CHAPTER V
CONCLUSION

In this study, adaptive laboratory evolution through strategic co-cultivation with drug-resistant pathogens effectively regulate secondary metabolism in *Streptomyces* sp. SSUT88A. Phenotypic assessments revealed that while most evolved strains retained wild-type-like morphology, one lineage (SSUT88A^{3P6-7}) isolated after the sixth cycle of three-pathogen co-culture, exhibited dramatic morphological changes, losing its characteristic yellow diffusible pigment and shifting to a red colony phenotype. This pigment loss coincided with a complete loss of antimicrobial activity, supporting the link between morphological traits and secondary metabolite production.

Pathogen-specific adaptation drove markedly different outcomes in bioactive compound yields: MRSA was the strongest pathogen, enhancing crude extract production by 46.3%, followed by *A. baumannii* with a 32.6% increase, whereas *P. aeruginosa* decreased output and the three-pathogen combination showed only a modest effect. Among adapted strains, SSUT88A^{Ab9-5} produced the highest yield (43% increase), followed closely by SSUT88A^{MR9-16} (40%). Antimicrobial activity assays further corroborated these findings: SSUT88A^{Ab9-5} and SSUT88A^{MR9-16} showed significant improvements in inhibition zones, particularly against Gram-positive pathogens. A time-course assay using MRSA confirmed that SSUT88A^{Ab9-5} initiated bioactivity as early as day 2 (faster than the wild-type and SSUT88A^{MR9-16}) and maintained potent inhibition, while the SSUT88A^{3P6-7} strain demonstrated a complete loss of bioactivity. MIC and MBC assays showed 4–8-fold improved potency in the adapted strains compared to the wild-type, especially against MRSA and MRSE, while SSUT88A^{3P6-7} exhibited no detectable antimicrobial activity.

All genome assemblies exhibited high completeness (99.94% as assessed by CheckM), ensuring the reliability of downstream comparative analyses. Taxonomic validation through digital DNA–DNA hybridization, average nucleotide identity, and phylogenomic tree analysis confirmed that all adapted strains remained taxonomically

identical to the wild-type, ruling out contamination during adaptive laboratory evolution or sequencing. Furthermore, antimicrobial resistance profiling via ResFinder revealed that the wild-type strain contains no AMR genes, and none were detected in any of the evolved strains despite prolonged exposure to multiple drug-resistant pathogens.

Comparative analysis of biosynthetic gene clusters predicted by antiSMASH revealed dynamic structural shifts that likely underlie the observed phenotypic divergence in antimicrobial activity across strains. While several BGCs were conserved across all strains, others were found to be differentially retained, either exclusively in the enhanced antibiotic-producing strains (SSUT88A^{MR9-16} and SSUT88A^{Ab9-5}) or uniquely preserved in the wild-type and the non-producing SSUT88A^{3P6-7}. These patterns suggest adaptive genomic remodeling favoring the retention of clusters with greater bioactive potential. Six clusters were consistently shared among the antibiotic-producing strains but absent in the non-producing strain., each of these BGCs encodes compound classes with known or potential antimicrobial properties, implying a functional role in the enhanced bioactivity observed. In particular, the aryl polyene cluster may also contribute to the pigment-associated phenotype, reinforcing the link between metabolite production and strain-specific coloration. Further distinguishing the high-producing strains, SSUT88A^{MR9-16} and SSUT88A^{Ab9-5} harbored two unique clusters: a NRPS cluster with low-confidence similarity to actinomycin D, and a T1PKS cluster with no clear homolog but strong candidate potential for novel antimicrobial activity. Although functional confirmation remains unclear, the restriction of these clusters to strains with elevated antimicrobial activity strengthens the hypothesis that they play a direct role in the observed phenotype. Conversely, a group of clusters was detected only in the wild-type and non-producer. These clusters, while potentially biologically active, are not commonly associated with antibacterial potency and may represent less relevant, their absence in the enhanced strains may reflect selective loss during adaptive evolution. Altogether, the antiSMASH-based comparative BGC analysis suggests that adaptive laboratory evolution leading to the emergence or enrichment of specific biosynthetic pathways.

Orthologous clustering of amino acid sequences across all four strains using OrthoVenn3 revealed broad conservation alongside key differences in gene content likely shaped by adaptive pressures. The relatively small number of unique clusters further supports the high degree of overall genomic conservation. However, among the remaining variable clusters, several were strain-specific or shared only among phenotypically similar groups, highlighting regions of potential relevance to adaptive divergence, particularly with respect to antimicrobial activity. Importantly, 667 clusters were shared among all three antibiotic-producing strains, potentially representing a functional core underpinning antibiotic biosynthesis. In contrast, 16 clusters were uniquely shared between the two evolved strains exhibiting enhanced antibiotic production. Among these were clusters associated with RNA secondary structure unwinding and trehalose catabolic processes, both of which may play roles in transcriptional regulation or stress response during co-culture adaptation. On the other hand, 618 clusters were uniquely shared between the wild-type and the non-producer strain, suggesting the retention of gene content unrelated to increased antimicrobial production. Phylogenetic tree reconstruction based on shared orthologous clusters supported a branching pattern consistent with observed phenotypes: the wild-type diverged first, followed by the loss-of-function strain, while enhached production strain clustered most closely, suggesting parallel evolutionary route.

To integrate functional and biosynthetic insights, antiSMASH-predicted BGCs were cross-referenced with OrthoVenn orthologous clusters based on core biosynthetic genes. Cross-comparison revealed that many key core biosynthetic genes predicted by antiSMASH could be reliably mapped to orthologous clusters, strengthening the connection between genotype and bioactive phenotype.

Lastly, genome-wide analysis using Breseq provided critical insight into both structural and fine-scale sequence changes that accompanied adaptation. Large-scale deletions identified in evolved strains helped explain some of the observed phenotypic differences, where deleted contigs corresponded to antiSMASH-predicted biosynthetic and Orthologous gene clusters.

In parallel, SNP analysis revealed surprisingly few high-confidence point mutations across evolved genomes. However, only three high-confidence SNPs were

ultimately retained after strict manual curation for read depth, quality, alignment confidence, and contextual biological plausibility. Of these, two occurred exclusively in the high-antibiotic-producing strains.

The first was located 37 bp upstream of *murE_2*, a gene involved in peptidoglycan biosynthesis, in the intergenic region between *valS* and *murE_2*. This SNP is positioned near the expected location of a -35-promoter motif, raising the possibility of transcriptional modulation. Since *murE_2* plays a role in cell wall precursor assembly, this mutation may reflect adaptation evolution to co-culture-induced cell wall alteration.

The second SNP was unique to the precocious producer SSUT88AAb9-5 and lies 14 bp upstream of a gene encoding a DUF2637-domain (*SpdA*-like) protein. Although the function of this gene remains uncharacterized, its conservation across *Streptomyces* species and the SNP's proximity to a likely -10 promoter region suggest a potential regulatory impact on the function.

The third SNP was found only in the pigment-deficient, non-producing strain SSUT88A3P6-7. This was a synonymous mutation within a gene annotated as a hypothetical protein (EAAMLAOK_05879), altering the codon CGG to AGG (both coding for arginine). Despite being silent at the protein level, this mutation affects the first codon position and may influence translation efficiency, mRNA structure, or codon usage factors increasingly recognized as functionally significant in gene regulation. Given its exclusive occurrence in the strain with complete loss of pigment and bioactivity, a subtle post-transcriptional or translational effect cannot be ruled out.

None of the mutations mapped to core biosynthetic genes identified by antiSMASH, implying that phenotypic divergence among strains was not driven by coding sequence changes within BGCs but altering the regulatory regions, alongside structural changes such as BGC loss. Taken together, Breseq results complement the functional and comparative genomic analyses by highlighting two major evolutionary trends: targeted gene loss in the non-producing strain and subtle, potentially regulatory sequence changes in the bioactive strains.

These findings underscore that enhanced antimicrobial phenotypes emerged not through directly modification of biosynthetic gene content, but through a

combination of selective gene retention, gene loss, and modest but impactful genomic refinements.

These results demonstrate that adaptive laboratory evolution through co-cultivation drives meaningful genomic changes, as revealed by whole-genome sequencing. Structural deletions and regulatory-region mutations were linked to observable phenotypic improvements, such as precocious bioactive compound production in SSUT88A$^{Ab9\text{-}5}$ and enhanced antimicrobial potency in SSUT88A$^{MR9\text{-}16}$. These evolved traits can potentially reduce production time and improve yields, making this approach a valuable strategy for generating industrially improved *Streptomyces* strains for antibiotic production.