Insights into the Cell-to-Cell Signaling and Iron Homeostasis in *Xanthomonas* Virulence and Lifestyle

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ABSTRACT

The *Xanthomonas* group of phytopathogens causes economically important diseases that lead to severe yield loss in major crops. Some *Xanthomonas* species are known to have an epiphytic and in planta lifestyle that is coordinated by several virulence-associated functions, cell-to-cell signaling (using diffusible signaling factor [DSF]), and environmental conditions, including iron availability. In this review, we described the role of cell-to-cell signaling by the DSF molecule and iron in the regulation of virulence-associated functions. Although DSF and iron are involved in the regulation of several virulence-associated functions, members of the *Xanthomonas* group of plant pathogens exhibit atypical patterns of regulation. Atypical patterns contribute to the adaptation to different lifestyles. Studies on DSF and iron biology indicate that virulence-associated functions can be regulated in completely contrasting fashions by the same signaling system in closely related xanthomonads.

Keywords: bacterial pathogens, biochemistry and cell biology, biofilm, chemotaxis, DSF, genetics, iron storage, iron toxicity, iron uptake, motility, quorum sensing, *Xanthomonas*

THE DIFFUSIBLE SIGNALING FACTOR (DSF) FAMILY OF CELL-TO-CELL SIGNALING SYSTEM IN *XANTHOMONAS*

Bacteria communicate with diffusible signaling factors that are synthesized and secreted in a density-dependent fashion and regulate several cellular and social behaviors in a process known as “quorum sensing.” Diverse classes of cell-to-cell signaling molecules are now characterized. The most common form of signaling system studied is the acyl-homoserine lactone-mediated cell-to-cell signaling particularly, in Gram-negative bacteria (Fuqua et al. 2001; Ng and Bassler 2009). In many species of *Xanthomonas*, an unrelated system, known as the regulation of pathogenicity factor (*rpf*),

is involved in the regulation of virulence-associated functions (Barber et al. 1997). This is done through the synthesis of DSF, a regulator of the production and secretion of several extracellular enzymes, including cellulose and endoglucanase (Slater et al. 2000). Interestingly, the *X. oryzae* pv. *oryzae* *rpfF* mutant (mutant in the diffusible signaling factor [DSF] synthase) was proficient in the production of secreted Type-II effectors; however, it exhibited virulence deficiency on rice (Chatterjee and Sonti 2002; Rai et al. 2015). Further characterization of the DSF family of signaling molecules in *X. campestris* pv. *campestris*, *X. oryzae* pv. *oryzae*, and *Burkholderia* revealed that the DSF synthase RpfF is a promiscuous enzyme belonging to the member of the crotonase superfamily with both dehydratase/desaturase and thioesterase activity (Boon et al. 2008; Bi et al. 2012; He et al. 2010; Wang et al. 2004). In *Xanthomonas* and several closely related bacteria, a DSF family of signaling molecules has been identified and characterized in an unusual fatty acid QS signal molecule (Deng et al. 2011; Dow 2017). In the *Xanthomonas* group phytopathogens and their close relative *Xylella*, at least four different species of the DSF family of signaling molecules have been reported as *cis*-11-methyl-2-dodecenoic acid, *cis*-2-dodecenoic acid, *cis,cis*-11 methyl-dodeca-2,5-dienoic acid, and *cis*-2-hexadecenoic acid (Beaulieu et al. 2013; Bi et al. 2012; Boon et al. 2008; He et al. 2010; Wang et al. 2004). In *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae*, mixed species of the DSF family of signaling molecules are made depending on the culture conditions and media composition (Dow 2017; He et al. 2010).

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RpfF, presumably, utilizes different substrates based on the growth conditions (Dow 2017; Zhou et al. 2015). Other members of Xanthomonadales, including *Burkholderia*, *Xylella*, and *Stenotrophomonas*, communicate with the DSF family of signaling molecules to regulate social behavior required for virulence and lifestyle transition (Chatterjee et al. 2008c, 2008a, 2008b; Dow 2017; He and Zhang 2008; Ryan and Dow 2011).

WORKING MECHANISM OF DSF SYSTEM IN XANTHOMONAS

DSF binds the hybrid sensor kinase RpfC to induce a conformational change that activates the response regulator RpfG (He et al. 2006). In *X. campestris* pv. *campestris*, dual signaling functions of the RpfC are involved in either phosphorelay or receiver domain-protein interactions (He et al. 2006). RpfG harbors an HD-GYP domain with phosphodiesterase activity that specifically hydrolyzes the bacterial secondary messenger cdiGMP (Fig. 1) (Ryan et al. 2006). The *rpfF*-, *rpfC*-, and *rpfG*-mediated quorum sensing regulates multiple virulence-associated functions including production of exoenzymes for motility and biofilm formation and exopolysaccharides (Fig. 1) (Guo et al. 2012; Ryan et al. 2006, 2010). The HD-GYP domain of RpfG interacts with the GGDEF domain protein, a diguanylate cyclase, and regulates motility in *X. campestris* pv. *campestris* (Ryan et al. 2010). However, a direct link between the level of RpfG phosphodiesterase activity-dependent cyclic-di-GMP (cdiGMP) and the regulation of RpfG-associated virulence factors needs to be further investigated.

Alteration of cdiGMP levels affects the binding of the second messenger to the global regulator Clp (homologous of cAMP nucleotide receptor protein Crp from *E. coli*), which in turn regulates the expression of several genes (Deng et al. 2010; He et al. 2006). Clp regulates the expression of zinc uptake regulator (Zur) and FhrR, a TetR-family transcription factor in *X. campestris* pv. *campestris* (He et al. 2007; Tao et al. 2010). Zur regulates extracellular polysaccharides (EPS) production, iron uptake, detoxification, and

multidrug resistance; and FhrR regulates the expression of genes encoding Hrp, flagella, and ribosomal proteins (Fig. 1) (He et al. 2007; Huang et al. 2009; Pandey et al. 2018; Tao et al. 2010; Zhang 2010). The RavS/RavR two-component system is also involved in regulation of virulence-associated functions by controlling the level of cdiGMP in xanthomonads (Fig. 1) (He et al. 2009). Research into DSF-mediated signal transduction in *Xanthomonas*, *Xylella*, and *Burkholderia* revealed a complex signal transduction process that involves other two-component sensors (An et al. 2013; Chatterjee et al. 2008c; Chatterjee and Sonti 2002; Rai et al. 2015). For example, in *X. campestris* pv. *campestris*, it has been shown that a second DSF sensor RpfS (a PAS domain containing Histidine kinase) is involved in the regulation of a subset of DSF-regulated genes in an RpfC (cognate DSF sensor)-independent fashion (An et al. 2014). Furthermore, it has been reported that in *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, the *rpfC* mutants do not completely phenocopy the *rpfF* mutant, suggesting that the DSF regulatory signal transduction pathway involves additional unidentified signal sensors or regulators (Rai et al. 2012, 2015). This further corroborates the complexity of the DSF-mediated cell-to-cell signaling regulation, as it appears that several other two-component sensors and response regulators may also be involved in fine-tuning the DSF-mediated regulation of gene expression (An et al. 2013; Rai et al. 2012, 2015).

ATYPICAL REGULATION OF VIRULENCE-ASSOCIATED FUNCTIONS BY DSF IN *X. CAMPESTRIS* PV. *CAMPESTRIS* AND *X. ORYZAE* PV. *ORYZAE*

In *X. campestris* pv. *campestris*, it has been reported that DSF is involved in the positive regulation of synthesis of extracellular enzymes and EPS, and promotes biofilm dispersal. DSF-deficient mutants exhibit virulence deficiency, reduced in planta growth, and enhanced biofilm formation compared with the wild-type strain. In contrast, DSF in *X. oryzae* pv. *oryzae* negatively regulates the production of several Type-II effectors such as cellulase, endoglucanase, lipase, and xylanase. A DSF-deficient *rpfF* mutant overproduces these secreted enzymes, and exhibits hypermotility and decreased biofilm formation. In planta infection assays indicated that *rpfF* mutant of *X. oryzae* pv. *oryzae* is deficient in both epiphytic modes of infection and in wound infection. Large-scale gene expression analysis and phenotypic assays of different DSF rpf system mutants in *X. oryzae* pv. *oryzae* revealed that DSF positively regulates functions required for biofilm formation such as adhesions (XadA, EPS) and iron metabolism, and negatively regulates motility, chemotaxis, and Type-II effectors production. These regulatory differences may have arisen from the different lifestyles and host environments encountered by each species during infection (Rai et al. 2012).

A model has been proposed for DSF-mediated regulation of virulence-associated functions and lifestyle transition in both *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*. At low cell density, presumably on the leaf surface, low DSF levels trigger chemotaxis-driven motility, which helps the *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* to locate hydathodes and stomata, their entry point on the leaf, respectively. Once they enter, *X. oryzae* pv. *oryzae* colonizes the xylem vessel and *X. oryzae* pv. *oryzicola* colonizes the space between parenchyma cells. Low cell density further triggers the secretion of cell-wall hydrolyzing enzymes to degrade the plant cell walls and release nutrients. As the nutrients are released, bacteria multiply and increase the cell density. DSF levels correspondingly increase and iron becomes limiting, which triggers the expression of high-affinity iron uptake genes involved in both siderophore-mediated ferric iron uptake and ferrous iron uptake (Rai et al. 2012, 2015). In addition, several low-affinity Ferric iron uptake and storage genes like bacterioferritin are activated. High cell density also triggers biofilm formation by increasing

FIGURE 1

QS-mediated regulation of virulence functions in *Xanthomonas*. The enoyl-CoA hydratase RpfF synthesizes the diffusible signaling factor (DSF). The threshold level of DSF production by the group of bacterial cells is sensed by RpfC, which activates the RpfG (a HD-GYP domain containing phosphodiesterase), which then hydrolyzes the bacterial secondary messenger cdiGMP to GMP. The RavS/RavR two-component system is also involved in cdiGMP hydrolysis. The high level of cdiGMP binds with DNA binding regulator Clp to inhibit binding to the targets. Clp activates the expression of Zur and FhrR. Therefore, hydrolysis of cdiGMP activates the expression of several virulence-associated genes. QS positively regulates T3SS, Hrp expression, EPS production, biofilm formation, and attachment and negatively regulates the motility, chemotaxis, and T2SS. The RpfF also activates the siderophore production and ferric iron uptake in *X. oryzae* pv. *oryzicola*, but up-regulate ferrous iron uptake in *X. oryzae* pv. *oryzae*.

the production of EPS and adhesin that contribute to cell-to-cell and cell-plant attachment (Fig. 2) (Rai et al. 2012, 2015).

In *Xylella fastidiosa*, which causes economically important disease in crops like grape and citrus, DSF regulates contrasting phenotypes both in planta and in its insect vector. The *rpfF* mutant of *X. fastidiosa* is hyper-virulent and causes disease faster than the wild type when mechanically inoculated into grapevines. However, the *rpfF* mutants of *X. fastidiosa* exhibit a severe deficiency in colonizing the insect vector mouth parts, a key step in transmission (Newman et al. 2004). Further analysis of genes and functions regulated by DSF in *Xylella* revealed that DSF positively regulates biofilm formation and negatively regulates motility. Because of this, *X. fastidiosa* *rpfF* mutants exhibit hyper-migration and systemic spread, leading to a faster disease development. In contrast, a *X. fastidiosa* mutant in the DSF sensor *rpfC*, which overproduces DSF, also exhibits virulence deficiency, enhances biofilm formation, and reduces migration from the point of inoculation (Chatterjee et al. 2008c, 2008a; Newman et al. 2008). Overproduction of DSF, or addition of exogenous DSF, induces production of adhesins such as XadA and EPS, which promote biofilm formation. This understanding of the dual role of DSF in the interaction of *X. fastidiosa* with its insect host and plant had prompted researchers to develop transgenic plants that produce large amounts of *Xylella* DSF to disrupt *X. fastidiosa* pathogenesis. The transgenic grapes are more resistant to Pierce's disease (a disease caused by *X. fastidiosa* on grapes) (Chatterjee et al. 2008b; Lindow et al. 2014).

In *X. citri* subsp. *citri*, transcriptome analysis suggests that DSF regulates modulation of bacterial adaptation, signal transduction, nutrition uptake and metabolism, stress tolerance, and virulence (Li et al. 2019). These alterations lead to a corresponding downregulation of host genes associated with defense response, aiding *X. citri* subsp. *citri* infection (Li et al. 2019).

FIGURE 2

The diffusible signaling factor (DSF)-mediated regulation of bacterial entry and establishment inside the host. The low level of DSF (as the smaller number of bacterial cells at early stage) promotes chemotaxis, and motility is executed toward the hydathodes to facilitate entry. A low level of DSF also allows the release of hydrolase enzymes (T2E) to extract nutrient from host. Further, high bacterial cell density results in induced expression of XadM and XadA, which are involved in cell-to-host surface extracellular polysaccharides (EPS) release, and cell-to-cell attachment, respectively. The high DSF density results in low production of T2E and high production of EPS, resulting in adhesins and consequent biofilm formation. DSF is represented in a blue/cyan star shape.

DSF AS A NOVEL PAMP TO INDUCE PLANT DEFENSE RESPONSE

Studies indicate that DSF can also serve as a pathogen-associated molecular pattern (PAMP) to induce plant innate immune response. Synthetic DSF can induce callose deposition, programmed cell death, and induction of expression of PAMP-responsive genes in diverse plants such as rice, *Arabidopsis*, and *Nicotiana benthamiana* (Boutrot and Zipfel 2017; Kakkar et al. 2015; Ranf 2017). The study showed that *X. campestris* pv. *campestris* EPS can act as a suppressor of DSF-induced plant defense response (Kakkar et al. 2015). The *rpfF* mutant of *X. campestris* pv. *campestris* induced less callose deposition compared with the wild-type cells, but mutants defective in EPS production induced a higher amount of callose. Importantly, a *gumD* (EPS)-*rpfF* (DSF) double mutant rescued the hyper-callose-inducing phenotype of the EPS mutant. Interestingly, expression of DSF synthase in *Pseudomonas syringae* B728a, which uses acyl-homoserine lactone-mediated quorum sensing, induced much more callose deposition and increased the host defense responses (Kakkar et al. 2015). These results indicate that during the course of *Xanthomonas*-host coevolution, plants acquired the ability to recognize DSF as PAMP, and that the *Xanthomonas* group of phytopathogen secretes EPS to suppress DSF-induced innate immunity, perhaps by chelating calcium ions. However, this phenomenon has not been investigated in other phytopathogenic bacteria-host interactions.

INSIGHTS INTO THE IRON HOMEOSTASIS AND XANTHOMONAS VIRULENCE

Iron is an essentially required element for almost all organisms. The iron present in two oxidation states, one ferric iron (Fe^{3+}), is abundant but insoluble at physiological pH and biologically inaccessible; and another ferrous iron (Fe^{2+}) is scarce but soluble and biologically accessible. Iron participates in the catalysis of several cellular redox reactions, including respiration, photosynthesis, DNA replication, and energy metabolism (Andrews et al. 2003; Lieu et al. 2001). However, excess iron induces the generation of reactive oxygen species through Fenton's reaction, which may cause cellular damage (Hatcher et al. 2009; Touati 2000; Vattanaviboon and Mongkolsuk 1998). Therefore, cellular storage and distribution of iron require strict regulation.

The limited availability of the soluble form of iron and the iron-holding strategies employed by the eukaryotic hosts creates a low iron environment for microbial pathogens. Adaptation to low-iron conditions inside the host is crucial for successful disease establishment by both animal and plant pathogenic bacteria (Cassat and Skaar 2013; Cornelis and Andrews 2010; Expert et al. 1996; Teper et al. 2021). *Xanthomonas* spp. employ various strategies to maintain iron homeostasis under different levels of iron availability. Most factors associated with iron homeostasis are widely conserved among xanthomonads but vary at the molecular level of regulation and their role in virulence (Pandey et al. 2016; Pandey and Sonti 2010; Rai et al. 2015).

XANTHOFERRIN-MEDIATED IRON UPTAKE AND VIRULENCE

Xanthomonads produce and secrete xanthoferrin, an α -hydroxy-carboxylate-type of siderophore, into the extracellular milieu, which chelates and solubilizes the ferric iron to make it accessible (Pandey et al. 2017a, 2017b; Pandey and Sonti 2010). The *Xanthomonas* siderophore synthesis (*xss*) gene cluster encodes the enzymes (*XssABCDE*) required for xanthoferrin production. It also encodes *XsuA*, which is required for the uptake of the xanthoferrin-ferric iron complex, and *MphE*, whose function is unknown. *XsuA*, a homolog of the TonB-dependent outer membrane receptor, is required

for Xanthoferrin-mediated iron uptake, but does not play a role in siderophore synthesis. The bacterial TonB-dependent receptor, with the TonB, ExbB, and ExbD protein complexes, plays a role in siderophore-mediated iron uptake (Fig. 3) (Ferguson and Deisenhofer 2004; Larsen et al. 1999; Wiener 2005; Wiggerich et al. 1997). The interaction of the siderophore–ferric iron complex with the TonB-dependent receptor causes a conformational change in the plug domain of the channel, which leads to the internalization of a protein complex into the periplasmic space (Buchanan et al. 1999; Miethke and Marahiel 2007). The protein complex XsuA of xanthomonads is homologous to the TonB-dependent transporters, which are made up of a C-terminal 22-stranded transmembrane β -barrel enclosing a N-terminal globular plug domain (Noinaj et al. 2010; Pandey et al. 2017a). The periplasmic protein TonB gets energized by integral

membrane proteins ExbB and ExbD using the electrochemical charge gradient of a cytoplasmic membrane to release the siderophore–iron complex (Fig. 3) (Larsen et al. 1999; Letain and Postle 1997; Woolldridge et al. 1992). Siderophore-specific periplasmic-binding proteins deliver the siderophore–iron complex to the cognate ABC transporter at the cytoplasmic membrane for transport into the cytoplasm (Fig. 3) (Köster 2001). The *xss* gene cluster is conserved among the xanthomonads and also shows close homology with the *pvs* operon of human pathogen *Vibrio parahaemolyticus* (Pandey et al. 2017a; Pandey and Sonti 2010; Tanabe et al. 2003).

The xanthoferrin-mediated iron uptake supports growth under iron-depleted conditions. A mutant strain lacking the siderophore biosynthesis gene ($\Delta xssA$) in the *X. campestris* pv. *campestris* 8004 (*X. campestris* pv. *campestris* 8004) background exhibits the

FIGURE 3

Iron homeostasis regulation in xanthomonads. The enzymes required for xanthoferrin siderophores biosynthesis are encoded by the *xss* gene cluster. The XibR coordinates with ferric iron and binds with the *xss* promoter. XibR negatively regulates the *xss* expression in response to the available iron sensed by an unknown sensor. The ferric uptake regulator (Fur) coordinates with ferrous iron and binds at the *xss* promoter to negatively regulate the siderophore production under high iron conditions. VgrS senses the extracytoplasmic iron and then phosphorylates the cognate response regulator VgrR, which negatively regulates the *tdvA* expression. TonB-dependent receptors with ExbB and ExbD, PBP, and ABC complexes are involved in xanthoferrin-ferric iron uptake. The ferrous iron uptake takes place by FeoB complex (FeoABC) transporter at the cytoplasmic membrane. The periplasmic glucan sequesters the iron to make a reservoir to support bacterial growth under low-iron conditions. EM, extracellular moiety; OM, outer membrane; P, periplasmic; IM, inner membrane; C, cytoplasmic; S, sensor; PBP, periplasmic binding protein; G, glucan; X, xanthoferrin siderophore.

growth defect under low-iron conditions, which can be amended by external supplementation of xanthoferrin or iron (Pandey et al. 2017a; Pandey and Sonti 2010). However, external supplementation of xanthoferrins fails to restore the low-iron growth defect of a mutant lacking the siderophore utilization gene *xssA*. The $\Delta xssA$ mutant can utilize the xanthoferrin–iron complex but exhibits a severe deficiency in siderophore production; although the $\Delta xssA$ mutant can produce siderophores, it is unable to uptake the xanthoferrin–iron complex (Pandey et al. 2017a).

Siderophore-mediated iron-uptake is a powerful strategy that evolved in pathogenic bacteria to access iron in an iron-depleted environment. However, the contribution of siderophore-mediated iron uptake in virulence is not consistent among pathogenic bacteria. Siderophores play a significant role in achieving optimum virulence in several phytopathogenic bacteria, including *Erwinia chrysanthemi*, *E. amylovora*, and *P. syringae* pv. *tabaci* 6605 (Dellagi et al. 1998; Enard et al. 1988; Franza et al. 2005; Taguchi et al. 2010). However, the siderophore-deficient mutants of *E. carotovora* subsp. *carotovora*, *Agrobacterium tumefaciens*, *Ralstonia solanacearum*, and *P. syringae* pv. tomato DC3000 remain virulent (Bhatt and Denny 2004; Bull et al. 1996; Jones and Wildermuth 2011; Rondon et al. 2004). The xanthoferrin production and utilization of xanthoferrin–iron complex are required for optimal virulence and *in planta* growth of *X. campestris* pv. *campestris*. In *X. campestris* pv. *campestris* 8004, the *xss* operon is induced under iron-depleted conditions (in the presence of iron chelator 2,2'-dipyridyl) and is repressed under an iron-replete condition. (Pandey et al. 2017a). Similarly, in *X. oryzae* pv. *oryzicola*, expression of the siderophore biosynthetic cluster is induced *in planta* and under iron-depleted conditions. In *X. oryzae* pv. *oryzicola*, loss of the siderophore biosynthesis gene *xssA* reduces virulence on rice plants (Rai et al. 2015). In *X. oryzae* pv. *oryzae*, siderophore production is not required for *in planta* growth and virulence and is not expressed during colonization inside the rice xylem vessels (Pandey and Sonti 2010). However, in *X. oryzae* pv. *oryzae*, the ferrous uptake system mediated by the ferrous iron transporter FeoB is required for optimal virulence (Pandey and Sonti 2010). FeoB interacts with FeoA and FeoC to form an active complex at the cytoplasmic membrane, which is required for the uptake of ferrous iron (Fig. 3) (Stevenson et al. 2016). However, the role of FeoB-mediated ferrous iron uptake in the virulence of other xanthomonads needs to be investigated.

Differences in the forms of iron uptake systems in closely related *Xanthomonas* phytopathogens may have arisen from adaptation strategies evolved to cope with differences in availability/abundance of different ferrous-versus-ferric iron molecules in the host plant and in their colonization sites (Rai et al. 2015).

REGULATION OF IRON HOMEOSTASIS IN XANTHOMONADS

Microorganisms require finely coordinated regulation of iron homeostasis especially for the deployment of various iron-sequestering strategies and protection against high iron-induced toxicity. The bacterial ferric uptake regulator (Fur) regulates coordinated gene expression in response to the level of iron availability (Mey et al. 2005; Troxell and Hassan 2013). Fur specifically coordinates to Fe^{2+} , causing conformational changes to bind at the 19-bp Fur box (Desai et al. 1996; Escobar et al. 1999). Under the iron-replete condition, the holo-Fur (Fur– Fe^{2+} complex) binds to the upstream of the target genes (e.g., the genes encoding proteins for iron uptake and siderophore biosynthesis) and represses the expression of Fe^{2+} . Under the iron-depleted condition, apo-Fur releases from the promoter, to allow RNA polymerase binding and expression of target genes (Fig. 4) (Troxell and Hassan 2013).

A genetic screen in *X. campestris* pv. *campestris* for altered siderophore production led to the identification of another iron-associated regulator: the *Xanthomonas* iron binding regulator (XibR). XibR belongs to the NtrC family of proteins, which are conserved among the xanthomonads and have homologs in *Pseudoxanthomonas dokdonensis*, *Lysobacter* sp. URHA0019, and *Bordetella bronchiseptica*. XibR regulates multiple virulence-associated functions including siderophore production, iron uptake and storage, motility, and chemotaxis occurring in response to iron availability. XibR negatively regulates siderophore production, and mutants lacking *xibR* produce a high amount of xanthoferrins under iron-restricted conditions. The specific binding of ferric iron (Fe^{3+}) with XibR causes conformational changes that allow it to bind to promoters of operons involved in siderophore production (*xss*), motility, chemotaxis, and flagellar biogenesis (*mot* and *flg*). XibR positively regulates motility and chemotaxis. The induced bacterial chemotaxis response for xylose and glutamic acid under iron-limiting conditions requires the presence of XibR. Further, XibR contributes to *X. campestris* pv. *campestris* virulence on cabbage (Pandey et al. 2016).

The VgrS/VgrR (ColS/ColR) two-component system controls the expression of *hrp* genes and is required for *in planta* colonization and virulence of xanthomonads (Subramoni et al. 2012; Yan and Wang 2011; Zhang et al. 2008). VgrS/VgrR (ColS/ColR) system responds to the availability of iron, zinc, manganese, and cadmium, and influence several functions, including iron uptake, biofilm formation, catalase activity, lipopolysaccharide production, and tolerance to environmental stresses (Subramoni et al. 2012; Teper et al. 2021; Wang et al. 2016; Yan and Wang 2011; Zhang et al. 2008). VgrS, the membrane-bound receptor histidine kinase, senses periplasmic iron limitation and the cognate sensor VgrR detects the iron-replete condition in the cytoplasm. Phosphorylated VgrR directly suppresses expression of the TonB-dependent receptor encoding gene, *tdvA*, by binding the promoter site (Wang et al. 2016). *tdvA* encodes a TonB-dependent receptor in xanthomonads. Loss of *tdvA* in *X. campestris* pv. *campestris* leads to enhanced *in planta* growth and virulence, while its overexpression reduces *in planta* growth and virulence on cabbage (Wang et al. 2016). The interaction between XibR (orphan response regulator) and VgrS/VgrR two-component system is yet to be investigated.

Besides Fur and XibR, the LysR-type carbohydrate-related transcriptional regulator LcrX and DNA methyltransferase EadM in *X. axonopodis* pv. *glycines*, the GntR family transcriptional regulator YtrA, and the RNA polymerase-binding transcription factor DksA in *X. citri*, all influence siderophore production (Park et al. 2019, 2020; Zhang et al. 2019; Zhou et al. 2017). *In silico* analysis predicts the presence of several iron homeostasis related homologs in *X. citri* subsp. *citri*, including *fur*, *xibR*, *xss*, *feca*, *feoABC*, *fluA*, and the TonB-dependent receptor encoding genes (Guerra and Balan 2020). DSF-mediated cell-to-cell signaling also regulates the iron homeostasis of xanthomonads (Chatterjee and Sonti 2002; Rai et al. 2015). An *rpjF* (DSF synthase-encoding gene) mutant of *X. oryzae* pv. *oryzae* overproduces siderophores and has reduced growth in low-iron conditions. Exogenous ferrous (Fe^{2+}) iron supplementation can rescue the *in planta* growth and migration defect of the *rpjF* *X. oryzae* pv. *oryzae* mutant (Chatterjee and Sonti 2002). In contrast to *X. oryzae* pv. *oryzae*, the *rpjF* mutant of *X. oryzae* pv. *oryzicola* exhibits a reduced expression of siderophore biosynthesis genes, a low ferric iron uptake, and a deficiency in *in planta* growth and migration (Rai et al. 2015). The differences in the DSF-mediated regulation of different iron uptake genes (ferrous versus ferric) in *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* may have evolved as an adaptation strategy based on the availability and abundance of the different forms of iron that they encounter in the xylem versus parenchyma, respectively.

Apart from DSF-mediated regulation of iron metabolism, studies have shown that chemotaxis-related genes and regulators also alter

the siderophore production and the intracellular level of iron in *X. oryzae* pv. *oryzae*. $\Delta cheA3$, $\Delta cheB2$, $\Delta cheD$, $\Delta cheR1$, $\Delta cheR2$, $\Delta cheW2$, $\Delta cheW3$, $\Delta cheY2$, $\Delta cheZ$, and $\Delta vieA$ mutants exhibit overproduction of xanthoferrin siderophores. Conversely, $\Delta cheA2$, $\Delta cheW1$, and $\Delta cheY1$ mutants show reduced siderophore production. Indirect measurement of iron while assessing streptonigrin sensitivity reveals high intracellular iron content in $\Delta cheA1$, $\Delta cheA2$, $\Delta cheB1$, $\Delta cheR3$, $\Delta cheV$, $\Delta cheW1$, $\Delta cheY1$, $\Delta cheY2$, $\Delta cheZ$, $\Delta mcp2$, $\Delta pdeA$, and $\Delta vieA$ mutants. However, $\Delta cheA3$, $\Delta cheB2$, $\Delta cheR1$, $\Delta cheR2$, $\Delta cheW2$, and $\Delta cheW3$ display a low intracellular level of iron (Kumar Verma et al. 2018).

ROLE OF CYCLIC β -(1, 2)-GLUCANS IN IRON HOMEOSTASIS OF XANTHOMONADS

Bacterial cyclic β -(1, 2)-glucans are present in periplasmic space (membrane-derived oligosaccharide) as well as secreted as a minor component of EPS (Javvadi et al. 2018; Wanke et al. 2021). EPS and cyclic β -(1, 2)-glucan-deficient mutants overproduce siderophores in *X. campestris* pv. *campestris*. Pharmacological analysis demonstrates the direct binding of *Xanthomonas* cyclic β -(1, 2)-glucan (membrane-derived oligosaccharide and secreted) with ferrous iron (Fe^{2+}). The iron-binding property is conserved among the glucans isolated from various sources including barley, euglena, and yeast. Periplasmic cyclic β -(1, 2)-glucan sequester iron to protect bacteria against the excess iron-induced toxicity. Iron-bound cyclic β -(1, 2)-glucan in the plasma membrane acts as a reservoir and supports bacterial growth under iron-depleted conditions (Javvadi et al. 2018).

IRON-LIMITING HOST ENVIRONMENT AND EXPRESSION OF HR FACTORS IN XANTHOMONADS

Transcriptome analysis shows the induced expression of several *hrp* (hypersensitivity and pathogenicity) genes including type-3 secretion system- and type-3 effector-encoding genes under iron-restricted conditions in *X. campestris* pv. *campestris* (Pandey et al. 2016, 2018). Interestingly, the pattern of induced expression of

type-3 secretion system- and type-3 effector-encoding genes under low iron varies among the xanthomonads. The *hrcV*, *hrcQ*, *hrpB1*, *hrcU*, *hrpB4*, *hrcN*, and *hrpG* in *X. campestris* pv. *campestris* 8004, the *hrcV*, *hrpB1*, *hrcU*, and *hrpB4* in *X. oryzae* pv. *oryzicola*, and the *hrcV* and *hrpB1* in *X. oryzae* pv. *oryzae* show induced expression under low iron conditions. *X. campestris* pv. *campestris* 8004 exhibits largest induction of *hrp* genes, followed by *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae*. However, siderophore production is much higher in *X. oryzae* pv. *oryzae* than in *X. oryzae* pv. *oryzicola* and *X. campestris* pv. *campestris* (Pandey et al. 2018). Intracellular iron levels may vary among xanthomonads, leading to differential expression of *hrp* genes in response to the low-iron environment. It is evident that xanthoferrin-mediated iron uptake is higher in *X. oryzae* pv. *oryzicola* than *X. campestris* pv. *campestris* (Pandey et al. 2017a; Rai et al. 2015). Iron supplementation suppresses HR induction in non-host plants as well as disease development in host plants by *X. campestris* pv. *campestris*. However, iron supplementation does not affect the HR induction by *X. oryzae* pv. *oryzae* (Pandey et al. 2018).

Several factors regulating the iron homeostasis in xanthomonads have been identified, from DSF-mediated cell-to-cell signaling to the influence of chemotaxis-associated genes on iron uptake and metabolism. Fur and XibR are the major contributors to iron homeostasis regulation in this group of phytopathogenic bacteria. Still, several possible iron-regulating factors remain unexplored. For instance, *X. oryzae* pv. *oryzae* and *X. campestris* pv. *campestris* display extracellular ferric reductase activity to convert insoluble ferric iron to soluble ferrous iron (Pandey et al. 2017a; Rai et al. 2015). However, the factors responsible for this activity have not yet been identified. Understanding iron homeostasis in phytopathogenic bacteria is crucial to devise the disease control strategies based on the iron-homeostasis-regulating functions. From the study from *X. campestris* pv. *campestris*, it is evident that the modulation of in planta iron can reduce the disease severity (Pandey et al. 2018). The deferrioxamine siderophore-mediated iron chelation induces plant immunity and host defense response in *A. thaliana* (Aznar et al. 2014). As closely related xanthomonads exhibit a substantially distinct mode of regulation of DSF and the iron metabolism-associated

FIGURE 4

Regulation of iron uptake genes by iron-responsive transcriptional regulator ferric uptake regulator (Fur) and quorum-sensing molecule diffusible signaling factor (DSF). The abundance of iron leads the holo-Fur (Fe^{2+} -Fur) binding to the promoter region of iron uptake genes and suppresses the expression. Under low iron condition, apo-Fur releases from promoter and then RNA polymerase (RNAP) binds to start the gene expression. The high DSF induces the ferric and ferrous iron uptake system, encoding genes in *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae*, respectively.

functions required for virulence, a detailed understanding of the mechanism would help to devise species- or pathovar-specific strategies to control diseases (Chatterjee and Sonti 2002; Pandey et al. 2018; Pandey and Sonti 2010; Rai et al. 2015; Thowthampitak et al. 2008).

INTERPLAY OF QUORUM SENSING AND IRON METABOLISM

DSF-mediated quorum sensing and cell-to-cell signaling influence the iron uptake and metabolism in xanthomonads. Interestingly, the impact of DSF on iron uptake and metabolism varies within closely related strains or pathovars of *Xanthomonas*. In *X. oryzae* pv. *oryzicola*, which causes bacterial leaf streak, DSF induces ferric iron uptake by positively regulating the production of xanthoferrin siderophore and low-affinity ferric iron transporters (Figs. 1 and 4). In *X. oryzae* pv. *oryzicola*, DSF-deficient mutants exhibited reduced ferric iron uptake under low iron conditions but were proficient in ferrous iron uptake. The deletion mutant of *xssA* phenocopies the $\Delta rpfF$ in *X. oryzae* pv. *oryzicola* for lower ferric iron uptake, growth deficiency under low iron, and virulence. However, DSF mutants were deficient in growth under low iron conditions and exhibited less intracellular storage of iron. Interestingly, a DSF mutant upregulates organic acids synthesis genes, and it over-produced oxalic acids (a low affinity ferric iron chelator) as an iron starvation response because of the deficiency in the production of the high-affinity ferric siderophore transport system (Rai et al. 2015). The Krebs cycle intermediate organic acids are secreted under iron-depleted conditions by a few bacteria from the genera *Bradyrhizobium* and *Rhizobium*, as well as some fungi, and might act as low-affinity iron chelators (Carson et al. 1992; Frawley et al. 2013; Howard 1999). Expression analysis suggests that DSF positively regulates several ferric iron-uptake-related genes in *X. oryzae* pv. *oryzicola*. The DSF-deficient mutant exhibits a 4- to 5-fold lower production of xanthoferrin siderophores compared to the wild-type strain (Rai et al. 2015).

In contrast, in the bacterial leaf blight pathogen of rice, *X. oryzae* pv. *oryzae*, DSF positively regulates the ferrous iron uptake system (Fig. 4) (Chatterjee and Sonti 2002; Rai et al. 2012). Transcriptome analysis suggests a general upregulation of iron uptake and metabolism by DSF in vascular pathogen *X. oryzae* pv. *oryzae*. The $\Delta rpfC$ and $\Delta rpfG$ mutants failed to phenocopy $\Delta rpfF$ for growth deficiency under low iron but displayed reduced EPS production and virulence (Rai et al. 2012). These results suggest that the RpfCG two-component system influences the virulence and EPS production by other, unidentified pathways.

It has been proposed that regulation of two different types of iron uptake systems by DSF may have evolved from differences in the availability of different types of iron in the host tissue. *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* both share a common host, but have distinct modes of infection, colonization in tissues, and disease symptoms. *X. oryzae* pv. *oryzae* enters through the hydathodes and colonizes vascular tissues, while *X. oryzae* pv. *oryzicola* enters through stomata and colonizes intercellular spaces of mesophyll tissues (Niño-Liu et al. 2006; Ryan et al. 2011). Because *X. oryzae* pv. *oryzae* is a xylem-limited pathogen, ferrous iron may predominate as the available iron source under these conditions. In contrast, *X. oryzae* pv. *oryzicola* colonizes the intercellular space of parenchyma, where it has been proposed that the ferric form of iron may predominate. Pathogens colonizing nonvascular tissues (e.g., apoplast) utilize iron mainly from storage vesicles, which contain mostly ferric iron (Expert et al. 2012; Palmer and Gueriot 2009).

The level of intracellular iron content plays a crucial role in bacterial tolerance against oxidative stress (Touati 2000). Quorum sensing contributes to the adaptation of bacteria under oxidative stress by regulating bacterial iron homeostasis and controlling the expression of catalase, superoxide dismutase, and oxidative

stress-associated transcriptional factors (Hassett et al. 1999; He and Zhang 2008; Rai et al. 2015). The *fur* mutant of xanthomonads exhibits hypersensitivity to hydrogen peroxide, reduced catalase activity, and poor survival under oxidative stress along with the constitutive siderophore production, high level of intracellular iron, and virulence deficiency (Jittawuttipoka et al. 2010; Liu et al. 2016; Subramoni and Sonti 2005). The bacterial response to oxidative stress is coordinated by several transcriptional regulators including OxyR (LysR family), PerR (Fur family), and OhrR (MarR family) by sensing the intracellular level of reactive oxygen species (Dubbs and Mongkolsuk 2012; Sukchawalit et al. 2001; Yu et al. 2016). The direct involvement of quorum sensing in bacterial adaptation under oxidative stress has been well studied in *Pseudomonas* spp. (García-Contreras et al. 2015; Hassett et al. 1999; Wagner et al. 2003). However, extensive investigations are required to understand the correlation between quorum signals and bacterial responses against oxidative stress in xanthomonads.

It is pertinent to note that many of the DSF-regulated virulence-associated functions such as motility, chemotaxis, and components of iron metabolism are also influenced by iron and iron-responsive regulators (Pandey et al. 2016, 2018). It is possible that the DSF and iron-sensing signal transduction pathway engage in cross talk, and several of these social behaviors are regulated or fine-tuned by both changes in cell density as well as availability of iron in the environment. Future work on the understanding the mechanism/s of coregulation of virulence-associated functions by DSF and iron could elucidate how complex social behaviors are fine-tuned by multiple environmental signals that control the complex lifestyles of several xanthomonads.

In conclusion, several virulence-associated functions such as motility, chemotaxis, biofilm formation, production of secreted siderophores, and other cell-wall-degrading enzymes as well as induction of HR response, are coregulated by both DSF-mediated cell-to-cell signaling as well as iron sensing and regulation in *Xanthomonas*. Future research will investigate the mechanism of how the sensing systems are coordinated in their regulation of pathogenicity, and seek to understand the factors that connect density-dependent signaling with iron sensing and homeostasis.

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