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Mini review

Virulence-related regulatory network of *Pseudomonas syringae*

Jiadai Huanga, Chunyan Yaoa, Yue Suna, Quanjiang Jic, Xin Denga,b,*

a Department of Biomedical Sciences, City University of Hong Kong, Kowloon Tong, Hong Kong SAR 999077 China
b Shenzhen Research Institute, City University of Hong Kong, Shenzhen 518057, China
c Gene Editing Center, School of Life Science and Technology, ShanghaiTech University, Shanghai 201210, China

**Abstract**

Transcription factors (TFs) play important roles in regulating multiple biological processes by binding to promoter regions and regulating the global gene transcription levels. *Pseudomonas syringae* is a Gram-negative phytopathogenic bacterium harbouring 301 putative TFs in its genome, approximately 50 of which are responsible for virulence-related gene and pathway regulation. Over the past decades, RNA sequencing, chromatin immunoprecipitation sequencing, high-throughput systematic evolution of ligands by exponential enrichment, and other technologies have been applied to identify the functions of master regulators and their interactions in virulence-related pathways. This review summarises the recent advances in the regulatory networks of TFs involved in the type III secretion system (T3SS) and non-T3SS virulence-associated pathways, including motility, biofilm formation, quorum sensing, nucleotide-based secondary messengers, phytotoxins, siderophore production, and oxidative stress. Moreover, this review discusses the future perspectives in terms of TF-mediated pathogenesis mechanisms and provides novel insights that will help combat *P. syringae* infections based on the regulatory networks of TFs.

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*Corresponding author at: Shenzhen Research Institute, City University of Hong Kong, Shenzhen 518057, China.
E-mail address: xindeng@cityu.edu.hk (X. Deng).*
1. Introduction

*Pseudomonas syringae* is a model phytopathogenic bacterium and can infect many plants, including major crops. To establish infection and incite disease in plants, *P. syringae* first enters the host plant, overcomes its immune system, and then causes disease [1]. *P. syringae* uses flagella- and type IV pili-mediated motility and performs active taxis for entering the host cells [2,3]. Once *P. syringae* cells enter the apoplasts, they secrete a group of hypersensitive response and pathogenicity (Hrp) type III secretion system (T3SS) effectors and phytotoxins to suppress host resistance and enhance disease development [4-6]. Additionally, *P. syringae* can exert its virulence via biofilm formation [7], siderophore production [8], and c-di-GMP-mediated signaling [9]. To successfully infect plants and cause disease, bacteria require many regulators, including nucleotide-based secondary messengers and transcription factors (TFs). Herein, we summarise the integrative virulence-related TFs and complex regulatory networks, providing insights into the regulatory mechanisms of *P. syringae* pathogenesis.

TFs bind genomic DNA and play crucial roles in regulating numerous biological pathways [10]. Several important virulence-related TFs have been previously identified and characterised in *P. syringae* [11–15]. For example, RhpRS, a two-component system (TCS), has been identified as a master regulator of T3SS genes by directly regulating hrpRS and other virulence-related genes by sensing environmental and polyphenol signals in plants [16–18]. T3SS genes are regulated by at least 12 additional regulators, including sigma factors (HrpL, RpoN, and AlgU) [16,19–21] and other regulators (AefR, CorR, HrpA, Lon, HrpV, GacA, CvsR, PrrA, and RhpPC) [22–30]. In recent years, owing to the development of research technologies, including RNA sequencing (RNA-Seq), chromatin immunoprecipitation sequencing (ChIP-Seq) [31-33], and high-throughput systematic evolution of ligands by exponential enrichment (HT-SELEX) [34], new regulators have been identified and predicted in *P. syringae* virulence-related pathways. We map the genome-wide regulons of 16 key virulence-related transcriptional regulators and designated their croststalk as an intricate network named ‘PSNNet’. We find five additional T3SS regulators (EnvZ-OmpR, ChrAB2, PhoPQ, PrrA, and MgrA) in this network [31]. In another study, we have identified binding motifs for 100 of the 301 putative annotated T3SS and non-T3SS pathways of *P. syringae* mainly relies on the T3SS [38], in the present review, we focus on summarising the critical and complicated regulatory networks related to the T3SS under different conditions (Figs. 1-2).

In *P. syringae*, T3SS activation largely depends on the HrpRSL (hrpRS and hrpL) pathway in planta or in MM. hrpR and hrpS are adjacent and located and share the same promoter that is present in front of hrpR. HrpR forms a hetero-hexamer and induces hrpL transcription under the assistance of ε32 (RpoN), which encodes an alternative RNA polymerase sigma factor [13]. HrpL binds the hrp box (GGACC-N15I5-GACANNA), which is found in the promoter regions of most T3SS effector genes. The induction of hrpRS operon transcription is mainly mediated by regulators such as AlgU, HrpA, and several TCss (RphR, CvsRS, and GacAS) [14,17,33,39,40]. For example, AlgU controls the transcription of hrpl, hrpRS, and other T3SS effector genes [32]. Moreover, HrpA can induce the expression of hrpRS, hrpL, and hrcC [39].

RhpRS is the best-studied master TCS that regulates T3SS genes and bacterial virulence. Our previous studies have shown that an hrpS mutant remarkably reduces disease severity [14,17,40,41]. hrpRS is located in the same operon as the hrpR operon. RhpS is a histidine kinase (HK) and functions as an autokinase upon itself and as a phosphatase on its cognate response regulator (RR) HrpR [14]. These components help *P. syringae* sense and respond to external environmental stress by harmonising T3SS gene expression. Phosphorylated HrpR (P-RhpR) has a high binding affinity to the inverted repeats at the 5’ end of the upstream hrpRS and directly suppresses hrpRS transcription. Our recent studies have identified three polyphenols (tannic acid; 1,2,3,4,6-pentagalloylglucose; and epigallocatechin gallate) that suppress both the kinase and phosphatase activities of RhpS in vitro and induce the expression of rhpRS operon by directly targeting RhpS.

Three above-mentioned polyphenols can be sensed by PrO40 of RhpS and repress its phosphatase activity, thereby inhibiting virulence through phosphorylating crosstalk with three other non-cognate HKs (PSPH_3550, 5115, and 3736) [18]. In T3SS-induced conditions, RhpS performs phosphatase activity on P-RhpR and thus reverses the inhibition of hrpRS operon, leading to induction of the downstream hrpRSL pathway. The non-cognate HKs phosphorylate HrpR, suppressing T3SS and virulence in *P. syringae*. CvsRS is a metal ion-sensing TCS, and its expression is induced by Ca2+, whose concentration is higher in the apoplast during bacterial infection [42]. CvsR can bind to the promoter of the hrpRS operon and activate T3SS-related genes expression in MM [26]. On the other hand, GacAS modulates *hrpL* transcription and contributes to hrpRS operon induction, which induces T3SS [39,43]. On the other hand, GacAS is considered a negative regulator of the T3SS in *P. syringae* and is not even required for its pathogenesis in *Arabidopsis* leaf tissue [29]. We propose that GacAS have different functions on T3SS in different subspecies [31]. On the leaf surface, GacAS is activated, leading to the repression of T3SS and induction of flagellarp-dependent motility. When bacteria enter the leaf apoplast, the GacAS system is deactivated, leading to desuppression of T3SS and virulence. Once bacteria reach a high density, GacAS might be re-activated to repress T3SS [44].

Similar to the EsaADCE system in *P. aeruginosa*, a HrpSVPN downbeat feedback regulatory system tunes the T3SS in *P. syringae* [45]. In *P. aeruginosa*, EsaA is a master transcription activator of T3SS. EsaD is an anti-activator that inhibits T3SS by disrupting the self-association of EsaA and its binding to DNA. EsaC is an anti-anti activator that forms a complex with EsaD to relieve the inhibitory effects of EsaD on EsaA [46]. In *P. syringae*, the crosstalk of HrpS/V/G in the regulation of T3SS is characteristically equiva-
HrpV directly binds HrpS via the AAA+ domain to destroy the HrpRS heterodimer, resulting in the negative regulation of the T3SS [48]. Meanwhile, HrpG can remove the anti-activator HrpV from HrpS [49], and HrpJ allows the de-repression of the T3SS by binding the HrpG/HrpV heterodimeric chaperone [50]. Moreover, HrpF plays two roles in the regulation of T3SS assembly; it either functions as a negative regulator by possibly participating in HrpSVG circuit regulation via interactions with HrpG or stabilises HrpA in the cytoplasm [47].

HrpJ is also co-regulated by other regulators, including AefR, PsaA, HrpT, and TCS CorRS. CorR can bind to the promoter region of hrpL and directly induce its early transcription, thereby impacting the expression of the T3SS [22]. Meanwhile, HrpG can remove the anti-activator HrpV from HrpS [49], and HrpJ allows the de-repression of the T3SS by binding the HrpG/HrpV heterodimeric chaperone [50].

Syringace employs 14 TFs to control motility

Motility is essential for a bacterial pathogen to spread on the plant surface and colonise host plants. Several types of motility occur in P. syringae: flagella-dependent liquid swimming, surface swimming, swarming motility, and type IV pili (T4P)-dependent twitching motility. Motility is an important virulence factor in P. syringae because the mutation of flagella-related genes may cause P. syringae to lose its motility, remarkably decreasing its virulence [56, 57]. The type of motility via which a pathogen causes infection
mainly depends on environmental factors, including wetness and viscosity. Both flagella- and T4P-mediated surface motilities are essential for the translocation of *P. syringae* in leaf apoplasts.

The motility regulatory network of *P. syringae* is centred around the transcriptional activator FleQ [58] (Fig. 3). It directly induces the expression of *fleSR* and two essential flagellar basal body rod protein operons – *flgBCDE* and *flgFGHIJKL* – as well as many other flagella-related genes, including *fliH, fliE, fliG*, and *fliJ* [58,59]. Moreover, the transcriptional levels of *flhA, flhF, fleN*, and *fliA* are lower in pathogens lacking FleQ than in wild-type pathogens, which provides basal concentrations of these proteins [59]. The regulators OmpR, GacA, and CvsR can bind to the promoter region of *fleQ* and positively regulate its transcription [31]. CbrB2 negatively controls *fleQ* by binding to its upstream region. ChIP-Seq results reveal that MgrA and PhoP simultaneously bind to the promoter of *fleQ* [31]. GacA regulates swimming [43], whereas CvsR contributes to swimming and swarming motility [26].

AlgU, an extracytoplasmic function sigma factor, is a global virulence regulator that plays essential roles in regulating the motility of *P. syringae*. Previous studies present the AlgU-dependent regulatory binding landscape and confirms that it downregulates many flagella-related and chemotaxis motility genes [33,60,61]. However, AlgU induces antisense transcript expression of the *fleQ* gene (*fleQ* <sub>α</sub>) in *P. syringae*, enhancing its swimming motility and positively controlling its flagellar activity [60]. In addition, P-RhpR can bind to the promoter region of *flhA*, encoding a flagellar export-related protein, thereby affecting swimming motility [40]. Motility plate culture experiments verify that P-RhpR negatively regulates the swimming motility of *P. syringae*. *fimA*, which encodes a type I fimbrial subunit and is related to twitching motility, is also controlled by P-RhpR. Twitching phenotype tests demonstrate that P-RhpR positively regulates twitching motility by directly binding the promoter region of *fimA* [40]. Thus, an essential regulatory network is involved in the motility of *P. syringae* (Fig. 3).

### 4. Twelve TFs modulate biofilm formation in *P. syringae*

When *P. syringae* enters the apoplasts of host plants, they need to establish colonies and form biofilms by secreting highly viscous compounds such as extracellular polysaccharides (EPSs), extracellular DNA, proteins, and lipids, which protects them from host immune resistance and attack by antibiotics [62,63]. *P. syringae* generally produces two types of EPS, namely alginate and levan; of the two, alginate is more common [64]. Alginate is a copolymer composed of O-acetylated β-1,4-linked d-mannuronic acid and its C-5 epimer, l-guluronic acid. Alginate contributes to the rigidity and flexibility of biofilms as it provides a robust and sticky skeleton, as well as interlocking chains that hold bacterial cells together. Alginate biosynthesis is controlled by 12 regulators – AlgU, AlgR, RpoN, HrpL, AmrZ, MgrA, TCSs CvsRS, GacAS, RhpRS, PilRS, OmpR/EnvZ, and PhoPQ; these are summarised as a regulatory network in Fig. 4.

AlgU not only suppresses flagella-dependent motility but also plays essential roles in regulating alginate production by activating the expression of genes such as *algD* and *alg8*, which are essential to this function [31]. AlgU also controls the expression of additional regulators (e.g., *AmrZ*) that positively participate in this pathway-regulatory crosstalk [65]. Moreover, GacS and SalA activate the modest expression of alginate production-related genes.
in planta and iron-enriched conditions while contributing significantly to their regulation in vitro in an iron-limited manner [21,23]. However, CvsR inversely controls biofilm formation by indirectly suppressing the algU transcription levels, thereby repressing alginate production in a Ca²⁺-dependent manner [26]. Additionally, OmpR plays a key role in the negative regulation of alginate synthesis by directly binding the promoter region of algD and algU, whereas HrpL induces algR3 expression to positively regulate alginate production [31]. AlgR, an alginate biosynthesis regulatory protein, regulates the expression of algC and argB under the assistance of sigma factor FpoN, which is required for bacterial colonisation and biofilm formation [66]. These regulators reveal the intricate regulatory mechanisms underlying biofilm formation in P. syringae.

5. Nucleotide-based secondary messengers play significant roles in many virulence-related pathways

In prokaryotes, nucleotide-based secondary messengers, including cyclic adenosine monophosphate (cAMP), guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (collectively known as (p)ppGpp), and cyclic di-guanosine monophosphate (c-di-GMP), have emerged as essential signal molecules that play important roles in regulating multiple survival- and virulence-related biological functions in bacterial pathogens [67]. A summary of the architecture of these secondary messengers is presented in Fig. 5, with the related regulators and genes indicated.

cAMP, catalysed by adenyl cyclases, is a cyclic nucleotide that regulates essential processes in bacteria [68]. In P. syringae, cAMP production is reduced in the vfr mutant and abolished in the cyaA mutant. Vfr is a cAMP-regulatory protein, and cyaA encodes an adenylate cyclase [69]. Both mutants show reduced virulence in their hosts, decreased surface motilities, and higher sensitivity to antibiotic compounds through the activation of the expression of multidrug efflux pump genes (mexAB and oprM), which are located in the same operon in P. syringae [69].

(p)ppGpp is a signalling compound that responds to limited nutrient conditions, including amino acid-, fatty acid-, carbon-, and iron-starved conditions [68]. In P. syringae, the intracellular concentration of (p)ppGpp is controlled by two homologous proteins, RelA and SpoT. RelA is a monofunctional synthase that uses GDP and ATP to produce (p)ppGpp. SpoT is an essential virulence protein–synthase that generates and hydrolyses (p)ppGpp [68]. (p)ppGpp participates in multiple bacterial physiological pathways, such as metabolic processes including DNA replication, transcription, and translation, and virulence-associated pathways by inducing virulence factors. In P. syringae, SpoT is an essential virulence effector that affects its motility as well as pyoverdine production and induces T3SS gene expression by activating the transcription of hrpRS and hrpL [70] (Fig. 5).

As one of the best-studied bacterial secondary messengers, c-di-GMP is produced by diguanylate cyclases and eliminated by phosphodiesterases, which are highly conserved in many bacterial pathogens [71]. Studies have revealed that c-di-GMP can modulate several virulence-related pathways in P. syringae, including the T3SS, biofilm formation, reactive oxygen species (ROS) resistance, and siderophore production. High levels of c-di-GMP repress the transcription of hrpR and hrpL, projecting adverse regulatory effects on the T3SS [72]. In addition, increased c-di-GMP levels reduce the swimming and swimming motilities of P. syringae by...
regulating the expression of flagellar synthesis-related genes. In contrast, c-di-GMP positively regulates biofilm formation by upregulating the expressions of *alg8* and *alg44*, which are required for EPS biosynthesis. Additionally, c-di-GMP can increase siderophore production by inducing the expression of *pvdE*, *pvdP*, and *pvsA*, which encode pyoverdine transporter and peptide synthases [72]. Moreover, a higher level of c-di-GMP allows *P. syringae* to exert higher tolerance to oxidative stress by upregulating *sodA* expression [72] (Fig. 5).

6. Three TFs control the QS system in *P. syringae*

QS involves the production, release, and group-wide detection of extracellular signalling molecules among bacterial communities. Once the QS molecules are secreted by the bacteria and accumulated to a threshold level, the bacteria change their global gene expression traits to exert their virulence and adapt to a high-level cell population density situation [73]. In *P. syringae*, 3-oxohexanoyl-homoserine lactone (C6-AHL) is the primary QS molecule synthesised by an AHL synthase, which is a product of *ahlI*. C6-AHL interacts with its receptor AhlR, which forms a stable complex to activate the expression of *ahlI*, further accumulating the concentration of QS molecules [52] (Fig. 5). In addition, *aeIR* mutants cannot produce AHL, thereby reducing the motility and virulence and increasing tolerance to antibiotics by activating MexEF/OprN gene expression [3,74]. TCS GacAS is also required for AHL production in *P. syringae* [43,75]. Of note, AeIR and GacAS regulate the QS pathway independently in *P. syringae* [52], as shown in Fig. 5.

7. Three TFs control phytotoxin secretion in *P. syringae*

Bacterial pathogens produce phytotoxins to injure host cells and cause diseases. The disease symptoms can be generated with the treatment of phytotoxins without bacterial infection. Pathogens that lose the ability to secret phytotoxins reduce or completely lose their virulence [1]. Phytotoxins, such as coronatine and syringomycin, are produced during infection with *P. syringae* and produce disease symptoms [76]. *P. syringae* can produce coronatine to re-open the stomata at biological concentrations during the night; moreover, the pathogen uses syringomycin to target the plasma membranes of host plants to form ion channels and cause cytolysis [77–79]. Coronatine yields are affected by temperature changes, and its synthesis is induced at 18°C but significantly repressed at 28°C [80,81]. A modified TCS with one HK (CorS) and two RRs (CorR and CorP) serves as a regulatory system that can modulate coronatine synthesis [82] (Fig. 5). The environmental sensor CorS senses temperature changes and allows response regulators to function as positive activators of the coronatine synthesis process by binding to the specific target sequences. In addition, the regulation of syringomycin synthesis is complicated because of nutritional factors and plant signal molecules. SyrB1, SyrB2, SyrC, and SyrE are responsible for catalysing syringomycin production [83]. SyrD, which functions as a transporter protein, allows *P. syringae* to export this toxin across the cytoplasmic membrane once cyclized [84]. SyrP, which encodes a regulatory protein, plays a significant role in controlling syringomycin production and virulence in a phosphorylated manner [85]. However, although the GacAS system is likely at the top of the regulatory hierarchy network,
the intermediary regulators remain unknown [86]. This regulatory pathway is presented in Fig. 5.

8. Siderophore production is controlled by 3 TFs in *P. syringae*

Because the concentration of Fe^{3+} is relatively low in the environment, iron is required for the growth and complete virulence of many pathogenic bacteria [87]. Mutants lacking siderophore-associated genes exhibit reduced EPS production but increased antibiotic tolerance, and express dysregulated QS and multidrug efflux [8]. In *P. syringae*, siderophore production is regulated by three TFs (Fur, GacAS, SalA) (Fig. 5). Fur, which encodes a ferric uptake regulation protein, is a master negative regulator of iron homeostasis and is found to control swarming motility and tabtoxin and AHL production [88]. Fur acts as an iron-responsive repressor by binding to its target gene elements, known as Fur box [89]. In iron-limited or iron-rich conditions, Fur slightly and strongly represses siderophore production [88]. In addition, GacAS and SalA play roles in the modulation of siderophore-related genes. In a previous study, genes related to pyoverdine production are downregulated in both ΔsalA and ΔgacS strains under low-Fe conditions, suggesting that SalA and GacS function as important activators of pyoverdine production responding to iron limitation [21].

9. OxyR participates in the regulation of ROS resistance in *P. syringae*

Plants are always surrounded by many microorganisms, including pathogens such as *P. syringae*. These pathogens have developed two layers of defence system to prevent themselves from infection. The first line is pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) that recognizes the conserved molecules from pathogens. The second line is effector-triggered immunity (ETI) under the control of individual plant resistance genes [90]. These two layers of defence system can induce similar plant defence responses, including the oxidative burst (superoxide and hydrogen peroxide). PTI induces a weaker and/or short ROS burst, while ETI induces a stronger one. The ROS leads to physical barriers or direct toxicity against the invading pathogens. Therefore, pathogenic bacteria like *P. syringae* must detoxify the apoplastic ROS and overcome ROS-mediated defence signalling in host plants. OxyR is an oxidative regulatory protein required for the complete virulence and protection of *P. syringae* from exogenous/endogenous ROS toxicity (Fig. 5). OxyR positively regulates the expression of thioredoxins and catalase genes, including katB, katE, and katG, in response to the effects of H_2O_2 or during infection in host plants [91]. Overall, we present siderophore production and the ROS resistance regulatory networks in Fig. 5.
10. Similarities, distinctions, and crosstalk between networks

Many TFs play different roles in multiple regulatory systems, while some TFs are specific to certain regulatory networks. For example, HrpRS and HrpSVG pathways are specific to the T3SS regulatory network, while four TCSs (RhpRS, GacAS, PiIR, and CbrAB2) are participating in T3SS, motility, and biofilm formation systems. Biofilm formation largely relies on the sigma factor AlgU and regulates motility by downregulating flagella-related genes. c-di-GMP plays an essential role in mediating the switch between motility and biofilm formation. Besides, GacAS is a flexible and plastic TCS that can regulate the QS, phytotoxin, and siderophore production system. In addition, AhIR and AefR are required for the QS system, and the latter can also induce T3SS while inhibiting swimming motility indirectly. Furthermore, OxyR and SyrP are specific to the ROS system and phytotoxin secretion, respectively.

c-di-GMP is essential for the crosstalk between motility and biofilm formation. During the initial attachment period, *P. syringae* needs flagellar motility to swim before entering apoplastic spaces and attaching to the leaf surface. Subsequently, the flagellar and plus motility allows *P. syringae* to form an early attachment stage. The motility is then suppressed, while the EPS production-related genes are upregulated by the increased c-di-GMP level to permit EPS biosynthesis and promote biofilm formation. The decrease in flagellar motility might contribute to stabilizing the complicated structure of the biofilm. Taken together, the proper crosstalk between motility and biofilm formation plays an important role in contributing to *P. syringae* virulence and survival.

11. HT-SELEX predicts 13 novel TFs involved in virulence-related regulatory networks

Although virulence-related TFs in *P. syringae* have been studied for decades using many methods, the direct and specific binding patterns of many TFs remain elusive. To this end, we have used HT-SELEX to globally profile the DNA interaction specificities of annotated TFs in *P. syringae*. The ligand libraries are first produced by PCR with 40-bp randomized oligo as a template. Purified TFs and selection ligands are incubated together, followed by equilibrating with resin beads. The bound DNA is subject to PCR. The PCR products from selection ligands are used for the next HT-SELEX cycle, which is repeated four times before next-generation sequencing. Autoseed [92] is used to identify initial seeds for generating position weight matrix (PWM) models. We successfully reveal 14 previously uncharacterised TFs that control virulence-associated pathways, including the T3SS, motility, c-di-GMP, siderophore production, phytotoxin secretion, and ROS resistance [34]. We determine the predicted virulence-related regulators within the above-mentioned regulatory networks.

HT-SELEX identifies two regulators involved in flagella-dependent motility: FleR and PSPPH_2720. These regulators harbour similar DNA binding motifs, suggesting that they co-regulate similar motility-related genes, including fliH, fliE, fliJ, and fliG. Moreover, we predict five TFs (MgrA, GacA, PSPPH_0442, PSPPH_3297, and PSPPH_3294) as regulators of T4P pathways. MgrA can bind the promoters of algU, mucA, and mucB, which are also controlled by another TF, PiIR [34]. In terms of c-di-GMP, we use HT-SELEX to identify three potential TFs (MetR, PSPPH_1800, and PSPPH_2693) that might play roles in regulating this pathway by binding to the promoter region of PSPPH_4247, PSPPH_4420, PSPPH_2693, and PSPPH_2693, which encodes a known phospodiesterase and putative diguanylate cyclases [34]. In addition, we show that KdpE, PSPPH_2800, and PSPPH_5204 might regulate the production of phytotoxins by binding genes related to PSPPH_A0071 and PSPPH_A0005. In the siderophore pathway, we predict six TFs, including PiIR, CbrB2, GltR, PSPPH_4828, PSPPH_3800, and PSPPH_3577, that might regulate this pathway. PiIR and PSPPH_3577 are predicted to regulate the operon of PSPPH_1923, 1924, and 1925, which encode pyoverdine sidechain peptide synthetase I, II, and III, respectively. GltR, TF PSPPH_4828, and PSPPH_3800 might play roles in regulating PSPPH_5184 and PSPPH_5185, which are related to the iron ABC transporter protein. Additionally, CbrB2 is predicted to bind to the promoter region of the operon fecBCDIE, suggesting that CbrB2 controls iron transport in *P. syringae* [34]. Our HT-SELEX analysis also has revealed that three regulators (LexA1, PSPPH_1960, and PSPPH_3004) might participate in the ROS resistance pathway by regulating the expression of katE, sodA, and sodB, respectively [34]. We review these predicted TFs in their respective regulatory networks.

12. Conclusions and perspectives

Bacterial pathogens primarily rely on the plasticity of transcription levels to exert their virulence in different growth periods. During the infection, *P. syringae* first utilizes flagellar motility to adhere to the plant’s cell surface and expand attachment. Subsequently, the attachment signals may change the *P. syringae* genes expression pattern (including T3SS) to secrete effectors injuring plant cells and EPS to form biofilm preventing the access of antimicrobial compounds. Meanwhile, *P. syringae* employs phytotoxins, ROS resistance system, and T3 effectors to suppress the plant defence. QS senses the threshold-level molecules and induces biofilm maturation. Finally, the bacterial cells at the surface layer of biofilm detach from the matrix and disperse to cause a new infection cycle [1].

In the past decades, several virulence-related regulatory mechanisms have been studied and illustrated in *P. syringae*. This review summarises these regulatory networks, including T3SS and non-T3SS pathways, to profile a global visualisation of crosstalk among master regulators in *P. syringae* (Table 1). The ChIP-Seq, RNA-Seq, and HT-SELEX approaches help us identify the binding sites, differentially expressed genes, and DNA-binding specificities of TFs, which demonstrate a vast regulatory landscape.

Many of the abovementioned virulent pathways are shared in other *Pseudomonas* species, but the associated TFs are largely different. Take the T3SS for example, different from the T3SS regulatory network (22 TFs and Hrp-T3SS) in *P. syringae* reviewed in this article, two key pathways (Gac/Rsm-T3SS and Vfr-ExsA-T3SS) regulate T3SS in *P. aeruginosa* [93]. GacAS is the vital switch for the upstream activation of T3SS. HptB is an activator of RetS, which can inhibit GacS [94]. Besides, GacA can induce rsmY and rsmZ, while PtrR and SuhB repress GacA. In the Vfr-ExsA-T3SS pathway, Vfr is influenced by the level of cAMP, and the transcription activator ExsA directly induces the T3SS-related genes expression in *P. aeruginosa*. ExsA is also modulated by nine other regulators (VqsM, Fis, RcoA1, PsrA, HigB, PtrA, ExsD, MvaT(J), and HigA) [93]. However, a self-feedback regulatory pathway is shared by both species, including ExsADCE in *P. aeruginosa* and HrpSVG in *P. syringae*.

The diseases caused by *P. syringae* have caused massive economic losses. It is essential to find measures to combat this bacterial pathogen. The most common treatment currently applied to combat *P. syringae* infection is copper-based bactericides. However, it is decreasingly popular because pathogens develop copper resistance, and the copper accumulation has caused environmental damage [95]. Targeting TFs can treat human diseases such as cancer or inflammation [96,97]. For example, phthalimide-based drugs can treat multiple myeloma cells by targeting TFs IKB1 and IKB2, both as the cereblon substrates. The most clinically approved antibiotics negatively affect bacterial viability in bacterial pathogens by targeting cell wall growth, DNA replication, transcription,
Therefore, several drugs have been designed to target virulence, which results in fewer antibiotic-resistant strains [99]. In addition, targeting virulence instead of viability [98] is advantageous because inhibiting virulence reduces the selective pressure and prevent drug resistance is targeting virulence instead of viability [98]. An advantageous method to combat bacterial infection becomes antibiotic resistance may have a selective growth advantage [26]. In this situation, bacteria that can mutate to become antibiotic resistant may have a selective growth advantage [26].

| Genes        | Produced proteins/ secondary messengers | Roles                                                                 |
|--------------|----------------------------------------|----------------------------------------------------------------------|
| hrpB         | HrpR and HrpS                          | HrpR and HrpS together activate T3SS by inducing hrpL transcription [13]. |
| hrpL         | HrpL                                   | HrpL regulates most T3SS genes and alginate production [31].         |
| algU         | AlgU                                   | AlgU regulates the transcription of T3SS effector genes [33]. AlgU negatively regulates flagella-dependent motility [33,40] and positively regulates alginate production [31]. |
| hrpA         | HrpA                                   | HrpA induces the expression of hypR, hypR, and hrcC [39]. P-RhpB negatively regulates the hrpRS by directly binding to its promoter region. P-RhpR also negatively regulates swimming, c-di-GMP, and biofilm formation by targeting fimL, PSSPH2590, and algD. It positively regulates twitching motility by targeting fimA [14,17,40,41]. |
| rhpRS        | RhpR and RhpS                          |                                                                       |
| cvrRS        | CvsR and CvsS                          | CvsRS upregulates TSS induction [26]. CvsR positively regulates swimming and swarming [26]. CvsR positively regulates alginate production in a Ca2+-dependent manner [26]. |
| gacAS        | GacA and GacS                          | GacAS is a negative regulator of T3SS [29]. GacA regulates swimming [43] and activates the modest expression of alginate-related genes [21]. GacAS is also required for the QS system [43,75], and positively regulates siderophore-related genes expression [21]. |
| hvrN         | HvrN                                   | HvrN negatively regulates T3SS [48].                                 |
| hvgG         | HvgG                                   | HvgG can remove the inhibitor HvrN from HrpS [49].                   |
| hrf         | Hrf                                    | Hrf degrades the T3SS genes by directly binding with the Hrpg/Hrpg heterodimeric chaperone [50]. |
| hrfF         | HrfF                                   | HrfF functions as a negative regulator of T3SS and stabilises Hrpa in the cytoplasm [47]. |
| corRS        | CorR and CorS                          | CorR induces the expression of hrp. [22]. CorS activates the catalysed synthesis. [82]. |
| araA         | AraA                                   | AraA indirectly and positively regulates hrl expression [27].         |
| aerR         | AerR                                   | AerR indirectly regulates the transcription of hrp [30,52]; AerR positively regulates motility, virulence, and antibiotic tolerance [3,47]. |
| hrtT         | HrtT                                   | HrtT suppresses hrpL expression [53].                                |
| lon          | Lon                                    | Lon negatively regulates T3SS by degrading HrpR and T3 effectors in KB. Lon also regulates motility by binding to the promoter of gacA [24,55]. |
| ompRenvZ     | OmpR and EnvZ                          | EnvZ-OmpR regulates the T3SS in KB [31]. OmpR positively and directly regulates the expression of fleQ and negatively regulates alginate synthase [31]. |
| cheB         | CheB                                   | CheB represses the T3SS in KB [31], CheB negatively controls fleQ [31] and regulates siderophore pathway and iron transport [34]. |
| pilRS        | PilR and PilS                          | PilRS represses the T3SS in KB [31] and regulates motility-related genes [34]. PilR modulates the siderophore pathway [34]. |
| PSSPH_3618   | PSSPH_3618                             | PSSPH_3618 downregulates the expression of avrB2 and rhpRC [34].    |
| fleQ         | FleQ                                   | FleQ is a transcriptional activator of motility [58].                |
| mrgA         | MrgA                                   | MrgA binds to the promoter of fleQ [31] and regulates the expression of motility-related genes [34]. |
| phoP         | PhoP                                   | PhoP binds to the promoter of fleQ [31]. PhoP binds to the promoter of fleQ [31]. |
| saaA         | SaaA                                   | SaaA activates the modest expression of alginate production-related genes [23] and positively regulates siderophore-related genes expression [21]. |
| algR         | AlgR                                   | AlgR is an alginate biosynthesis regulatory protein that regulates the expression of algC and argB [66]. |
| vfr          | Vfr                                    | Vfr positively regulates cAMP production [69].                      |
| ahrR         | AhrR                                   | AhrR participates in QS regulation, activating the expression of ahr [52]. |
| corP         | CorP                                   | CorP modulates coronation production [82].                          |
| syrP         | SyrP                                   | SyrP plays a significant role in controlling syringomycin production and virulence in a phosphorylation-dependent manner [85]. |
| fur          | Fur                                    | Fur is a master negative regulator of iron homeostasis. Fur controls motility, tal-toxin, and AHL production [88]. Fur also represses siderophore production [88]. |
| oxyR         | OxyR                                   | OxyR positively regulates ROS resistance [91].                      |
| fleR         | FleR                                   | FleR positively regulates motility-related genes [34].              |
| PSSPH_2720   | PSSPH_2720                             | PSSPH_2720 positively regulates motility-related genes [34].        |
| PSSPH_0442   | PSSPH_0442                             | PSSPH_0442 regulates T4P pathway [34].                              |
| PSSPH_3297   | PSSPH_3297                             | PSSPH_3297 regulates T4P pathway [34].                              |
| PSSPH_3294   | PSSPH_3294                             | PSSPH_3294 regulates T4P pathway [34].                              |
| metR         | MetR                                   | MetR regulates the expression of c-di-GMP-related genes [34].        |
| PSSPH_1800   | PSSPH_1800                             | PSSPH_1800 regulates the expression of c-di-GMP-related genes [34]. |
| PSSPH_2693   | PSSPH_2693                             | PSSPH_2693 regulates the expression of c-di-GMP-related genes [34]. |
| kdpE         | KdpE                                   | KdpE regulates the production of phytotoxins [34].                  |
| PSSPH_2800   | PSSPH_2800                             | PSSPH_2800 regulates the production of phytotoxins [34].            |
| gilR         | GilR                                   | GilR modulates the siderophore pathway [34].                        |
| PSSPH_4828   | PSSPH_4828                             | PSSPH_4828 modulates siderophore pathway [34].                      |
| PSSPH_3800   | PSSPH_3800                             | PSSPH_3800 modulates siderophore pathway [34].                      |
| PSSPH_3577   | PSSPH_3577                             | PSSPH_3577 modulates siderophore pathway [34].                      |
| lexA         | LexA                                   | LexA participates in ROS resistance pathway [34].                   |
| PSSPH_1960   | PSSPH_1960                             | PSSPH_1960 participates in ROS resistance pathway [34].             |
| PSSPH_3004   | PSSPH_3004                             | PSSPH_3004 participates in ROS resistance pathway [34].             |

and translation. In this situation, bacteria that can mutate to become antibiotic resistance may have a selective growth advantage [98]. An advantageous method to combat bacterial infection and prevent drug resistance is targeting virulence instead of viability because inhibiting virulence reduces the selective pressure and results in fewer antibiotic-resistance strains [99]. In addition, therapies targeting TFs would not harm the normal microbiota of hosts. Therefore, several drugs have been designed to target virulence-related TFs to inhibit bacterial infection. For example, structure-based small-molecule can inhibit several TFs (such as MarA, SoxS, and Rob), which regulate virulence in pathogenic Escherichia coli [100]. Besides, virstatin and benzimidazole compounds can limit infection by targeting vital virulence TFs ToxT and LcrF in Vibrio cholerae and Yersinia pseudotuberculosis, respectively [101,102]. Given these studies, we propose that targeting virulence-related TFs to inhibit bacterial infection.
associated TFs in *P. syringae* is a potential method to combat its infection.

We propose the following approaches to inhibit *P. syringae* virulence:

1) The drugs or compounds that directly inhibit T3SS effectors or switch off upstream master regulators such as RhpRS. 2) The measures to alleviate bacterial motility, such as swimming, swarming, and surface attachment, by targeting the master motility regulators (e.g., FleQ and AlgI). We further predict that drugs targeting virulence-related TFs in *P. syringae* may act synergistically with conventional antibiotics to control diseases caused by *P. syringae*.

Taken together, this review demonstrates that eight virulence-related regulatory networks are key to successful *P. syringae* infection. Given the crucial roles of the transcriptional regulatory networks (TRN) of *P. syringae*, an exploration of a more comprehensive TRN with all TFs in the genome would help us to further understand its pathogenicity and metabolism. We believe that our findings will contribute to the development of environmentally friendly and sustainable strategies to relieve the infections caused by *P. syringae*.  

CRediT authorship contribution statement

**Jiadai Huang:** Conceptualization, Investigation, Visualization, Writing – original draft, Writing – review & editing.

**Chunyan Yao:** Investigation, Writing – review & editing.

**Yue Sun:** Investigation, Writing – review & editing.

**Xin Deng:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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