CHAPTER III
RESEARCH METHODOLOGY

3.1 Materials and Instruments

3.1.1 Microbial Isolates and Tested Pathogens

The bacterial of interest *Streptomyces* sp. strains, SSUT88A and SSUT88Red, were isolated from soil samples collected from Sakaerat Environmental Research Station (14°25′-14°33′ N 100°48′-100°56′ E), Wang Nam Khieo District, Nakhon Ratchasima, Thailand. The strains were kindly provided by A'liyatur Rosyidah, a former graduated Ph.D. student in the same research group (Rosyidah et al., 2021).

The pathogenic strains were obtained from, Thailand Institute of Scientific and Technological Research (TISTR), Department of Medical Sciences Thailand (DMST), and Suranaree University of Technology Hospital (SUTH). The list of pathogens are *Bacillus cereus* TISTR687, *Bacillus subtilis* TISTR008, *Enterobacter aerogenes* TISTR1540, *Escherichia coli* TISTR780, *Proteus mirabilis* TISTR100, *Pseudomonas aeruginosa* TISTR1287, *Salmonella typhimurium* TISTR292, *Staphylococcus aureus* TISTR1466, *Staphylococcus epidermidis* TISTR518, methicillin-resistant *Staphylococcus aureus* DMST20651 (MRSA), methicillin-resistant *Staphylococcus epidermidis* (MRSE) SUTH-isolate, *Acinetobacter baumannii* SUTH-isolate, *Escherichia coli* 2026 SUTH-isolate, *Klebsiella pneumoniae* 1617 SUTH-isolate, *Pseudomonas aeruginosa* N90PS SUTH-isolate, and *Serratia marcescens* SUTH-isolate.

3.1.2 Culture Media and Solutions

- International *Streptomyces* Project-2 Medium (ISP-2) contains 4.0 g of yeast extract, 10.0 g of malt extract, 4 g of dextrose (Glucose), 1 L of deionized water, pH was adjusted to 7.2. 20 g of agar is added to the mixture in case of making the agar medium.
- The liquid nutrient broth (NB) medium is prepared by dissolving 3 g of beef extract, 10 g of bacteriological tryptone, and 5 g of NaCl in

- 1 L of deionized water. The pH of the NB medium is adjusted to approximately 7.0.
- Starch Casein Medium (SC) per 1 liter contains 10.0 g of soluble starch, 0.30 g of casein hydrolysate, 2.0 g of KNO_3, 0.05 g of $MgSO_4{\cdot}7H_2O$, 2.0 g of K_2HPO_4, 2.0 g of NaCl, 0.02 g of $CaCO_3$, 0.01 g of $FeSO_4{\cdot}7H_2O$ in deionized water, pH = 7.0. 18 g of agar is added to the mixture in case of making the agar medium.
- The Luria-Bertaini (LB) medium is prepared with deionized water containing (per 1 L) 10 g of bacteriological tryptone, 5 g of yeast extract, and 10 g of NaCl, adjusting the pH to 7.2.
- Mueller-Hinton medium (MH) is purchased from Himedia, India. Suspend 21.0 g of MH in 1 L deionized water and add 15 of g agar if needed. pH = 7.3.
- Saline water (SW) is prepared by dissolving 8.5 g of NaCl (0.85%, w/v) with 1 L of deionized water, and the pH is adjusted to 7.2
- All media and solutions are sterilized by autoclaving at 121 °C, 15 psi for 15 min.

3.2 Adaptive Laboratory Evolution Experimental protocol

3.2.1 Preliminary Experiment: Strain of Interest Selection

Strain of *Streptomyces* used in this study was selected by their antimicrobial activity against test pathogens using perpendicular streak method (Bhat and Nayaka, 2023). Briefly, the *Streptomyces* strains were inoculated on one sector of Mueller-Hinton agar (MHA) plate and incubated at 30 °C for 7 days. Following incubation, 0.5 McFarland Standard concentrations of test pathogens are streaked in straight line starting 1 cm apart perpendicularly to the lined *Streptomyces* colony. The plates are incubated at 37 °C for 12 h. An antimicrobial activity is evaluated based on the distance of inhibition between the colony margin of *Streptomyces* and test pathogen.

The condition for co-culture method was chosen by optimizing *Streptomyces* growth condition that yields highest antibacterial activity. The *Streptomyces* strains were cultured in various media and incubated at 30 °C and 37 °C under 200 rpm shaking condition. After that, the cell-free supernatant(s) was then used to evaluate an antimicrobial activity by using agar well diffusion method.

Based on the preliminary results of antimicrobial activities, the following was used for co-culture ALE experiment.

- Bacterial strain: *Streptomyces* sp. SSUT88A.
- Co-culturing Pathogens: MDR-*Acinetobacter baumannii* SUTH-Isolate, methicillin-resistant *Staphylococcus aureus* DMST20651 (MRSA), and MDR-*Pseudomonas* aeruginosa SUTH-Isolate N90PS.
- Media: International *Streptomyces* Project-2 Medium (ISP-2).
- Temperature: 30 °C.

3.2.2 Co-Cultivation: Adaptive Laboratory Evolution

The co-cultivation method was be adapted form Dharmesh Harwani et al., 2022. *Streptomyces* sp. SSUT88A was inoculated into 5 different flasks (No.1, No.2, No.3, No.4, No.5) each containing sterilized 50 mL of ISP-2 broth to an initial OD_{600} of 0.01 and incubated at 30 °C, 180 rpm. After 72 hours of incubation and visible growth of SSUT88A, the flasks were inoculated with 100 µL of target pathogen(s) at OD_{600} = 0.50, following these conditions: Flask No.1 was not co-cultured with any pathogen and served as a negative control. While flask No.2, 3, and 4 were inoculated with MDR-*Acinetobacter baumannii* SUTH-Isolate, MRSA DMST20651, and MDR-*Pseudomonas* aeruginosa SUTH-Isolate N90PS, respectively. Flask No.5 was inoculated with all *A. baumannii*, MRSA, and *Pseudomonas aeruginosa* N90PS.

All the flasks were incubated at 30 °C, 200 rpm for 10 days to complete the first adaptation cycle (Cycle 1). Following co-culture incubation, cultures from each flask were serially diluted in normal saline and plated on Starch Casein agar (SCA) using spread plate technique. The plates were incubated at 30 °C for 5 days, or until the colonies of SSUT88A appear.

At least 40 colonies from each flask were randomly selected and streaked separately onto ISP-2 agar plates containing exactly 25 mL of medium per plate and incubated at 30 °C for 7 days. The colonies were tested for their antimicrobial activities via agar plug method (Rütten et al., 2022). In brief, a 6 mm cork borer was used to cut out the agar plugs from the grown cultures. To minimize variation due to biomass differences, the agar plugs were carefully selected from lawn with visually similar bacterial density, as different biomass may lead to inconsistent antibiotic production. The plugs were subsequently placed on MHA pre-seeded with MRSA DMST20651, used as representative pathogens to indicate antimicrobial activity. The strains of SSUT88A isolated from flask No.1, 2, 3, 4 and 5 exhibiting highest antimicrobial activity by zone of inhibition around agar plug were served as cycle 1 adapted strains. These strains were used to initiate cycle 2 of adaptation by following the same procedure as described for cycle 1 (Harwani et al., 2022). This experimental procedure was repeated for a total of 9 cycles (Figure 3.1). All strains were preserved in 25% glycerol (v/v) and kept at -80 °C until further experiment.

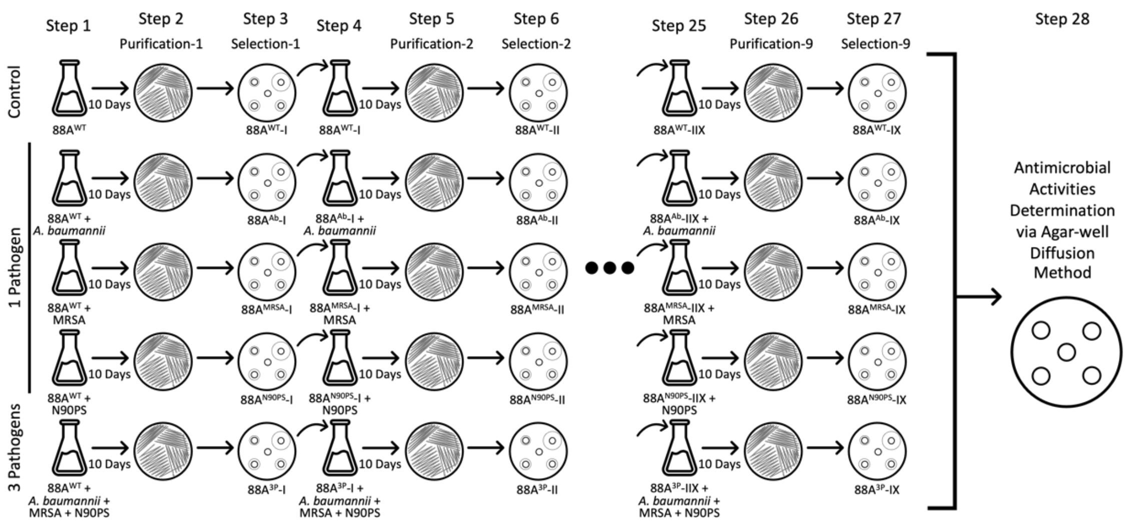

Figure 3.1 Flowchart depicting the Adaptive Laboratory Evolution process aiming to enhance antimicrobial compound production in co-cultured *Streptomyces* sp. SSUT88A and multidrug-resistant pathogens.

3.3 Crude Bioactive Compound Production

A dense spore suspension, pregerminated in ISP-2 media for 6-8 h at 30 °C, 200 rpm, was used to inoculate new ISP-2 media to the initial OD_{600} of 0.03, and incubated at 30 °C, 200 rpm for 10 days. The cell-free supernatant containing bioactive compounds of both wild-type and evolved strains were extracted using ethyl acetate-based liquid extraction method. Briefly, the cell-free supernatant obtained after centrifugation was mixed with an equal volume of ethyl acetate (1:1, v/v) in a separatory funnel and shaken vigorously for 10 minutes and left to allow phase separation, the extraction was performed twice (Ahsan et al., 2017). The combined ethyl acetate phases were collected, evaporated to dryness using a rotary evaporator, weighed and stored at -20 °C until further analysis.

3.4 Antimicrobial Activity Assessment

3.4.1 Primary Antimicrobial Activity Assay: Agar Well Diffusion

Agar well diffusion method was used to determine a primary antimicrobial activity of crude extract. A 0.5 McFarland standard concentration of test pathogen was evenly swabbed onto MHA plates. Wells were then punched aseptically with a sterile cork borer, and a 50 µL of the crude extract at desired concentration is pipetted into the well. The plates will be incubated at 37 °C for 12 h. After incubation, the zone of inhibition in diameter (mm) will be observed and recorded (Abirami and Kannabiran, 2016).

3.4.2 Antimicrobial Activity Monitoring of Selected Strains

To further monitor the onset and progression of bioactive compound production, a time-course study was performed on the adapted strains SSUT88A those exhibited the strongest antimicrobial activity in the primary agar well diffusion assay. Each strain was cultured in ISP-2 medium under the same conditions as described previously in section 3.2.3. Cell-free supernatants were collected daily by centrifugation and kept at -20 °C until subjected to antimicrobial activity screening against MRSA DMST20651 using the agar well diffusion method, as described in section 3.2.4.

3.4.3 Secondary Antimicrobial Activity Evaluation: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

3.4.3.1 Minimum Inhibitory Concentration (MIC)

The MIC was determined following EUCAST guidelines (2024). A single colony of the test organism was inoculated into 5 mL of nutrient broth and incubated overnight at 37 °C, 200 rpm. The overnight culture was adjusted to an OD_{600} of 0.008 in sterile normal saline to prepare a standardized inoculum. Crude extract was initially dissolved in dimethyl sulfoxide at a stock concentration of 10 mg/mL. This stock was then diluted in Mueller–Hinton Broth (MHB) to prepare a working solution, with a maximum test concentration of 2,048 µg/mL. All solutions containing the crude extract were filter-sterile using a 0.22 µm syringe filter prior to use in the assay to ensure sterility and homogeneity. A two-fold serial dilution series was prepared in a 96-well plate. Positive control (no antibiotic), negative control (sterility) and DMSO control (to account for solvent effects) were included. Plates were incubated at 37 °C for 16–24 h. The MIC was defined as the lowest concentration at which no visible bacterial growth was observed (Kadeřábková et al., 2024).

3.4.3.2 Minimum Bactericidal Concentration (MBC)

To determine MBC, 50 µL from wells showing no visible growth in the MIC assay were spot-plated onto Mueller-Hinton Agar plates. Plates were incubated at 37 °C for 24 h. The MBC was defined as the lowest concentration resulting in no colony growth on the agar surface.

3.5 Whole Genome Sequencing, Assembly, and Visualization

3.5.1 Genomic DNA Extraction, Sequencing, and Quality Control

The strain demonstrating the highest level of adaptation, as determined by increased antimicrobial activity against multidrug-resistant (MDR) pathogens, was carefully chosen for further genomic analysis.

The genomic DNA (gDNA) of the selected strains was extracted from cells grown in ISP-2 broth at 30°C for 72 h. The salting-out method described by Kieser et al., 2000 was modified for optimized gDNA isolation (Kieser et al., 2000). Bacterial cells were collected by centrifugation at 3,000 ×g for 5 min and transferred to 50 mL centrifuge tubes. The cell pellet was washed twice and suspended in 4 mL of TE buffer (10 mM Tris-HCl and 1 mM EDTA, final pH = 8.0). 1 mL of lysozyme solution (50 mg/mL) and 125 µL of RNAse A solution (10 mg/mL) were then added, the mixture was incubated at 37°C for 60 min with occasional inversion. Subsequently, 140 µL of proteinase K solution (20 mg/mL) and 600 µL of 10% of sodium dodecyl sulfate (SDS) were added, the solution was incubated at 55°C for 30 min. To extract gDNA, 2.2 mL of 5M sodium chloride (NaCl) was added and allow to cool to room temperature before adding 5.2 mL chloroform: Isoamyl alcohol (24:1), mixed by gently inversion for 5-10 min. before being centrifuged at < 3,000 ×g for 15 min. The upper layer was carefully transferred to a new clean tube with cut P1000 pipette tip (If the solution is still opaque, additional NaCl, SDS and Chloroform: Isoamyl alcohol were added, and the extraction step was repeated). The extract was then incubated on ice for at least 2 min. before adding 2 volumes of ice-cold absolute ethanol. gDNA was precipitated as visible clumps by very gentle inversion. The gDNA was carefully spooled into a new microcentrifuge tube containing 70% ethanol using a sealed Pasteur pipette. (If the DNA clump was not intact or the amount was too small to spool, the DNA was instead collected by centrifugation at <3,000 ×g for 10 min.). The pellet was washed 2 additional times with 70% ethanol, air-dried in a biosafety cabinet and resuspended in TE buffer or nuclease-free water.

The quality of gDNA was assessed using agarose gel electrophoresis and NanoDrop^{TM} One spectrophotometry (Thermo Scientific, USA).

This selected strain was subjected to whole genome sequencing, which will be conducted by Theragen Bio Co.,LTD., Republic of Korea, using the Illumina Novaseq 6000 sequencer (Illumina, CA, USA).

To achieve high quality reads, quality assessments, adapter trimming, and quality filtering were performed using fastp v0.23.4, with default settings (Chen, 2023; Chen et al., 2018).

3.5.2 Genome Assembly and Assessment

The quality-filtered short reads were *de novo* assembled using Unicycler v0.5.0, an assembly pipeline for bacterial genomes (Wick et al., 2017). Default parameters were applied with -conservative assemble mode. The completeness and quality of the assembled genome were assessed using two tools: (1) QUAST v5.2.0 to generate assembly statistics such as N50, total length, number of contigs, and GC content (Gurevich et al., 2013) and (2) CheckM v1.2.2 to evaluate the genome's completeness and contamination based on lineage-specific marker genes (Parks et al., 2015).

3.5.3 Genome Annotation and Visualization

Genome annotation was performed using Prokka v1.14.6, to identify coding sequences (CDS), predict encoding protein, and other genomic features (Seemann, 2014).

Proksee, which used CGView engine, was used to generate circular genome maps for all strains and also to annotate the genomes with Prokka (Grant et al., 2023).

3.6 Functional and Comparative Genomic Analysis

To characterize and compare key functional elements and genomic changes among the wild-type and evolved strains, set of tools were applied to analyze strains' taxonomy, antibiotic resistant profile, mobile genetic element, secondary metabolites, comparative clustering, variant calling, and k-mer validation.

3.6.1 Taxonomic Comparisons

Digital DNA-DNA hybridization (dDDH) analysis and a phylogenomic tree derived from the whole genome sequences of wild-type and adapted strains with their related type strains were conducted using the Type (Strain) Genome Server (TYGS), which utilizes Genome BLAST Distance Phylogeny (GBDP) methods (Meier-Kolthoff and Göker, 2019).

The Average Nucleotide Identity (ANI) values between wild-type and evolved strains were calculated using JSpeciesWS, to quantitatively measure genomic similarity (Richter et al., 2015).

3.6.2 Antimicrobial Resistance Gene Exploration

Antimicrobial resistance (AMR) genes were identified using ResFinder (v4.7.2), a curated database and detection pipeline hosted by the Center for Genomic Epidemiology (Florensa et al., 2022), on annotated genome with default thresholds for sequence identity (> 90%) and minimum gene length.

3.6.3 Biosynthetic Gene Clusters Detection and Comparisons

The secondary metabolite biosynthetic capacity of each strain was analyzed using antiSMASH (v8.0). All assembled genomes were submitted for complete BGC detection with strict detection strictness to avoid false positives. The resulting annotations provided information on the types, locations, and predicted functions of Biosynthetic Gene Clusters (BGCs), such as Non-Ribosomal Peptide Synthetase (NRPS), Polyketide Synthase (PKS), Ribosomally synthesized and post-translationally modified peptides (RiPPs), and terpenes. These results were used for presence/absence BGC analysis (Blin et al., 2025).

3.6.4 Orthologous Gene Clustering based on Translated Amino Acid

Orthologous gene clusters were analyzed using OrthoVenn3 (Sun et al., 2023), a platform for comparative functional genomics provided a functional framework for interpreting which gene sets were gained or lost. Translated amino acid sequences predicted by Prokka from each assembled genome were submitted for clustering using OrthoFinder algorithm, E-value and Inflation value of 10^{-2} and 1.50, respectively.

3.6.5 Structural and Point Mutational Analysis of Adapted Strains

Genomic variants between the evolved strains and the wild-type reference were analyzed using Breseq v0.38.1, a tool specifically designed for microbial genome variations detection (Deatherage and Barrick, 2014). Because the reference genome used in this study was a draft assembly composed of multiple contigs, relying solely on reference mode (-r) could lead to inaccurate or excessive detection of structural differences those might reflect incomplete or fragmented assemblies rather than true biological variation. To overcome this limitation and better distinguish between small, high-confidence mutations and possible larger-scale rearrangements or deletions, both consensus mode (-c) and reference mode (-r) were employed.

- Contigs mode (-c), a primary focused mode due to time constraints and its robustness in identifying point mutations such as single nucleotide polymorphisms (SNPs). In this mode, the reference genome was treated as an independent draft assembly, and high-confidence differences from the wild-type were reported. Only a small number of variants were detected per evolved strain, which highlighted the overall genetic similarity and stability of the core genome across conditions. The focused scope and lower susceptibility to assembly artifacts made this mode the most reliable for confident mutation calling.
- Reference mode (-r) was used to complement SNP detection and assess potential larger-scale genomic changes, such as deletions or structural losses. In this mode, reads from each evolved strain were aligned directly to the wild-type draft assembly to recapture the SNPs detected in -c, while additionally identifying numerous putative large deletions. This approach flagged the apparent loss of over 100 contigs in some strains (a result that initially seemed implausible and raised concerns

about misalignment or artifacts due to the fragmented reference). Deletion predictions from this mode were retained for downstream validation and comparative analysis.

To distinguish true genomic loss from assembly or mapping artifacts, we conducted 31-mer frequency analysis on raw FASTQ reads using Jellyfish v2.3.0 (Marçais and Kingsford, 2011). Unique 31-mers derived from each reference contig were queried against evolved strains k-mer databases (Lee et al., 2020). Contigs with zero 31-mer hits with overall high sequencing depth were classified as high-potential true deletions (Zheng et al., 2022). Those with residual 31-mers or low depth were deprioritized as likely artifacts.

3.6.6 Mobile Genetic Elements

Mobile Genetic Elements (MGEs) were annotated with MobileOG-DB through the Proksee platform. Regions of Interests were observed for integrases, transposases, conjugation machinery, or replication/recombination proteins.

3.7 Data Analysis and Visualization

Statistical analyses were performed using R (version 4.4.2). One-way ANOVA followed by Tukey's HSD post hoc test was used to assess differences between groups. A significance difference was determined by $p < 0.05$ throughout. Unless otherwise stated, all experiments were performed in triplicate (n = 3). Graphs, including box plots and line graphs, were generated using the ggplot2, reshape2 and dplyr package.