Reverse Engineering and Assaying the Transcriptional Regulatory Network of *S. pneumoniae* for Antibiotic Potentiation

The state of a bacterial cell greatly influences its response to antibiotic treatment. Similar to how cancer treatment disproportionately affects rapidly dividing cells, antibiotic treatment is most effective when bacteria are spending significant energy on the process the antibiotic is designed to disrupt. The behavior of a cell is dependent on the regulatory logic encoded in its genome, and understanding this logic within pathogenic bacteria would allow for the rational design of antibiotic potentiators.

Streptococcus pneumoniae is the leading cause of community-acquired pneumonia, the 7th leading cause of death in the USA[1]. Alarmingly, 30% of pneumococcal infections are resistant to one or more antibiotics[2], and its transcriptional regulatory network (TRN) remains uncharacterized. Our work here is comprised of two main aims: first, to comprehensively characterize the TRN of *S. pneumoniae* and, second, to interrogate this network to understand the complex interactions between the current state of the transcriptome and antibiotic outcomes.

To achieve these aims, we first designed and cloned 89 Transcription Factor Induction (TFI) strains in our model strain, TIGR4. Each TFI strain encodes a barcoded IPTG-inducible transcription factor. We scraped the RefSeq annotation for TIGR4 for terms representative of TFs, including "helix-turn-helix", "transcriptional regulator" and "repressor". We identified 110 TFs and were able to clone a TFI strain for 89 of them. RNAseq was completed on all TFI strains to assess each individual TF's effect on the transcriptome.

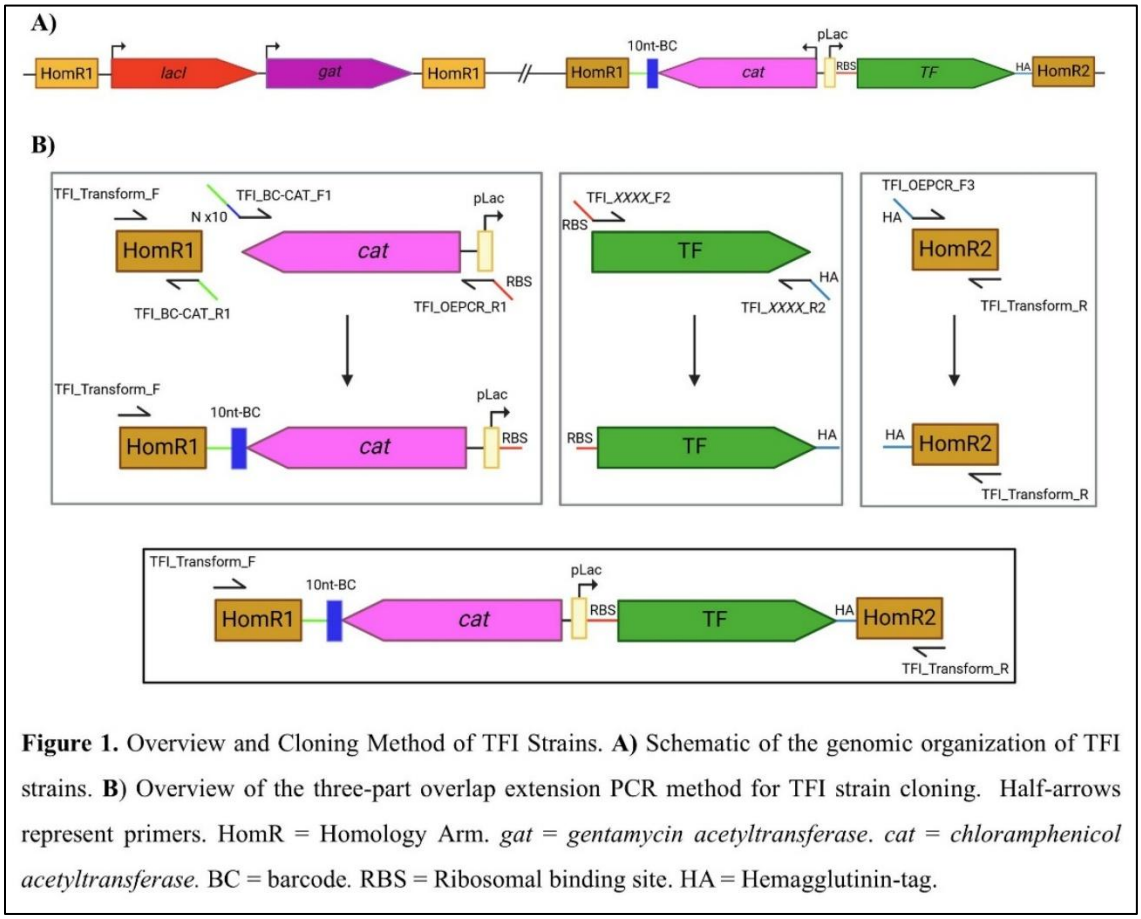

Figure 1. Overview and Cloning Method of TFI Strains. **A)** Schematic of the genomic organization of TFI strains. **B)** Overview of the three-part overlap extension PCR method for TFI strain cloning. Half-arrows represent primers. HomR = Homology Arm. *gat* = *gentamycin acetyltransferase*. *cat* = *chloramphenicol acetyltransferase*. BC = barcode. RBS = Ribosomal binding site. HA = Hemagglutinin-tag.

To reverse engineer a comprehensive TRN for TIGR4, we compiled a large gene expression dataset of over 600 experiments and utilized an ensemble of network inference methods to predict regulatory interactions. Each experiment provides a snapshot of the transcriptome and includes all induced TFI strains and wild-type TIGR4 under diverse conditions. We input this dataset into eight network inference methods[3–10], and each method was graded by calculating the AUPR relative to predicted interactions from RegPrecise. All interactions predicted by our optimized set of inference methods and all significantly regulated target genes per TFI RNAseq were combined into a list of candidate interactions. To remove false positives, all candidate target promoters for a TF were interrogated with XSTREME to predict each TF's binding motif[11,12], and only interactions with an identified motif were accepted. Our final network contains 3,867 interactions between 88 TFs and 1,607 target genes, representing the most comprehensive TRN of *S. pneumoniae* to date.

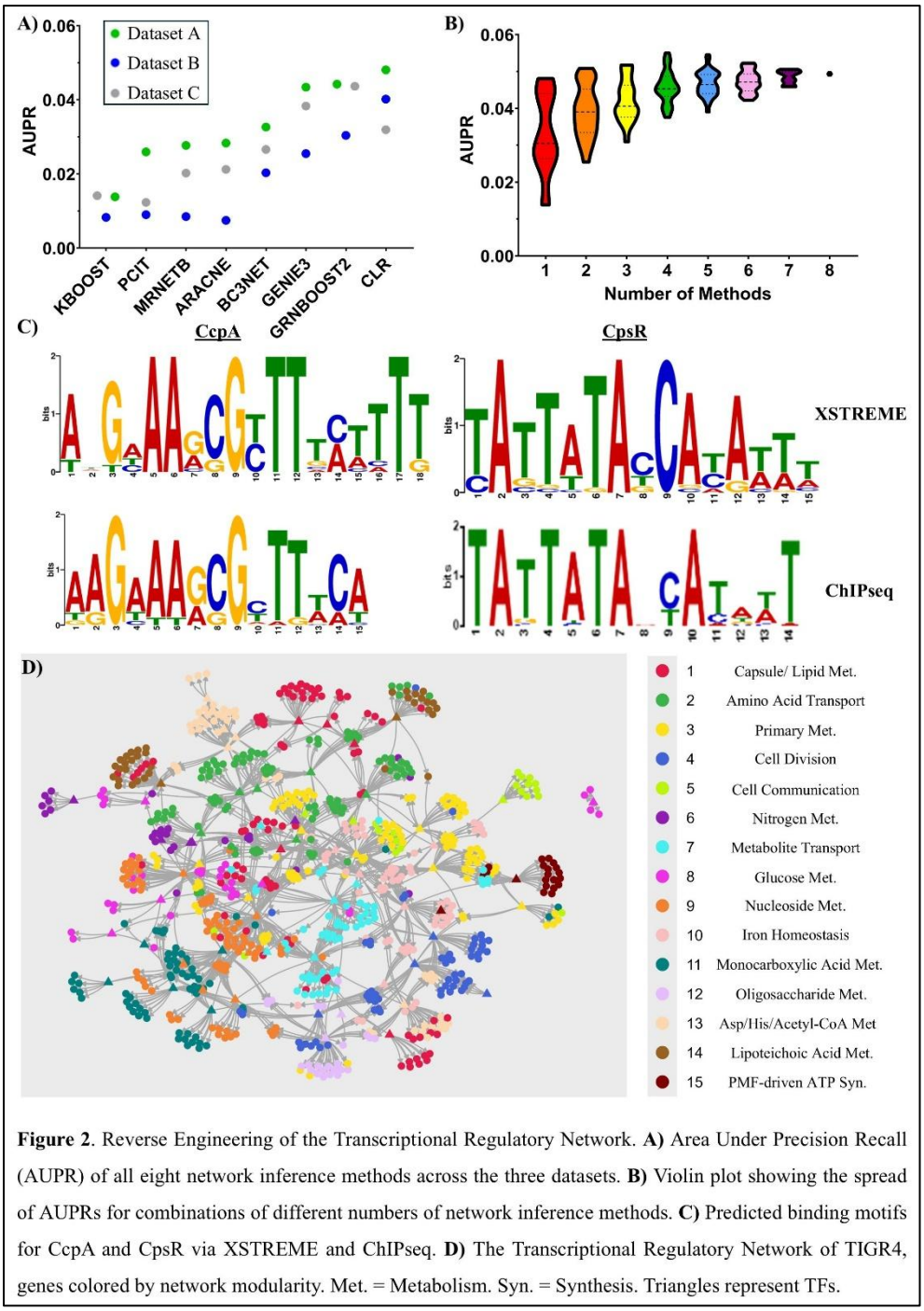

Figure 2. Reverse Engineering of the Transcriptional Regulatory Network. **A)** Area Under Precision Recall (AUPR) of all eight network inference methods across the three datasets. **B)** Violin plot showing the spread of AUPRs for combinations of different numbers of network inference methods. **C)** Predicted binding motifs for CcpA and CpsR via XSTREME and ChIPseq. **D)** The Transcriptional Regulatory Network of TIGR4, genes colored by network modularity. Met. = Metabolism. Syn. = Synthesis. Triangles represent TFs.

Transcriptional Regulator Induced Phenotype (TRIP) screens were then completed under various antibiotic stress to assess how the transcriptome influences antibiotic fitness[13]. Briefly, all TFI strains are combined into a TRIP pool, induced with IPTG and cultured across varying antibiotic treatments. The relative fitness effect of each TFI strain is calculated by tracking each strain's relative abundance across time through barcode sequencing. TRIP screens identified three regulators, CpsR (*SP_0058*), SusR (*SP_1799*) and TreR (*SP_1885*), with significantly increased resistance to cell-wall synthesis inhibitors but decreased resistance to both protein and DNA synthesis inhibitors. Overexpression of these TFs was found to repress peptidoglycan synthesis by rerouting carbon metabolism, resulting in resistance to cell wall synthesis inhibition, but in turn increase cellular acidity, resulting in susceptibility to other antibiotics. For antibiotic tolerance, we found strong correlation between the fitness results between different classes of antibiotics, suggesting that the same mechanisms are used to survive antibiotic stress irrespective of initial target. We identified that overexpression of the Clp protease repressor, CtsR (*SP_2195*), lead to massive antibiotic tolerance. This aligns with recent findings that an activator of the Clp protease, ADEP4, can effectively kill persisters[14].

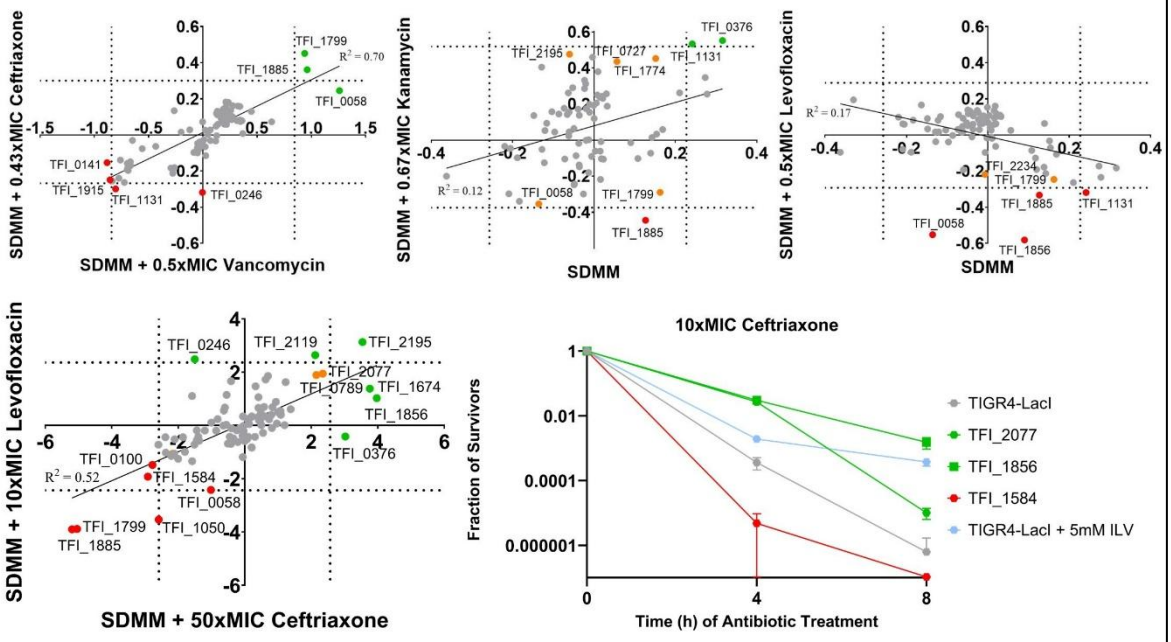

Figure 3. TRIP Screens Quantify the Fitness Effect the Transcriptome has on Antibiotic Fitness.

Top: Specific fitness plots comparing TRIP results in Ceftriaxone and Vancomycin **(Left)**, baseline SDMM and Kanamycin **(Middle)** and baseline SDMM and Levofloxacin **(Right)**. **Bottom:** Correlation of Tolerance TRIP screen results for Levofloxacin (DSI) and Ceftriaxone (CWSI) **(Left)** and Monoculture time-kill assay validation of Tolerance TRIP screen results for select strains or supplementation with Isoleucine, leucine and Valine (ILV). Average of three biological replicates shown.

Overall, we present the most comprehensive TRN of *S. pneumoniae* to date and quantified the fitness effects of the network under various antibiotic stresses. We identified the pivotal role of metabolic regulation in antibiotic stress response and proteostasis as a main determinant of antibiotic tolerance. Many of our insights align with previous observations in other pathogens, highlighting conserved and targetable mechanisms that provide a unified framework for overcoming antibiotic resistance.

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