CHAPTER II: MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals and reagents

Most chemicals and reagents were purchased from Nice Chemicals, Himedia, Sigma Aldrich, Millipore and Merck.

2.1.2. Bacterial strains and plasmids

List of bacterial strains used and their source are mentioned in **Table 2**.

2.1.3. Consumables: Polypropylene tubes (1.5, 2.0, 15 and 50 ml) and 96 well polystyrene ELISA plates were purchased from Tarsons; Vivaspin 15R (5 kDa MWCO) ultra-filtration device was purchased from Sartorius; syringe filters were purchased from Millipore; injection needles from DispoVan Hindustan Syringes & Medical Devices Ltd (India); all glass wares including glass beakers, graduated measuring cylinders, bottles, petri dishes, culture tubes were purchased from Borosil.

2.1.4. Instruments: Fluorescence microscope (Leica), stereo microscope (Leica), scanning electron microscope (JEOL), EDTA-vaccutainers and Non-EDTA vaccutainers (BD-Bisciences), cell culture plates (tarson), digital weighing balance (OHAUS), screw gauge (Schut), centrifuge (Beckman Couter AvantiÒJ-26 XP, HettichZentrifugen, Eppendorf), gel rocker (Genei), magentic stirrer (REMI), vortex mixer (REMI), microfuge (Eppendorf), agarose gel eletctrophoresis unit (Bio-Rad), poly-acrylamide gel electrophoresis unit (Bio-Rad), 37°C incubator (Sanyo), 37°C shaking incubator (Infors HT Multitron), gel documentation system (Bio-Rad), laminar air flow hood (BSL2, Kleanzone systems); -80°C freezer (Sanyo), X-ray machine (CMB-2; Softex Co., Tokyo, Japan), microplate reader (BioTek-Synergy), microtome (Leica).

Table 2: Bacterial strains and plasmids

Bacteria	**Strains/ Plasmids**	**References / Source**
S. aureus	SA113	[125]
	Δ*oatA*	[79]
	Δ*spa*	[126]
	Complemented mutant (Δ*oat*A: pTX*oat*A)	[127]
S. carnosus	TM-300	[128]
E. coli	BL-21 (DE3)	Novagen
Plasmids	pET:*oatA*; Kan^R	Gene script

2.1.5. Assay kits/reagent:

Table 3: Assay and kits

Items	Vendor
Ni-NTA His.Bind® Resin	Novagen - Merck Millipore, India
Mouse IL-1β/IL-6/TNF-α/RANKL ELISA kit	RayBiotech
EmeraldAmp® GT PCR Master Mix	Takara Bio Inc., Japan
O'Gene Ruler DNA ladder	Life Technologies, Thermo Fisher Scientific, India
Prestained Protein Molecular Weight Marker	Life Technologies, Thermo Fisher Scientific, India
Freund's complete and incomplete adjuvant	Genei - Merck Millipore, India

2.2. Methods

2.2.1. Bacterial culture: *S. aureus* wild type strain SA113 (ATCC 35556), *oatA* deletion mutant (Δ*oat*A) and the complemented mutant (Δ*oat*A: pTX*oat*A) strain [79, 129] were maintained in Trypticase soy agar (TSA; TSB+1.5% agar) or manitol salt agar (MSA; 5.0 g enzymatic digest of casein, 5.0 g enzymatic digest of animal tissue, 1.0 g beef extract, 10.0 g D-mannitol, 75.0 g sodium chloride, 0.025 g phenol red, 15.0 g agar, 1 litre of distilled water) plates (selective media for *S. aureus*; HiMedia). In broth culture all the strains were grown in autoclaved Trypticase soy broth (TSB; 17 g of Tryptone, 3 g of Phytone, 5 g of NaCl, 2.5 g of dipotassium phosphate (K2HPO4), 2.5 g of glucose, 1 liter of distilled water) medium at 37 °C with 160 rpm shaking. The Δ*oat*A mutant was maintained in presence of Kanamycin (HiMedia) (30 µg/ml); and the complemented mutant Δ*oat*A: pTX*oat*A was maintained in presence of Kanamycin (HiMedia) (30 µg/ml) and Tetracycline (HiMedia) (12.5 µg/ml) [129]. Glycerol stock of all the cultures were made by adding 50% of overnight culture with autoclaved 50% glycerol (freezing medium), for future use.

2.2.2. Bacterial growth curve of SA113 and Δ*oat*A in presence or absence of lysozyme: 1% inoculums of the bacterial cells from their overnight cultures were added in TSB medium containing increasing concentrations of lysozyme (Sigma) (0, 50, 150, 300, 600 and 1200 µg/ml). The tubes were incubated at 37°C for 96 h with agitation. To assess the bacterial growth, absorbance was taken at regular interval. The addition of lysozyme was done only after 4 h of initial inoculation [129].

2.2.3. Bacterial survival assays/ Lysozyme induced lysis assay:

Lysozyme induced lysis of SA113, Δ*oat*A mutant and the complemented mutant (Δ*oat*A: pTX*oat*A) was measured in phosphate buffer saline (PBS) medium. Stationary phase cultures of *S. aureus* strains were pelleted down. The bacterial cells were washed twice with PBS buffer and re-suspended in the same buffer containing 0, 50, 150, 300, 600 and 1200 µg/ml of lysozyme. The initial O.D (600 nm) was adjusted to 0.5 and bacterial survival was measured at frequent intervals for 96 h, serially diluted, and plated on TSA plate for enumeration of surviving colonies at different time points [129].

2.2.4. Scanning electron microscopy:

To monitor the bacterial cell integrity due to lysozyme action, scanning electron microscopy was used. Bacterial cells were treated with 1200 µg/ml lysozyme in PBS for 8 h, centrifuged and fixed with 2% glutraldehyde. Further. The samples were washed 4 times with phosphate buffer saline (PBS; NaCl 8.0 g, KCl 0.2 g, Na_2HPO_4 1.42 g, KH_2PO_4 0.24 g, 1 liter distilled water) to remove any glutraldehyde content. The cells were dehydrated using graded series of ethanol (50, 70 and 90%, each for 10 minutes) and finally re-suspended in 100% ethanol. The suspension was made as smear on a glass cover slip and kept for drying at 37°C for 2 h so that all the ethanol content gets evaporated. The cells were gold spur coated and imaged [120].

2.2.5. Survival of *S. aureus* strains in human whole blood and human synovial fluid:

Blood was withdrawn from healthy individuals (n=5) in a vaccutainer containing EDTA. Overnight culture of SA113, Δ*oat*A mutant and the complemented mutant (Δ*oat*A: pTX*oat*A) strains was washed twice with PBS to remove the media components and the initial O.D. of the bacterial suspensions were adjusted to 0.5 at 600 nm and from this bacterial suspension 1% inoculums were added in 1 ml of blood. The tubes were incubated at 37°C for 96 h with agitation. Samples were collected in frequent interval, diluted, and plated on TSA plate for enumeration of surviving colonies. At same time intervals 20 µl of the blood, were used for Giemsa staining. The slides were observed under oil emersion objective, using Leica microscope and images were captured [130].

Additionally, bacterial survival in human synovial fluid (SF) samples (n = 5) were also measured using the same protocol.

2.2.6. Intracellular survival in macrophages and endothelial cells:

The intracellular bacterial survivals were monitored in human umbilical vein derived endothelial cells (HUVECs) and RAW 264.7 macrophage cell line. RAW 264.7 cells were grown in RPMI-1640 medium containing 10% FBS and HUVECs in Endothelial cell growth medium (Lonza) with 20 % FBS. To achieve adherence, 3 x 10^5 cells/ml (HUVECs) 2 × 10^6 cells/ml RAW 264.7 were seeded in 12-well plates for at least 24 h at 37 °C in a 5% CO2 incubator prior to the experiment. The bacterial cells were re-suspended in RPMI medium without antibiotics and macrophage cells were infected with *S. aureus* at multiplicity of infection (MOI) of 1:10 and

incubated for 2 h for bacterial internalization. The infected HUVECs and macrophages were washed with PBS and then treated with 100 µg/ml of gentamicin for 30 min to kill any extra-cellular bacteria. Further HUVECs and RAW 264.7 cells were re-supplemented with antibiotic free endothelial cell medium (with 20% FBS) /RPMI-1640 medium (10% FBS). After 24 h the HUVECs/macrophages were lysed using 0.1% Triton X 100 and the intracellular bacteria was enumerated by serially dilution and plating [97, 131].

2.3. Survival of SA113 and Δ*oat*A in *Drosophila melanogaster*

2.3.1. *Drosophila melanogaster* strains and maintenance:

The flies were raised at 26 °C, 60 % humidity, and 12h light/dark cycle on classical banana agar medium and flies were transferred into new culture vials with fresh medium every 3–5 days. Male/females flies were selected, 2–5 days after eclosion for experiments [120].

2.3.2. Fly infection:

SA113 or Δ*oat*A cultures were grown overnight in TSB broth from frozen glycerol stock. Following an overnight culture, cultures were re-inoculated in fresh TSB medium tube and incubated at 37°C with shaking to achieve the exponential growth phase (4–5h). Bacterial culture was pelleted down and then washed twice with PBS. The washed pellet was re-suspended in PBS to an O.D. of 2.0 at 600 nm. A whatman filter paper was kept on top of the banana culture medium in fly culture vial on top of which 60 µl of the bacterial suspensions (SA113 or Δ*oat*A) were spread and PBS was used as control. The flies were starved for 4 h and following starvation period 20 flies were transferred in each vial containing bacterial suspensions or PBS and incubated at 28°C. The flies were allowed to feed for 48 h bacterial suspension/PBS containing medium and then transferred to fresh culture tubes with only banana agar medium [120].

2.3.4. Fly survival:

After feeding assay, consequent number of surviving flies was recorded every day for the study period of 10 days. Three independent experiments were performed in order to get statistical significance of the data [120].

2.3.5. Rapid iterative negative geotaxis (RING):

RING assay protocol was adopted from Kaynar *et al.*, with slight modification. Briefly, customized empty glass vials of 20 cm length were used for experiments and marked at a distance of 10 cm. 20 flies/group were transferred into the vials and allowed to acclimatize to the new setting for 5 min before conducting the assay [132]. Flies were gently tapped down to the bottom of the vial for 10s with the same interval and strength by the same operator throughout the whole experiment. Pictures of the flies were taken with a digital camera at 5s. Each geotaxis experiment was repeated three times, allowing for 1 min rest periods between each trial and pictures were analyzed by counting the number of flies that climb above the 10 cm mark in 5s after the tap. The average of the number of flies crossing the 10 cm threshold was counted and the results were expressed as percentage of the total number of flies in the tube (=% climbing index). Each geotaxis experiment was performed 1h before the feeding assay (0 h baseline) and at day 0, 5, and 10 after feeding. The data are presented as percent of the baseline at time 0h.

2.4 Murine model of sepsis and septic arthritis:

Three murine models were used to analyze the *in vivo* effects of SA113 and Δ*oat*A induced infection-

(A) Murine model of sepsis (BALB/c mice)

(B) Hematogenous arthritis model (NMRI mice) – this study was done in Prof Jin Tao's lab, University of Gothenburg, as a collaborative project where both the bacterial strains were send and tested in standardized hematogenous arthritis model.

(C) Murine model of local infection of septic arthritis

2.4.1. Mice:

4-6 week old BALB/c female mice were used for the experiments. All mice were maintained under standard condition of temperature, light; and had free access to laboratory chow (food) and water.

2.4.2. Determination of the fitness of SA113 and Δ*oat*A in murine model of bacterial sepsis:

In vivo sepsis model or murine model of intravenous infection was used to compare the virulence of SA113 and Δ*oat*A. Three group of animals were used (n=10/ group), Group 1 were injected

with 100 µl (50% lethal dose: 2×10^5 CFU) of SA113, and Group 2 Δ*oat*A were injected with 100 µl (50% lethal dose: 2×10^5 CFU) respectively, through tail vein, while in Group 3 same volume of PBS was injected which served as negative control. The total study period was for 10 days. Bacterial load in internal organs heart, liver, kidney and in blood was determined at different time points (0, 2, 4 and 10 days). Tissues were aseptically removed, homogenized in PBS and serially diluted. The diluted fractions were plated on LB agar plates to estimate the presence of bacterial load in each organ [133].

2.4.3. Histopathology:

The *S. aureus* mediated kidney abscess formation was assessed by histopathological analysis of SA113 and Δ*oat*A infected mice kidneys. SA113 and Δ*oat*A mice were sacrificed humanely, 10 days after the infection, by Ketamine:Xylazine (10:1) administration. Kidneys were excised and stored in 10% formalin.

Thin sections were made from the kidney tissues and treated with 95% alcohol for 2 min followed by 2 min of 70% ethanol treatment. The section was washed with distilled water and treated with hematoxylin for 8 min followed by washing under running tap water for 5 min. Tissue was differentiated in 1% acid alcohol for 30 sec followed by washing under running tap water for 1 min. The tissue section was subsequently treated with 0.2% ammonia water for 30 sec and washed under running tap water for 5 min. The section was rinsed by dipping in 95% alcohol ten times. Counterstaining with eosin-phloxine solution was performed by for 1 min followed by dehydration with 95% alcohol, 2 changes of absolute alcohol in between, 5 min each. The section was cleared with 2 changes of xylene, 5 min each and mounted with xylene based mounting medium. The slides were analyzed under the light microscope to evaluate inflammatory changes.

For knee joint histology, mice were sacrificed after 28 days and the whole knee joints were dissected, and fixed in 4% paraformaldehyde buffered with phosphate buffered saline (PBS) (pH 7.4) for 24 h at room temperature. The specimens were decalcified for 2 weeks with 10% ethylene-diaminetetraacetic acid (EDTA; pH 7.4) at 4°C. After dehydration with an increasing concentration of ethanol and embedding in paraffin, 5 µm sagittal sections were cut from the whole medial compartment of the joints and stained with hematoxylin and eosin. The specimens were evaluated with regard to synovitis and cartilage/bone destruction [134].

2.4.4. Mouse model for hematogenous *S. aureus* arthritis:

Pre-made batches of bacteria (SA113 and Δ*oat*A strains) were thawed, washed and diluted to desired concentration ($1.5x10^8$cfu/ml). Twenty NMRI mice (10 mice/group) were inoculated intravenously (i.v.) into the tail vein with 0.2 ml of *S. aureus* with expected dose ($3x10^7$cfu/mouse). The mice were then regularly weighed and examined for clinical arthritis by observers blinded to the groups. On day 10, the mice were sacrificed, kidneys were obtained for assessment of bacterial persistence, and the paws were obtained for radiological examination of bone erosions.

Observers blinded to the treatment groups visually inspected all 4 limbs of each mouse [135]. The arthritis index was calculated by adding the scores from all 4 limbs for each animal as described before [20, 135, 136]. Since the signs of septic arthritis in deeper joints (e.g. the knee and elbow joints) are impossible to evaluate clinically, µ-CT of joints were further used to confirm the clinical arthritis data.

Joints were fixed in 4% formaldehyde for 3 days and then transferred to PBS for 24 hours. Afterwards all 4 limbs were scanned and reconstructed into a three-dimensional structure with Skyscan1176 micro-CT (Bruker, Antwerp, Belgium) with a voxel size of 35 µm. The scanning was done at 55kV/455 mA, with a 0.2 mm aluminum filter. Exposure time was 47 ms. The X-ray projections were obtained at 0.7° intervals with a scanning angular rotation of 180°. The projection images were reconstructed into three-dimensional images using NRECON software (version 1.5.1; Bruker). After reconstruction, the 3D structures of each joint were blindly assessed by 2 observers using a scoring system from 0-3 (0- healthy joint; 1- mild bone destruction; 2- moderate bone destructions; 3- marked bone destructions) [137].

2.4.5. Mice model of local septic arthritis:

Animals were divided into three groups; Group-1: control uninfected; Group-2: SA113 infected and Group-3: Δ*oat*A infected (n =12/group). Lyophilized bacterial cells were suspended in PBS at a concentration of 2 X 10^8 CFU/ ml. Mice were anaesthetized with an intra-peritoneal injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). 10 µl bacteria containing 2 X 10^6 CFU of the bacterial suspension were injected intra-articularly (IA) into the right knee joint of each anesthetized mouse [86, 124]. The IA injections were performed on mice approaching from the frontal aspect of the knee joint, just below the patella. The left knee joint was used as a

negative control and similar volume PBS was injected. Following injections, the mice were observed for changes in body weight, knee swelling, extensor posterior thrust and locomotion pattern for the next 28 days. Bacterial colonies present in the knee joints were enumerated at different time intervals.

2.4.6. Body weight and knee swelling measurement:

Regular alternative day measurement of body weight and knee swelling were recorded for a period of 28 days. A calibrated screw gauge (Schut) was used to assess knee swelling by a single blinded investigator. Care was taken to measure the outer transarticular thickness of the knee at the level of the patella [138, 139].

2.4.7. Extensor posterior thrust analysis (EPT):

Motor performance function was evaluated by measuring extensor postural thrust (EPT). For this test, the entire body of the animal, with exception of the hind-limbs, was wrapped in a surgical towel. EPT was induced by supporting the animal by the thorax and lowering the test hind-limb towards the platform of a digital balance. The force applied by the hind-limb of the mice to the digital platform balance was recorded [140, 141]. Each hind-limb was tested 3 times, with an interval of 2 min between consecutive tests, and resulting values were averaged to obtain a final result. Percent motor deficit in tested hind-limb was calculated using the equation [(NEPT – EEPT) / NEPT] × 100 where, NEPT = Normal EPT and EEPT = experimental EPT.

2.4.8. Locomotion pattern estimation using open-field test arena:

Open field tests are widely used to measure locomotory behavior [142, 143]. This test was carried out to evaluate if pattern of locomotion of SA113 infected animals was different from Δ*oat*A in terms of its velocity and distance travelled. The open-field box used for the test measuring 40 × 40 × 40 cm, had black colored floor and four walls to get the good contrast as our subject BALB/c mice coat color was white. Individual mouse was placed into one of the four corners of the open field and allowed to move around within the box for 5 min, which was recorded by a video camera and later the locomotion was scored by two independent blinded observers with the help of EthoVision XT (Noldus Information Technology, Leesburg, VA). The arena was thoroughly cleaned using 70% ethyl alcohol in between each trial.

2.4.9. Clinical scoring of arthritis:

Mice were individually evaluated for development of arthritis by three blinded observers. The development of clinical arthritis was monitored at regular intervals till the end of study. Arthritis was defined as visible joint swelling or erythema of the joints and paws. To evaluate the intensity of arthritis, a clinical scoring was carried out by using a system where macroscopic inspection yielded a score of 0-3 points for each limb (1 point = mild swelling and/or erythema; 2 points = moderate swelling and erythema; 3 points = marked swelling, erythema) [144, 145].

2.4.10. Bacterial load estimation in the knee joint:

Bacterial load was estimated in the knee joints after 7, 14 and 28 days. The mice were euthanized, and the knee joints with the surrounding muscle tissues were aseptically excised. Joint tissue was weighed and homogenized in PBS (500 mg tissue/ml of PBS) using a mortar and pestle. The homogenates were serially diluted, and plated on MSA plates for enumeration of surviving colonies. The plates were incubated overnight at 37ºC, for colony enumeration [146-148].

2.4.11. Radiology:

Radiographs of the mouse knee joints were taken in the anteroposterior and lateral projections before; and 2 and 4 weeks after intra-articular injection under general anesthesia using a soft X-ray apparatus (CMB-2; Softex Co., Tokyo, Japan) and were evaluated by two individual observer who was blinded with regard to the experimental group [149, 150].

2.4.12. Joint cytokine measurements (ELISA):

Levels of Tumor necrosis factor- α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and receptor activator of nuclear factor-κB ligand (RANKL) from whole knee joints (including synovium, adjacent tissues and bones) were determined. Whole joints including synovium, adjacent tissues and bones were pulverized using a mortar and pestle filled with liquid nitrogen [18, 151]. Pulverized tissues were re-suspended as 200 mg of tissue /ml PBS containing protease inhibitor cocktail (Roche). The mouse joint homogenates were centrifuged at 15000 X *g* for 10 min at 4°C and the supernatants were collected and used for cytokine analysis. The cytokine

measurement was done using ELISA kits following the manufacturer's protocol (RayBiotech). Data were expressed as pg cytokine/g tissue.

2.4.13. Development of anti-lysozyme antibody:

Human lysozyme protein (Sigma) was used to immunize 1 year old rabbit, to develop the anti-lysozyme antibody. Following protocol was followed:

Day	Procedure
1	Immunize with 0.2 mg/200 µl Lysozyme in CFA, SQ 4 sites
14	Boost with 0.2 mg/200 µl Lysozyme in IFA, 4 SQ sites
42	Boost with 0.2 mg/200 µl Lysozyme in IFA, 4 SQ sites
56	Boost with 0.2 mg/200 µl Lysozyme in IFA, 4 SQ sites
70	Collect blood, separate serum and store at -20^{o}C

https://www.thermofisher.com/in/en/home/life-science/antibodies/custom-antibodies/custom-antibody-production/custom-polyclonal-antibody-production/custom-rabbit-polyclonal-antibody-production-protocols.html

2.4.14. Binding of lysozyme with cell wall:

A fluorescent assay was performed to confirm about binding site of lysozyme protein in bacterial cells using an anti-lysozyme antibody. The bacterial cells were spread as smear on the surface of a glass slide, heat fixed. The 0.5% milk powder (in PBS) was added on the slide to block non-specific binding. The smear was incubated with lysozyme solution for 15 min. Further anti-lysozyme antibody in 1:5000 dilution and secondary antibody used was anti-rabbit IgG tagged with alexa-flour red.

2.4.15. Survival of wild type *S. aureus* in presence of anti-lysozyme antibody in human blood:

In order to understand the efficiency of OatA to be used as therapeutic target, human blood samples were incubated with anti-lysozyme antibody to block the lysozyme present in blood and inoculated it with SA113 and Δ*oat*A mutant strain. In the first group non-immunized mice serum was added as control while second group same volume of immunized blood serum containing

anti-lysozyme antibody was added. Further, both the tubes were inoculated with 1% SA113/Δ*oat*A. The bacterial count was taken at 0, 12, 24, 48 h.

2.4.16. Ethics statement:

All animal experiments using BALB/c mice and collection of human SF samples were done after obtaining the approval from the Amrita Institute Animal Ethics Committee (Reference number: Acad/2015/29) and Amrita Institute Human Ethics Committee (IAEC/2015/2/3), Amrita Institute of Medical Sciences, Kochi-682041, India. Female BALB/c mice were bred as homozygotes and were kept in the animal house of the Amrita Institute of Medical Sciences, Kochi, India. Human SF samples were obtained from the Orthropedic department, Amrita Institute of Medical Sciences, Kochi, India. Among the human SF samples two samples were obtained from rheumatoid arthritis patients and the remaining three were from the osteoarthritis patients. All animal experiments using NMRI mice (purchased from Charles River Laboratories, Sulzfeld, Germany) were approved by the Animal Research Ethical Committee of Gothenburg, Sweden. All animal experimentation guidelines were strictly followed.

2.4.17. Statistical analyses:

All statistical analysis was performed using GraphPad 7 software (Prism 7 for windows, Version 7.00, GraphPad Software, La Jolla California USA, www.graphpad.com). Statistical comparison between experimental groups for assessing bacterial counts was performed using Students unpaired 't' test, while knee diameter, EPT, and ELISA results were analyzed using multiple comparison test with Holm-Sidak correction, with an α level of 0.05. Further, body-weight change, and results from the open field test were statistically tested using an ordinary one-way analysis of variance (ANOVA) with Sidak correction for multiple comparison. All other non-parametric data-sets were analyzed using the Mann-Whitney U test and the $\chi 2$ test. All data are expressed as mean ± S.D., unless otherwise specified and p value of <0.05 was considered as statistically relevant.