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Global transcription factors analyses reveal hierarchy and synergism of regulatory networks and master virulence regulators in *Pseudomonas aeruginosa*

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eLife Assessment

This study provides an **important**, comprehensive, large-scale dataset on transcription factor binding in Pseudomonas aeruginosa, along with analyses of its regulatory network, key virulence and metabolic regulators, and a pangenomic examination of transcription factors. Utilizing large-scale ChIP-seq and multi-omics integration, the research **convincingly** supports the hierarchical regulatory structures and offers insights into virulence mechanisms. This dataset, made available through an online database, should be an invaluable resource to the research community studying P. aeruginosa, a key pathogen at risk for hospital infections and development of antibiotic resistance.

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Abstract

The transcription factor (TF) regulatory network in *Pseudomonas aeruginosa* is complex and involves multiple regulators that respond to various environmental signals and physiological cues by regulating gene expression. However, the biological functions of at least half of its 373 putative TFs remain uncharacterised. Herein, chromatin immunoprecipitation sequencing (ChIP-seq) was used to investigate the binding sites of 172 TFs in the *P. aeruginosa* PAO1 strain. The results revealed 81,009 significant binding peaks in the genome, more than half of which were located in the promoter regions. To further decode the diverse regulatory relationships among TFs, a hierarchical network was assembled into three levels: top, middle, and bottom. Thirteen ternary regulatory motifs revealed flexible relationships among TFs in small hubs, and a comprehensive co-association atlas was established, showing the enrichment of seven core associated clusters. Twenty-four TFs were identified as the master regulators of virulence-related pathways. The pan-genome analysis revealed the conservation and evolution of TFs in *P. aeruginosa* complex and other species. A Web-based database combining existing and new data from ChIP-seq and the high-throughput systematic evolution of ligands by exponential enrichment was established for searching TF-binding sites. This study provides important insights into the pathogenic mechanisms of *P. aeruginosa* and related bacteria and is expected to contribute to the development of effective therapies for infectious diseases caused by this pathogen.

Introduction

Transcription factors (TFs) are key regulators of transcription and thus play crucial roles in mediating multiple biological pathways and events in eukaryotes and prokaryotes. Based on their DNA-binding domains (DBDs), TFs can be classified into different families, such as the LysR, AraC, and LuxR families[1]. TFs can activate and repress the expression of a set of genes in response to various environmental and/or specific signal triggers[2,3]. Several approaches can be used to study TFs, including chromatin immunoprecipitation sequencing (ChIP-seq)[4] and ChIP-exo[5] in *in vivo* research and DNA affinity purification sequencing (DAP-seq)[6] and high-throughput systematic evolution of ligands by exponential enrichment (HT-SELEX)[7] in *in vitro* research. ChIP-seq is a powerful *in vivo* technique that enables more accurate genome-wide mapping of TF–DNA interactions than *in vitro* methods because TFs may interact with other co-regulators in an environment-specific pattern, thereby altering binding preferences *in vivo*[8,9].

These sequencing approaches have been used to illustrate the biological functions of TFs in several bacterial species, including *Mycobacterium tuberculosis*, *Vibrio cholerae*, *Salmonella enterica*, *Escherichia coli*, and *Clostridium thermocellum*[10–15]. In addition, several databases and models have been constructed based on experimental validation or machine learning for studying TFs in bacteria, such as CollecTF, PredicTF, PRODORIC, DBTBS, and RegulomePA[16–20]. However, the functions and spatiotemporal interactions of most bacterial TFs remain to be clarified.

The gram-negative bacterium *Pseudomonas aeruginosa* is a major human opportunistic pathogen that grows ubiquitously. *P. aeruginosa* tends to cause infection in burn victims, patients with cystic fibrosis (CF), and individuals hospitalised for long periods[21,22]. It utilises versatile pathways to exert its virulence, such as biofilm formation, quorum sensing (QS), type III and type VI secretion systems (T3SS and T6SS, respectively), motility, siderophore production, oxidative stress resistance, and antibiotic resistance, all of which are under the control of a complicated TF regulatory network (TRN). The metabolic changes regulated by the TRN enhance the pathogen's adaptability under different infection and survival conditions.

P. aeruginosa employs diverse virulence pathways to establish successful infection, with QS being one of the major mechanisms involving the expression of many virulence genes. Its regulation occurs hierarchically, comprising the interconnected *las*, *rhl*, and *pqs* systems[23,24]. Through its transcriptional regulator, LasR, and in collaboration with its corresponding autoinducer, 3-oxo-C_{12}-HSL (encoded by *lasI*), the *las* system triggers the activation of the *rhl* and *pqs* systems[23,25]. Additionally, *P. aeruginosa* utilizes motility systems for surface attachment and colonization, including: (i) swarming and swimming powered by flagella, which are multicellular and individual cell movements, respectively, and (ii) twitching powered by type 4 pili (T4P)[26], wherein T4P promote surface attachment by twitching to pull the cell closer to the attachment sites[27]. The motility of *P. aeruginosa* is thus the initial step in its pathogenic process and is associated with the upregulation of virulence and the induction of host defence. Both QS and motility pathways are precisely controlled by the sophisticated TRN in *P. aeruginosa*. Understanding how these virulence pathways are integrated and regulated is crucial for developing novel therapeutic strategies against *P. aeruginosa* infections.

Despite the TRN being essential for understanding how bacterial genes are regulated synergistically, only a limited number of binding targets of TFs have been identified in *P. aeruginosa* over the past decades. For example, ChIP-seq and RNA sequencing (RNA-seq) co-analysis has been used to map a *P. aeruginosa* genomic regulatory network (PAGnet) of 20 key virulence-related TFs, while HT-SELEX has been used to characterise the DNA-binding specificities of 182 TFs[28]. DAP-seq has been used to reveal a regulatory network of 55 response regulators (RRs) of two-component systems in *P. aeruginosa*, 51 of which are from the PAO1 strain[29]. However, almost half of the TFs remain functionally uncharacterized, and their downstream targets and upstream regulators are unknown. To fill this knowledge gap, we performed ChIP-seq of 172 TFs, representing the majority of TFs not covered by HT-SELEX, to map their genomic binding sites *in vivo* (Table S1). This comprehensive dataset revealed a hierarchical and co-association regulatory network among TFs and newly identified virulence-related regulators. We integrated both the

ChIP-seq and HT-SELEX data into a web-based database for querying TF-binding patterns, which is publicly available. Our findings and tools will significantly contribute to future mechanistic studies on and a comprehensive understanding of the TRN in *P. aeruginosa* and other bacterial species.

Results

ChIP-seq of 172 uncharacterised TFs reveals a global transcriptional atlas of *P. aeruginosa*

In this study, 373 proteins in *P. aeruginosa* were annotated as TFs with DNA-binding activity based on the existing annotations in the *Pseudomonas* Genome Database[30] (Fig. 1A). To profile the whole TF–DNA binding landscape and construct a comprehensive regulatory network of *P. aeruginosa*, ChIP-seq experiments were performed on 172 TFs to determine the TF-binding sites (TFBSs) (Fig. 1A). Libraries were constructed using vesicular stomatitis virus glycoprotein tagging, and raw sequence reads were mapped to the PAO1 genome using bowtie2 (Version 2.3.4.1)[31]. Using MACS2[32], 81,009 significant binding peaks with a cut-off p-value of 0.001 were identified. Subsequently, the R package ChIPpeakAnno[33] was used to define the peak locations and find the nearest genes. The peak location can be divided into six features: upstream, overlapStart, inside, overlapEnd, downstream, and includeFeature. The percentages of binding peak locations for each TF indicated that more than half of the TFs preferentially bind to the upstream (upstream intergenic regions) and overlapStart (overlapped with the transcription start sites [TSSs] of genes) regions (Fig. 1B). Apart from these regions, some TFs bind to the inside regions (coding regions of genes). Few TFs were found to preferentially bind to the rest of the peak location features.

To further provide insights into the functional roles of different TFs in gene regulation, we analysed the peak distance to the TSS, which revealed several patterns (Fig. 1C). First, most of the TFs exhibited peak distributions that were concentrated in the proximal region around the TSS. Some TFs, such as Cor_CI, showed a broader distribution of peak distance, indicating that these TFs might also be active at positions farther from the TSS. Furthermore, certain TFs, including RpiR and DeoR, displayed distinct bimodal or multimodal distributions, while other TFs, such as LysR and AraC, showed smoother and more narrowly focused distributions, suggesting their different binding preferences. The number of peaks of each TF and its DBD types are summarised in a treemap in Fig. 1D. TFs in the LysR and AraC families accounted for almost half of all of the peaks. PA2718, classified in the MerR family, yielded the highest number (2,480) of binding peaks, followed by the TF PA0756 (2,080 binding peaks) from the OmpR family. Furthermore, eight TFs showed no more than 10 peaks, and the TFs PA4074 and PA4806 had only two peaks each. To preliminarily identify the different regulatory functions of TFs via their different binding preferences, we performed Gene Ontology (GO) functional enrichment analysis for each TF and presented the top 10 GO terms (Fig. 1E). The significantly enriched GO terms (BH-adjusted $P < 0.05$) for all of the 172 TFs analysed by ChIP-seq are listed in Table S2, and these terms suggest divergent functions of the TFs. Of these functional categories, most were related to metabolism, such as transcription, translation, ribosome structure, and GTPase activity, while some of the functional categories were associated with virulence, including T4P, O antigen biosynthesis, and biofilm formation (Fig. 1E).

Hierarchical networks of TFs based on pairwise interactions

TF-coding genes can also serve as targets for other TFs, leading to interactions between the DNA-binding profiles of TFs. To clarify these relationships, lists of binding targets of the TFs obtained using ChIP-seq and HT-SELEX were used to assemble a system-level hierarchical regulatory network. After filtering by TFs that bind to promoter regions, 947 unique pairwise patterns between TFs were identified from a total of 13,375 promoter interactions.

We next defined a statistic index, h, based on the out-degree (O) and in-degree (I) of interactions to measure the direction and hierarchical level of TFs [$h = (O-I)/(O+I)$] in our established hierarchical regulatory network and thus identify hubs and information-flow bottlenecks[34]. The h value of

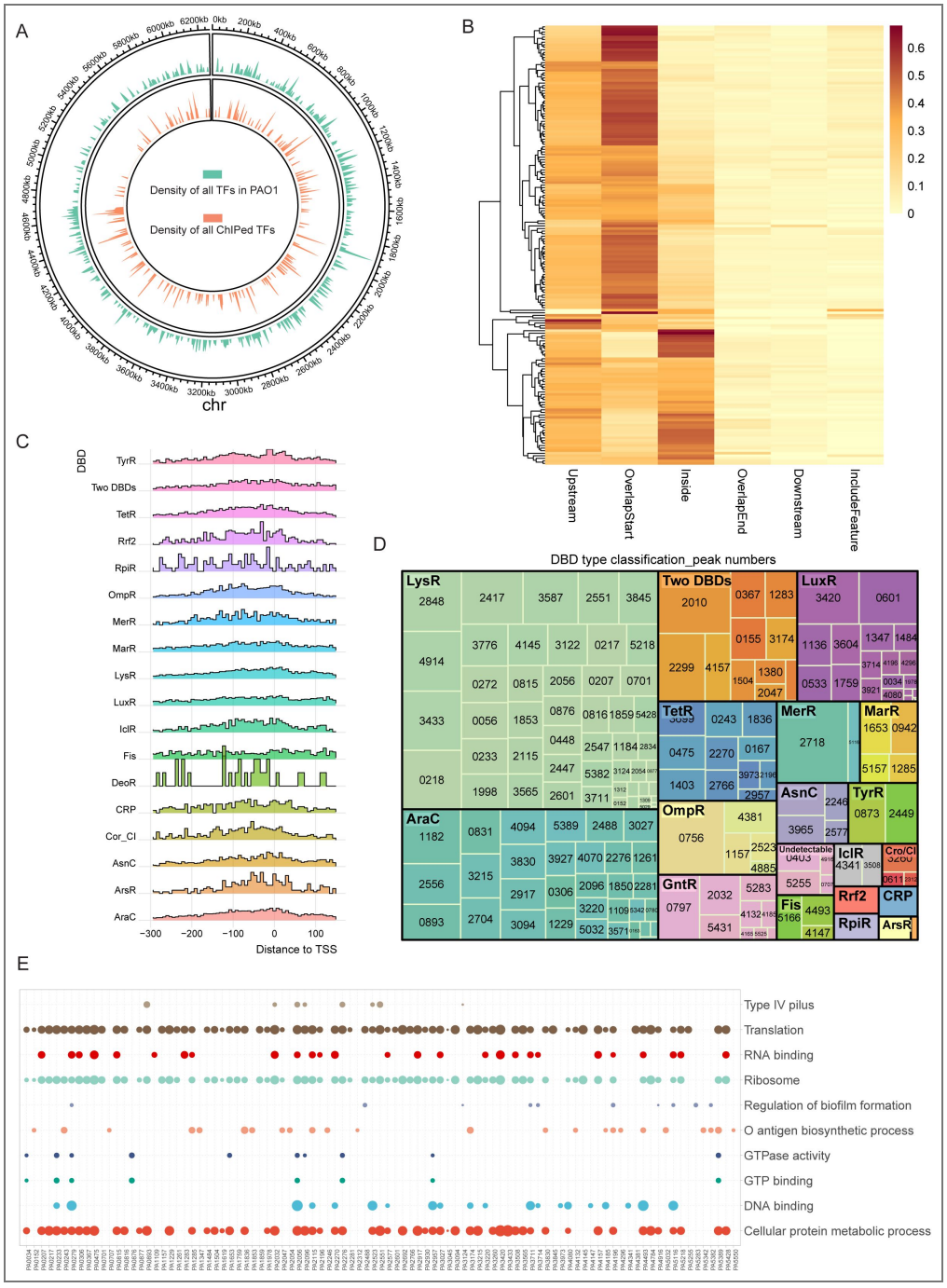

Figure 1. Overview of ChIP-seq results.

(**A).** Density of all TFs (green) and ChIPed TFs (orange) in this study throughout the *P. aeruginosa* genome. (**B).** Annotation heatmap of all peak distribution with 6 locations: Upstream, where the peak is located entirely upstream of the gene; Downstream, where the peak is positioned completely downstream of the gene; Inside, where the peak is entirely contained within the gene body; OverlapStart, where the peak overlaps with the 5′ end of the gene; OverlapEnd, where the peak overlaps with the 3′ end of the gene; and IncludeFeature, where the peak completely encompasses the gene. **(C).** Peak distance to the translational start site (TSS) of each DBD family. **(D).** Treemap of the 172 TFs peak numbers based on DBD family. Each box's size represents the family's size (number of peaks), and the explained variance of each DBD type means the color shades of each box. DBD families of ChIPed TFs are classified into 20 different categories: LuxR, LysR, Two DBDs (Two DNA binding domains), AraC, TetR, ArsR, CRP, OmpR, GntR, MarR, AsnC, Cro/CI, TyrR, Rrf2, MerR, IclR, Fis, RpiR, DeoR, and undetectable. **(E).** The dot plot shows the top 10 gene ontology (GO) terms from the PseudoCAP annotation of ChIPed TFs. The size of the dots indicates the significance of each functional category, quantified by –log10 (p. adjust).

each TF can reflect the direction and extent of information flow. A positive h indicates that the TF acts 'upstream', whereas a negative value suggests that it acts 'downstream'. The higher the absolute h value, the stronger the implication of the direction. A density plot of all of the TFs with their h values revealed that they can be approximately divided into three groups: top (h ∈ [0.75, 1]), middle (h ∈ [-0.75, 0.75]), and bottom (h ∈ [-1, 0.75]) levels (Fig. S2A). The top-level TFs are those that tend to control many other TFs, while the bottom-level TFs are those that are more regulated by other TFs than acting as regulators themselves. The middle-level TFs are those that connect the top- and bottom-level TFs. Of the 231 TFs in this hierarchy, 100, 46, and 85 TFs were defined as top-, middle-, and bottom-level TFs, respectively. The complete hierarchical information of all of the TFs is provided in **Table S3**. To avoid extreme cases and aid visualisation, we summarised the hierarchical regulatory network by removing TFs for which (*O+I*) was more than 10 (Fig. 2A and Fig. S2B**)**. The network was visualised using Cytoscape software[35].

Additionally, we investigated the factors influencing the hierarchical level of TFs, such as DBD types (e.g., DBD families containing more TFs). TFs from the AraC, GntR, and TetR families tended to cluster at the top level. TFs from the MarR family and two-DBD-type TFs (i.e., TFs with two DBDs) were mostly found in the bottom level. Additionally, among the characterised TFs, 58% were clustered at the bottom level, probably because these TFs directly regulated the downstream genes involved in important phenotypes, such as AlgR[36], AmrZ[37], MvfR[38], FleQ[39], and VqsM[40]. Overall, the organised hierarchical regulatory network profiling at the three levels showed complex pairwise interactions among TFs in *P. aeruginosa*.

Ternary regulatory motifs show flexible relationships among TFs in *P. aeruginosa*

Apart from their global hierarchical regulatory structure, we investigated the constituent regulatory network motifs of TFs, which revealed small connectivity patterns associated with canonical functions[41]. Positive and negative feedback loops are prevalent in bacterial regulatory networks, which generally involve combinations of several genes to appropriately respond to stimuli. One of the regulatory patterns was a single auto-regulator that can self-regulate its expression or activity. Through analysis, nine auto-regulator TFs were identified (Fig. 2B). This number likely represents a conservative estimate, as experiments may not optimally capture auto-regulatory events that depend on native expression levels or specific physiological conditions. Compared with non-auto-regulators, auto-regulators usually tend to be repressors, which play important roles in maintaining a steady state[42,43]. For example, the key T3SS activators PsrA and HrpL can repress their own expression in *P. aeruginosa* and *P. savastanoi*, respectively[44–46].

In addition, the most fundamental regulatory pattern was the ternary TF motif. We computed all possible motifs, which are listed in Fig. 2C and Table S4. The basic triangular motifs were monodirectional regulatory structures with five different motifs. The most frequently observed motif (6,535 occurrences) was motif_2, wherein two different TFs co-regulate another TF. The remaining eight types of motifs, which contained toggle switches, occurred much less frequently than the basic motifs. The more complex the relationships among TFs, the lesser their occurrence. In particular, motif_10, motif_12, and motif_13, characterised by two or three mutually regulating TFs, were not found even once (Fig. 2C). The high occurrence of different TFs in diverse motifs revealed a complex and flexible regulatory network in *P. aeruginosa*. Next, we constructed a basic motif_3 regulatory network based on potential TF–TF interactions to detect the regulatory preferences based on DBD types (Fig. 2D). Basic motif_3 was a typical hierarchical regulatory network in which TF1 regulates TF2, which in turn regulates TF3. We found that the AraC, Fis, and GntR families tended to harbour top-level TFs rather than middle- and bottom-level TFs, while the OmpR family tended to harbour TFs from middle- and bottom-levels more than those from the top level. Furthermore, the LysR and LuxR families revealed non-significant differences among these three TF levels (Fig. 2D). Taken together, our integrated hierarchical regulatory profiling revealed a multifaceted TF–TF connection, reflecting the regulatory preferences of TFs and DBD types in *P. aeruginos*a.

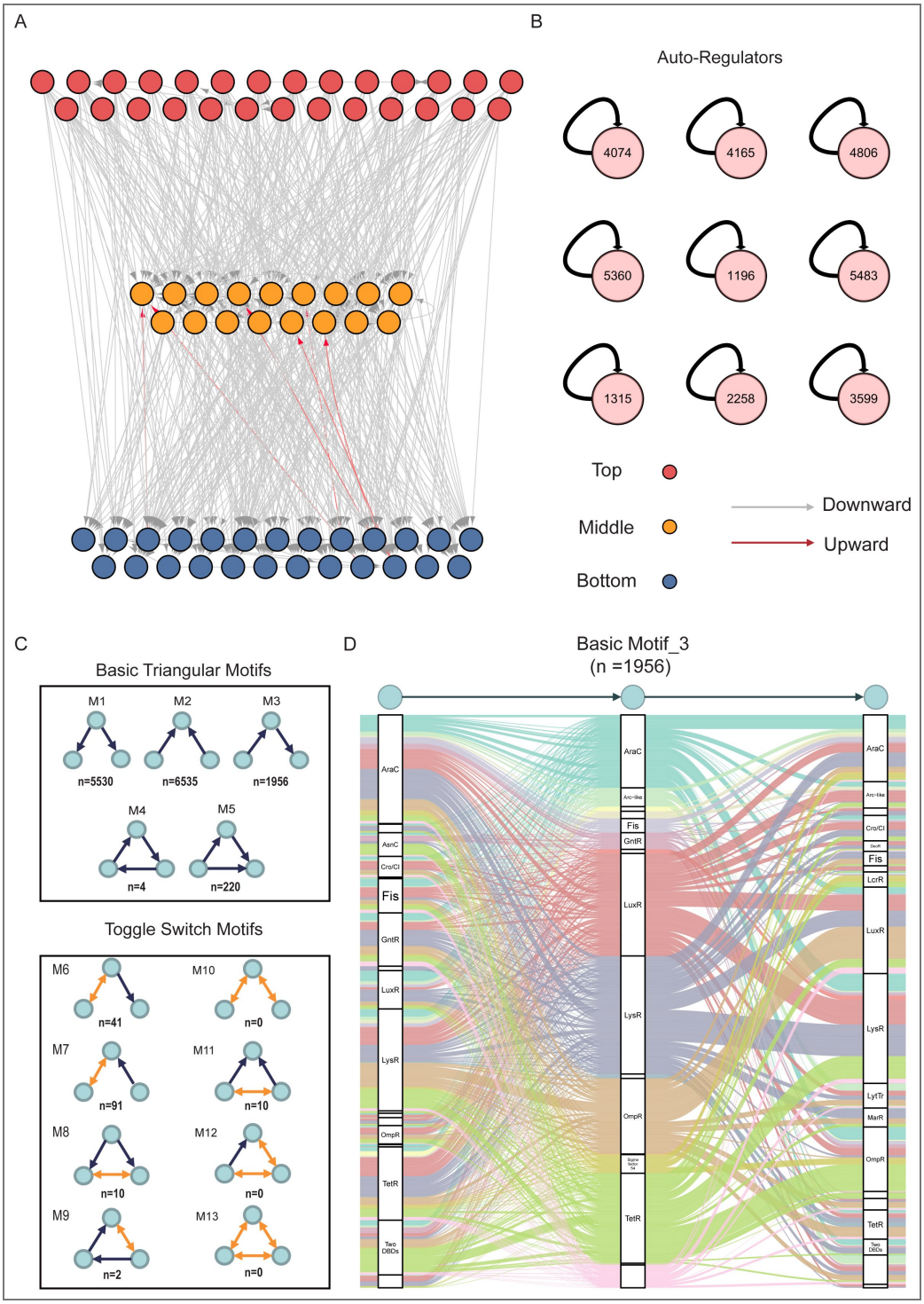

Figure 2. **Hierarchical networks and network motifs.**

(A). Overview of hierarchy regulatory network after removing TFs with degree O+I <= 10. Nodes indicate TFs, and the color represents the hierarchical level. The top level was highlighted in red, the middle in yellow, and the bottom highlighted in blue. The edges with arrows indicate the regulatory direction. Gray means downward-pointing, and red means upward-pointing. **(B).** The auto-regulator motif of 9 TFs. **(C).** Three transcription-factor motifs occurrences with five basic triangular motifs and eight toggle switch motifs. The circle presents TF, and the arrow indicates the regulatory direction**. (D).** Alluvial diagram reveals basic triangle motif 3 (n = 1956) depending on DBD types. The color of splines is highlighted in different DBD families, and the name of DBD families are labeled.

Clustering of 103 TF-binding motifs

According to their binding sites, a total of 103 binding motifs were determined. The TFs were clustered based on the positional weight matrix similarity of their motifs. A circular phylogenetic tree was constructed using hierarchical clustering of the pairwise similarity matrix (Fig. S3A and Table S5). Each node in the tree represents a TF, colour-highlighted according to its DBD family classification. The clustering analysis revealed four distinct clusters of TFs, one each coloured light green (Cluster 1), light blue (Cluster 2), purple (Cluster 3), and pink (Cluster 4). These clusters indicate groups of TFs with similar binding properties or regulatory functions. To validate the identified motifs, we compared them against existing motifs in the RegPrecise database[47], and we found the motif of PA3587 exhibited similarity to motifs of its orthologs in other *Pseudomonadaceae* species (Fig. S3B).

Within each identified cluster, TFs tend to share conserved motif patterns, indicating potential functional relationships. For instance, it was found that the TFs in cluster 1 were enriched in adenine (A) and thymine (T) bases. Further, cluster 4 was more likely to have an inverted repeat (IR) sequence, such as PA0367 and PA1015. Clustering of TF binding motifs identified groups of TFs with similar intrinsic DNA-binding specificities. As expected, many clusters contained TFs from the same DBD families, reflecting evolutionary conservation and potential functional redundancy or competitive binding at shared regulatory elements. Notably, the clustering also uncovered associations between TFs from different DBD families, suggesting convergent evolution of binding specificity or novel regulatory interactions that warrant further investigation.

Genome-wide synergistic co-association of TFs in *P. aeruginosa*

To further investigate the crosstalk among TFs in *P. aeruginosa*, we computed the co-association scores based on the overlaps between the peaks of all pairs of TFs and ChIP-seq data (Fig. S4A and Table S6). Among a total of 16,175 TF pairs, we found 5,716 significant TF interactions with a co-association score of up to 0.1 based on an elbow statistic (Fig. S4B). The relationships among TFs can be further determined as core clusters if their co-association scores are more than 0.4. The clustering analysis of core networks using Glay clustering[48] resulted in seven associated modules, each containing a diverse number of TFs with the DBD families highlighted in different colours (Fig. 3A).

Next, we summarised the potential functional crosstalk among the middle-scale co-associated five TFs (PA2417, PA2718, PA0756, PmiR, and AgtR) in cluster 3. Among a total of 1,241 target genes, 372 were co-bound by at least three TFs, and 35 were co-bound by all five TFs, indicating that these TFs have a high level of collaboration in regulatory patterns (Fig. 3B). There were smaller intersections among different combinations of TFs, highlighting potential co-regulation or shared binding sites. Binding targets co-bound by more than four TFs are summarised in Fig. 3C. The dense network and connectivity indicate a complex regulatory landscape, where these TFs may orchestrate a coordinated regulation of gene expression. For example, peak visualization of PA2504 revealed distinct binding patterns of these five TFs compared with the control group (Fig. 3D). PA2504 is the sole partner of the PppH RNA hydrolase related to several important cellular factors[49]. In addition, four of the five TFs (barring PA2718) can bind to the promoter region of *pqsH*, which is involved in the *pqs* pathway (Fig. 3E). Notably, PmiR has previously been verified to bind to the *pqsH* promoter region and thereby regulate virulence[50]. PA2504 and *pqsH* are highlighted in blue and green, respectively, in Fig. 3C.

Identified potential master regulators are associated with virulence

After elucidating the binding targets of 172 TFs, we investigated their potential functions in the virulence-related pathways and pathogenesis of *P. aeruginosa*. For this purpose, we performed an enrichment analysis using a hypergeometric test to define the master regulators (BH-adjusted $P < 0.05$) that might play important roles in regulating specific pathways. We primarily focused on TF-binding profiles involving the promoter regions of genes involved in nine pathways, namely QS,

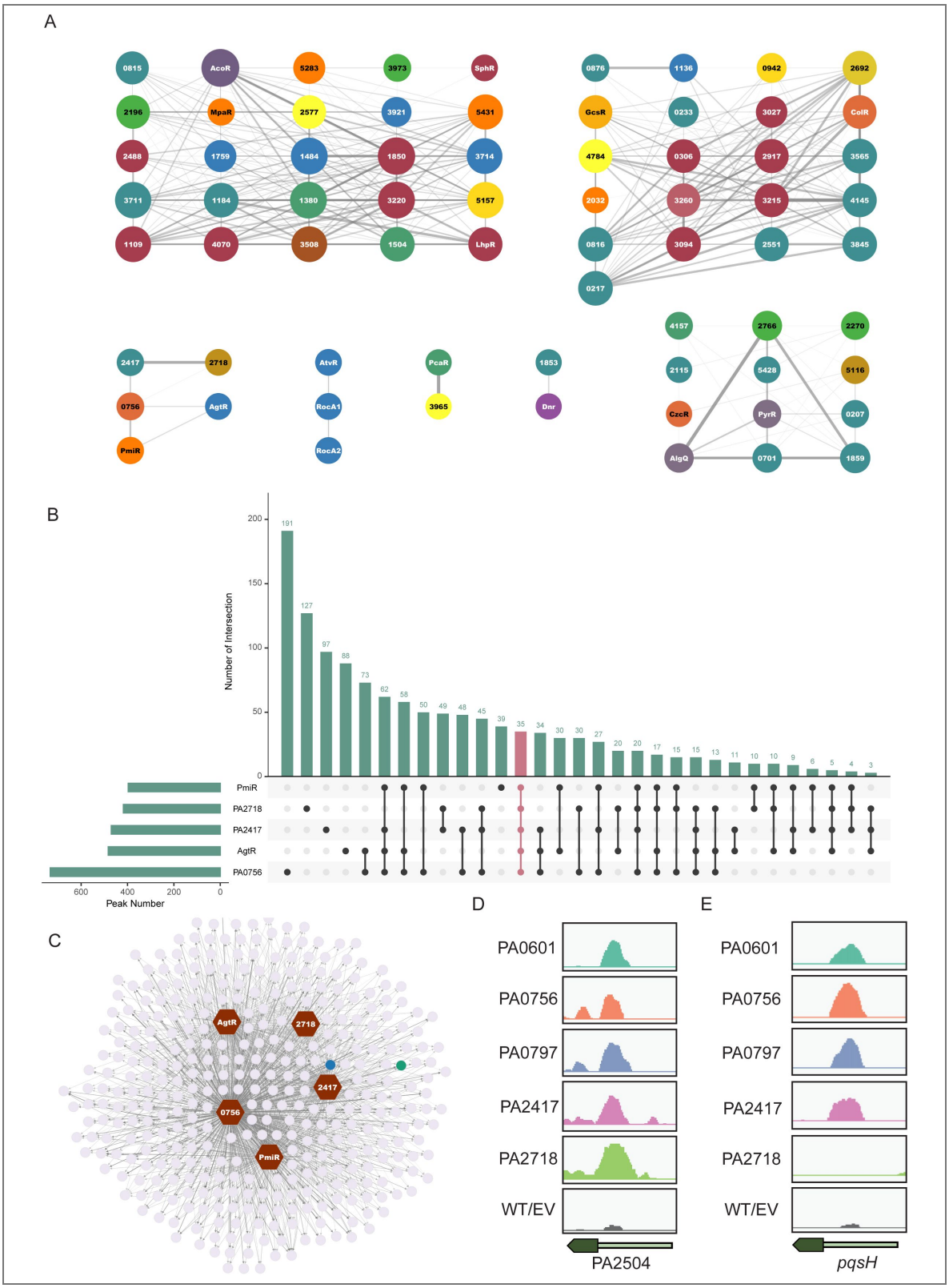

Figure 3. **Core co-association regulatory networks.**

(A). Core clusters of significant co-binding patterns of TFs. Each TF is highlighted in different colors based on DBD types. The co-association score by pair of TFs was calculated by Jaccard statistics, which measures the ratio of the number of base pairs in overlapped binding peaks on both TFs to the number of base pairs in their union. **(B).** The histogram's overlapped target genes of TFs in cluster 3 represent the number of target peaks in the individual/overlapped set. **(C).** The network of co-regulation of TFs in cluster 3 with co-bound targets of more than 4. **(D-E).** Genome browser view of TFs in cluster 3 binding intensities at the PA2504 and *pqsH* locus.

motility, biofilm production, antibiotic resistance, T6SS, T3SS, reactive oxygen species (ROS) resistance, pyocyanin production, and siderophore production. Accordingly, 134 of the 172 TFs were found to bind to at least one target gene associated with the abovementioned virulent pathways, and 24 were identified as master regulators of genes involved in six pathways, namely siderophore production, biofilm production, pyocyanin production, QS, motility, and ROS resistance pathways (Fig. 4A and Table S7).

To experimentally validate the regulatory interactions identified by ChIP-seq, we performed biochemical and genetic analyses on selected TFs. First, we conducted Electrophoretic Mobility Shift Assays (EMSA) for four TFs, including PA0167, PA0815, PA1380, and PA3094, using DNA fragments containing their predicted binding sites from target gene promoters. These TFs showed specific binding to their cognate DNA sequences (Figures S5A-D), confirming the direct binding of the ChIP-seq-identified interactions.

To further validate the functional regulatory roles of these TFs, we constructed clean deletion mutants of PA1380 and PA3094 (ΔPA1380 and ΔPA3094) along with their complemented strains (ΔPA1380/p and ΔPA3094/p). RT-qPCR analysis revealed that PA1380 positively regulates the expression of *cupB1* and *cupB3* (Figure S5E), two genes within the CupB fimbrial cluster identified as ChIP-seq targets. Similarly, PA3094 was confirmed to positively regulate *lecA* expression (Figure S5F), which encodes a lectin involved in biofilm formation and host interactions[51]. Expression of these target genes was restored to wild-type (WT) levels in the complemented strains, validating the regulatory relationships predicted by ChIP-seq. These combined biochemical and genetic validations demonstrate the accuracy and biological relevance of our TF binding data.

The intersection of master regulators across these virulent pathways revealed that six TFs (PA0167, PyrR, PA0707, PA0877, PA1504, and PA1484) only played an important role in regulating the motility pathway, and four TFs (PA1380, PA0815, PA5428, and PA3973) jointly regulated the motility and other pathways, including biofilm production, pyocyanin production, and QS pathways (Fig. 4B). Specifically, PA0815 co-regulated motility, biofilm production, and QS pathways. PA1380 jointly regulated motility and pyocyanin production pathways. PA3973 and PA5428 jointly regulated motility and biofilm production pathways. The regulatory network of these four master regulators and their target genes is summarised in Fig. S5G, represented by lines connecting the TFs to the targets involved in specific pathways.

Predicted functions of regulators characterised in two metabolic pathways

P. aeruginosa uses different global regulators associated with metabolism that ensure its survival and enhance its adaptability under fluctuating environmental conditions. For instance, it uses different regulatory mechanisms to respond to nutrient changes, oxygen limitation, or CF-associated lung infection conditions[52–55].

To define the core TFs that regulate metabolism, we performed master regulator analysis in metabolic pathways (*p.adjust* < 0.05) based on the TFBSs located in the promoter regions revealed by ChIP-seq for all TFs. We identified 14 TFs potentially involved in two pathways, namely the tricarboxylic acid (TCA) cycle and ribosome function pathways (Fig. S6A-B). The radial plot highlights the master regulators within the TCA cycle and ribosome function pathways, and their joint regulation with their targets is presented in the regulatory network below. PA2957 and PA5438 were found to play putative roles in the TCA cycle by potentially binding to the promoter of certain genes, including *icd*, *sucA*, and *sdhC*, indicating that they coordinate the expression of genes crucial for central metabolism and energy production (Fig. S6B). Furthermore, certain TFs, such as PA0611, PA0403 (PyrR), and PA1283, were found to be involved in regulating a broad array of ribosomal genes, such as *rpsB*, *rpsA*, and *rplL*. Notably, TF PyrR was found to play a putative role in regulating motility in *P. aeruginosa*. Overall, these regulators, which are related to

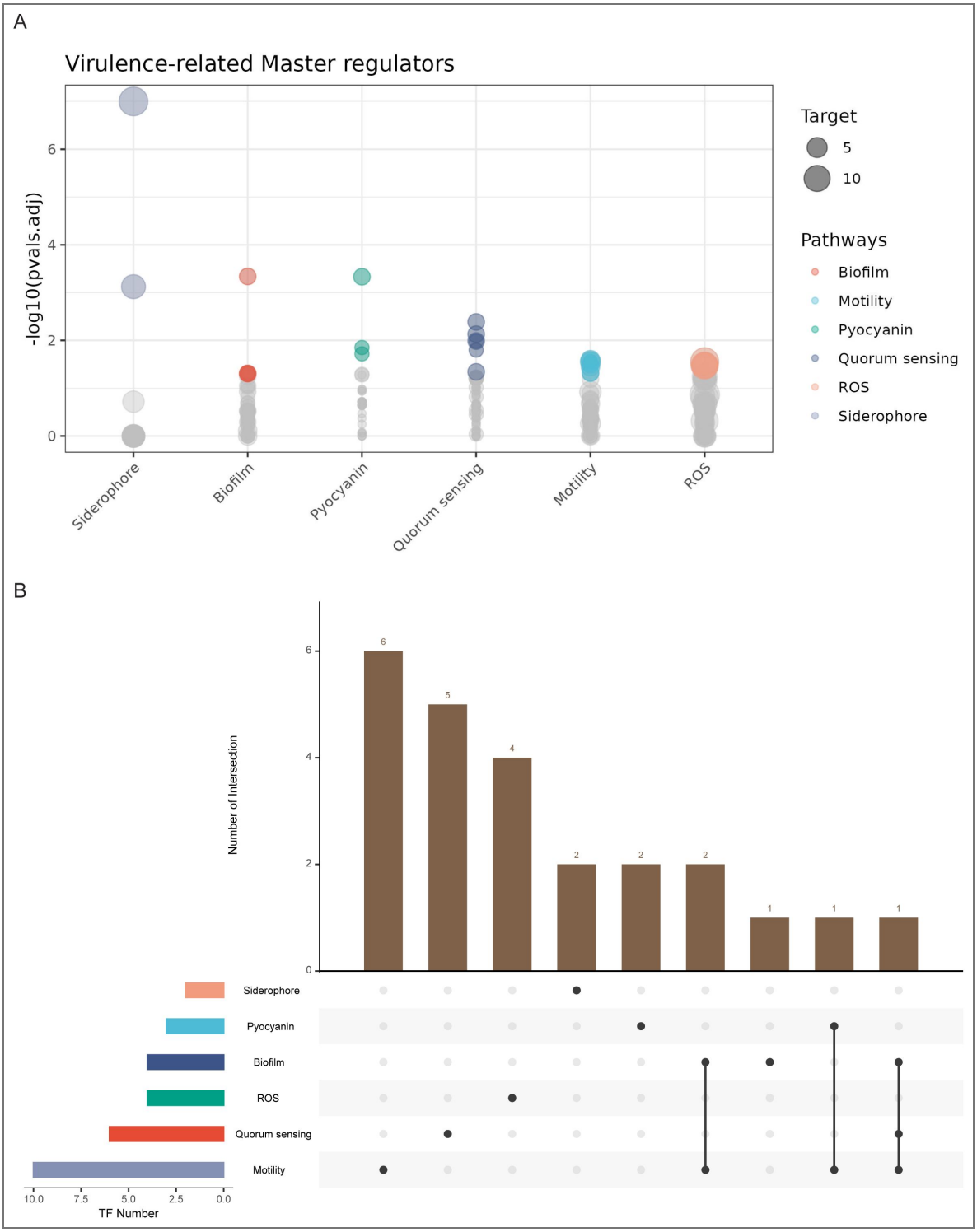

Figure 4. Newly identified virulence-related master regulators.

(A). Overview of all identified master regulators related to 6 pathways, including QS, motility, biofilm, siderophore, pyocyanin, and ROS. Each circle represents one TF, with the height indicating the significance, quantified by –log10 (p.adjust), and the size indicates the number of targets associated with the virulence pathway. **(B).** Intersection of master regulators in 6 virulence pathways. The bar chart on the top shows the number of intersections, while the matrix below indicates which TFs are involved in specific biological processes such as siderophore production, pyocyanin production, biofilm formation, ROS response, QS, and motility.

the core metabolic pathways that enhance the adaptation of *P. aeruginosa* to different infection conditions and contribute to its pathogenicity, could offer potential drug targets for tackling *P. aeruginosa* infection in the future.

A global summary of transcription regulatory networks and functions in *P. aeruginosa*

After studying the TFBSs of most of the TFs in *P. aeruginosa* both *in vivo* and *in vitro*, we organised these datasets to yield a comprehensive connection of virulence-related master regulators. The QS and motility pathways consisting of 18 and 20 TFs, respectively, were the key pathways in the virulent landscape and showed a close collaboration with the other five pathways (Fig. 5A). Four TFs (ExsA, CprR, AlpR, and PA2449) were involved in T3SS, a direct host infection mechanism used by *P. aeruginosa*. Additionally, six TFs (PA4436, PA0929, PA3771, PA2534, PA3699, and PA5218) were involved in siderophore production, as identified using HT-SELEX, and showed correlations with only one TF, PA2534, involved in the ROS resistance pathway. Four TFs (CprR, PA0708, PA1520, and PA2479) were characterised in the stringent response and persister cell pathways and were correlated with other virulence-related pathways, namely QS, ROS resistance, T3SS, and antibiotic resistance pathways.

To investigate the phylogenetic classification of the binding targets of the identified TFs on a genome-wide scale, we derived a global atlas revealing the relationships between the protein functions of target genes and the family categories of TFs (Fig. 5B). The Clusters of Orthologous Groups of proteins (COGs) database was used to classify proteins based on the orthology concept[56]. Based on the annotated COGs of *P. aeruginosa*, we found that most of the TF targets were involved in metabolism, followed by poorly characterised categories. The *P. aeruginosa* PAO1 genome is 6.3 Mbp long, and approximately 40% of it remains uncharacterised but is believed to encode well-conserved hypothetical proteins (HPs), which might have indispensable and similar functions. Given that most of the TF targets are poorly characterised, it is crucial to study these HPs in the future to provide more insights into *P. aeruginosa* pathogenicity. Altogether, our results provide a resource of TFs and their putative target genes in *P. aeruginosa* and, consequently, a basis for understanding the regulatory network of virulence and orthological functions.

A Web-based database of all TFBSs in *P. aeruginosa*

To enable a quick search of the TFBSs in *P. aeruginosa*, we developed a Web-based database containing our ChIP-seq and HT-SELEX results at *https://jiadhuang0417.shinyapps.io/PATF_Net/*, which is available to the public. It has four parts: homepage, data page, upload, and contact. The homepage briefly describes the background information and schematic workflow of ChIP-seq and HT-SELEX. The data page provides two types of data: a network plot of TFs and targets, and detailed TF–target information in a table format. The network plot illustrates the top 50 peaks for ease of visualisation. The code was generated using R, and the figures were created using BioRender (ChIP-seq: *https://biorender.com/zru6mz7*; HT-SELEX: *https://biorender.com/2mwef9b*).

The database offers multiple search modalities to facilitate data exploration: users can perform TF-centric searches to query binding sites, target genes, and regulatory networks for individual TFs, or utilize the target gene search function to identify all TFs that regulate any gene of interest by entering its locus tag. To connect regulatory data with biological function, we have implemented a virulence pathway browser that allows users to explore TF binding patterns across curated gene sets for major *P. aeruginosa* virulence pathways. Interactive visualization tools, including network graphs and binding profile plots, facilitate intuitive exploration of regulatory relationships. The primary purpose of PATF_Net is to store, search, and mine valuable information on *P. aeruginosa* TFs for researchers investigating *P. aeruginosa* infection. The current resource is based on the reference strain PAO1, which serves as the foundation for most *P. aeruginosa* molecular studies and allows direct integration with existing genomic annotations and functional data. However, *P. aeruginosa* exhibits substantial genomic diversity across clinical isolates, and strain-specific differences in TF binding patterns may contribute to phenotypic variation in

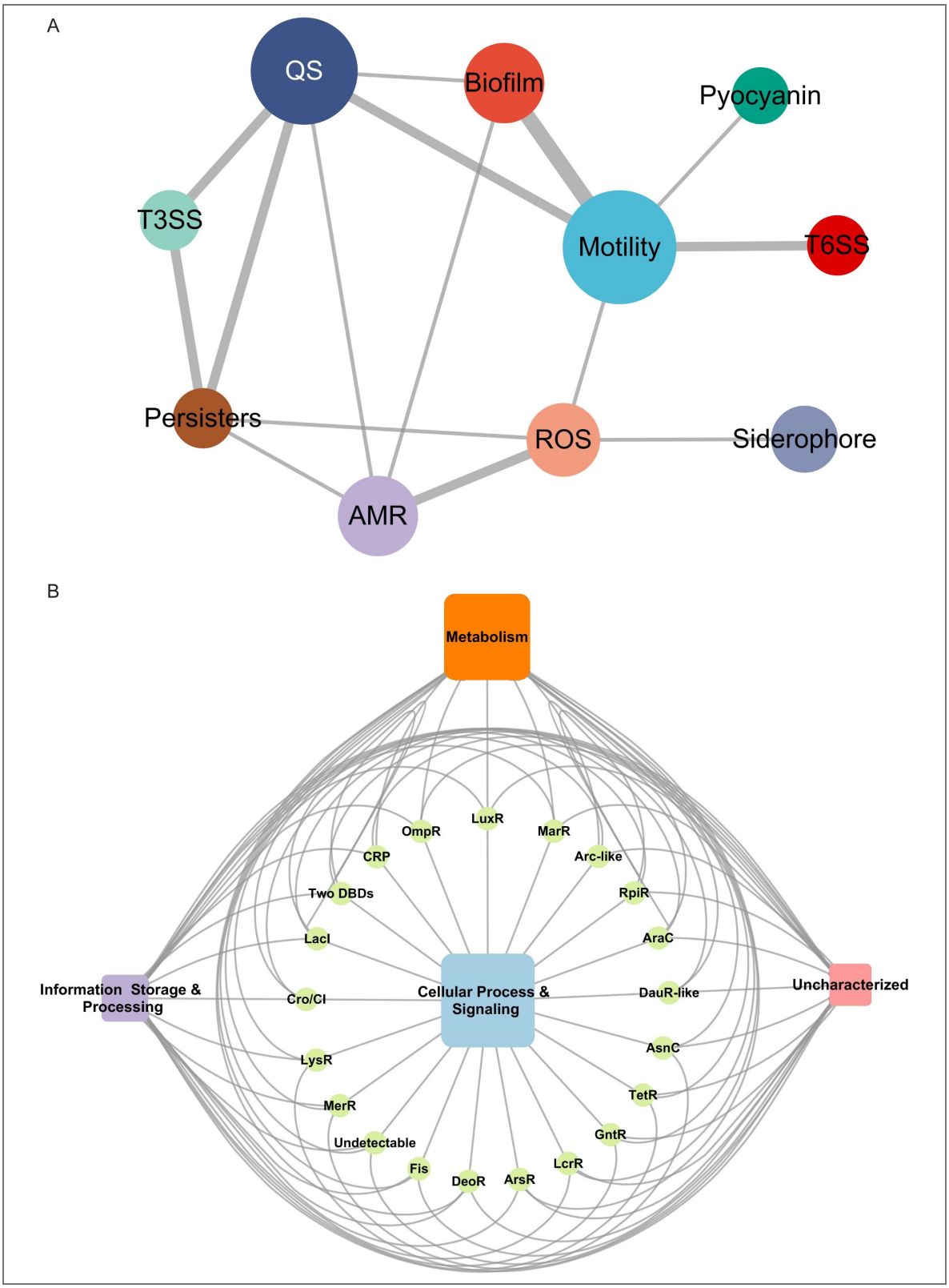

Figure 5. Overview of transcriptional network of TFs in *P. aeruginosa*.

(A). The interaction network of virulence-related master regulators in *P. aeruginosa*. The ten virulence pathways are highlighted in different colors. The size of the circle represents the degree, and the width of the edges indicates the overlapped number of TFs between two pathways. **(B).** The target annotated using the COG database shows four orthology classification profiling from different DBD types TFs. The size of rectangle indicates the number of targets.

virulence, antibiotic resistance, and host adaptation. Extension of this resource to include strain-specific regulatory maps from diverse clinical isolates would provide valuable insights into the regulatory basis and represents an important direction for future investigation.

Conservation and evolution of TFs in *P. aeruginosa*

Differences between species are mainly due to differences in genes, whereas essential genes are usually well conserved, especially in subspecies. To investigate the conservation of TFs in *P. aeruginosa*, we performed a pan-genome analysis of 100 strains containing model strains and clinical strains using the Roary software[57]. A total of 21,432 genes were identified across the 100 *P. aeruginosa* strains, of which only 4,544 genes were classified as core genes (existing in more than 99% of strains), revealing that most of the genes were cloud genes (existing in less than 15% of strains) (Fig. 6A). However, when focusing on the TFs among these 100 strains, almost 89% of TFs (331 of 373) were found to be well-conserved (Fig. 6B), which implied that these recognised TFs from PAO1 play essential roles in biological activities in all of the *P. aeruginosa* subspecies.

The bar plot provides detailed information on the rest of the non-core TFs in PAO1, in addition to several important TFs, such as LasR and ExsA, that are lacking in some strains (Fig. 7C). As is known, the *lasR* mutant of *P. aeruginosa* is associated with CF lung disease progression and is considered a marker of an early CF-adaptive phenotype[58]. ExsA is considered an important activator of the T3SS pathway in *P. aeruginosa*, which is characterised by an acute infection phenotype. Additionally, VqsM might have a relatively special function in PAO1 among *P. aeruginosa* subspecies in addition to playing an important role in the QS and antibiotic resistance pathways. Taken together, the core- and non-core TFs were both vital and worth studying for understanding the metabolism and virulence-related regulatory mechanisms in *P. aeruginosa*.

To further investigate the conservation of TFs in *P. aeruginosa*, we re-analysed the ChIP-seq data of PhoB and RpoN in the PA14 strain[59,60] and compared them with those in the PAO1 strain. Fig. S7A shows genome-wide binding peaks of PhoB and RpoN throughout the genomes of PAO1 and PA14. We found that in PA14 and PAO1, PhoB had 725 and 63 peaks, while RpoN yielded 363 and 1,238 peaks, respectively. Next, we used MEME[61] to compare the motifs of PhoB and RpoN in PAO1 and PA14 based on their binding sequences from ChIP-seq (Fig. S7B). PhoB and RpoN shared a well-conserved motif in the two strains even though they showed different peak distributions in the genomes. They had different numbers of peaks, and of the 690 and 345 respective annotation targets of PhoB and RpoN in PA14, 114 and 66 targets, respectively, were unique to PA14. The rest of the targets were homologous between PAO1 and PA14. We next performed target annotation and observed that the genes regulated by these two TFs were significantly (Fisher's test) overrepresented in the two tested strains (Fig. S7C). For example, RpoN has been found to play an important role in regulating T6SS in both PAO1 and PA14[60,62]. Overall, the conservation of the representative TFs PhoB and RpoN in PAO1 and PA14 indicates that conserved TFs may share similar functions in different species. This knowledge is expected to guide drug development for different infectious strains.

TFs play a pivotal role in regulating gene expression, and studying their conservation and evolution could provide insights into how gene expression patterns are conserved or modified across different species and help to predict gene regulatory networks in different species. For example, the conservation of a TF across multiple species suggests that its targets and regulatory interactions are likely to be conserved as well. Therefore, to study the conservation and evolution of *P. aeruginosa* TFs across other species, we conducted phylogenetic analyses of the 373 TFs of *P. aeruginosa* across other bacteria, fungi, archaea, plants, and animals. We found that three *P. aeruginosa* species showed the most conserved TFs, followed by *P. savastanoi*, a phytopathogen (Fig. 6D). Compared with bacteria, the conservation values of the TFs were lower in fungi, archaea, plants, and animals. However, several orthologues of TFs were still noted among these organisms. For instance, PA2032 had more than 25% and approximately 30% identity with aminoadipate aminotransferase in humans and mice, respectively. The phylogenetic tree of PA2032 across bacteria, archaea, fungi, plants, and animals, with PAO1 as the root revealed that the bacterial TFs (purple) indicates a high degree of conservation within prokaryotes, suggesting a

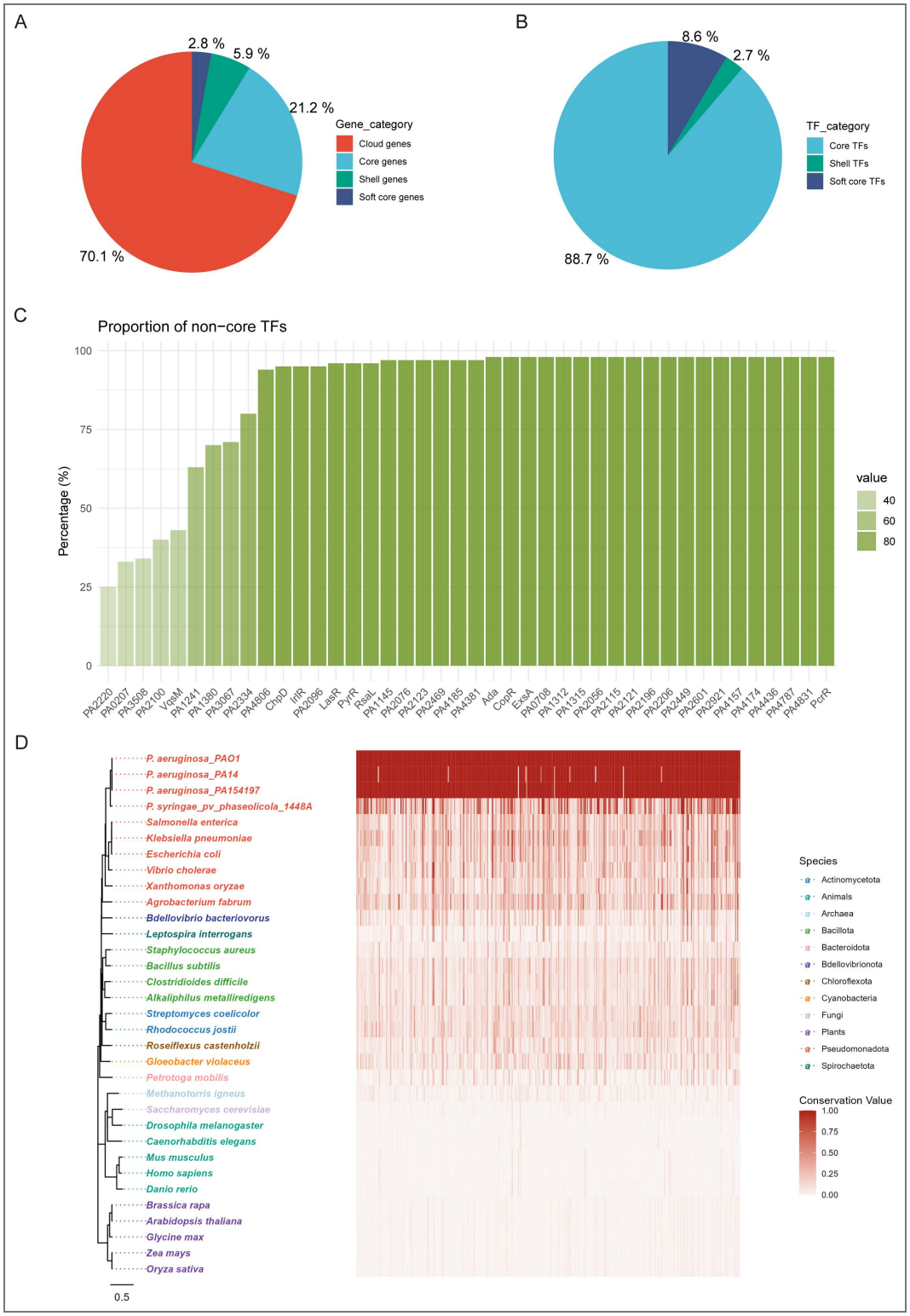

Figure 6. Conservation and variability of TFs in PAO1.

(A). The pie chart shows the proportions of genes categorized by their presence across *P. aeruginosa* strains for all genes. **(B).** The pie chart shows the distribution of TFs identified from PAO1 across different conservation categories. **(C).** The bar plot of the proportion for non-core TFs. Genes are categorized based on their presence frequency across P. aeruginosa strains: Core genes (present in 99% ~ 100% strains), Soft core genes (present in 95% ~ 99% strains), Shell genes (present in 15% ~ 95% strains), and Cloud genes (present in 0% ~ 15% strains). **(D).** The conservation and evolutionary relationship of all 373 TFs in PAO1 among bacteria, archaebacteria, fungi, plants, and animals. The conservation value was normalized after blastp alignment[90]. The phylogenetic trees were constructed using MEGA11[91] and plotted via R package ggtree[92].

fundamental role in core regulatory processes (Fig. S7D). In contrast, eukaryotic TFs (fungi, plants, and animals) form distinct clades with longer branch lengths, indicating significant divergence and specialization during eukaryotic evolution. These findings suggest that while TF is conserved across domains of life, its functional roles and regulatory mechanisms have undergone substantial diversification in eukaryotes. Altogether, the high-level evolutionary conservation of TFs in *P. aeruginosa* strongly suggests that the regulatory mechanisms are common to a wide range of bacteria.

Discussion

Bacterial pathogens use many mechanisms to establish infection and cause diseases in human hosts, making them a major public health concern worldwide. These bacteria can secrete a wide range of molecular particles that recognise and bind to host cell targets, thus damaging or preventing host responses[63]. Pathogenic bacteria use strategies to adapt to environmental stressors such as nutrient limitation or antibiotic treatment[64]. The underlying mechanism of these strategies involves sensing external signals and then responding to different environmental conditions. TFs are key molecules involved in responding to host or environmental signals by precisely modulating transcription levels; hence, their pivotal roles in infection conditions cannot be overstated. Nevertheless, our comprehension of bacterial TFs remains considerably limited, even in extensively examined model organisms such as *P. aeruginosa*. To address this knowledge gap and shed more light on TFs in *P. aeruginosa*, we conducted ChIP-seq experiments aimed at elucidating the TFBSs for 172 TFs with relatively unexplored biological functions.

ChIP-seq is a valuable technique to obtain high-resolution DNA–protein interaction mapping on a genome-wide scale to study the potential binding targets of TFs. The technique is highly sensitive and can detect low-abundance DNA-binding events, allowing the detection of weak TF–DNA interactions. To enhance the visualisation of more subtle binding peaks, particularly when investigating co-associations and TFs lacking well-defined signals, we devised a strategy involving TF-overexpressing plasmids. *In vitro* assays, such as DAP-seq or HT-SELEX, offer superior scalability to ChIP-seq but present their own challenges[29,65]. Our analysis revealed that TF binding events occur within coding regions, which is consistent with previous study demonstrating that bacterial TFs possess binding capabilities for coding regions and can regulate transcription through multiple mechanisms[66]. Besides, it may also regulate RNA stability, warranting integration with translatomics or epigenomics data. These findings extend the basic function of TFs is to recognise specific DNA sequences (i.e., motifs) at the promoter region and then upregulate or downregulate the transcription of the target gene[67]. Notably, the literature underscores the collaborative nature of TFs, which often exhibit interactions with other auxiliary proteins at multiple promoter sites[68]. As shown in Figures 3 and S3, we present a TF-binding motifs clustering tree and a TF co-association regulatory network based on ChIP-seq data and core clusters of TFs with high co-association scores. Overall, our above-described analysis has illustrated a regulatory atlas to study the biological functions of *P. aeruginosa* TFs in specific contexts.

However, several limitations of the ChIP-seq approach should be acknowledged. Firstly, TF overexpression ensures sufficient protein levels for ChIP-seq signal detection but does not guarantee that all TFs are in their active conformational states, as many bacterial TFs require allosteric activation by metabolites, cofactors, or post-translational modifications. The cells under standard laboratory conditions which may not activate all TFs to their maximal regulatory states, potentially leading to underestimation of condition-specific binding peaks. Secondly, while we observed TF binding at thousands of genomic sites, binding per se does not equate to functional regulation, as chromatin context, cofactor availability, and competitive binding all influence regulatory outcomes.

P. aeruginosa uses TFs to control biological functions, including metabolism and virulence. Given the public health burden of *P. aeruginosa* infection, many studies have investigated the roles of TFs in this pathogen. These studies have characterised the regulatory mechanisms of individual TFs, such as FleQ, AlgR, LasR, VqsM, and PvrA[40,69–72]. Additionally, a TRN comprising 690 genes and

1,020 regulatory interactions with six biological modules and main motifs was established in 2011 using published data[73]. Furthermore, we developed a PAGnet containing 20 key virulence-associated TFs based on ChIP-seq and RNA-seq data[74]. A previous study used machine learning to identify modules of a group of genes related to known TFs from published data[75]. Another study used DAP-seq to determine a regulatory network of 55 RRs in *P. aeruginosa*[29]. To further identify and characterise unknown TFs, we used HT-SELEX to describe the binding specificities of 182 TFs[28]. Compared with the abovementioned studies, the present work profiled a comprehensive regulatory network of *P. aeruginosa*, combining existing and new data with experimental verification.

Additionally, although the TRN analysis revealed organizational patterns in *P. aeruginosa* regulatory network, the functional significance these topological features, including their specific contributions to pathogenicity, metabolic adaptation, and antibiotic resistance remains to be experimentally determined in the future work. The hierarchical structure and regulatory motifs we identified represent objective network properties derived from our binding data, but translating these structural observations into mechanistic understanding will require condition-specific functional studies, genetic validation, and phenotypic characterization. Our analysis provided a systematic framework and generating testable hypotheses rather than definitive functional conclusions. Nevertheless, these network-level organizational principles provided value to the community as a foundational reference, similar to other regulatory network maps[73] that were useful even before comprehensive validation.

We confirmed the functions and provided potential molecular mechanisms of a number of previously identified TFs to explain their phenotypes. PA0797 is known to regulate the *pqs* system and pyocyanin production[76]. In the present study, it was also found to bind to the *pqsH* promoter region and its motif was visualised. PA5428 was found to bind to the promoter regions of *aceA* and *glcB* genes[77], which was also demonstrated in our ChIP-seq results. PA4381 (CloR) was found to be associated with polymyxin resistance in a previous study[78] and to be possibly related to ROS resistance in the present study. Furthermore, PA5032 plays a putative role in biofilm regulation and also forms an operon with PA5033, an HP associated with biofilm formation[79].

While our phylogenetic analysis reveals varying degrees of TF conservation across bacterial species, the functional implications of this conservation remain to be fully explored. Many *P. aeruginosa* TFs have clear orthologs in both Gram-negative (e.g., *Klebsiella pneumoniae*) and Gram-positive pathogens (e.g., *Bacillus cereus*), yet whether these orthologs regulate similar target genes and biological processes is largely unknown. Future comparative profiling of orthologous TFs could reveal the extent to which regulatory network architecture is conserved versus rewired during bacterial evolution, potentially identifying core regulatory modules governing universal bacterial strategies versus species-specific innovations. Such cross-species comparisons would enhance our understanding of regulatory network evolution and enable functional prediction in less well-characterized pathogens based on homology to experimentally validated *P. aeruginosa* regulators.

Our study is the first to illustrate the hierarchical regulatory network and ternary motifs of and genome-wide co-association relationships among TFs in *P. aeruginosa*, contributing to a better understanding of the fundamental traits of pathogenicity of this species. Traditional bacterial eradication strategies involving antibiotics often focus on pathogen extermination, yet this approach can inadvertently foster selective growth advantages and precipitate the emergence of drug-resistant strains. Therefore, prioritising TF-targeted drugs could potentially alleviate selective growth pressure and enhance future preventative measures against *P. aeruginosa* infection. The extensive datasets generated in this study offer valuable insights into understanding and targeting *P. aeruginosa* pathogenicity. The genome-wide binding profiles can be systematically analyzed through our hierarchical regulatory network framework to decode complex virulence mechanisms. The virulence-related master regulators and core regulatory clusters identified in this study highlighted key nodes of transcriptional control. Understanding these regulatory relationships is particularly valuable for identifying targets whose modulation would significantly impact virulence while accounting for potential compensatory mechanisms. This knowledge base

thus provides a foundation for developing targeted approaches to combat *P. aeruginosa* infections, moving beyond traditional antibiotic strategies toward more sophisticated interventions based on regulatory network manipulation.

Methods

Strains, plasmids, and primers

The bacterial strains, plasmids, and primers used in the present study are listed in **Table S8**. The *P. aeruginosa* PAO1 strain, and its derivatives were grown at 37 °C in LB (Luria-Bertani) broth with shaking at 220 rpm or on LB agar plates. Antibiotics were used for *Escherichia coli* at the following concentrations: kanamycin at 50 μg/ ml and carbenicillin at 60 μg/ml.

ChIP-seq and analysis

The chromatin immunoprecipitation (ChIP) procedures were modified from a previous study[80]. Briefly, for the VSVG-tagged, the ORF was amplified by PCR from the PAO1 genome and cloned into pAK1900 plasmid by HindIII/BamHI for the overexpressed TFs through HindIII site by using ClonEx- press MultiS One Step Cloning Kit (Vazyme, China). Wild-type *P. aeruginosa* containing empty pAK1900 or pAK1900-TF-VSV-G was cultured in LB medium supplemented at 37°C with shaking until mid-log phase (OD_{600} = 0.6), then we cross-linked the samples with 1% formaldehyde for 10 min. Subsequently, cross-linking was stopped by the addition of 125 mM glycine. Samples were centrifuged and washed thrice with a Tris buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl). The resulting pellets were resuspended in 500 μl IP buffer (50 mM HEPES–KOH [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 % Triton X- 100, 0.1% sodium deoxycholate, 0.1% SDS, and mini-protease inhibitor cocktail (Roche), and then the DNA was broken into pieces (100 to 300 bp) with an ultrasonic processor. Insoluble cellular debris was removed by centrifugation at 4 °C, and the supernatant was used as the input sample in IP experiments. We next added 25 μl agarose-conjugated anti-VSV antibodies (Sigma) in the IP buffer. Washing, reverse cross-linked, and purification of the ChIP DNA were conducted. We used the NEXTflex ChIP-Seq kit (Bio Scientific) to construct the DNA fragment library, and agarose gel was used to cut DNA fragments between 150 and 250 bp. After sequencing, the raw reads were trimmed by Trim Galore[81] with default parameters. Then, the filtered reads were aligned to the PAO1 genome (NC_002516) using bowtie2. Only the unique reads after alignment will be used to perform peakcalling with MACS2 ($P < 0.001$). MEME-ChIP was used to identify consensus motifs with all peaks. TF target genes were then annotated by the R package ChIPpeakAnno.

Identification of virulence-related master regulators

We adopted the definition of “master regulator” from developmental biology[82], where it refers to TFs that control the expression of multiple downstream genes governing a specific biological process or lineage commitment. In our context, we use “master regulator” to designate TFs that coordinately regulate multiple genes within specific virulence-related pathways based on statistical enrichment criteria as described[83]. Briefly, we first generated gene lists associated with nine pathways[84], including QS, motility, biofilm production, antibiotic resistance, T6SS, T3SS, ROS resistance, pyocyanin, and siderophores. These gene lists are provided in full detail at our PA_TFNet database. Then, we calculated the number of overlap genes between target genes of TF and genes involved in the above eight pathways. The statistical significance of the master regulator was identified using the Hypergeometric test (BH-adjusted $P < 0.05$). R package ggplot2[85] was used to visualize the result.

GO analysis

GO enrichment analysis of TFs target genes was identified using R package clusterProfile[86]. The enriched GO terms with BH-adjusted $P < 0.05$ were defined as significantly enriched.

Pan-genome analysis

The information on the model and clinical *P. aeruginosa* strains[87,88] can be found in Table S9. Briefly, the genome sequence of all strains were annotated by prokka[89] and the outputs were then used as input files for roary.

Statistical analysis

Student's t-test was performed to analyze the RT-qPCR and lux production results using Microsoft Office Excel 2019. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, and results represent means ± SD. All experiments were repeated at least twice.

Electrophoretic Mobility Shift Assay (EMSA)

DNA probes corresponding to predicted TF binding regions were PCR-amplified from PAO1 genomic DNA. For binding reactions, 20 ng of purified DNA probe was incubated with varying concentrations of recombinant TF protein in 20 μL binding buffer containing 10 mM Tris-HCl (pH 7.4), 50 mM KCl, 5 mM $MgCl_2$, and 10% (v/v) glycerol. Reactions were incubated at room temperature for 20 min to allow protein-DNA complex formation. Samples were then resolved on 6% native polyacrylamide gels by electrophoresis at 90 V for 90 min in TBE buffer. Following electrophoresis, gels were stained with GelRed for 5 min and visualized using a ChemiDoc imaging system (Bio-Rad).

Reverse Transcription Quantitative PCR (RT-qPCR)

Bacterial cultures were grown in LB medium to mid-log phase (OD_{600} = 0.6), and total RNA was extracted using the Bacterial Total RNA Isolation Kit (Sangon Biotech). RNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). cDNA synthesis was performed using 600 ng of total RNA with HiScript III RT SuperMix (Vazyme). Quantitative PCR reactions were carried out using ChamQ Universal SYBR qPCR Master Mix (Vazyme) on a QuantStudio Real-Time PCR System (Applied Biosystems). Relative gene expression levels were calculated using the $2^{\wedge(-\Delta\Delta Ct)}$ method, with expression in wild-type strain set as 1.0. All RT-qPCR experiments were performed with three biological replicates, each with three technical replicates.

Supplementary information

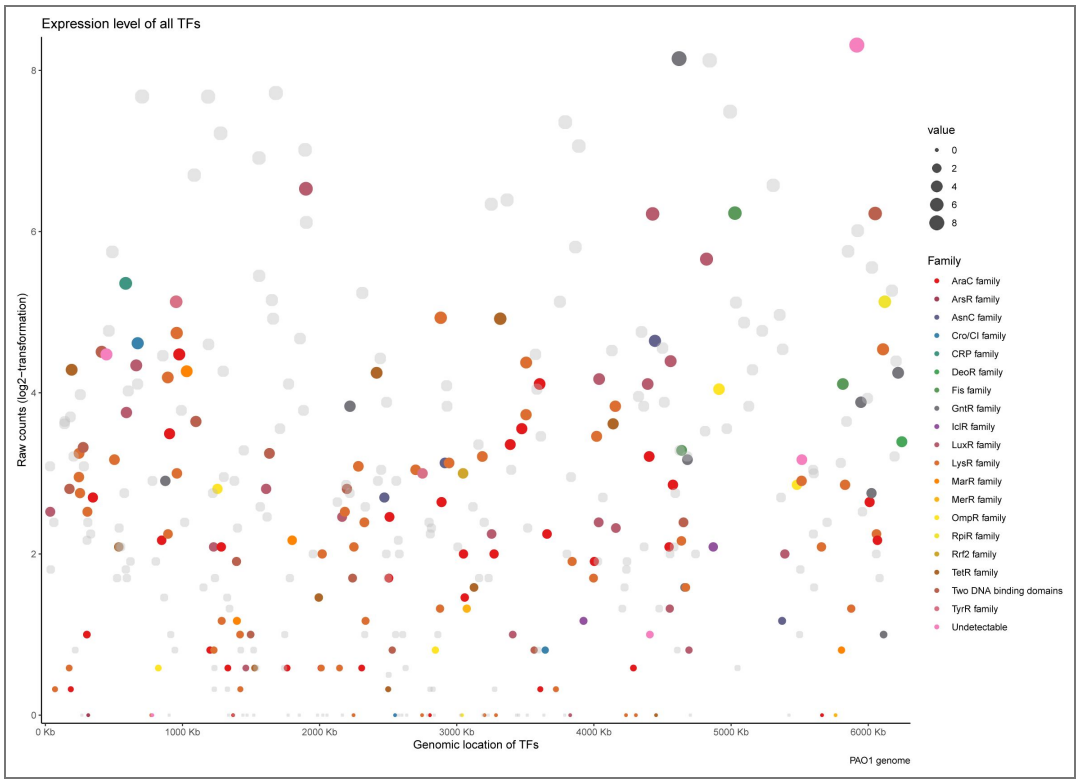

Fig. S1. RNA-seq for all TFs and ChIPed TFs. Previous RNA-seq data shows the expression level of all TFs and ChIPed TFs. The circle size indicates the log2-transformation raw counts, and the color highlighted for ChIPed TFs represented different DBD families.

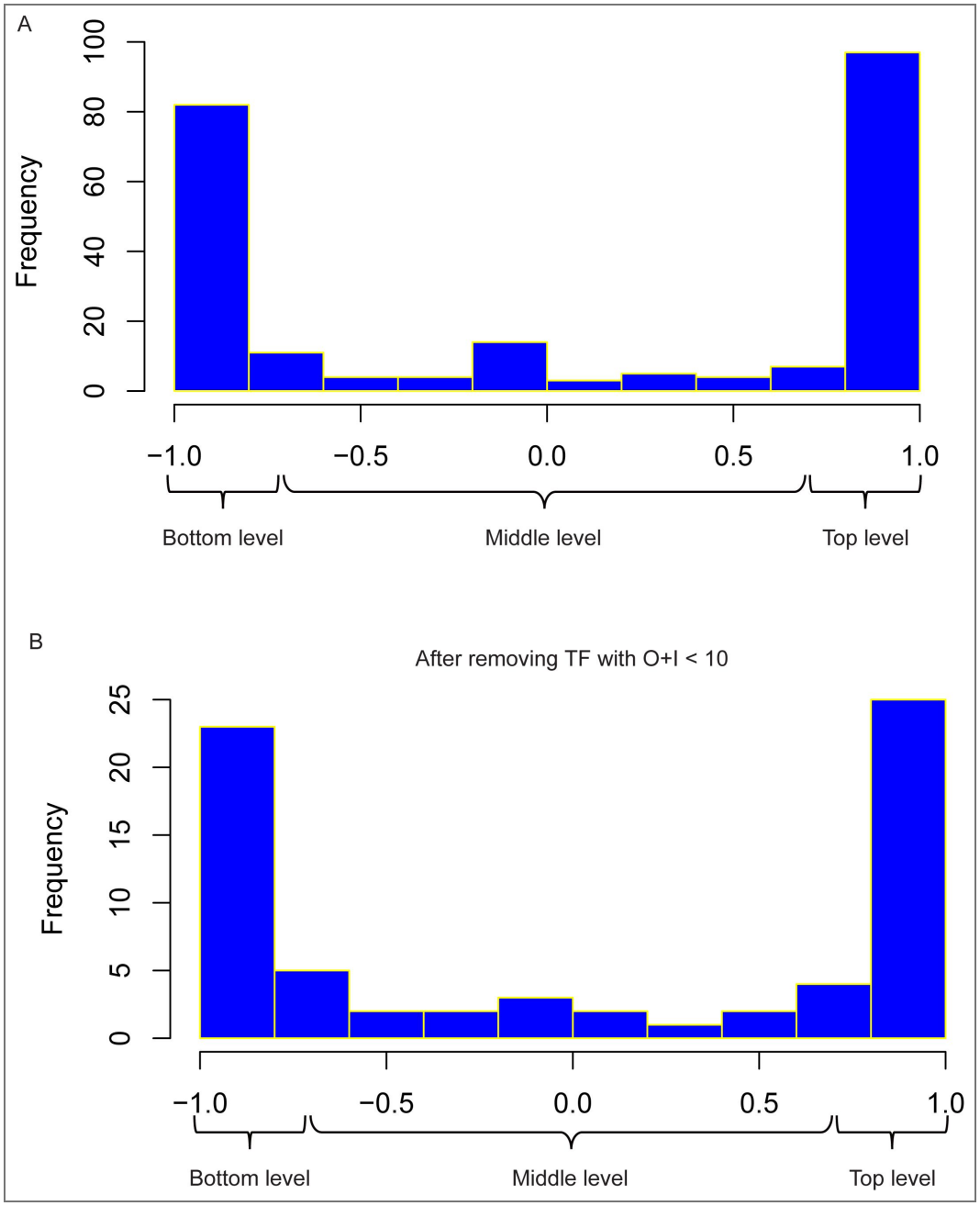

Fig. S2. Distribution of Hierarchy Height

h. *h = (O-I)/(O+I)*, *O* presents the extent they regulate other TFs, and *I* indicates the level other TFs regulate them. **(A).** TFs can be divided into almost equal three levels depending on index h: Bottom level (−1.0 < h < 0.75), Middle level (−0.75 < h < 0.75), and Top level (0.75 < h < 1.0). **(B).** The number of TFs resided in three levels after removing TFs with *O+I* <10.

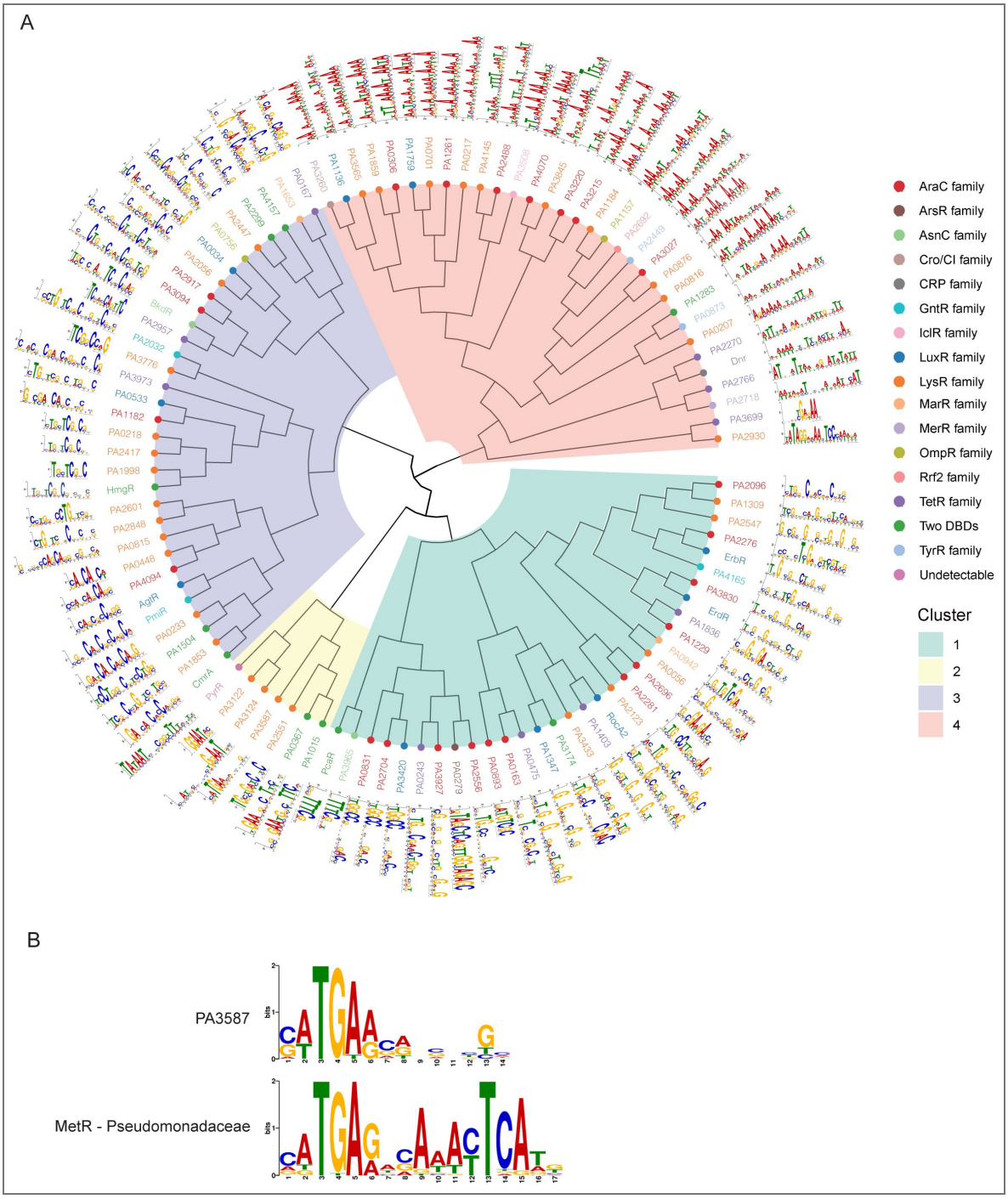

Fig. S3. Circular phylogenetic tree of TF binding motifs.

(A). Circular phylogenetic tree representing the clustering of TFs based on their motif similarity. The tree was constructed using hierarchical clustering with the Ward.D2 method applied to the pairwise similarity matrix of motifs. The tree were visualized using ggtree[93]. Each node represents a TF, color-coded by its DBD family classification as indicated in the legend on the right. The motif logos are aligned with the respective TFs, with their orientations adjusted to maintain legibility around the circular layout. **(B).** PA3587 and MetR display similar DNA binding motifs.

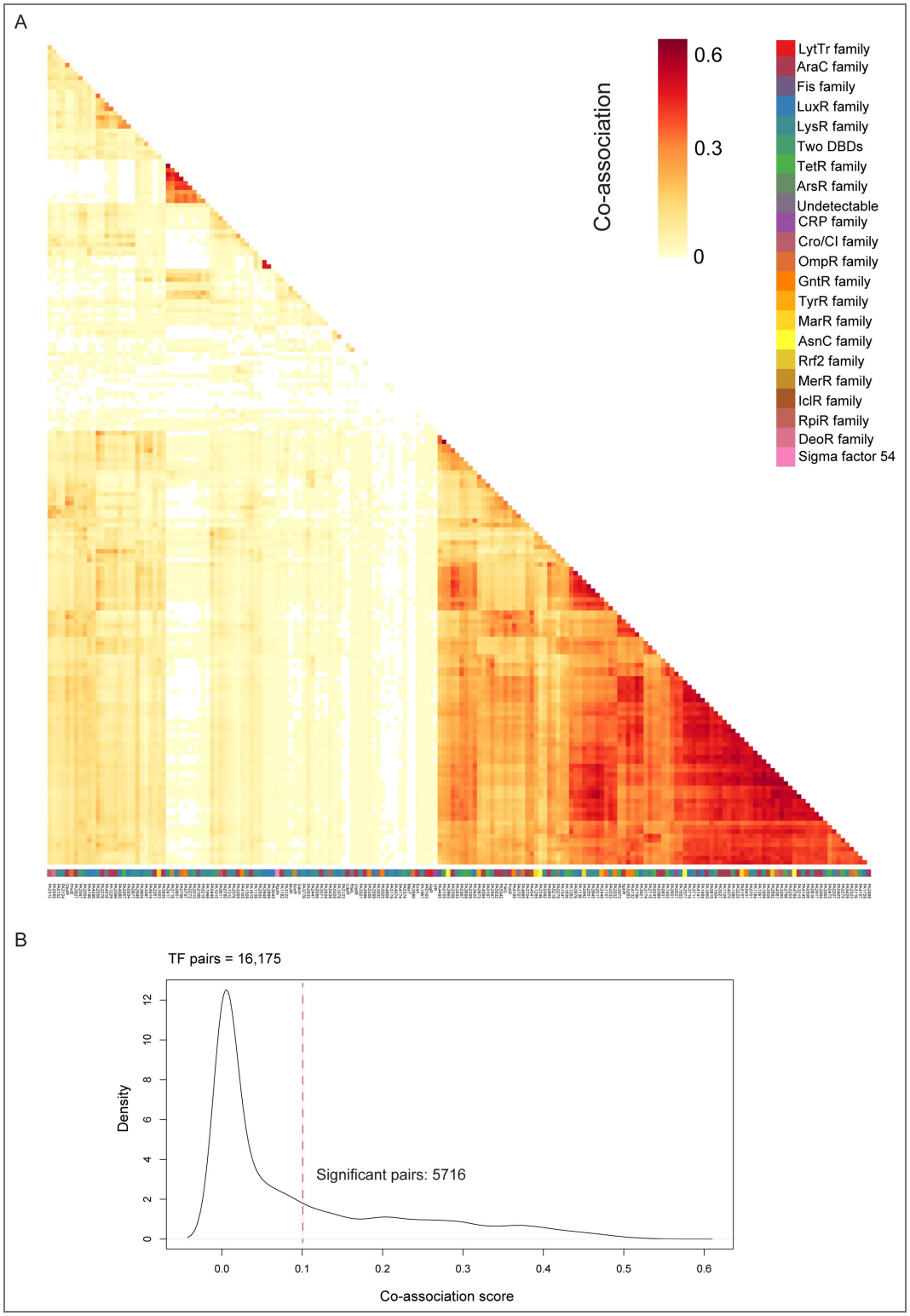

Fig. S4. Co-association network of TFs.

(A). Heatmap reveals a full co-association pattern of all TFs. **(B).** The density of co-association score for all TF pairs. Determining co-association score as 0.1 (dashed in red) of significant TF co-associations based on an elbow statistic.

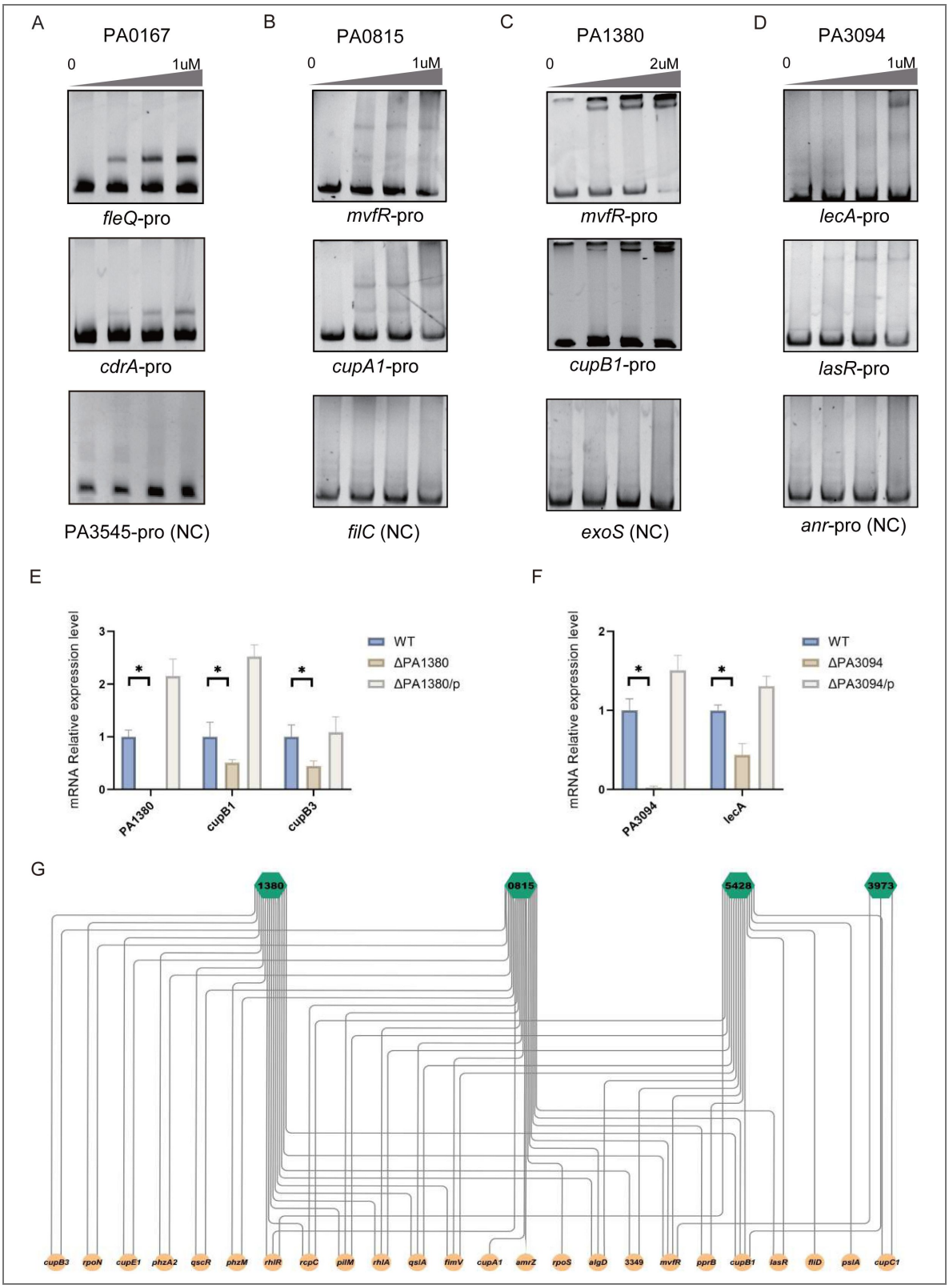

Fig. S5. Validation and co-regulation of virulence-related master regulators.

(A). The validation of the binding sites of PA0167 by EMSA. **(B).** The validation of the binding sites of PA0815 by EMSA. **(C).** The validation of the binding sites of PA1380 by EMSA. **(D).** The validation of the binding sites of PA3094 by EMSA. **(E).** The detection of expression of target genes PA1380, *cupB1*, and *cupB3* in WT, ΔPA1380 and complementary strain by RT-qPCR. **(F).** The detection of expression of target genes PA3094 and *lecA* in WT, ΔPA3094 and complementary strain by RT-qPCR. **(G).** Graph diagram of interactions involving target genes of 4 TFs, including PA1380, PA0815, PA5428, and PA3973. NC indicates negative control. Student's *t* test, $*P \leq 0.05$.

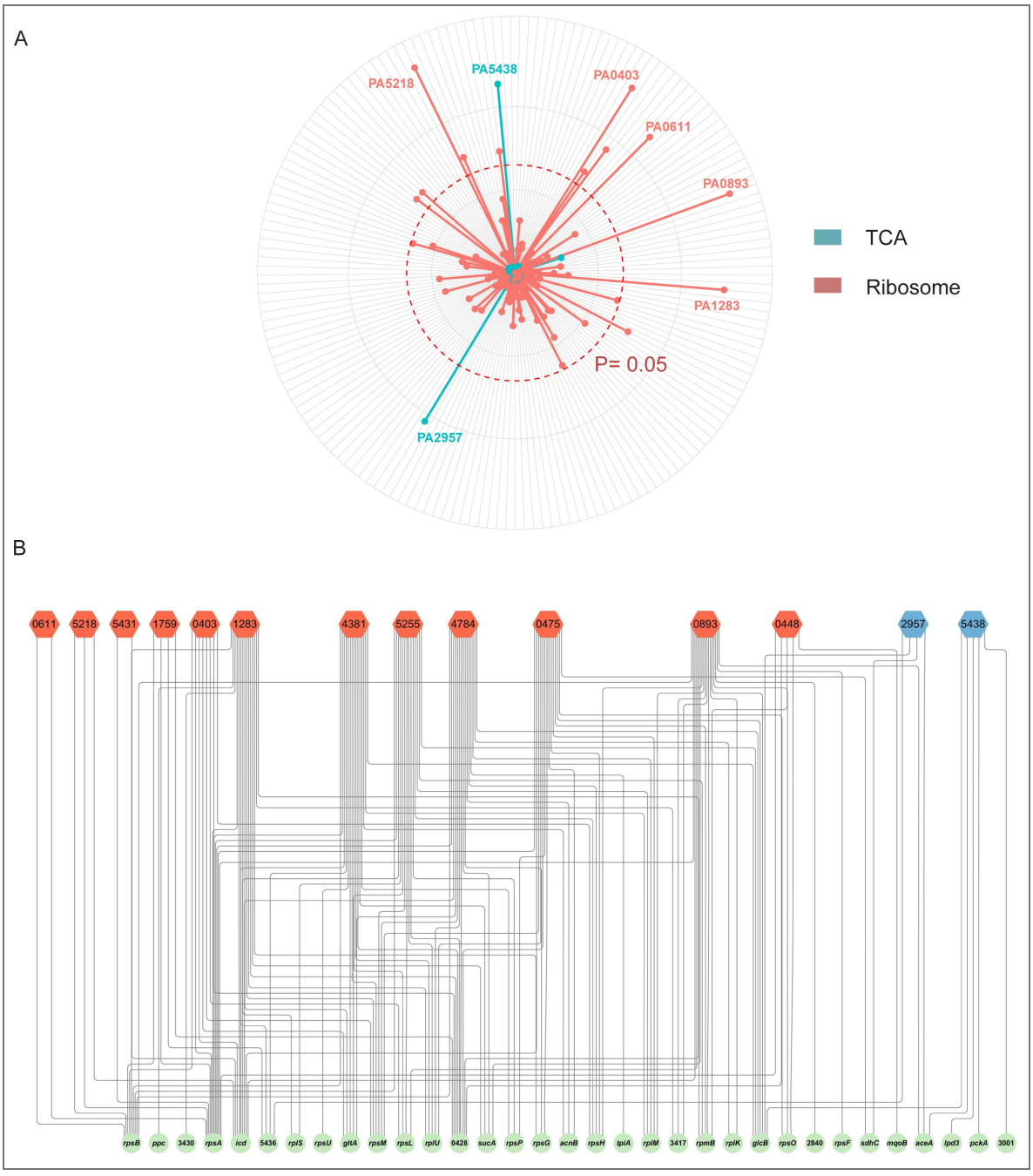

Fig. S6. Regulators involved in TCA and Ribosome pathways.

(A). Radar plots show the putative regulator in TCA and ribosome pathways. **(B).** Graph diagram of interactions involving target genes of 14 TFs, including PA0611, PA5218, PA5431, PA1759, PA0403 (PyrR), PA 1283, PA4381, PA5255 (AlgQ), PA4784, PA0475, PA0893, PA0448, PA2957 and PA5438.

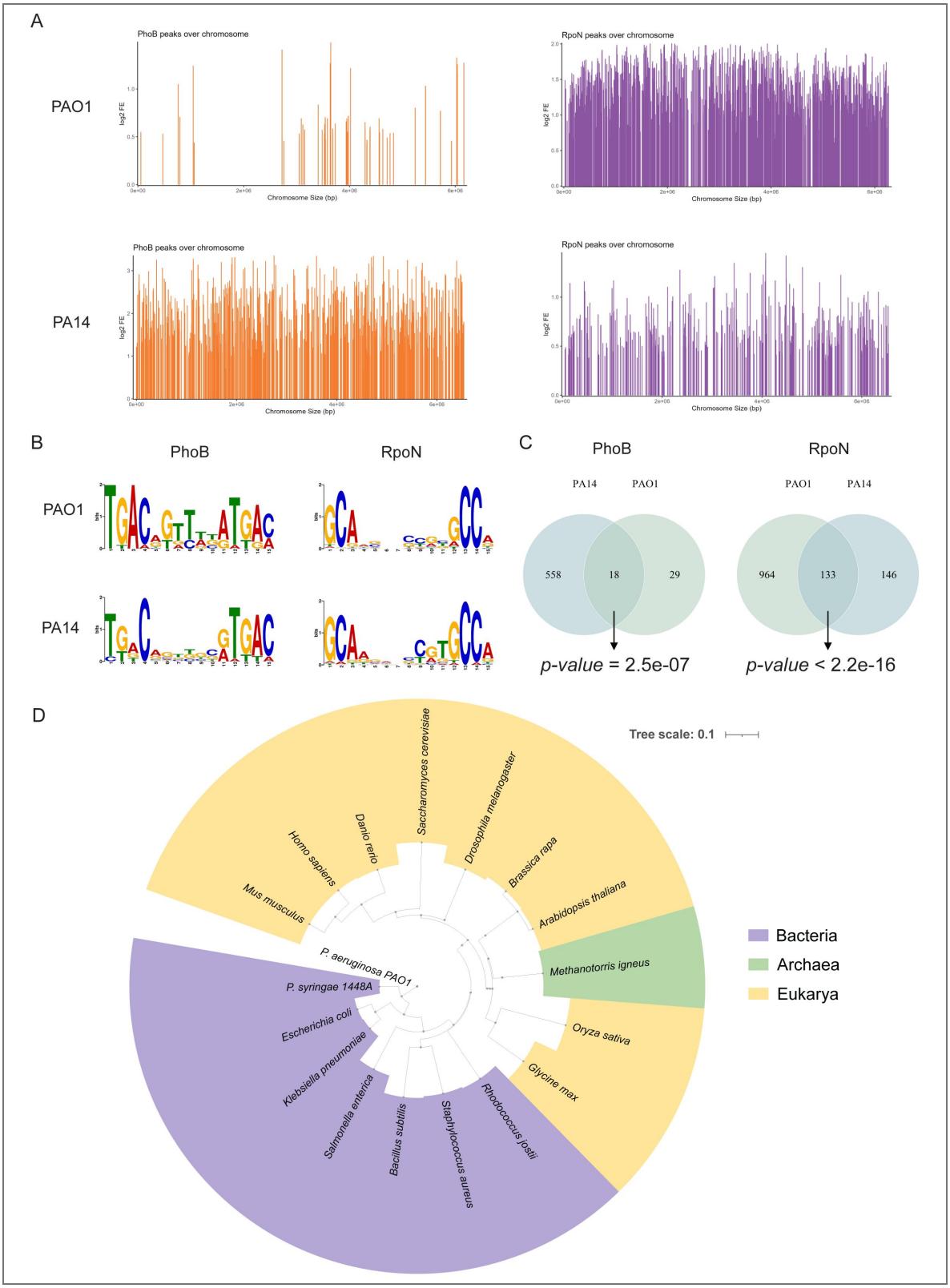

Fig. S7. Conserved TFs in *P. aeruginosa*.

(A). The coverage of PhoB and RpoN peak regions over PAO1 and PA14 chromosomes. Each line shows the location and log2 Fold Enrichment of peaks signal in the chromosome. **(B).** The binding motifs of PhoB and RpoN were analyzed via MEME-ChIP. All peaks were used to define the binding motif. **(C).** Comparison of overlapped targets enriched in PAO1 and PA14 of PhoB and RpoN. The Fisher test made the significance of the overlap ratio. **(D).** Phylogenetic tree of PA2032 across different domains of life. The phylogenetic tree was constructed using PA2032 sequence from PAO1 as the root.

Data availability

The ChIP-seq data was uploaded to National Center for Biotechnology Information Gene Expression Omnibus database under accession GSE241603 and GSE271817. Analysis codes have been deposited at *https://github.com/dengxinb2315/PS-PATRnet-code*.

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Additional information

Contributions

X.D. designed the project and obtained funding, material support and study supervision. X.D., J.H. wrote the manuscript and generated the figures and tables. J.H. performed bioinformatic analysis. J.H., Y.S., F.C., X.Y., and J.L performed the experiments. S.L. helped to write the code. Y.S. and L.H. helped to edit the manuscript. All authors reviewed the manuscript.

Declare of interest

The authors declare no competing interests.

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Additional files

Supplemental Tables. **Table S1.** List of 172 ChIPed TFs. **Table S2.** Enriched GO terms of 172 ChIPed TFs. **Table S3.** Hierarchical regulatory network. **Table S4.** Ternary regulatory motifs. **Table S5.** PWM pairwise similarity scores. **Table S6.** Co-association network. **Table S7.** Virulence-related master regulators. **Table S8.** Strains and primers used in this study. **Table S9.** Reference list of strains for pan-genome analysis.

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Peer reviews

Reviewer #1 (Public review):

Summary:

In this work, Huang et al. revealed the complex regulatory functions and transcription network of 172 unknown transcriptional factors (TFs) in Pseudomonas aeruginosa PAO1. They have built a global TF-DNA binding landscape and elucidated binding preferences and functional roles of these TFs. More specifically, the authors established a hierarchical regulatory network and identified ternary regulatory motifs, and co-association modules. Since P. aeruginosa is a well known pathogen, the authors thus identified key TFs associated with virulence pathways (e.g., quorum sensing [QS], motility, biofilm formation), which could be potential drug targets for future development. The authors also explored the TF conservation and functional evolution through pan-genome and phylogenetic analyses. For the easy searching by other researchers, the authors developed a publicly accessible database (PATF_Net) integrating ChIP-seq and HT-SELEX data.

Strengths:

(1) The authors performed ChIP-seq analysis of 172 TFs (nearly half of the 373 predicted TFs in P. aeruginosa) and identified 81,009 significant binding peaks, representing one of the largest TF-DNA interaction studies in the field. Also, The integration of HT-SELEX, pan-genome, and phylogenetic analyses provided multi-dimensional insights into TF conservation and function.

(2) The authors provided informative analytical Framework for presenting the TFs, where a hierarchical network model based on the "hierarchy index (h)" classified TFs into top, middle,

and bottom levels. They identified 13 ternary regulatory motifs and co-association clusters, which deepened our understanding of complex regulatory interactions.

(3) The PATF_Net database provides TF-target network visualization and data-sharing capabilities, offering practical utility for researchers especially for the P. aeruginosa field.

Weaknesses:

(1) There is very limited experimental validation for this study. Although 24 virulence-related master regulators (e.g., PA0815 regulating motility, biofilm, and QS) were identified, functional validation (e.g., gene knockout or phenotypic assays) is lacking, leaving some conclusions reliant on bioinformatic predictions. Another approach for validation is checking the mutations of these TFs from clinical strains of P. aeruginosa, where chronically adapted isolates often gain mutations in virulence regulators.

(2) ChIP-seq in bacteria may suffer from low-abundance TF signals and off-target effects. The functional implications of non-promoter binding peaks (e.g., coding regions) were not discussed.

(3) PATF_Net currently supports basic queries but lacks advanced tools (e.g., dynamic network modeling or cross-species comparisons). User experience and accessibility remain under-evaluated. But this could be improved in the future.

Achievement of Aims and Support for Conclusions

(1) The authors successfully mapped global P. aeruginosa TF binding sites, constructed hierarchical networks and co-association modules, and identified virulence-related TFs, fulfilling the primary objectives. The database and pan-genome analysis provide foundational resources for future studies.

(2) The hierarchical model aligns with known virulence mechanisms (e.g., LasR and ExsA at the bottom level directly regulating virulence genes). Co-association findings (e.g., PA2417 and PA2718 co-regulating pqsH) resonate with prior studies, though experimental confirmation of synergy is needed.

Impact on the Field and Utility of Data/Methods

(1) This study fills critical gaps in TF functional annotation in P. aeruginosa, offering new insights into pathogenicity mechanisms (e.g., antibiotic resistance, host adaptation). The hierarchical and co-association frameworks are transferable to other pathogens, advancing comparative studies of bacterial regulatory networks.

(2) PATF_Net enables rapid exploration of TF-target interactions, accelerating candidate regulator discovery.

Comments on revisions:

The authors have done a good job of revising their manuscript. The manuscript is now more concise and logical for readers.

https://doi.org/10.7554/eLife.103346.3.sa2

Reviewer #3 (Public review):

Summary:

The authors utilized ChIP-seq on strains containing tagged transcription factor (TF)-overexpression plasmids to identify binding sites for 172 transcription factors in P.

aeruginosa. High-quality binding site data provides a rich resource for understanding regulation in this critical pathogen. These TFs were selected to fill gaps in prior studies measuring TF binding sites in P. aeruginosa. The authors further perform a structured analysis of the resulting transcriptional regulatory network, focusing on regulators of virulence and metabolism, in addition to performing a pangenomic analysis of the TFs. The resulting dataset has been made available through an online database. While the implemented approach to determining functional TF binding sites has limitations, the resulting dataset still has substantial value to P. aeruginosa research.

Strengths:

The generated TF binding site database fills an important gap in regulatory data in the key pathogen P. aeruginosa. Key analyses of this dataset presented include an analysis of TF interactions and regulators of virulence and metabolism, which should provide important context for future studies into these processes. Experimental validation has been included in the revised version. The online database containing this data is well organized and easy to access. As a data resource, this work should be of significant value to the infectious disease community.

Weaknesses:

Drawbacks of the study, which have been mitigated in a revised version, include 1) challenges interpreting binding site data obtained from TF overexpression due to unknown activity state of the TFs on the measured conditions (discussed by the authors), and 2) remaining challenges in the practical utilization of the TRN topological analysis.

https://doi.org/10.7554/eLife.103346.3.sa1

Author response:

The following is the authors' response to the previous reviews

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
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> *Summary:*
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> *In this work, Huang et al. revealed the complex regulatory functions and transcription network of 172 unknown transcriptional factors (TFs) in Pseudomonas aeruginosa PAO1. They have built a global TF-DNA binding landscape and elucidated binding preferences and functional roles of these TFs. More specifically, the authors established a hierarchical regulatory network and identified ternary regulatory motifs, and co-association modules. Since P. aeruginosa is a well known pathogen, the authors thus identified key TFs associated with virulence pathways (e.g., quorum sensing [QS], motility, biofilm formation), which could be potential drug targets for future development. The authors also explored the TF conservation and functional evolution through pan-genome and phylogenetic analyses. For the easy searching by other researchers, the authors developed a publicly accessible database (PATF_Net) integrating ChIP-seq and HT-SELEX data.*
>
> *Strengths:*
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> *(1) The authors performed ChIP-seq analysis of 172 TFs (nearly half of the 373 predicted TFs in P. aeruginosa) and identified 81,009 significant binding peaks, representing one of the largest TF-DNA interaction studies in the field. Also, The integration of HT-SELEX, pan-*

> *genome, and phylogenetic analyses provided multi-dimensional insights into TF conservation and function.*
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> *(2) The authors provided informative analytical Framework for presenting the TFs, where a hierarchical network model based on the "hierarchy index (h)" classified TFs into top, middle, and bottom levels. They identified 13 ternary regulatory motifs and co-association clusters, which deepened our understanding of complex regulatory interactions.*
>
> *(3) The PATF_Net database provides TF-target network visualization and data-sharing capabilities, offering practical utility for researchers especially for the P. aeruginosa field.*

Thank you for your positive feedback!

> *Weaknesses:*
>
> *(1) There is very limited experimental validation for this study. Although 24 virulence-related master regulators (e.g., PA0815 regulating motility, biofilm, and QS) were identified, functional validation (e.g., gene knockout or phenotypic assays) is lacking, leaving some conclusions reliant on bioinformatic predictions. Another approach for validation is checking the mutations of these TFs from clinical strains of P. aeruginosa, where chronically adapted isolates often gain mutations in virulence regulators.*

Thank you for this valuable suggestion. We have performed the EMSA experiment to validate the binding result and also constructed the mutants for further functional validation. The details can be found in Figure S5.

> *(2) ChIP-seq in bacteria may suffer from low-abundance TF signals and off-target effects. The functional implications of non-promoter binding peaks (e.g., coding regions) were not discussed.*

Thank you for this insightful comment regarding ChIP-seq data quality and non-promoter binding events. While we acknowledge that completely eliminating all non-specific binding signals is technically challenging in bacterial ChIP-seq experiments, we implemented stringent quality control measures including replicates, negative controls, and FDR cutoffs to minimize false positives.

Although the coding binding peaks represent a smaller fraction of total binding events, they are functionally significant rather than mere technical artifacts. Our previous work systematically demonstrated that bacterial TFs can bind to coding sequences and regulate gene expression through multiple mechanisms, including modulating cryptic promoter activity and antisense RNA transcription, hindering transcriptional elongation, and influencing translational efficiency[1]. We have now expanded the Discussion section to address these regulatory mechanisms.

> *(3) PATF_Net currently supports basic queries but lacks advanced tools (e.g., dynamic network modeling or cross-species comparisons). User experience and accessibility remain underevaluated. But this could be improved in the future.*

Thank you for this constructive feedback on PATF_Net. We acknowledge that more advanced features would further enhance the platform's utility. To enhance the utility of PA_TFNet, we have implemented two new features: (1) a virulence pathway browser that allows users to explore TF binding across curated gene sets for key virulence pathways (quorum sensing, secretion systems, biofilm, motility, etc.), and (2) a target gene search function that enables rapid identification of all TFs regulating any gene of interest by locus tag query.

> *Achievement of Aims and Support for Conclusions*

> *(1) The authors successfully mapped global P. aeruginosa TF binding sites, constructed hierarchical networks and co-association modules, and identified virulence-related TFs, fulfilling the primary objectives. The database and pan-genome analysis provide foundational resources for future studies.*
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> *(2) The hierarchical model aligns with known virulence mechanisms (e.g., LasR and ExsA at the bottom level directly regulating virulence genes). Co-association findings (e.g., PA2417 and PA2718 co-regulating pqsH) resonate with prior studies, though experimental confirmation of synergy is needed.*

Thank you for your positive feedback! We have added experimental validation in the Results section.

> *Impact on the Field and Utility of Data/Methods*
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> *(1) This study fills critical gaps in TF functional annotation in P. aeruginosa, offering new insights into pathogenicity mechanisms (e.g., antibiotic resistance, host adaptation). The hierarchical and co-association frameworks are transferable to other pathogens, advancing comparative studies of bacterial regulatory networks.*
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> *(2) PATF_Net enables rapid exploration of TF-target interactions, accelerating candidate regulator discovery.*

Thank you for your positive feedback!

> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *The authors utilized ChIP-seq on strains containing tagged transcription factor (TF)-overexpression plasmids to identify binding sites for 172 transcription factors in P. aeruginosa. High-quality binding site data provides a rich resource for understanding regulation in this critical pathogen. These TFs were selected to fill gaps in prior studies measuring TF binding sites in P. aeruginosa. The authors further perform a structured analysis of the resulting transcriptional regulatory network, focusing on regulators of virulence and metabolism, in addition to performing a pangenomic analysis of the TFs. The resulting dataset has been made available through an online database. While the implemented approach to determining functional TF binding sites has limitations, the resulting dataset still has substantial value to P. aeruginosa research.*
>
> *Strengths:*
>
> *The generated TF binding site database fills an important gap in regulatory data in the key pathogen P. aeruginosa. Key analyses of this dataset presented include an analysis of TF interactions and regulators of virulence and metabolism, which should provide important context for future studies into these processes. The online database containing this data is well organized and easy to access. As a data resource, this work should be of significant value to the infectious disease community.*

Thank you for your positive feedback!

> *Weaknesses:*

> *Drawbacks of the study include 1) challenges interpreting binding site data obtained from TF overexpression due to unknown activity state of the TFs on the measured conditions, 2) limited practical value of the presented TRN topological analysis, and 3) lack of independent experimental validation of the proposed master regulators of virulence and metabolism.*

We thank the reviewer for summarizing these key concerns. We acknowledge the limitations raised regarding TF overexpression, TRN topological analysis interpretation, and experimental validation. We provide detailed point-by-point responses to each of these concerns in our replies to the specific comments below, where we explain our rationale, the measures taken to address these limitations, and our plans for improvement.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations for the authors):***
>
> *Future Directions for the authors to consider for next steps:*
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> *(1) Key TFs (e.g., PA1380, PA5428) should be validated via gene knock out experiments, fluorescent reporter assays, or animal models to confirm roles in virulence pathways.*

Thank you for this important suggestion. We agree that experimental validation is essential to confirm their regulatory roles and biological functions.

Firstly, we selected a subset of key TFs, including PA0167, PA1380, PA0815, and PA3094, and performed Electrophoretic Mobility Shift Assays (EMSA) experiments to validate their direct binding to target promoters. These results confirmed the ChIP-seq-identified interactions and are now included as Figure S5A-F.

We also constructed a clean deletion mutant of PA1380 and PA 3094 (ΔPA1380 and ΔPA3094) and their complementary strains (ΔPA1380/p and ΔPA3094/p). We then performed RT-qPCR analysis to validate their regulatory effects on key target genes. We found that PA1380 positively regulate the expression of *cupB1* and *cupB3* genes (Figure S5F). While the CupB cluster was known not be as important as CupA cluster in the biofilm information, so we did not find significant difference in biofilm formation between WT and ΔPA1380. Additionally, we found TF PA3094 also positively regulate *lecA* expression, which were shown in Figure S5G.

We agree that comprehensive functional validation, including animal model studies, would further strengthen the biological significance of these findings. Such experiments are currently underway in our laboratory and will be the subject of follow-up studies.

We have revised the Results section and Method section to include these validation experiments and their implications. Please see Figure S5 and Lines 283-300.

“To experimentally validate the regulatory interactions identified by ChIP-seq, we performed biochemical and genetic analyses on selected TFs. First, we conducted Electrophoretic Mobility Shift Assays (EMSA) for four TFs, including PA0167, PA0815, PA1380, and PA3094, using DNA fragments containing their predicted binding sites from target gene promoters. These TFs showed specific binding to their cognate DNA sequences (Figure S5A-D), confirming the direct binding of the ChIP-seq-identified interactions.

To further validate the functional regulatory roles of these TFs, we constructed clean deletion mutants of PA1380 and PA3094 (ΔPA1380 and ΔPA3094) along with their complemented strains (ΔPA1380/p and ΔPA3094/p). RT-qPCR analysis revealed that PA1380 positively regulates the expression of cupB1 and cupB3 (Figure S5E), two genes within the CupB fimbrial cluster identified as ChIP-seq targets. Similarly, PA3094 was confirmed to positively

regulate lecA expression (Figure S5F), which encodes a lectin involved in biofilm formation and host interactions[2]. Expression of these target genes was restored to wild-type (WT) levels in the complemented strains, validating the regulatory relationships predicted by ChIP-seq. These combined biochemical and genetic validations demonstrate the accuracy and biological relevance of our TF binding data."

> *(2) Non-promoter binding events (e.g., coding regions) may regulate RNA stability, warranting integration with translatomics or epigenomics data.*

Thank you for this suggestion. We have now expanded the Discussion section to address this comment. Please see Lines 478-482.

"Our analysis revealed that TF binding events occur within coding regions, which is consistent with our previous study demonstrating that bacterial TFs possess binding capabilities for coding regions and can regulate transcription through multiple mechanisms [1]. Besides, it may also regulate RNA stability, warranting integration with translatomics or epigenomics data."

> *(3) Incorporate strain-specific TF data (e.g., clinical isolates) and dynamic visualization tools to broaden PATF_Net's applicability.*

Thank you for this constructive suggestion. To enhance the utility of PA_TFNet, we have implemented two new features: (1) a virulence pathway browser that allows users to explore TF binding across curated gene sets for key virulence pathways (quorum sensing, secretion systems, biofilm, motility, etc.), and (2) a target gene search function that enables rapid identification of all TFs regulating any gene of interest by locus tag query. These features are now live on the database and described in the revised manuscript.

Regarding strain-specific TF data, we agree this would be valuable for understanding regulatory diversity in clinical isolates. However, such an expansion would require ChIP-seq profiling across multiple strains. The current dataset is based on the reference strain PAO1, which serves as the foundation for most *P. aeruginosa* research and allows direct comparison with existing genomic and functional studies. We have added a statement in the revised manuscript acknowledging this limitation and highlighting strain-specific TF analysis as an important future direction for the field. Please see Lines 372-390.

"The database offers multiple search modalities to facilitate data exploration: users can perform TF-centric searches to query binding sites, target genes, and regulatory networks for individual TFs, or utilize the target gene search function to identify all TFs that regulate any gene of interest by entering its locus tag. To connect regulatory data with biological function, we have implemented a virulence pathway browser that allows users to explore TF binding patterns across curated gene sets for major *P. aeruginosa* virulence pathways. Interactive visualization tools, including network graphs and binding profile plots, facilitate intuitive exploration of regulatory relationships. The primary purpose of PATF_Net is to store, search, and mine valuable information on *P. aeruginosa* TFs for researchers investigating *P. aeruginosa* infection. The current resource is based on the reference strain PAO1, which serves as the foundation for most *P. aeruginosa* molecular studies and allows direct integration with existing genomic annotations and functional data. However, *P. aeruginosa* exhibits substantial genomic diversity across clinical isolates, and strain-specific differences in TF binding patterns may contribute to phenotypic variation in virulence, antibiotic resistance, and host adaptation. Extension of this resource to include strain-specific regulatory maps from diverse clinical isolates would provide valuable insights into the regulatory basis and represents an important direction for future investigation."

> *(4) Phylogenetic analysis highlights TF conservation in bacteria; future work could explore functional homology in other Gram-negative pathogens (e.g., E. coli).*

Thank for this insightful suggestion. Our phylogenetic analysis revealed that *P. aeruginosa* TFs exhibit varying degrees of conservation across bacterial species, with some showing broad distribution across Gram-negative pathogens while others are lineage-specific.

We agree that exploring functional homology of orthologous TFs across species would be highly valuable. Such comparative studies could address whether conserved TFs regulate similar target genes and biological processes across species, or whether regulatory networks have been rewired during evolution. For example, comparative ChIP-seq analysis of *P. aeruginosa* TFs and their orthologs in *Klebsiella pneumoniae* or even Gram-positive pathogen like *Bacillus cereus* could reveal conserved regulatory modules governing universal virulence or metabolic strategies versus species-specific adaptations. This represents an important direction for future investigation and would be facilitated by the comprehensive TF binding dataset we provide here. We have expanded the Discussion section to highlight this future direction. Please see Lines 539-550.

"While our phylogenetic analysis reveals varying degrees of TF conservation across bacterial species, the functional implications of this conservation remain to be fully explored. Many *P. aeruginosa* TFs have clear orthologs in both Gram-negative (e.g., *Klebsiella pneumoniae*) and Gram-positive pathogens (e.g., *Bacillus cereus*), yet whether these orthologs regulate similar target genes and biological processes is largely unknown. Future comparative ChIP-seq profiling of orthologous TFs could reveal the extent to which regulatory network architecture is conserved versus rewired during bacterial evolution, potentially identifying core regulatory modules governing universal bacterial strategies versus species-specific innovations. Such cross-species comparisons would enhance our understanding of regulatory network evolution and enable functional prediction in less well-characterized pathogens based on homology to experimentally validated *P. aeruginosa* regulators."

> ***Reviewer #3 (Recommendations for the authors):***
>
> *Major comments*
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> *- Limitations of the ChIP-seq approach: With overexpression plasmids as an approach to TRN elucidation, there are always a set of concerns. First, TF expression is not enough to ensure regulatory activity - metabolite effects must be such that the TF is active which requires growing the cells in activating conditions. Second, the presence of a binding event does not mean that the binding has a regulatory effect - the authors are clearly aware of this as they specify binding sites in promoter regions, which should be helpful, but they also mention the possibility of regulatory binding events in coding regions. These issues should be listed as weaknesses of the approach in the Discussion.*

Thank you for these important suggestions. We agree that these limitations should be explicitly discussed. We have now added a dedicated paragraph in the Discussion section addressing these concerns. Please see Lines 492-501.

"However, several limitations of the ChIP-seq approach should be acknowledged. Firstly, TF overexpression ensures sufficient protein levels for ChIP-seq signal detection but does not guarantee that all TFs are in their active conformational states, as many bacterial TFs require allosteric activation by metabolites, cofactors, or post-translational modifications. The cells under standard laboratory conditions which may not activate all TFs to their maximal regulatory states, potentially leading to underestimation of condition-specific binding peaks. Secondly, while we observed TF binding at thousands of genomic sites, binding per se does not equate to functional regulation, as chromatin context, cofactor availability, and competitive binding all influence regulatory outcomes."

> *- Lack of independent validation: The study seems to lack substantial independent validation of either the functional nature of the binding sites as well as the proposed*

> *physiological regulatory role of the TFs. For example, for the 103 identified TF motifs, do any of these agree with existing motifs in motif databases that may be homologous to P. aeruginosa TFs? The authors claim to have discovered master regulators of virulence and associated core regulatory clusters - but there does not seem to be any independent validation of the proposed associations. The authors selected the TF targets to cover TFs that had not yet been characterized; however, it would have been nice to have some overlap with previous studies so that consistency and data quality could be assessed.*

Thank you for raising these critical points about validation.

As for motif validation, we compared the existing motifs in the RegPrecise database[3] and we found that the motif of PA3587 show significant similarity to homologous TFs in Pseudomonadaceae. We have added the related description in the Results section. Please see Figure S3B and Lines 228-231.

As for the validation of master regulators, we have performed EMSA experiments for validating the binding events and constructed the mutants for function validation. We have added the related contents in Results section. Please see Figure S5 and Lines 283-300.

We have discussed the overlap between our results and previous studies in the Discussion section. Please see Lines 530-538.

“PA0797 is known to regulate the pqs system and pyocyanin production[4]. In the present study, it was also found to bind to the pqsH promoter region and its motif was visualised. PA5428 was found to bind to the promoter regions of aceA and glcB genes[5], which was also demonstrated in our ChIP-seq results. PA4381 (CloR) was found to be associated with polymyxin resistance in a previous study[6] and to be possibly related to ROS resistance in the present study. Furthermore, PA5032 plays a putative role in biofilm regulation and also forms an operon with PA5033, an HP associated with biofilm formation[7].”

> *- Uncertain value of TRN topology analysis: The relationship between ternary motifs and pathogenicity of P. aeruginosa, and why the authors argue these results motivated TF-targeting drugs (the topic of the last paragraph of the Discussion), are unclear to me. The authors allude to possible connections between pathogenicity, growth, and drug resistance, but I don't see concrete examples here of related TF interactions that clearly represent these relationships. The sections "Hierarchical networks of TFs based on pairwise interactions" and "Ternary regulatory motifs show flexible relationships among TFs in P. aeruginosa" seem to not say much in terms of results that are actionable or possible to validate. A topological graph is constructed based on observed TF-TF connections in measured binding sites - however, it's unclear if any of these connections are physiologically meaningful. Line 178 - Why would there be any connection between the structural family of TF and its location in the proposed TRN hierarchy?*

Thank you for this valuable comment on TRN topology analysis. It is hard to quantify precisely how much this resource will accelerate *P. aeruginosa* research or drug development, but we believe providing this foundational network architecture has inherent value for the community, which is valued for enabling hypothesis generation even before comprehensive functional validation. We would like to clarify our perspective on these findings and have added the discussion in the revised manuscript to better describe their nature and value. Please see Lines 517-528.

“Additionally, although the TRN analysis revealed organizational patterns in *P. aeruginosa* regulatory network, the functional significance these topological features, including their specific contributions to pathogenicity, metabolic adaptation, and antibiotic resistance remains to be experimentally determined in the future work. The hierarchical structure and regulatory motifs we identified represent objective network properties derived from our

binding data, but translating these structural observations into mechanistic understanding will require condition-specific functional studies, genetic validation, and phenotypic characterization. Our analysis provided a systematic framework and generating testable hypotheses rather than definitive functional conclusions. Nevertheless, these network-level organizational principles provided value to the community as a foundational reference, similar to other regulatory network maps[8] that were useful even before comprehensive validation."

> *- Identification of "master" regulators: Line 527 on virulence regulators: "We first generated gene lists associated with nine pathways" - is this not somewhat circular, i.e. using gene lists generated from (I assume) co-regulated gene sets to identify regulators of those gene lists? I can't tell from the cited reference (80), which is their own prior review article, what the original source of these gene lists was. Somewhat related to this point - Line 32: 24 "master regulators" - if there are that many, is it still considered a master regulator? Line 270: This term "master regulator" would seem to require some quantitative justification. Identifying 24 (a large number of) "master" regulators of virulence would seem to dilute the implied power of the term.*

We apologize for the lack of clarity regarding the virulence pathway gene lists, and we have provided complete gene lists for virulence-related pathways, which were compiled from functional annotations, in our online PA_TFNet database.

Additionally, we appreciate your concern about the use of "master" regulator. The usage is based on previous studies[9,10], and the master regulator is commonly known in the development of multicellular organisms as a subset of TFs that control the expression of multiple downstream genes and govern lineage commitment or key biological processes. We employed the term "master regulator" in an analogous manner to specify a class of functionally crucial TFs that participate in a pathway or biological event by regulating multiple downstream genes statistically enriched in that pathway. In line with this definition, we identified TFs whose targets were significantly enriched in genes associated with specific virulence pathways (hypergeometric test, $P < 0.05$).

We understand the concern that identifying 24 master regulators might seem to dilute the term. However, we would like to clarify that each of these 24 TFs is a "master regulator" with respect to specific virulence pathways based on statistical criteria, not necessarily a global master regulator of multiple pathways of *P. aeruginosa*. We have revised the Method section. Please see Lines 604-612.

> *- Line 234: "Genome-wide synergistic co-association of TFs in P. aeruginosa." This section was an interesting analysis. As I mention above, the weakness of an overexpression approach is not knowing whether the TF is active on the examined conditions. By looking at shared binding peaks across overexpression of different TFs, it should indeed be possible to glean some regulatory connections across TFs. Furthermore, the authors discuss specific examples that appear physiologically reasonable, which is appreciated.*

We thank the reviewer for this positive assessment of our co-association analysis. We agree with the limitation of the overexpression approach, which have been discussed in the Discussion section. We are pleased that the reviewer found the approach and specific examples valuable.

> *Minor comments*
>
> *- Line 35 - "high-throughput systematic evolution of ligands by exponential enrichment" - no idea what this means. Is this related to the web-based database, or why is it mentioned in the same sentence?*

We apologize for the unclear presentation. To clarify: “High-throughput systematic evolution of ligands by exponential enrichment” (HT-SELEX) is an in vitro technique for determining TF DNA-binding motifs, which our group previously applied to a subset of *P. aeruginosa* TFs in a prior publication[11]. In the current study, we performed ChIP-seq for 172 TFs, which represent the majority of TFs not covered by the previous HT-SELEX study. Together, these two complementary approaches (HT-SELEX for in vitro binding motifs, ChIP-seq for in vivo genomic binding sites) provide near-complete coverage of the *P. aeruginosa* TF repertoire. Both datasets are integrated into our PA_TFNet database.

Due to space constraints in the abstract, we could not provide detailed explanation of HT-SELEX, but we have now improved the clarity in the Introduction to better explain the relationship between our previous HT-SELEX work and the current ChIP-seq study, and why both are mentioned together in the context of the database. Please see Lines 99-105.

> *- Line 193 - Only 9 auto-regulating TFs seems like a low number, given the frequency of negative auto-regulation in other organisms like E. coli. Could the authors comment on their expectations based on well-curated TRNs?*

Thank you for this comment. We agree that 9 auto-regulating TFs is lower than might be expected based on E. coli, where auto-regulation is more prevalent. This likely reflects technical limitations of ChIP-seq approach that our detection was limited to standard growth conditions rather than the diverse physiological states where auto-regulation often occurs. Therefore, the 9 TFs we report represent a high-confidence subset, and the true frequency of auto-regulation in *P. aeruginosa* likely is higher. We added the content in the revised manuscript. Please see Lines 193-196.

“This number likely represents a conservative estimate, as experiments may not optimally capture auto-regulatory events that depend on native expression levels or specific physiological conditions.”

> *- Line 230 - "This conservation suggests that TFs within the same cluster co-regulate similar sets of genes." - Why would clustering of TF binding site motifs need to be done to make this assessment? Couldn't the shared set of regulated genes be identified directly from the binding site data? Computing TF binding site motifs has obvious value, but I am struggling to understand the point of clustering the motifs. Is there some implied evolutionary or physiological connection here? No specific physiological roles or hypotheses are discussed in this section.*

Thank you for this important question. We agree that shared target genes can be identified directly from ChIP-seq binding data, which we also analyzed (co-association analysis). The motif clustering analysis serves a complementary and distinct purpose that provides information not directly obtainable from overlapped targets alone. Specifically, target overlap is inherently condition dependent, and motif clustering captures this intrinsic binding specificity, which reflects the structural similarity of DBDs, evolutionary relationships, and potential for functional redundancy or cooperativity under specific conditions. We have revised the related content in the manuscript, and please see Lines 236-242.

“Clustering of TF binding motifs identified groups of TFs with similar intrinsic DNA-binding specificities. As expected, many clusters contained TFs from the same DBD families, reflecting evolutionary conservation and potential functional redundancy or competitive binding at shared regulatory elements. Notably, the clustering also uncovered associations between TFs from different DBD families, suggesting convergent evolution of binding specificity or novel regulatory interactions that warrant further investigation.”

> *- Line 284 - should "metabolomic" be "metabolic"? I didn't see metabolomic data*

Yes, we have revised. Please see Line 311.

> *- Several of the figures are too small (e.g. Fig S4A) or complex (Fig 2A) to see clearly or glean information from.*

Thank you for this comment. We acknowledge that Figure 2A and Figure S4A contain dense information due to the comprehensive nature of the regulatory network and the large number of TFs analyzed. We believe these overview figures serve an important purpose in conveying the scale and organization of the regulatory network, while the tables (Table S6 for Fig. S4A and Table S3 for Fig. 2A) provide the granular data needed for specific inquiries. We have also made the figures available in higher resolution and increased font sizes where possible without compromising the overall layout.

> *- I don't understand the organization of the "Ternary regulatory motifs" in Supplementary Data File 4 - A table of contents explaining the tabs and columns would be welcome (for this as well as other supplementary files, some of which are more straightforward than others).*

Thank you for this suggestion. We have now revised all supplementary data files to include header and necessary annotations in the first row. Specifically for Supplementary Data File 4, the three columns (Top, Middle, Bottom) represent the left, middle, and right node, respectively, in each ternary regulatory motif.

> *- I would have expected genomic locations of TF binding sites would have been one of the Supplementary Tables, to increase the accessibility of the data. However, the data is made available through their website, https://jiadhuang0417.shinyapps.io/PATF_Net/, which was easy to access and download the full dataset, so this is a minor issue.*

Thank for accessing our PA_TFNet database and for the positive feedback on data accessibility. We agree that providing genomic locations of TF binding sites is crucial. These data are fully available and downloadable through the web interface, which allows flexible searching, filtering, and batch download of binding sites. We felt that the interactive and database format provides more functionality than static supplementary tables (e.g., dynamic filtering by TF, genomic region, or binding strength), given the large scale of this dataset.

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