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REVIEW ARTICLE

The transcriptional regulators of virulence for *Pseudomonas aeruginosa*: Therapeutic opportunity and preventive potential of its clinical infections

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Abstract In *Pseudomonas aeruginosa* (*P. aeruginosa*), transcription factors (TFs) are important mediators in the genetic regulation of adaptability and pathogenicity to respond to multiple environmental stresses and host defences. The *P. aeruginosa* genome harbours 371 putative TFs; of these, about 70 have been shown to regulate virulence-associated phenotypes by binding to the promoters of their target genes. Over the past three decades, several techniques have been applied to identify TF binding sites on the *P. aeruginosa* genome, and an atlas of TF binding patterns has been mapped. The virulence-associated regulons of TFs show complex crosstalk in *P. aeruginosa*'s regulatory network. In this review, we summarise the recent literature on TF regulatory networks involved in the quorum-sensing system, biofilm formation,

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pyocyanin synthesis, motility, the type III secretion system, the type VI secretion system, and oxidative stress responses. We discuss future perspectives that could provide insights and targets for preventing clinical infections caused by *P. aeruginosa* based on the global regulatory network of transcriptional regulators.

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a gram-negative, opportunistic, human-infecting pathogen that can cause chronic lung infections in susceptible hosts, lung inflammation, infections of soft tissues and burn wounds and other types of infection.[1] *P. aeruginosa* accounts for 10% of nosocomial infections.[2] Chronic infections and lung inflammation caused by *P. aeruginosa* are key causes of death in patients with cystic fibrosis.[1] The respiratory tract is a frequent site of infection with a wide range of viruses, bacteria, or co-infection. The microbiota promotes resistance to respiratory infection. Viral or bacterial infections can induce alterations of the microbiome and secondary bacterial pneumonia.[3] The invasion of pathogenic bacteria or viruses also affects the ability of each other to infect. Respiratory viruses (such as influenza, respiratory syncytial virus, and parainfluenza) co-infected with *P. aeruginosa* have a higher prevalence (16.6%) and death rate (10.5%) among the patients compared with the infection without *P. aeruginosa*.[4] *P. aeruginosa* isolates with extensive biofilm production also enhance their antibiotic resistance and *in vivo* colonization in COVID-19 patients.[5] *P. aeruginosa* employs diverse metabolic and virulence systems, such as the quorum-sensing system (QS), type III secretion system (T3SS), type VI secretion system (T6SS), flagella and type IV pili, to adapt to internal and external environmental challenges.[6,7,8] Other virulence factors (e.g., biofilm, exopolysaccharide, lipopolysaccharide, elastase, protease, esterase and rhamnolipids) form a highly complex but ordered virulence system.[9–12] Although investigations of the pathogenic mechanisms of *P. aeruginosa* have led to the development of therapeutic approaches, only a few drugs have been approved to treat *P. aeruginosa* infections.[13–17] We previously proposed that targeting key virulence regulators has great potential for developing novel anti-*P. aeruginosa* drugs.[18] Here, we review the literature on *P. aeruginosa*'s integrative virulence-associated regulatory networks, which are mediated by a group of transcription factors (TFs).

DNA-binding TFs play a crucial role in mediating the adaptability and pathogenicity of pathogenic bacteria. The *P. aeruginosa* genome harbours 371 putative TFs that belong to 29 families, such as the LysR family (113 TFs), Arc family (56 TFs), LuxR family (30 TFs), OmpR family (24 TFs), TetR family (23 TFs) and GntR family (23 TFs).[19] Several experimental techniques, such as chromatin immunoprecipitation in conjunction with protein-binding microarray (PBM),[20] DNA affinity purification sequencing (DAP-seq),[21–23] chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq),[24–29] high-throughput analysis of *in vitro* protein—DNA interactions using massively parallel sequencing (Bind-n-seq)[30] and high-throughput systematic evolution of ligands by exponential enrichment (HT-SELEX) assay, have been applied to study the bacterial TFs of *P. aeruginosa*.[31] We and our collaborators have characterized eight regulators (VqsR, VqsM, AlgR, CdpR, CysB, RsaL, AnvM and RpoN) and elucidated their molecular pathogenic mechanisms.[25,27,28,32–36] A genomic virulence-associated network containing 20 key TFs and their targets is mapped, providing an online analysis platform for peer researchers.[26] An online regulatory-interaction database of *P. aeruginosa*, named RegulomePA, is also developed.[37] RegulomePA contains 4,827 interactions stemming from the interactions of 27.27% of TFs and 54.16% of TFs with their target genes (50.8% of the total genes). An additional transcriptional regulatory network containing 364 transcriptomes of *P. aeruginosa*, identifying 104 independently modulated sets of genes involved in biosynthetic gene clusters and associated secretion systems, was reconstructed.[38] We recently mapped an atlas of the binding specificities of 182 TFs in *P. aeruginosa* using HT-SELEX.[31] In addition, we construct nine virulence-associated regulatory networks and found 32 newly identified TFs involved in multiple virulence pathways and physiological processes. More recently, the regulatory atlas of two-component systems (TCSs) containing 55 TCS response regulators is mapped using DAP-seq, revealing numerous new regulatory interactions in several virulence-associated and metabolic pathways.[22]

In this review, we integrate the virulence-associated transcriptional regulators, especially TFs, and map seven regulatory networks, including the QS, pyocyanin synthesis, motility, biofilm formation, T3SS, T6SS and oxidative stress response networks. We also discuss the challenges and trends in studying the pathogenesis, drug targets and infection prevention of *P. aeruginosa*, which will provide insights for preventing clinical infections caused by *P. aeruginosa* based on the global regulatory network of transcription regulators.

QS-related regulatory networks control *P. aeruginosa* communication and virulence

The QS of *P. aeruginosa*, which controls most virulence factors, has been continuously studied for three decades. Although virulence phenotypes are mainly determined by four QSs (Las, Rhl, Pqs and Iqs),[12,39] a group of regulators mediates the integrated regulatory network-associated QS. For instance, LasR, acting as the master regulator of the

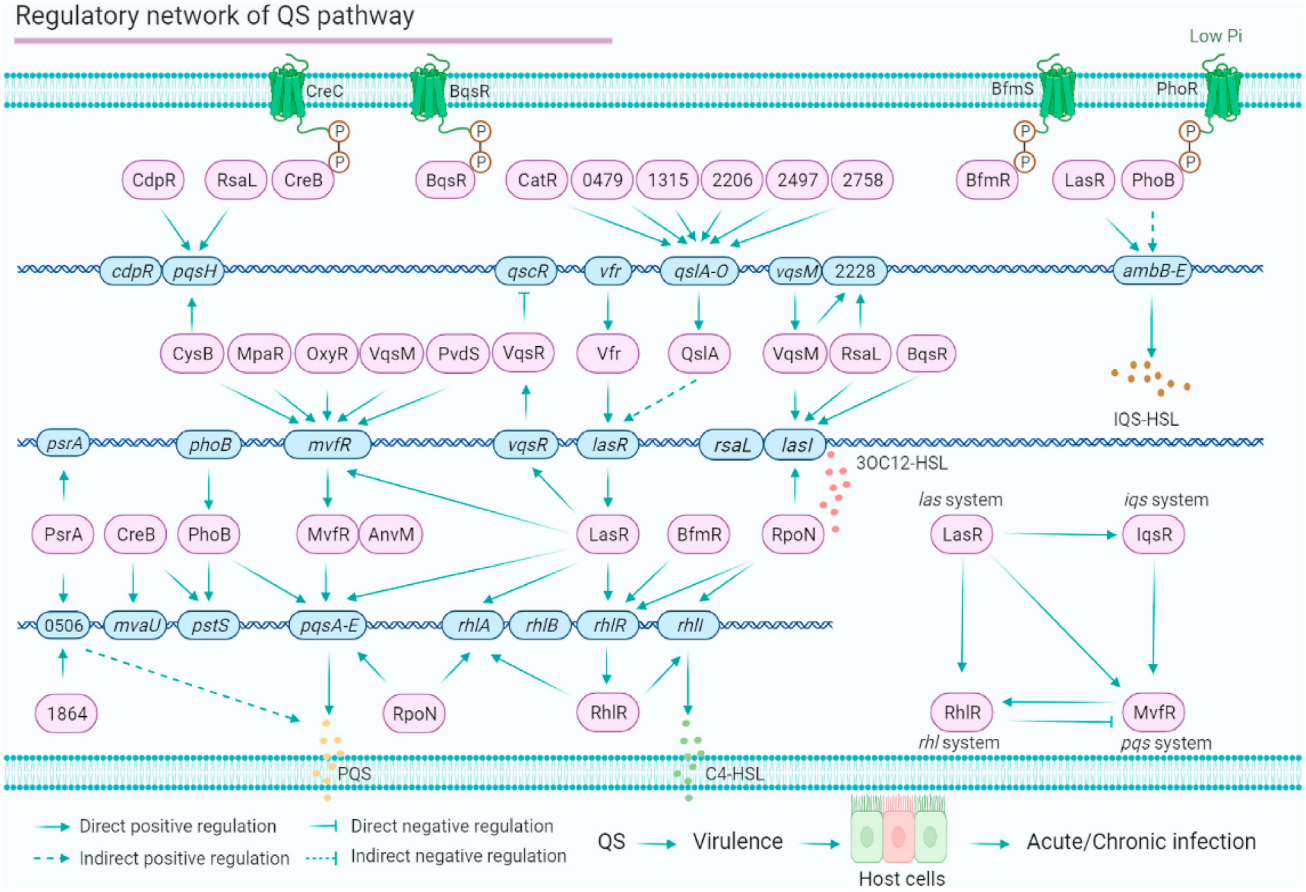

Figure 1 The QS pathway regulatory network of *P. aeruginosa*. The QS consists of four systems, the Las system, Rhl system, Pqs system and Iqs system, which are regulated by more than 28 transcriptional regulators with daedal networks. This regulatory network indicates the binding target genes and the crosstalk among these regulators.

QS, directly controls the gene expression of Las, Rhl, Pqs, and Iqs.[3,40–44] LasR binds to the *lasI* promoter to activate the production of 3-oxo-dodecanoyl-HSL (C12). C12 can bind to the N-terminal of LasR to enhance the DNA-binding affinity of LasR to the *lasI* promoter as well as the promoters of Rhl and Pqs via a positive-feedback mechanism. LasR also directly binds the *ambB-E* promoter to control IQS.[20] Vfr and AmpR specifically regulate the expression of *lasR*.[45,46] PtxR inhibits the transcription levels of the *pqsA-E* operon and *rhlI* and activates *lasI*.[47] QscR inhibits the expression of *lasI* and thus delays the transcription of QS genes.[48] QteE can lock the QS by destabilizing LasR and independently reducing RhlR levels when the cell density (OD_{600}) is higher than 2.3.[49] In addition, CspC can upregulate Las-QS by binding to the 5′ UTR (untranslated regions) of *rsaL* mRNA.[50]

We and our collaborators have determined the direct binding sites of eight QS-related regulators, VqsR, VqsM, AlgR, CdpR, RsaL, CysB, AnvM and RpoN.[25,27,28,32–36] The functions of these regulators overlap with other virulence-related pathways. For instance, VqsM simultaneously mediates Las-QS, the T3SS and antibiotic resistance by binding to the promoters of *lasI*, *exsA*, and *nfxB*, respectively.[28] CdpR, an important QS regulator, interacts with the ClpAS-P system to regulate virulence factors and pathogenicity.[34] RpoN has a direct regulatory effect on all three QS systems as well as T6SS.[27] AnvM, encoding a hypothetical protein, mediates pathogenicity under anaerobic conditions and host defences.[51]

We used a genome-wide network-based approach to uncover the crosstalk of 20 key virulence-related TFs and constructed a genomic regulatory network (PAGnet) that includes 347 direct target genes and their regulatory relationships.[26] Our HT-SELEX data show that PA2497 directly regulates the expression of *qslA*, which encodes a QS anti-activator. QslA is a negative regulator of multiple virulence phenotypes, including QS (*lasR*, *rhlR* and *rhlI*), H2-T6SS (*hsiA2*), T2SS (*xcpP*), elastase and pyocyanin in PAO1.[52] PA2206, PA2758, PA1315, PA0479, and CatR are identified as upstream regulators of *qslA*,[31] revealing a new regulatory network upstream of the QS. PA1864 and PsrA directly co-regulate PA0506, which encodes a probable acyl-CoA dehydrogenase involved in PQS,[31,53,54] CreB can bind to the promoter of *pstS*. Thus, we conclude that the QS architecture is controlled by a regulatory network containing 28 regulators (Fig. 1). Among these QS-related regulators, 12 co-target genes (*pqsH*, *qslA*, PA2228, *mvfR*, *lasR*, *lasI*, PA0506, *pstS*, *pqsA*, *rhlA*, *rhlR* and *rhlI*) are regulated by at least two regulators, indicating multiple co-regulatory networks in the QS (Fig. 1). For example,

qslA is co-regulated by six TFs, and *mvfR* is co-regulated by five TFs.

Pyocyanin biosynthesis is controlled by 19 TFs

Phenazines are a class of antibiotics secreted by multiple *Pseudomonas* spp., displaying a broad spectrum of antimicrobial activity to various organisms.[12] The phenazine blue pyocyanin is only secreted by *P. aeruginosa*. Pyocyanin is one of the key virulence factors in *P. aeruginosa* and is involved in both acute and chronic infections.[55,56] Pyocyanin biosynthesis is encoded by two gene clusters, *phzA1-phzG1* and *phzA2-phzG2* (Fig. 2). It has been reported that *phzA1* is directly regulated by LasR, RhlR, RsaL, BfmR and CzcR,[22,57,58] and *phzA2* is directly bound by RhlR and AmrZ.[29] Some pyocyanin repressors, such as QscR, PtxR, QteE and sigma factor RpoS, have also been reported. QscR, a quorum-sensing control repressor, negatively regulates pyocyanin production.[48] PtxR only inhibits *phzA1-G1* expression but not *phzA2-G2* expression.[47] Our SELEX assay showed that PA1314 directly binds to the promoter of *phzH* (encoding a potential phenazine-modifying enzyme).[31] CreB binds to the promoter of *mvaU*, which is involved in pyocyanin synthesis.[59] HuxI activates the production of pyocyanin.[60] QteE inhibits pyocyanin production and QS-related phenotypes in *P. aeruginosa*.[61] RpoS inhibits the expression of *phzA1* but activates the expression of *phzA2* at the post-transcriptional level, which might be due to the diverse host environments.[36] Because the production of pyocyanin is regulated by QS, the crosstalk among multiple pyocyanin-related regulators is common in *P. aeruginosa*'s regulatory network. For instance, LasR, RhlR, RsaL, BfmR, CzcR, PtxR and QteE share their targets in the QS, T3SS and T6SS. In conclusion, the two gene clusters (*phzA1-G1* and *phzA2-G2*) involved in pyocyanin biosynthesis are stringently controlled by these global regulators (Fig. 2). Similar to biocontrol bacteria, such as *Bacillus* spp., *Lysobacter* spp. and *Pseudomonas* spp., pyocyanin produced by *P. aeruginosa* can also be called a 'long-range weapon' as it is indirectly toxic to host cells, independent of cell—cell contact.[62]

P. aeruginosa employs a regulatory network to regulate motility

P. aeruginosa has a full set of motility-related genes that control flagella and type IV pili, which are required for swarming, swimming, and twitching motility. Six TCSs and a chemosensory system are involved in the motility-related regulatory network, including FleS/R, GacS/A, CreC/B, CarS/R, PilS/R, AlgZ/AlgR and the Chp system (Fig. 3). In the motility regulatory network, the Gac/Rsm signalling

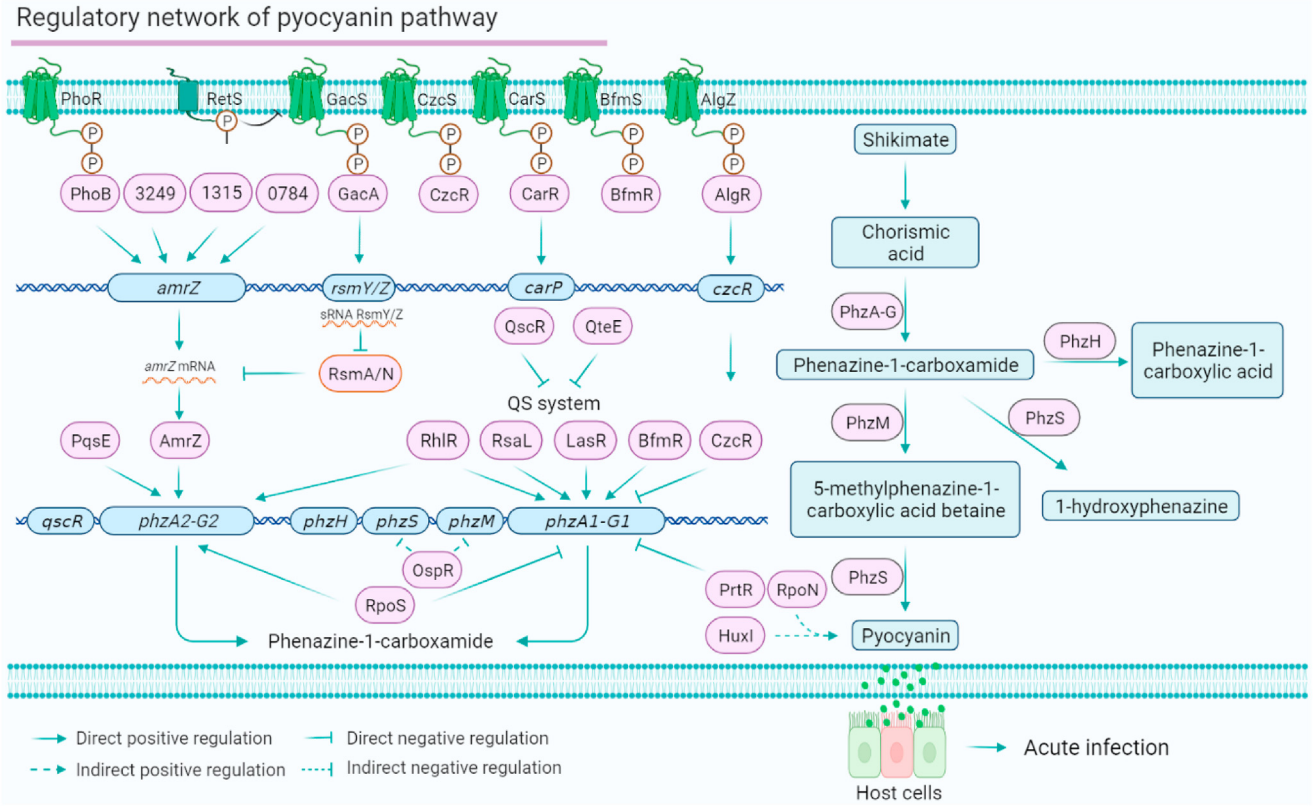

Figure 2 The pyocyanin synthesis pathway regulatory network of *P. aeruginosa*. (**Left panel**) The pyocyanin synthesis regulatory network is mediated by 19 regulators (PhoB, PA3249, PA1315, PA0784, GacA, CarR, PqsE, AmrZ, QscR, QteE, RhlR, RsaL, LasR, BfmR, CzcR, RpoS, PrtR, AlgR and RsmA/N) and three co-target genes (*amrZ*, *phzA1-G1* and *phzA2-G2*). (**Right panel**) The biosynthesis pathway of phenazines (including pyocyanin, 1-hydroxyphenazine and phenazine-1-carboxylic acid).

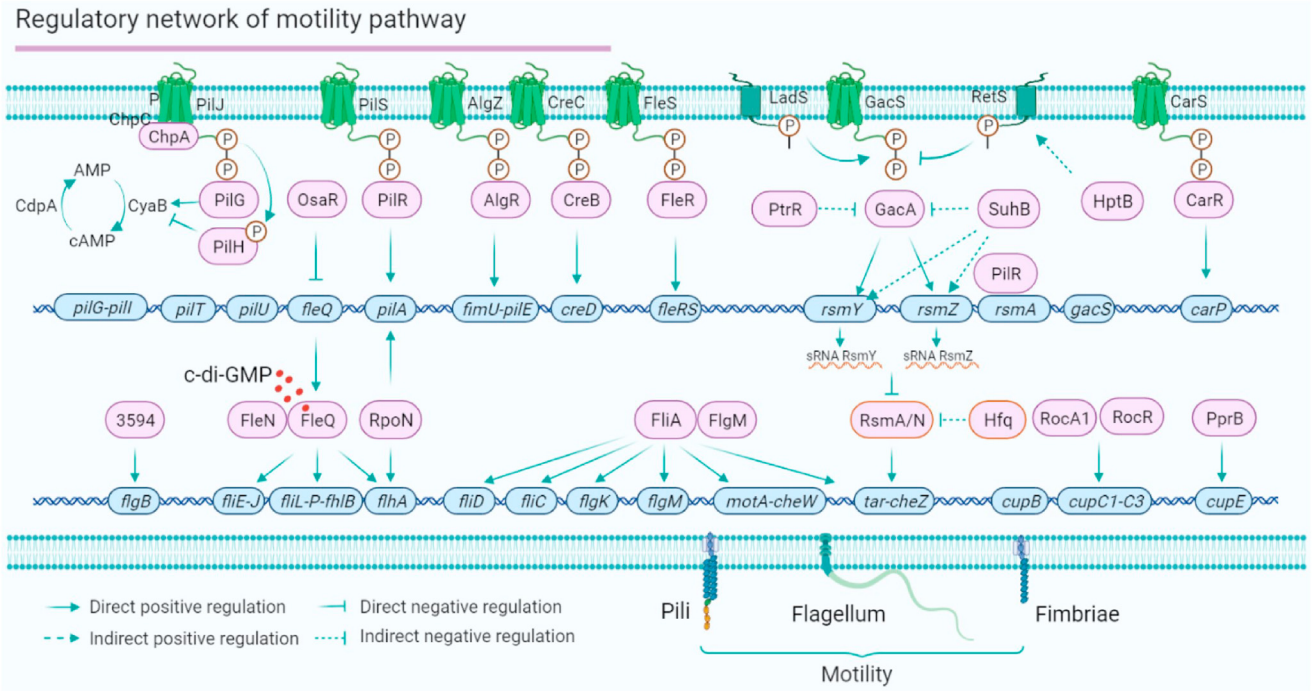

Figure 3 The motility regulatory network of *P. aeruginosa*. *P. aeruginosa* motility includes swarming, swimming and twitching. Swarming motility is driven by flagella, which are controlled by FleQ, two sigma factors (FliA and RpoN), two anti-sigma factors (FleN and FlgM), PA3594, and the Gac/Rsm signaling system. Swimming and twitching motility are driven by the type VI pili, which is regulated by PilRS, AlgRZ, RpoN and the Chp system. RocA1/RocR and PprB regulate the fimbriae, which are required for adherence. The motility network is mediated by 16 direct transcription regulators (such as FleQ, RpoN and PilR) and shares three co-target genes (*rsmY/Z*, *flhA* and *tar-cheZ*).

pathway functions as a global regulatory system that mediates *P. aeruginosa* motility. PtrR, SuhB and HptB inhibit the GacA/S system. Sigma factors and anti-sigma factors provide another level of motility regulation. Sigma factor FliA directly binds to the promoters of *fliD*, *fliC*, *flgK*, *motA-cheW* operon and *tar-cheZ* operon.[63,64] The function of FliA is also affected by the anti-sigma factor FlgM.[65] The sigma factor and global regulator RpoN directly regulate the expression of *pilA* and *flhA* to control flagellar motility. Vfr directly binds to the promotor of *fleQ* (encoding the major flagellar regulator).[66] FleQ binds to the promoters of *flhA*, *fliE-J* operon and *fliL-flhB* operon. The binding affinity of FleQ is mediated by the anti-sigma factor FleN and the intracellular c-di-GMP level.[67] PA3594 can directly regulate the transcription of *flgB*.[31] Thus, 16 regulators directly regulate the motility behaviours of *P. aeruginosa* (Fig. 3). Three target genes (such as *pilA*, *flhA* and *tar-cheZ*) are co-regulated by at least two regulators, such as FleQ, RpoN, FliA and RsmA/N, which have been proposed to be master regulators in the *P. aeruginosa* motility regulatory network.

Twenty-eight TFs form a complex biofilm-related regulatory network

P. aeruginosa biofilm formation is one of the chief culprits of chronic infection in the respiratory tract of cystic fibrosis patients and has adaptive resistance to antibiotics in hospitals. *P. aeruginosa* biofilms act as a helper to improve the living environment and assist the virulence system to adapt to the complex microbial gaseous-fluid environment of the respiratory tract. *P. aeruginosa* biofilm formation is coordinated by multiple mechanisms involving more than 20 regulators, such as PhoB, GacA, AmrZ, FleQ, RsmA/N, BfmR, MucA, AlgU, AlgR, QsaR, PA4547 and PprB. These regulators have been individually studied, and their crosstalk has also been analysed.[18,26] BfmR positively regulates biofilm formation and cell death by activating the expression of *phdA*, which encodes prevent-host-death protein A.[68] All of these regulators are involved in TCS, QS, and c-di-GMP networks, which respond to the regulation and synthesis of biofilm matrix components alginate, Psl and Pel (Fig. 4).

The production of Psl and Pel is dynamically regulated by multiple virulence-related TFs. In Figure 4, we map the *P. aeruginosa* biofilm regulatory network, visualising the whole atlas constructed by several virulence-related TFs. The Gac/Rsm signalling pathway is the master mediator of *P. aeruginosa* biofilm formation. GacS senses the environmental signals to rapidly activate its kinase activity and then transfers its phosphate group to GacA. The phosphorylated GacA directly controls the expression of small RNAs (RsmY and RsmZ) that directly bind to RsmA to form a RsmYZ—RsmA complex. RsmYZ—RsmA subsequently binds to the mRNA of *amrZ* or the 5′ UTR of *pslA* mRNA to inhibit Psl production. The *pslA* promoter is also bound by LasR and the sigma factor RpoS. AmrZ binds to the *pelA* promoter to directly control Pel production. The expression of the *pelA-G* operon is positively regulated by PA0225.[69] The PA0225-deletion mutant produces less biofilm than the wild-type strain PAO1. The intracellular c-di-GMP concentration

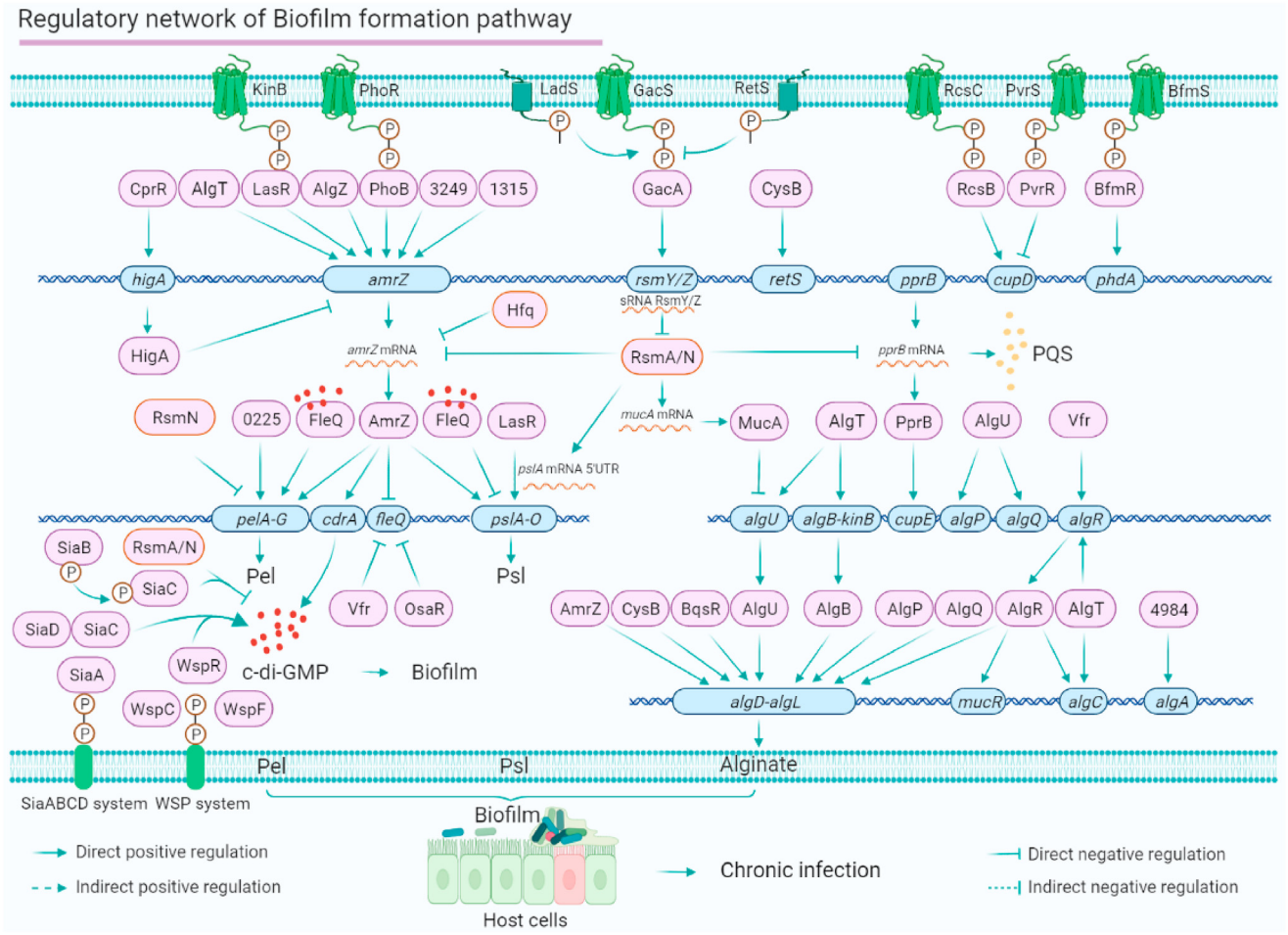

Figure 4 The biofilm formation pathway regulatory network of *P. aeruginosa*. The *P. aeruginosa* biofilm matrix mainly consists of three kinds of extracellular polysaccharides (Pel, Psl and alginate extracellular polysaccharide), which are synthesised by the *pelA-G* operon, *pslA-O* operon and *algD-L* operon, and is regulated by 29 direct transcription regulators. We have summarised the direct regulators involved in biofilm formation and re-mapped the regulatory network underlying these regulators. The network shows 10 co-target genes: *amrZ*, *cupD*, *pelA-G*, *fleQ*, *pslA-O*, *algU*, *algR*, *algA*, *algD-L* and *algC*.

dynamically regulates *P. aeruginosa* biofilm formation. The chemotactic system Wsp controls c-di-GMP production by sensing the extracellular environmental signals. WspR, a diguanylate cyclase, can receive phosphate groups from the Wsp system to enhance the intracellular concentration of c-di-GMP. Meanwhile, AmrZ directly regulates *cdrA*, which encodes an intracellular c-di-GMP concentration reporter. Interestingly, OsaR directly binds to the promoter of *fleQ* and inhibits its expression.[70] FleQ is a master regulator in the c-di-GMP-controlled biofilm pathway.[71,72] FleQ binds to the promoters of *pelA*, *pslA* and *cdrA*. FleQ and c-di-GMP can form a complex that mediates the bacterial behaviour between motility and biofilm formation. At low c-di-GMP concentrations, FleQ which is not bound by c-di-GMP interacts with the promoter of flagellar genes (such as *fleS* and *fliF*) to activate the flagella to promote bacterial motility.[71] At high c-di-GMP concentrations, more c-di-GMP/FleQ complex is formed, which shows stronger affinity to the promoters of biofilm synthesis genes, such as *pslA* and *pelA*.[71] As a result, c-di-GMP/FleQ positively regulates the expression of the *pel* operon, and negatively regulates the *psl* operon, showing that c-di-GMP/FleQ is a double-edged sword for biofilm formation.[71,73]

Alginate biosynthesis is another major biofilm matrix component that is directly regulated by eight TFs. RsmA binds to the *mucA* mRNA to control the expression of MucA, which can directly bind to the promoter of *algU*.[74,75] AlgU directly controls alginate biosynthesis by binding to the *algD-L* operon promoter. More than 10 TFs directly regulate the transcription activity of *algD*. Among them, the global regulator AmrZ binds to the *algD* promoter. TCS response regulators AlgB, AlgR and BqsR also directly control *algD* expression via the various signals transmitted by their corresponding sensor kinases. The TCS AlgB-KinB is required for biofilm formation in different regulatory pathways.[76,77] Meanwhile, sigma factors AlgU, AlgT and RhlR co-regulate *algB-kinB* expression. Other alginate-related genes can be directly regulated by several TFs. For instance, *algA* is regulated by PA4984, and AlgU co-regulates the expression of *algP* and *algQ*.

Adherence is an early step of biofilm formation. Adhesive factors required for biofilm formation are globally

regulated by RsmA/N, which binds to *pprB* mRNA to positively regulate its expression.[78] PprB regulates bacterial adherence and biofilm maturation by directly binding to the *cupE* promoter. TCSs RcsBC and PvrRS show opposite effects on *cupD* expression,[79] which may be caused by their opposite sensing signals. Here, we show that 30 regulators can directly regulate the expression of biofilm-related genes (Fig. 4). Additionally, 10 co-target genes, *amrZ*, *cupD*, *pelA-G*, *fleQ*, *pslA-O*, *algU*, *algR*, *algD-L*, *algC* and *algA*, are included in this network (Fig. 4).

The regulatory network of the T3SS mediates *P. aeruginosa* virulence

P. aeruginosa injects T3SS exoenzyme effectors into host cells, causing ventilator-associated pneumonia.[80–82] Although previous studies have investigated the regulation of the Vfr–ExsA–T3SS pathway and the functions of effectors, the complete regulatory network of the T3SS has yet to be uncovered. Here, we map an up-to-date regulatory network of the T3SS in *P. aeruginosa* based on the direct regulation between TFs and their targets involved in the T3SS. In the complex T3SS regulatory network, a group of TFs play important roles in mediating T3SS gene transcription, post-transcriptional modification and effector secretion. As shown in Figure 5, the T3SS is regulated by two main pathways, namely the Gac/Rsm–T3SS pathway and the Vfr–ExsA–T3SS pathway. In the Gac/Rsm–T3SS pathway, the global regulator GacA is strictly regulated by its sensor kinase GacS, which can be inhibited by RetS, but HptB (histidine phosphotransfer protein) is an activator of RetS.[83] PtrR and SuhB are two GacA repressors. MvaT/U represses the expression of *rsmY*,[84–86] and *rsmZ* is

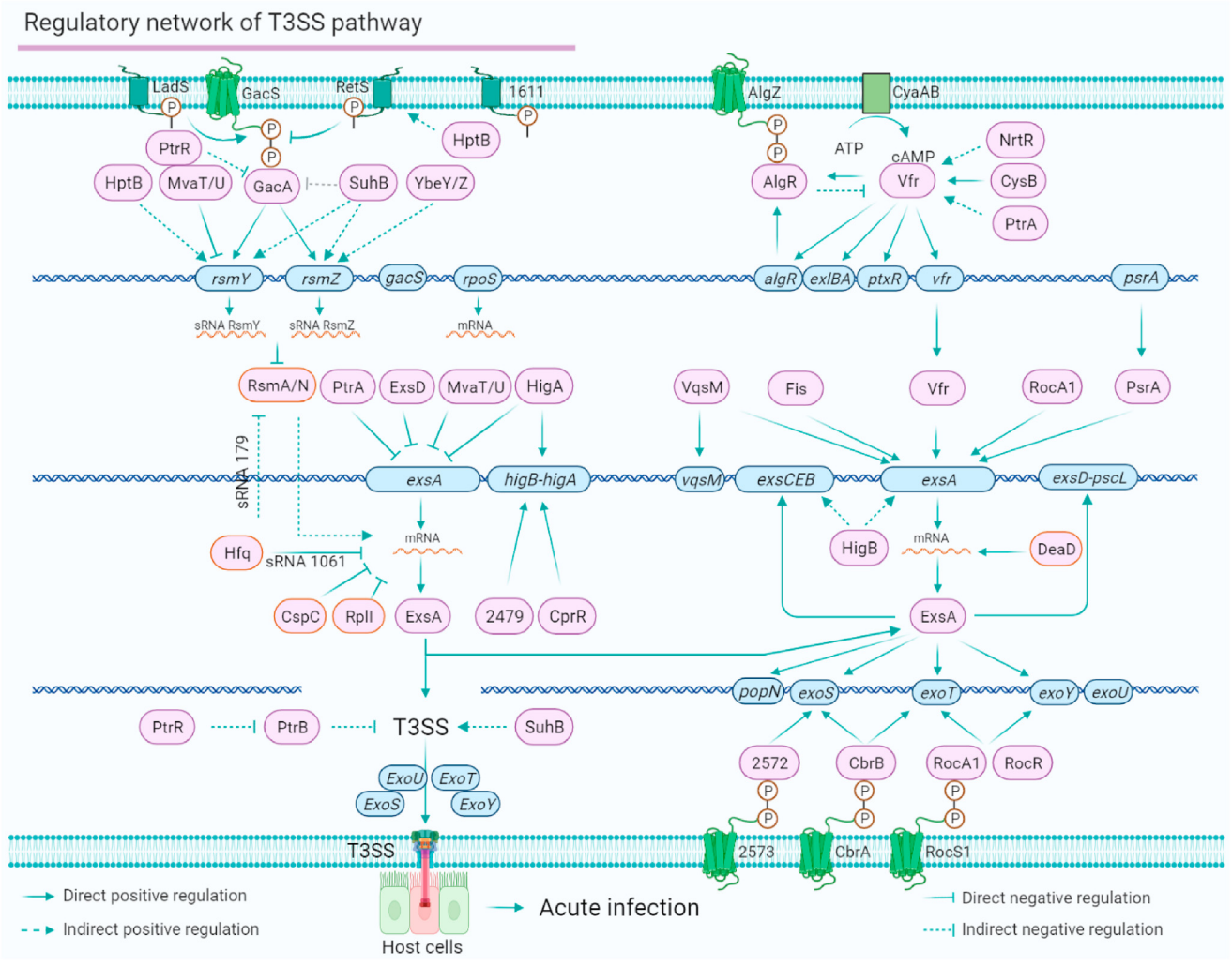

Figure 5 The T3SS pathway regulatory network of *P. aeruginosa*. The T3SS in *P. aeruginosa* is regulated by two main regulatory signalling systems, Gac/Rsm-ExsA-T3SS and cAMP/Vfr-ExsA-T3SS. ExsA is the master regulator of the T3SS. The expression of ExsA is inhibited by PtrA, ExsD, MvaT/U and HigA at the transcriptional level and by Hfq, CspC and RpII at the post-transcriptional level. ExsA acts as a co-target that can be directly activated by five regulators, VqsM, Fis, Vfr, RocA1 and PsrA. The downstream T3SS effectors (such as *exoS*, *exoT* and *exoY*) are also directly regulated by ExsA, PA2572/PA2573, CbrAB and RocS1/A1. The T3SS is directly mediated by 21 direct regulators and six co-target genes (*rsmY/Z*, *exsA*, *higB-A*, *exoS*, *exoT* and *exoY)*.

controlled by GacA, SuhB, and YbeY/Z.[87] In the Vfr—ExsA—T3SS pathway, the DNA-binding affinity of Vfr is affected by the level of cAMP, which is controlled by CyaAB. Vfr is also positively regulated by NrtR,[88] CysB and AlgR.

As the master regulator of the T3SS in *P. aeruginosa*, the self-feedback TF ExsA is mediated by six positive regulators (Vfr, VqsM, Fis, RcoA1, PsrA, and HigB) and four negative regulators (PtrA, ExsD, MvaT/U, and HigA) at the transcriptional level. ExsA is positively regulated by DeaD[89] but negatively regulated by Hfq,[90] CspC,[91] and RplI[69] at the translational level. In addition, the transcription of T3SS effectors can be directly regulated by a group of TFs. TCSs PA2573/PA2572 and CbrAB directly regulate the transcription of *exoS*.[22] RocS1-RocA1/RocR and CbrAB directly regulate the transcription of *exoT*, and PA4080 directly regulates the expression of *exoU*.[22] Therefore, the T3SS is mediated by a complex TF-dependent network that controls the T3SS device assembly, gene regulation, and effector secretion by responding to signals from host cells (Fig. 5). Twenty-one regulators directly regulate the expression of T3SS genes. Six target genes (*rsmY/Z*, *exsA*, *higB-A*, *exoS*, *exoT*, and *exoY)* are co-regulated by more than two regulators.

The T6SS regulatory network enhances *P. aeruginosa* virulence and competitiveness

The bacterial T6SS is a powerful weapon that injects toxic effectors into eukaryotic host cells and prokaryotic competitor bacteria, causing host damage or providing a competitive advantage in diverse microbial environments. The *P. aeruginosa* genome harbours three T6SS gene islands,[92] H1-T6SS, H2-T6SS, and H3-T6SS. Here, we review the TF-dependent networks involved in T6SS regulation (Fig. 6). First, the T6SS is mainly regulated by the Gac/Rsm-T6SS signalling system. The RNA-binding proteins RsmA/N directly bind to *tssA1* and *fha1* mRNAs.[78,93] Meanwhile, NrtR inhibits H1-T6SS expression by binding to the promoters of *rsmY*, *rsmZ* and *tssA1*.[94] Second, the T6SS is regulated by the PhoB-AmrZ-T6SS pathway. AmrZ is regulated by a group of TFs, including PhoB, PA3249, PA1315, AlgZ and AlgT. AmrZ directly binds to the promoter of *fleQ*, and FleQ directly inhibits the expression of two H1-T6SS effector genes (*hcp1* and *tse1*) via a c-di-GMP-dependent mechanism.[95] In addition, our recent study reveals that AnvM positively regulates the expression of *hsiA2*,[51] suggesting the regulatory role of AnvM in H2-T6SS. RpoN directly binds to the promotors of T6SS effectors *hcpA* and *hcpB* to

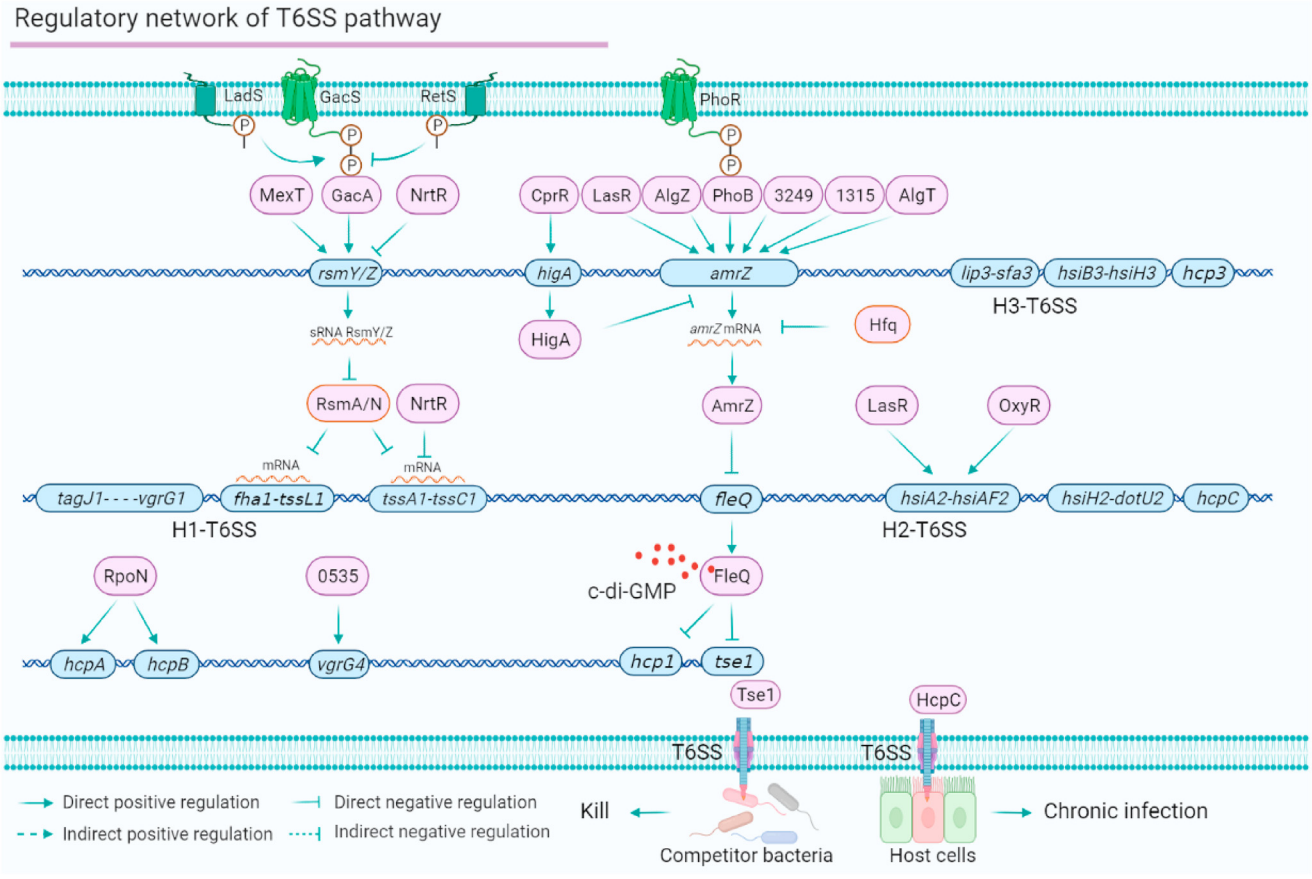

Figure 6 The T6SS pathway regulatory network of *P. aeruginosa*. The T6SS signalling system is mainly controlled by Gac/Rsm-H1-T6SS and AmrZ-FleQ-H1-T6SS, and is responsible for providing competitive advantages in environments with diverse bacteria. RpoN directly binds to the promoter of two effector genes (*hcpA* and *hcpB*). H2-T6SS is directly regulated by LasR and OxyR. Effector HcpC can be secreted in host cells to cause chronic infection. The T6SS network is mediated by 18 regulators and four co-target genes (*rsmY/Z*, *tssA1-C1*, *amrZ* and *hisA2-F2*).

positively regulate their expression.[27] Additionally, PA0535 directly regulates the expression of *vgrG4*. Eight regulators (RsmA/N, NrtR, AmrZ, LasR, OxyR, RpoN, PA0535 and FleQ) directly regulate T6SS genes (Fig. 6). The T6SS is directly mediated by relatively few regulators compared with the regulatory networks described in previous sections. These TFs are regulators of H1-T6SS and H2-T6SS; however, the regulators involved in H3-T6SS remain largely elusive.

The regulatory network of *P. aeruginosa* oxidative stress response

To maintain sustained virulence, bacterial pathogens must overcome the reactive oxygen species (ROS) barriers produced by the host immune system, such as H_2O_2 and —OH. Many ROS-sensing components have been reported, including various ROS-deactivating enzymes, such as catalase, superoxide dismutase, thioredoxin, and glutaredoxin. Pathogenic bacteria use multiple TFs that harbour ROS-sensing cysteines, such as OxyR, MgrA, OhrR, and SarA, to respond to and overcome host-derived ROS.[96,97,98] In *P. aeruginosa*, five regulators and three metabolic proteins have been reported to sense oxidation stress, including LasR, SoxR, MexR, OxyR, OspR, ExaC, ArcA and GapA (Fig. 7). SoxR directly regulates the expression of three genes, including *mexGHI-ompD* operon (involved in QS signal homeostasis), PA3718, and PA2274.[99] SoxR can be activated by the redox-active antibiotic pyocyanin.[100] OxyR directly upregulates antioxidant genes to defend against H_2O_2 stress as well as iron uptake.[101,102] OxyR also positively regulates swarming motility and rhamnolipid production and negatively regulates pyocyanin production, which may have a protective effect on the *oxyR* mutant.[103] OspR is also found to regulate phenazine biosynthesis, beta-lactam resistance, and dissemination in a mouse acute infection model.[104] The other oxidation-sensing regulator, MexR, regulates antibiotic resistance by binding to the promoter of the *mexAB-oprM* operon.[105] In addition, we have discovered ~200 ROS-sensing proteins in *P. aeruginosa* and identified four roles of oxidation-sensitive cysteines: i) Cys79 is required for the oxidation response of LasR; ii) H_2O_2 inhibits the activity of ExaC; iii) H_2O_2 activates the activity of ArcA; iv) GapA functions as a redox-sensing metabolic switch. Pathogenic bacteria use multi-layered responses to ROS to rapidly adapt to oxidative stress.[106] Cys201 and Cys203 are also required for LasR to sense oxidative stress.[107,108] Taken together, the ROS regulatory network is essential for maintaining *P. aeruginosa* virulence and overlaps with metabolic pathways and antibiotic resistance.

SELEX and DAP-seq predict interactions between novel TFs and virulence

Although the regulatory atlas of virulence-related transcription regulators in *P. aeruginosa* has been studied for more than three decades, the direct and specific binding targets and binding patterns have still not been fully uncovered. Recently, we have used an HT-SELEX assay to predict 32 uncharacterized TFs with new regulatory roles in multiple virulence pathways.[31] Moreover, the binding motifs of 55 TCS response regulators and their crosstalk were

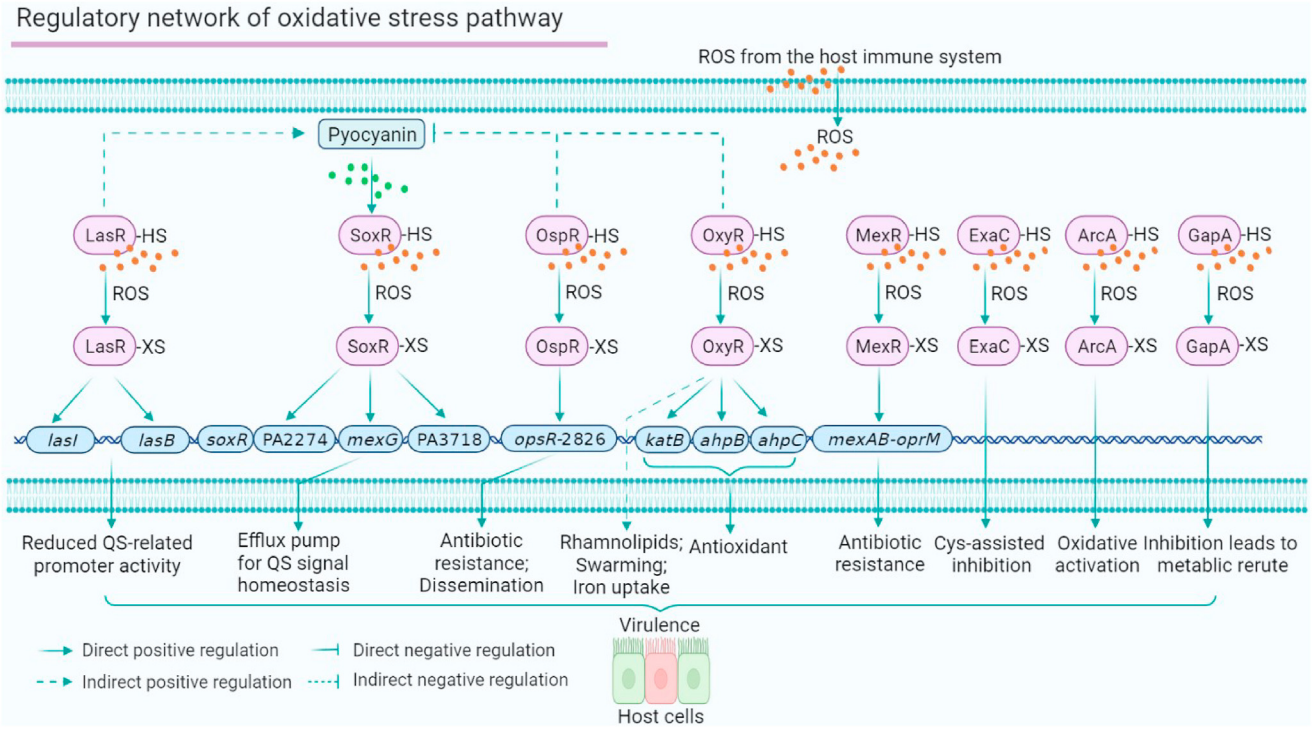

Figure 7 The oxidative stress response regulatory network of *P. aeruginosa*. Five TFs (LasR, SoxR, MexR, OxyR and OspR) involved in the QS, antibiotic resistance and motility sense ROS and translocate the signals to the downstream virulence-related phenotypes. Three metabolic proteins (ExaC, ArcA and GapA) also sense and respond to ROS from the host immune system.

revealed using DAP-seq.[22] Here, we summarise the potential TF-related regulatory network involved in these virulence pathways. We mainly focus on the predicted regulatory networks involved in the QS, biofilm formation, motility, T3SS, and T6SS.

We recently predict the new functions of five TFs involved in the QS.[31] For example, PA1241 is identified as a potential QS regulator that binds to the promoters of *anr*, *anvM*, *pqsH*, and *cdpR*. SouR can bind to the promoters of *lecB*, *cdpR*, *pqsH*, *phzA2* and *lasR*, suggesting potential roles in the QS. DAP-seq also reveals that 14 response regulators are involved in QS regulation.[22] For example, the *mvfR* promoter harbours the binding motifs of BqsR, KdpE, and AmgR.[22] CzcR is predicted to bind to the *phzA1* promoter, and seven TFs are predicted to mediate biofilm formation.[31] *rsmY/Z* was predicted to be regulated by a group of TFs identified by DAP-seq, including CopR, PirR, AgtR and CzcR.[22] The new functions of the 10 TFs involved in T6SS are predicted by SELEX.[31] For example, SouR is predicted to bind to the promoters of *tssA1*, *fha1*, and *hsiH2*. PA1241 is predicted to bind to the promoters of *anvM*, *hcpB*, *hsiA2*, and *lipA*. Eight TFs are predicted to regulate motility.[31] DAP-seq results indicate that PirR is a direct regulator of *fleQ*.[22] DAP-seq also suggests new functions of 7 TFs related to T3SS. RocA1, ErbR, BfmR, PilR, PA4080, AgtR and PirR have conserved potential binding sites in the *exsA* promoter of *P. aeruginosa* PA14.[22] In summary, SELEX and DAP-seq predict the involvement of 19, 13, 10, 15, and 7 potential TFs in the pathways of the QS, biofilm formation, T6SS, motility, and T3SS, respectively.

Conclusions and future perspectives

In this review, we summarise the seven main virulence-associated regulatory networks of *P. aeruginosa*, the QS, pyocyanin synthesis, motility, biofilm formation, T3SS, T6SS and oxidative stress response networks, revealing the global network and crosstalk between multiple pathways. We conclude that the virulence regulatory network is dynamically regulated according to environmental signals and the host immune system. The binding sites for half of the TFs have been identified using technologies such as PBM, ChIP-seq, HT-SELEX, Bind-n-seq, and DAP-seq,[22,26,31] but the binding sites of the remaining TFs remain elusive.

Nevertheless, the abovementioned technologies each have shortcomings: i) ChIP-seq data are limited by antibody specificity and indirect protein-DNA binding; ii) HT-SELEX and Bind-n-seq data do not generate native genomic motif-generating sequences and lack secondary DNA modifications *in vitro*; and iii) although DAP-seq uses the genomic DNA library, the binding reaction is performed *in vitro*. Therefore, it is worth exploring how to accurately identify binding sites by establishing *in vitro* bacterial cell models that better mimic *in vivo* environments. For instance, TFs can be expressed in the native bacterial cells, and cell lysates can be used as reaction conditions for binding between TFs and the genomic DNA library. This strategy not only mimics the *in vivo* environment but identifies transcriptional enhancers, which are short DNA sequences with TF-binding sites.[109] TF binding may be controlled by specific conditions, such as carbon or nitrogen resources, microelements, ROS, and host defences. A recent review noted that when host cells perceive pathogen invasion, immune cells affect a change in the metabolic pathway.[110] Immune cells promote anti-inflammatory and antibacterial reactions, which can act as host immune signals to activate virulence gene expression in pathogens. This interaction between immune cells and pathogens suggests that the functional study of *P. aeruginosa* TFs and pathogenic mechanisms should not be limited to the culture environment or *in vitro* biochemical reactions but also explore co-interactions with host immune cells. A comprehensive analysis of binding sites and transcription regulator functions would provide new insights into *P. aeruginosa* virulence-associated and metabolic networks.

Based on these virulence-associated TFs, we propose the following directions to develop effective strategies against *P. aeruginosa* infection. i) Metabolic networks are related to virulence-associated pathways in *P. aeruginosa*,[111,112] thus, targeting metabolic networks is a promising antimicrobial strategy.[111,113–115] ii) Targeting virulence-associated regulators is another approach to prevent infections caused by *P. aeruginosa*.[18] High-throughput screening assays based on microtiter plates or transcriptional reporters are well used for identifying new drugs to target specific virulence-associated TFs.[116–121] In addition, *in silico* computational screening method is also a good approach to identify new inhibitors for virulence factors, such as LasB, LasR, PqsA, PqsR, and SagS.[122–128] A master regulator analysis based on the virulence networks (such as PAGnet) can be used to prioritize these transcription regulators.[26] The obtained master regulators can be preferentially used to screen antimicrobial drugs via the high-throughput screening assays abovementioned. The antimicrobial activities of these candidate drugs can be evaluated by phenotypic analysis. Besides, the combined therapy of candidate drugs and existing antibiotics may have a more synergistic effect on *P. aeruginosa* infections. iii) The regulation of post-transcriptional modification is a promising technique for preventing infections caused by prokaryotic pathogens. Inducing RNA methylation, such as by N^6-methyladenosine, is another possible approach for overcoming the virulence of *P. aeruginosa*.[129] iv) Secondary structure modifications (such as G-quadruplex) provide new perspectives for studying the infections caused by *P. aeruginosa*. The specific secondary structures of RNA affect the transcription and translation of virulence genes. One hundred and sixty-one RNA G-quadruplex (rG4) sites at the genome-wide level have been identified using rG4-seq. Among them, 6.9% of rG4 sites are located in virulence-related genes, such as those involved in the T3SS, T6SS, QS, motility and biofilm formation.[130] For instance, rG4 mediates biofilm production and motility by enhancing *bswR* expression.[130] Thus, RNA secondary structure in virulence-related TFs warrant further study. Nevertheless, the above proposed potential strategies are still in laboratory trials and are far from clinical application, especially in the regulation of post-transcriptional and secondary structure modifications. Many QS-deficient variant strains, such as *lasR*, *rpoN*, *mucA*, *rhlRI* and *mexT* mutants, have been isolated clinically, and they still remain virulent.[3,131–139] Although QS inhibitors may not be suitable antimicrobial

targets for *P. aeruginosa* clinical isolates, specific anti- *P. aeruginosa* therapies are needed for these clinical isolates.[18] For example, combination therapies of antimicrobials and antivirulents also show promising synergistic antibiofilm effects.[140—142]

Overall, this review summarises the seven main virulence-associated regulatory networks in *P. aeruginosa* and the direct regulatory network of transcription regulators. Many TFs are predicted to be involved in virulence pathways, which will provide potential insight into new interactions among TFs and target genes. We propose that the whole metabolic network, in combination with post-transcriptional RNA secondary structure, contributes to the regulation of *P. aeruginosa* virulence.

Conflict of interests

The authors declare no conflict of interests.

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