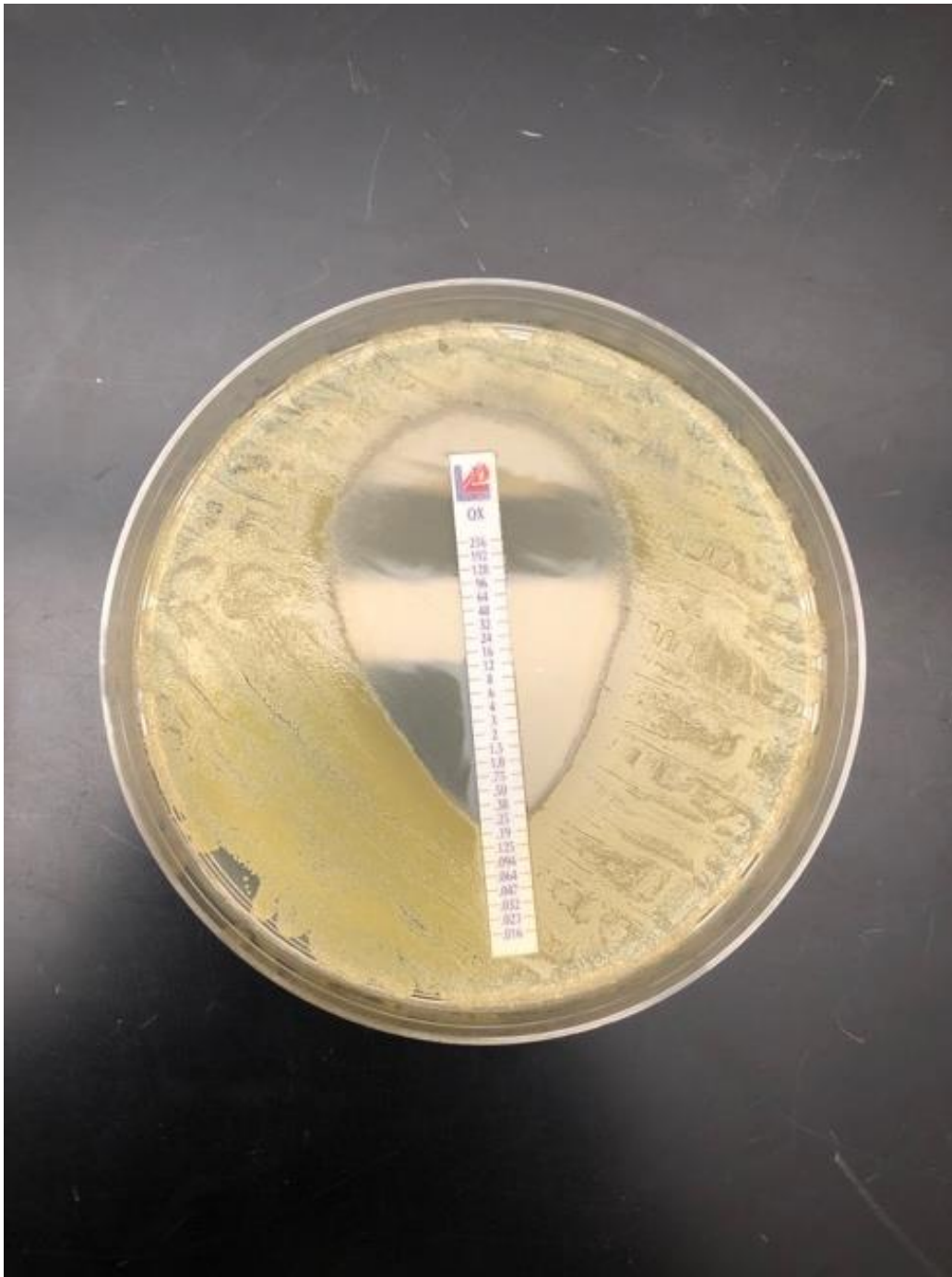
**Figure 2**: MIC test for *Staphylococcus aureus* strain ATCC 29213. Results are read at the intersection of growth with the oxacillin test strip (the bottom of the tear drop shape) and was determined to be around 0.25mg/mL.

**Figure 3**: 1mL syringes used to make agarose plugs. The syringe on the left is unmodified. The syringe on the right had the end cut off and was used as a mold to form agarose plugs (a piece of the plug is sticking out of the open end).
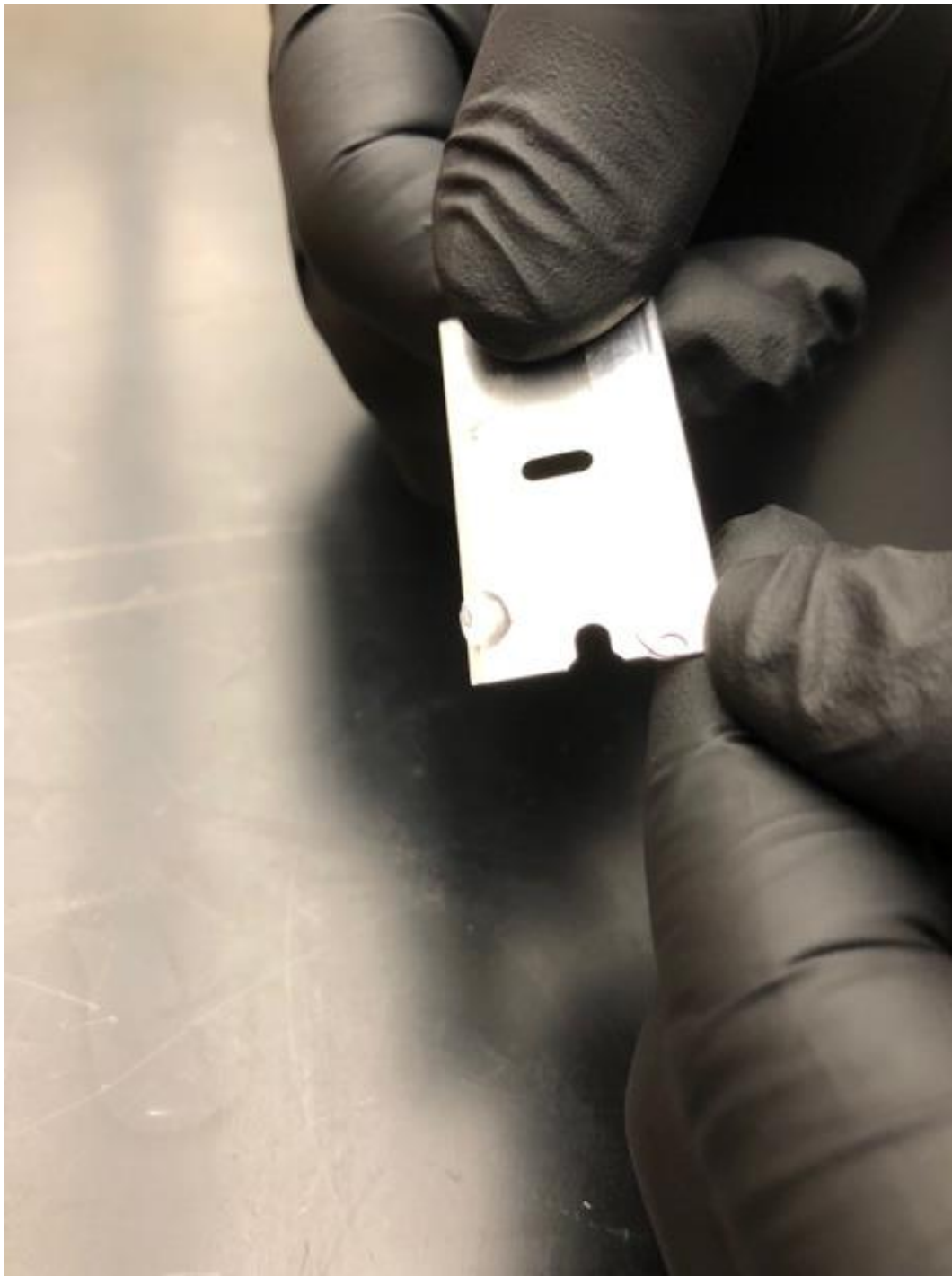
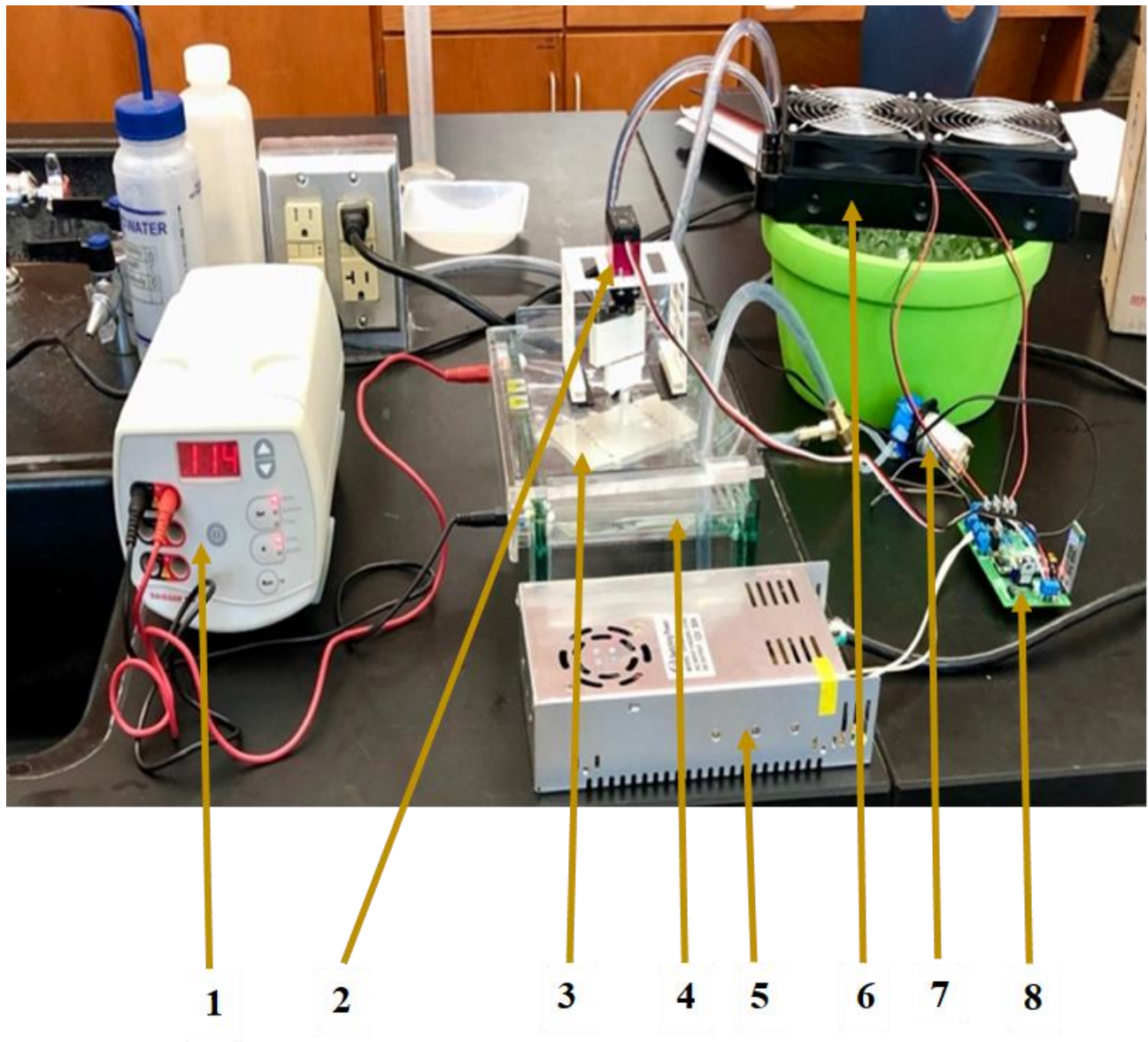**Figure 4**: A 1-2mm agarose plug slice using a straight razor blade.

**Figure 5**: Equipment for running the PFGE. 1: high voltage power source for the electrophoresis chamber, 2: gel rotational mechanism and support, 3: gel tray inside of the electrophoresis chamber, 4: electrophoresis chamber, 5: 12V power supply, 6: buffer cooling system, 7: peristaltic pump for buffer circulation, 8: assembled circuit to power and run the components. 3-D printed parts are the white components used to support the rotational motor and gel tray along with the gel tray itself.

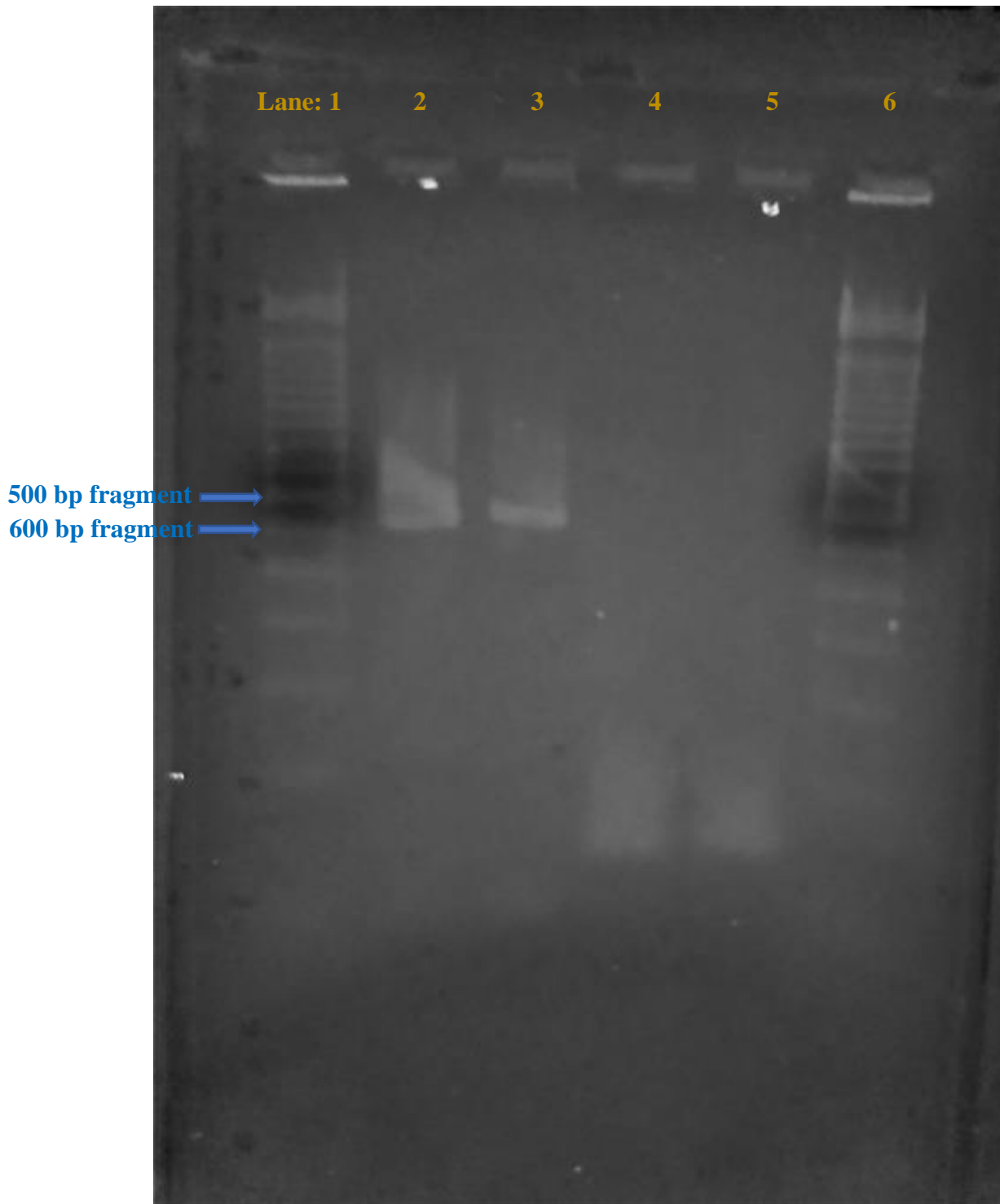**Figure 6**: Amplified growth of ATCC 29213 0.75mg mutant on a nonantibiotic plate.

**Figure 7**: Agarose gel electrophoresis of the amplified mecA gene. Lanes 1 and 6 contain a 1000 base pair ladder containing fragments of known sizes. Lane 2 is the unmutated ATCC 43300, lane 3 is the mutated ATCC 43300, lane 4 is the unmutated ATCC 29213, and lane 5 is the mutated ATCC 29213. Lanes 2 and 3 show a fragment in between the 500 and 600 base pair ladder fragment. No fragments were present in lanes 4 and 5.
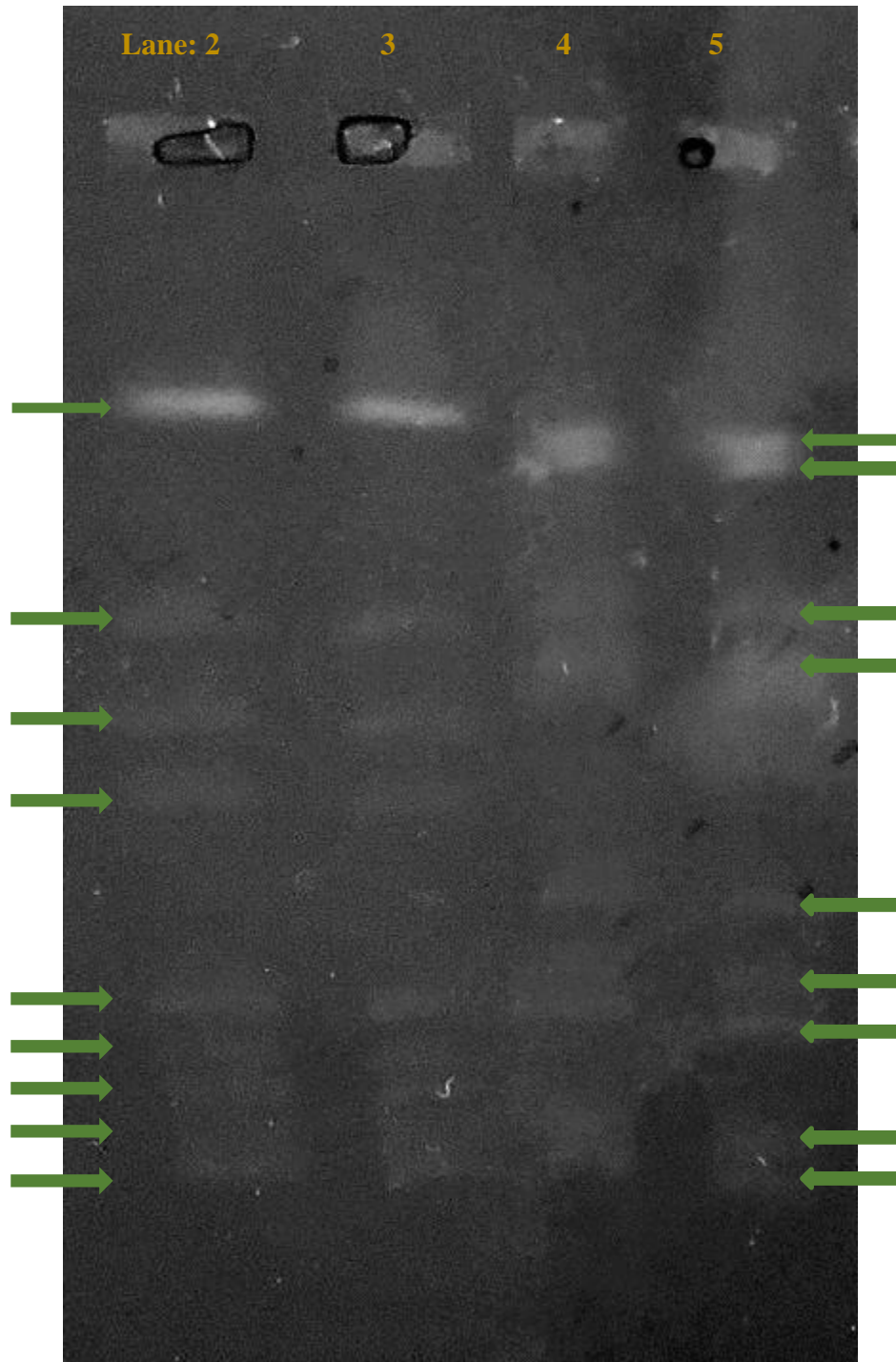
**Figure 8:** PFGE image of the SmaI digest. Lanes 1 and 6 are blank samples (not shown), lane 2 is nonmutated ATCC 43300, lane 3 is mutated ATCC 43300, lane 4 is nonmutated ATCC 29213, and lane 5 is mutated ATCC 29213. From this image, there are 9 visible fragments (indicated by the green arrows) for each sample.
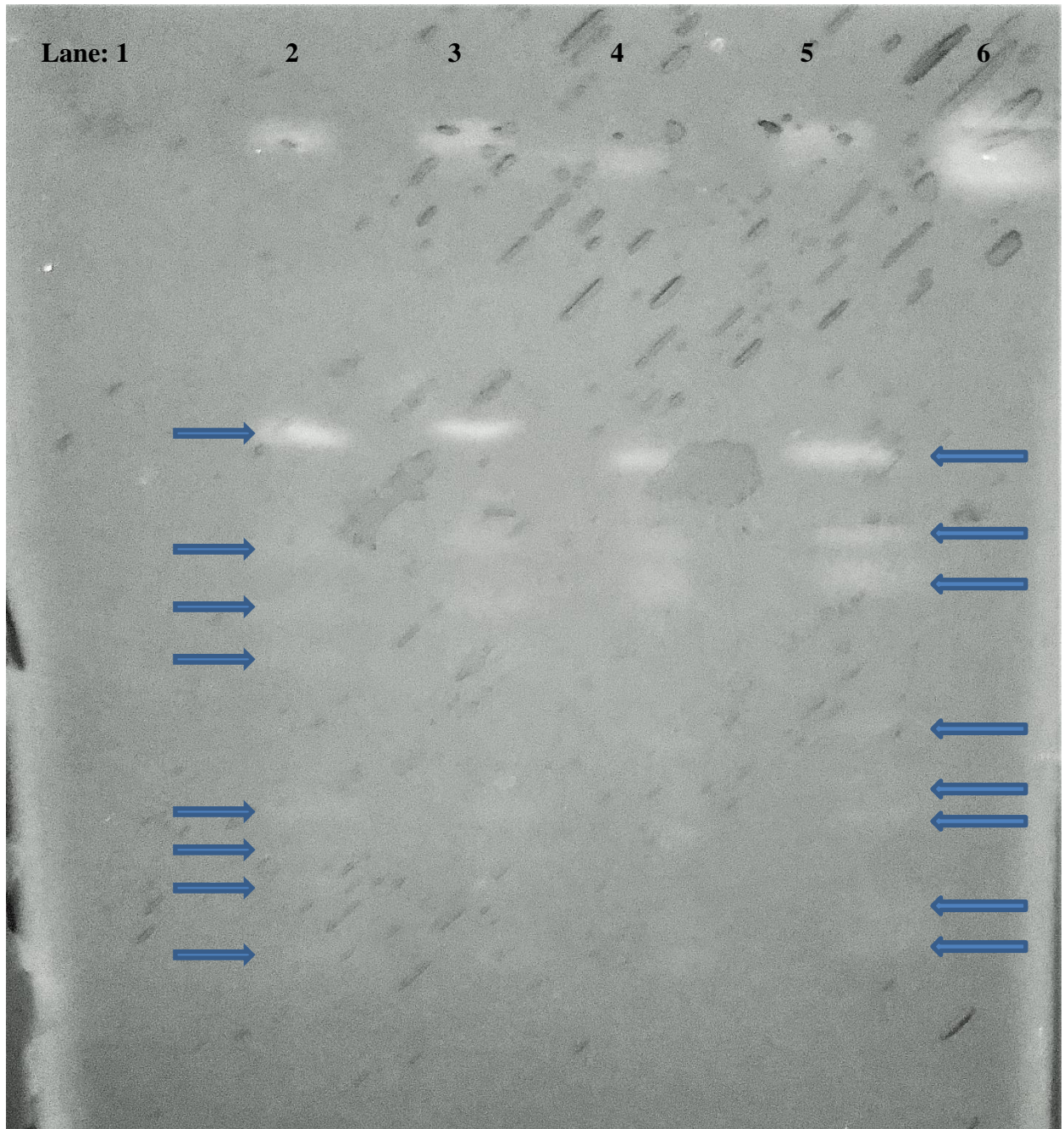
**Figure 9:** PFGE image of XmaI digest. Lanes 1 and 6 are blank samples, lane 2 is nonmutated ATCC 43300, lane 3 is mutated ATCC 43300, lane 4 is nonmutated ATCC 29213, and lane 5 is mutated ATCC 29213. From this image, there are 8 visible fragments (indicated by the blue arrows) for each sample.

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