Supplementary Methods

Machine learning analysis of type VII secretion system expression and its relationships with virulence traits and antibiotic resistance in Staphylococcus aureus

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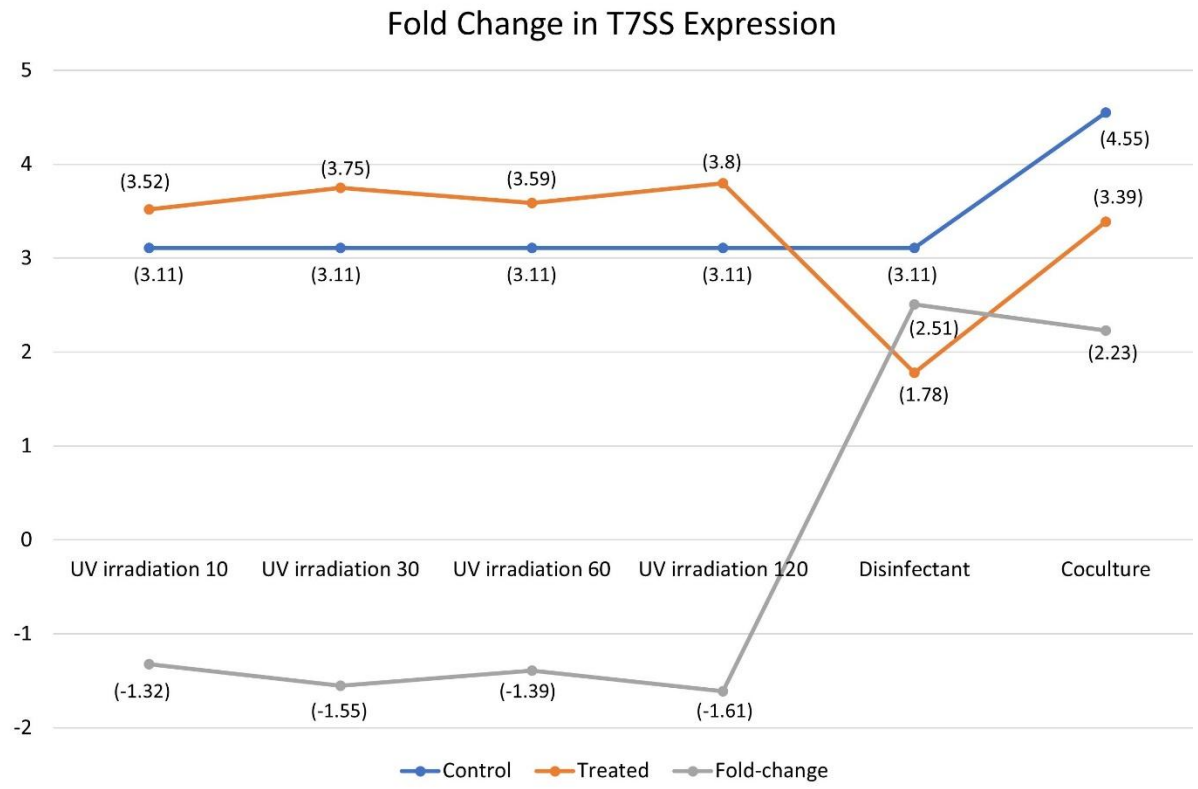
PCR cycle parameters

The presence of the T7SS in clinical isolates of *S. aureus* was confirmed by polymerase chain reaction (PCR) using an Applied Biosystems Veriti 96-well thermal cycler (Thermo Fisher Scientific, Waltham, Massachusetts). DNA was isolated from a 24-hour fresh culture of *S. aureus* grown on blood agar. Bacterial suspensions were prepared in nutrient broth, incubated at 37°C until log phase, and adjusted to the 0.5 McFarland standard. DNA extraction was performed using the HiPurA Bacterial Genomic DNA Purification Kit according to the manufacturer's protocol (HIMEDIA Laboratories LLC, PA, USA). The quality of the isolated DNA was verified by agarose gel electrophoresis using 0.8% agarose and a Tecan NanoQuant Plate™ (Infinite® 200 Pro; Tecan Life Sciences, Switzerland) with an absorbance ratio of 260/280. For PCR, a 25 µl reaction mixture was prepared containing 12.5 µl of Emerald dAMP GT PCR Master Mix K, 1.5 µl of template DNA, 0.8 µl of forward primer, 0.8 µl of reverse primer, 1 µl of MgCl2, and 8.4 µl of nuclease-free water. The PCR cycle parameters were as follows: initial denaturation at 94°C for 2 minutes; 30 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 1 minute; and a final extension at 72°C for 10 minutes. The PCR products were visualised using 1.3% agarose gel electrophoresis, with 5 µl of EtBr per 100 ml of gel. A 100 bp ladder (Gene to Protein Pvt. Ltd.) was used for size comparison. For each sample, 3 µl of DNA was mixed with 3 µl of Green Track 6X Loading Dye (Gene to Protein Pvt. Ltd.) and loaded into the gel wells. Electrophoresis was conducted at 130 volts. The 16S rRNA gene of *S. aureus* was used as the positive control, and *Escherichia coli* ATCC 25922 was used as the negative control.

cDNA synthesis and qPCR cycle parameters

A sample size of 84 was determined using the G*Power statistical tool for a correlation bivariate normal model test with a 95% confidence interval to achieve a power of 0.8. However, 90 clinical isolates of *S. aureus* were randomly selected for quantification of the T7SS using reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). RNA was isolated from a 24-hour fresh culture of *S. aureus* grown on blood agar. Bacterial suspensions were prepared in nutrient broth, incubated at 37°C until log phase, and adjusted to the 0.5 McFarland standard. RNA extraction was performed using the HiPurA Bacterial RNA Purification Kit according to the manufacturer's protocol (HIMEDIA®). The purity and quantity of the isolated RNA were measured using a Tecan NanoQuant Plate™ (Infinite® 200 Pro) with an absorbance ratio of 260/280. The RNA was reverse transcribed into cDNA using a synthesis kit from G-Biosciences (St. Louis, MO, USA) following the manufacturer's instructions. The RT reaction mixture (10 µl) consisted of 5 µl of 2X RT Easy Mix, 0.5 µl of random primer, 0.5 µl of oligo (dT) primer, total RNA (<2.5 µg), and nuclease-free water to a total volume of 10 µl. The program for reverse transcription was set to 42°C for 20 minutes, followed by inactivation at 85°C for 5 minutes.

The primers used for the esxA gene are listed in Table 1. Quantitative PCR was performed using SYBR™ Green qPCR Master Mix following the manufacturer's (G-Biosciences) protocol. The master mix (20 µl) included 10 µl of 2X AB HS SYBR Green qPCR Mix, 2 µl of forward primer, 2 µl of reverse primer, 2 µl of cDNA template, and 4 µl of double-distilled water. qPCR was performed using a C1000 Touch Thermal Cycler (Bio-Rad CFX96 Real-Time System; Bio-Rad Laboratories, Hercules, CA) with a program comprising initial denaturation at 94°C for 2 minutes, followed by 35 cycles of 94°C for 30 seconds (denaturation), 54°C for 30 seconds (annealing), and 72°C for 1 minute. The 16S rRNA gene of *S. aureus* was used as an internal positive control (primer sequences in Table 1), and a nontemplate control served as the negative control. The qPCR products were visualised using 1.3% agarose gel electrophoresis to verify the specificity of the band size (198 bp).



Supplementary Fig. 1 Fold change in *S. aureus* T7SS gene expression in response to environmental factors.