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Quorum Sensing, Its Role in Virulence and Symptomatology in Bacterial Citrus Canker

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Additional information is available at the end of the chapter

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Abstract

Xanthomonas citri subsp. *citri* (Xcc) is the etiological agent of citrus canker, a disease that affects almost all types of citrus crops. Production of Xcc virulence factors is controlled by a gene cluster *rpf*, which encodes elements of a cell-cell communication system called quorum sensing (QS). Perturbation of cell-cell signaling systems either by signal degradation or by overproduction significantly reduces symptoms and thereby the severity of the disease. Pathogenicity assays in *Citrus sinensis* showed that some bacterial species natural inhabitants of citrus phyllosphere have the strong ability to disturb QS system mediated by diffusible signal factor (DSF) molecule in Xcc and to reduce disease severity. The lessening of symptoms was associated with alteration in bacterial attachment and biofilm formation. These factors are known to contribute to Xcc virulence in the early stages of disease. The aim of this chapter is to review QS system in Xcc, the virulence factors affected by QS disturbing, as well as the main secretion systems that participate actively in virulence and its effect on the symptomatology of citrus canker.

Keywords: *Xanthomonas citri*, DSF, quorum quenching, biofilm, adhesin

1. Introduction

Of the entire bacterial population that comprise the plant microbiome, both phyllosphere and rhizosphere, the vast majority live as saprophyte on the plant surfaces and a few of them get internalized into the plant tissues, without causing any damage to their host; only about 100 bacterial species has become pathogenic to their host. Pathogenicity of microorganisms is due to the expression of virulence factors, which could be attributed to their genetic, biochemical, or structural traits in the attempt to produce host infection. Quorum sensing (QS) is a cell-

cell communication system, which leads to the regulation of specific genes, which in bacteria control essential biological functions as bioluminescence, antibiotic production, virulence, motility, and biofilm formation. In the bacterium *Xanthomonas citri* subsp. *citri* (Xcc), the etiological agent of citrus canker, the production of particular pathogenicity factors is controlled by a cluster of genes called *rpf* (for regulation of pathogenicity factors), which encodes elements of a QS system mediated by molecules of the diffusible signal factor (DSF) [1]. Interference with cell-cell signaling, also termed quorum quenching, drastically decreases disease symptomatology and is a promissory tool for biological control [2]. The aim of this chapter is to review what is known so far about mechanistic details of QS system in Xcc, the virulence factors affected by QS disturbing in Xcc, and its effect on the symptomatology of citrus canker.

2. *X. citri* subsp. *citri* virulence factors

The genus *Xanthomonas* (from Greek xanthos which means yellow) comprises a large group of plant pathogenic bacteria belonging to the group of Gamma proteobacteria. The xanthomonas bacteria infect 124 species of monocot plants and 268 species of dicot plants [3]. They are Gram-negative rod-shaped bacteria with a single polar flagellum, colonies are yellow because of a pigment named xanthomonadin and have a glossy appearance due to exopolysaccharide (EPS) called xanthan [4]. Although the genus itself has a very wide range of hosts, individual members are generally specialized to cause disease in a limited number of taxonomically related hosts. Several specialized virulence factors are employed by *Xanthomonas* bacteria to successfully invade the tissues of their susceptible hosts and multiply within them and cause disease.

The bacterium *X. citri* subsp. *citri* (Xcc) is the etiological agent of bacterial citrus canker (CC), a disease that affects almost all types of citrus crops. The invasion and colonization of the host occur through stomata and wounds in plant tissues, infecting leaves, fruits, and stems. Xcc harbors a wide range of virulence factors as surface attachment structures, cell-wall-degrading enzymes, several secretion systems and their effectors, and a diffusible signal factor-mediated quorum-sensing (QS) system [5].

2.1. Adhesins

A fundamental step in the bacterial colonization of host is its ability to attach to the surface of host cells. Bacterial surface structures anchored in its outer membrane that enables adhesion are termed adhesins, which are mostly of polysaccharidic nature (lipopolysaccharides (LPS) and exopolysaccharides) but may also be of proteinaceous nature (type IV pili, chaperone/usher pili, two-partner secretion) [6].

2.1.1. Lipopolysaccharide and exopolysaccharide

The lipopolysaccharides (LPS) play a role in adhesion, but it is difficult to determine whether it affects directly or indirectly the bacterial adhesion [7]. LPS is a crucial constituent of the

outer membrane in Gram-negative bacteria and plays multiple roles in plant-microbe interactions [8]. LPS acts as a barrier for protecting bacteria from adverse environmental factors such as antibacterial compounds produced by the host cell, besides triggering or enhancing plant-defenses responses, because it acts as pathogen-associated molecular pattern (PAMP) identified in a widespread variety of phytopathogenic bacteria [9]. A recent study by Casabuono et al. [10] identified the structure of LPS in Xcc as a penta- or tetra-acylated diglucosamine backbone attached to either two pyrophosphorylethanolamine (PP-EtNH₂) groups or to one PP-EtNH₂ group and one phosphorylethanolamine group. The core region is composed by a branched oligosaccharide and two phosphate groups, whereas the O-antigen consists of a rhamnose homo-oligosaccharide. Xcc mutants of *wxacO* and *rfbC* genes form an impaired biofilm on glass or host plant leaves reducing resistance to antimicrobial compounds such as polymyxin B and hydrogen peroxide. Both mutants also showed deficiency in virulence and growth on host leaves of susceptible host after spray inoculation [11]. Moreover, additional six LPS genes, that is, *wzm*, *wzt*, XAC3591, XAC3593, XAC3595, and XAC3597, were described to be involved in biofilm formation [12]. The *wzm* and *wzt* genes encode an ATP-binding cassette (ABC) transporter system that exports the O-antigen polysaccharide of LPS [13]. Mutation of *wzm* and *wzt* decreases the virulence of Xcc and significantly reduces bacterial populations in the host plant [5, 10].

The exopolysaccharide (EPS) in *Xanthomonas* is known as xanthan gum, which is another bacterial polysaccharide released as an extracellular slime in the late stationary growth phase. The EPS in Xcc is involved in biofilm formation that facilitates intimate association of bacteria to abiotic and biotic surfaces [13]. EPS in xanthomonads is composed by a backbone of β -1,4-linked D-glucose with trisaccharide side chains of mannose-(β -1, 4)-glucuronic acid-(β -1,2)-mannose attached to alternate glucose residues in the backbone by a 1,3 linkages [14]. Xanthan production in *Xanthomonas* is hierarchically regulated by *rpf* (regulation of pathogenicity factors) gene cluster [15]. Biosynthesis and exportation of EPS in Xcc are encoded by *gum* gene cluster (i.e., *gumB* to *gumP*), *gumCDEFJK* genes also are involved in biofilm formation in biotic and abiotic surfaces [12]. An early study has showed the expression in the plant of *gum* operon of Xcc using a reporter β -glucuronidase [16]. The authors found that bacteria isolated from leaves and subsequently inoculated into mesophilic tissue have an increased expression of *gum* gene in the later stages of the bacterial growth, suggesting a requirement for EPS production at later stages of infection. Xcc *gumB* deletion mutant has shown a significant reduction in the EPS production as well as an impaired ability to form a well-structured biofilm, leading to a deep reduction in severity disease [17]. Nevertheless, the defective mutant *gumD* remains fully pathogenic when it has been inoculated by infiltration pressure to the susceptible host, but displayed impaired survival capacity in citrus leaves and a reduction in symptoms when abaxial surfaces of leaves citrus were sprayed with a solution containing the deletion mutant *gumD*, suggesting a significant role of biofilm formation in the epiphytic fitness of Xcc, critical event in the early stages of pathogenicity development of citrus canker [18]. EPS also acts as plant-defense suppressor by preventing the callose deposition in the plant cell wall, because it operates as a chelator of divalent calcium ions present in the plant apoplast [19].

2.1.2. Proteic attachment structures

Pili (fimbriae) are filamentous appendices linked to cell surface; it has been categorized by their potential to induce hemagglutination, their anchoring site in bacteria (i.e., polar or omnidirectional) and their size. Several types of pili in *Xanthomonas* genus bacteria have been demonstrated or hypothesized to exist such as type IV pili, chaperone/usher pili, and pili linked to different protein secretion systems [20].

Homopolymeric ensemble of the pilin proteins termed PilA and PilE is the main constituent of *type IV pili* whose structure is associated to the type II secretion system [21]. Completely assembled structure projects from the bacterial surface to contact biotic or abiotic surfaces and once firmly attached retracts to bacterial cell wall, leading to bacterial cell movement known as twitching motility [22]. Type IV pili-related genes are consistent in the vast majority of sequenced strains of *Xanthomonas*; these genes are highly diverse and do not have any association with the phylogenetic relationship of strains. In Xcc, a tandem duplication of pilin has befallen and its consequences remain uncertain (i.e., whether there is an antigenic switch due to differential expression of the two genes) [23]. The changeability among major pilins could be related with specific interactions with their hosts, evocative of the situation in human and animal pathogens. The role of type IV in attachment and pathogenicity to their host plant is crucial in *Xanthomonas* bacteria. Studies using scanning electron microscopy (SEM) have shown that defective PilA mutants of several *Xanthomonas* species compromised its adhesion capacity, cell aggregation, and decrease its survivor and colonization of leaves [23].

Genes that encode for type I pili or CU pili (chaperone/usher) were first demonstrated in *Xylella fastidiosa* [24] in *Xanthomonas* there is a conserved gene cluster for type I pili. In *Xanthomonadaceae* family type I pili play a central role in cell attachment, cell aggregation, and biofilm formation [25].

2.2. Pigment xanthomonadin

Members of the group of *Xanthomonas* genus produce a yellow pigment where chemical structure is mono- or di-bromo-aryl polyene. This pigment is bounded to the outer membrane and is termed xanthomonadin. A group of genes *pigA-pigG* are required for the biosynthesis of pigment and were first identified in *X. campestris* pv. *Campestris* [26]. The xanthomonadin's main function is to protect bacteria against photobiological damage. Actually, there are no conclusive studies on the protective effect of xanthomonadin against UV radiation injury. Comparison between *pigB*-defective mutants and wild-type strain *X. campestris* pv. *Campestris* does not display differences in sensitivity to UVA and UVC radiation damage. Xanthomonadin provides defense against visible light injury, because the pigment protects the lipid component of cell membrane from reactive oxygen species generated by the exposure to visible light [27]. Defective mutants of *pigB* gene in *X. campestris* pv. *Campestris* were unable to survive on the surface of the leaves and also the infection of leaves was severely reduced when the infection is through stomata and hydathodes. Interestingly, the same mutant does not display variation in virulence after inoculation by infiltration pressure or spray in wounded leaves, indicating that the pigment is necessary to epiphytic infection, but is not required for growth in the inner tissue of susceptible host plant [28]. In addition to the loss of xanthomonadin production, defective mutants of *pigB* strains were also impaired in EPS production [28].

2.3. Virulence-related protein secretion systems and their effectors

Bacteria belonging to *Xanthomonas* genus display at least six different types of protein secretion system (i.e., T1SS to T6SS), which diverge in their arrangement, function, and in a recognition of secretion substrates [21]. As numerous Gram-negative phytopathogenic bacteria, Xcc employs mainly T2 secretion system (T2SS), T3SS, T4SS, and T5SS and their effectors as effective tools in an attempt to invade and to multiply in a susceptible host.

2.3.1. T2 secretion system (T2SS)

The T2SS was initially discovered in *Shigella flexneri*, a human pathogen bacterium [29]. T2SS is the principal secretion system that enables protein transport from bacterial periplasm to the extracellular milieu. It secretes extracellular enzymes as lipases, proteases, and cell-wall-degrading enzymes. It is feasible that type II-secreted enzymes cooperate to the degradation of the plant cell wall, which is the foremost difficulty for plant pathogenic bacteria. T2SS apparatus is made up of 12–15 components, most of which are linked with the bacterial inner membrane [30]. A member of secretin protein family forms a transmembrane channel in the outer membrane. It is expected that the secretion across the outer membrane depends on a predicted periplasmic pilus that is uninterruptedly assembled and disassembled and thus impulses T2SS substrates through the secretin channel [31]. It is assumed that the substrates transported by T2SS could enable the assemblage of extracellular appendages of virulence associated with T3SS, T4SS, and T6SS systems that are dedicated to effector protein translocation, thereby synthesis of T2SS is co-regulated with T3SS expression genes; it suggests a functional interplay between both secretion systems [32]. Genome sequence analysis disclosed that Xcc is outfitted with two predicted T2SS systems, which are encoded by *xcs* and *xps* gene clusters [20]. Remarkably, T2SS besides associated to bacterial virulence also can induce plant-defense response such as the deposition of callose in the cell wall; it has been evidenced in *X. oryzae* pv. *oryzae*. Induction of basal plant-defense response T2SS dependent is suppressed by *X. oryzae* pv. *oryzae* that contains a functional T3SS system [33]. This allows us to assume that T3SS effectors proteins act against the basal plant-defense response elicited by T2SS.

2.3.2. T3 Secretion system (T3SS)

Pathogenic bacteria employ the T3SS secretion system termed “needle,” in order to provide virulence factors (effectors) directly into host cells and consequently influence cell host activities [20]. Initially, the T3SS has been identified and studied in pathogenic animal bacteria, such as *Yersinia* spp., *Shigella* spp., and *Salmonella*, which were found to secrete a number of virulence determinants called Yops (*Yersinia* Outer Proteins), IPAS (Invasion Plasmid Associated Protein), and SIPS (*Salmonella* Invasion Proteins) into host cells. An early study has reported the presence of a homolog of T3SS in *Ralstonia solanacearum*, a phytopathogen bacterium of Solanaceae family as well as in several phytopathogenic bacteria including Xcc [34]. Xcc includes a *hrp* (hypersensitive response and pathogenicity) gene cluster, which comprises 26 genes from *hrpF* to *hpa2*, which encodes the T3SS proteins [35]. Deletion mutant strains *hrpB*, *hrpB4*, *hrcV*, and *hrcN* in Xcc completely abolished the bacterial ability to cause citrus canker symptoms on susceptible citrus host [5], thus confirming the critical role of T3SS in virulence of Xcc.

In the Xcc genome, 24 known and putative effectors have been identified [36]. One of the principal effectors carried by the T3SS in Xcc belongs to the family AvrBs3/PthA. These effectors contain functional domains characteristic of eukaryotic transcriptional activator and are thus named TALE (Transcription Activators Like Effector) [37]. Particularly, TALE contains a central repeat domain that recognizes the host DNA in a highly specific fashion. Each repeating unit contains 34 amino acids, with 12 and 13 hyper-variable amino acids termed VRD (Variable Repeat Di-residue). Thus, the composition and arrangement of different VRDs provide TALEs with tremendous capacity to recognize DNA host, and binding occurs with a high degree of specificity to a particular region at the promoter of target gene known as EBE (Effector Binding Element). Bioinformatics and experimental approach sophisticatedly demonstrated that each VRD type acts as a code recognizing a specific nucleotide [38]. Xcc contains four genes (*PthA*) that encode TALE, of which *pthA4* is known to be necessary for the formation of citrus canker lesions. Hypothetically, the TALE in Xcc encoded by *pthA4* gene induces a gene in the susceptible host resulting in the formation of erumpent lesions. Research done by Yang Hu et al. [39], on susceptible host gene expression, under TALE effect encoded by gene *pthA4* in *Xanthomonas* species, revealed a remarkable and constant searching for vulnerabilities in the host physiology. Based on these findings, it was proposed that erumpent lesions involve recruitment and expression of a single gene in the host, termed CsLOB. Induction of this gene by the effector encoded by the *pthA4* gene was assessed in sweet orange and grape, two susceptible host species to Xcc infection [39]. Although specific functions of CsLOB1 gene are unknown, previous studies have revealed that LOB protein domains are involved in the regulation of development of lateral organs, nitrogen, and anthocyanin metabolism. CsLOB1 gene also responds to plant hormones such as auxin, cytokinin, and gibberellin, as well as to environmental stimuli [40].

2.3.3. T4 secretion system (T4SS)

The translocator apparatus designated as type IV secretion system (T4SS) is an important virulence factor in several animals and plants bacterial pathogens, T4SS involving the secretion of proteins or DNA into the host cells [41]. The T4SS apparatus extends from the bacterial inner membrane through the periplasm; it ends at the outer membrane into a pilus-like structure, which protrudes from the surface of the bacterial cell. Several studies made on the *Agrobacterium tumefaciens vir B* locus revealed that 11 proteins form the T4SS apparatus [42]. Xcc has two sets of genes that individually are potential encoders for T4SS components [35], one placed in chromosome and the other on the plasmid pXac64. The T4SS translocator apparatus consists of a core 12 proteins, called VirB1-VirB11 and VirD4. Structural organization of T4SS currently is well elucidated: (i) a set of three cytoplasmic ATPase (VirB4, VirB11, and VirD4), which energizes the secretion process; (ii) a complex periplasmic core consisting of 14 repetitions of a trimer: VirB7-VirB9-virB10, in which virB10 is inserted in both inner and outer membranes and VirB7 is an outer membrane lipoprotein; (iii) an inner membrane complex that includes VirB3, VirB6, and VirB8; (iv) an extracellular pili formed by VirB2 and VirB5; and (v) VirB1 which is a periplasmic transglycosylase [43]. A differential feature of T4SS in Xcc is the presence of one protein called VirD4 (VirD4XAC2623), VirD4 recruit effectors for

T4SS secretion. A set of uncharacterized proteins termed VirD4-interacting proteins (XVIPs) is recruited by VirD4. A recent study by Souza et al. [44] has shown that XVIPs proteins secreted by the carrier apparatus T4SS of Xcc are toxins. These toxins kill contender bacteria in the niche. Peptidoglycan in bacterial cell wall is the target of XVIPs proteins.

3. Citrus canker, life cycle, symptoms, and types of diseases

One of the most important citrus diseases is citrus canker, affecting almost all types of citrus crops. The disease causes extensive damage to the cultivars and severity of infection varies with different bacterial species and the predominant weather conditions. The geographical origin of citrus canker is a matter of controversy. Lee et al. [45] reported that citrus canker may have emerged in southern China. However, several authors believe that the disease had its origin in particular regions of India and Java [46]. These reports suggest, therefore, that the origin of the disease has occurred in tropical areas of Asia, where it is assumed that citrus species have originated and been distributed to other areas through vegetative propagation material. Currently, citrus canker occurs in over 30 countries in Asia, Indian and Pacific Ocean islands, South America, and Southeast of USA [47]. Copper-based products are routinely used as a standard control measure for citrus canker.

The invasion and colonization of the host occur through natural openings of the leaves (the stomata) and wounds in the plant tissues. The pathogen multiplies within the intercellular spaces, inducing cell hyperplasia, leading to rupture of the leaf epidermis and resulting in raised corky and spongy lesions surrounded by a water-soaked margin, that is, the characteristic canker lesion. Yellowish chlorotic rings are also often observed on leaves and fruits and, when conditions are highly favorable to disease development, could produce general defoliation, tree decline, and premature fruit drop [48, 49].

There are three different forms of citrus canker produced by two species of *Xanthomonas*: citrus canker types A, B, and C. The differentiation of these forms is principally based on geographical distribution and a variety of pathogen hosts [50]. Asian form of citrus canker is caused by Xcc [48]. It is the most common and widespread, and its geographical distribution continues to increase. Disease is endemic in more than 30 countries: Asia, India, Pakistan, the islands of the Indian Ocean, Southeast Asia, South America, and Southeast China and Japan. Xcc has extended its host range, producing disease in most citrus species, that is, *C. paradisi*, *C. aurantifolii*, *C. sinensis*, and *C. reticulata*. Citrus canker type B is caused by *X. fuscans* subsp. *aurantifolii* type B (XauB) [36]. It has similar symptoms to symptoms present in type A citrus canker; nevertheless, the symptoms take longer to appear as a consequence of XauB slower growth. Host range is restricted to *C. limon*; however, it has also been sporadically isolated from *C. sinensis* and *C. paradisi* [51]. Type B citrus canker was first isolated in Argentina and a few isolated infection cases have been reported also in Uruguay and Paraguay [51]. Type C citrus canker has only been identified in the state of São Paulo, Brazil [52]. It presents the same symptoms as type A citrus canker and is produced by *X. fuscans* subsp. *aurantifolii* type C (XauC). Its host range is restricted to *C. aurantifolii* [36].

4. Quorum sensing in Xcc

Recognition of altruistic behavior, as those actions that increase the adaptation of another individual, their own cost, is a major challenge for evolutionary biologists because natural selection seems to favor selfish and uncooperative individuals [53]. Nevertheless, there are many examples in the animal kingdom, where this form of cooperation was successfully demonstrated. However, it is only recently that social behavior in microorganisms has been studied in relation to evolutionary theory [54]. Research over the last 20 years has expanded the view of bacteria as unicellular organisms having the ability to participate in complex social and cooperative behavior.

The development of an intercellular communication system is a hallmark characteristic that enables bacteria to colonize new habitats, adapt to environmental changes, resist host defense and antibiotic action, strengthen competitiveness, and take advantage of new food sources [55]. This talent for cooperative multicellular behavior depends on the implementation and recognition of diffusible signal molecules by a system known as quorum sensing (QS). Functionally, QS is a signal translation mechanism to coordinate the expression of genes at the population level. The process of QS relies upon the production, release, and detection of small signaling molecules termed auto-inducers (AIs). Each bacterial cell produces a basal amount of AIs, which are exported to the extracellular environment and reflect bacterial population density. At high cell densities, the AIs reach a critical concentration, at which point they are recognized by their cognate receptor, triggering a cascade of biological functions [56]. Bacteria within the genus *Xanthomonas* encode a cell-cell signaling or QS system which uses as AI, molecules from the diffusible signal factor (DSF) family. The DSF family are cis-2-unsaturated fatty acids, of which the paradigm is DSF itself, first identified in *X. campestris* pv. *campestris* and characterized as cis-11-methyl-2-dodecenoic acid. Detection of the DSF molecule activity as AI was first reported for over two decades by MJ Daniels Research Group [15], when these researchers were analyzing a cluster of genes in *X. campestris* called *rpf* (regulation of pathogenicity factors). It was found that the activity of the protease and endoglucanase enzymes in *rpfF* deletion mutant could be restored when this mutant was grown in the vicinity of its parental wild strain [1]. Observation of this effect led to speculation that *X. campestris* wild strain could produce a diffusible factor (DSF), which induced the production of protease and endoglucanase, and that the protein encoded by *rpfF* gene was linked with DSF biosynthesis. The signaling molecule DSF has a cis-unsaturated double bond at the two positions. It is a key structural feature for its activity; this motif is considered as signature for the DSF family. Additionally, it is believed that methyl branching plays an important role in signaling, for example, unbranched cis-2 dodecenoic acid and cis-3 tridecenoic acid; they are between 60 and 120 times less active than cis-11-methyl-2-dodecenoic acid [57].

The QS system in Xcc as well as in other species that comprise the *Xanthomonas* genus is encoded by *rpf* gene cluster. Biosynthesis of DSF auto-inducer in Xcc is dependent on *rpfF* and *rpfB* genes; these two genes encode, respectively, a putative enoyl-CoA hydratase (RpfF) and an acyl-CoA ligase of fatty acid long chain (RpfB); however, their catalytic mechanisms and corresponding substrates, as well as the reaction products, require further investigation [1]. A recent analysis of RpfF-crystallized structure has shown that it is structurally similar to

members of crotonase superfamily [58]. Sequences alignment and structural analysis enabled the identification of two putative catalytic glutamate residues (Glu141 and Glu161), which are preserved in enoil-CoA hydratase/dehydratase. Cheng et al. [58] demonstrated that the substitution of these two residues in RpfF completely abolished the DSF production, emphasizing its critical role in DSF biosynthesis.

DSF detection and transduction in bacteria involve a complex kinase sensor positioned in the cytoplasmic membrane, which is associated to a cytoplasmic regulator. The best studied of these systems is RpfCF/RpfG of *X. campestris*, which is shared by all *Xanthomonas* species [57]. RpfC is a complex sensor kinase composed of (i) sensory domain, which contains five transmembrane helices with periplasmic and endoplasmic loops, which has the function to sense DSF levels, (ii) a histidine kinase domain (HisKA) coupled to an ATPase, (iii) a CheY-like two components domain (REC), and (iv) histidine phosphotransferase domain (HPT) (**Figure 1**). The regulator RpfG comprises (i) an REC domain and (ii) a HD-GYP domain, which is a phosphodiesterase involved in the degradation of the second messenger cyclic di-GMP.

Responsible residues for DSF bond are not known yet. It is assumed that the binding of signal molecule triggers RpfC auto-phosphorylation, within the histidine-kinase domain, followed by phosphorelay, involving an aspartic acid residue in the REC domain and a histidine residue in HPT domain, and finally the transfer of one phosphate group to the REC domain in

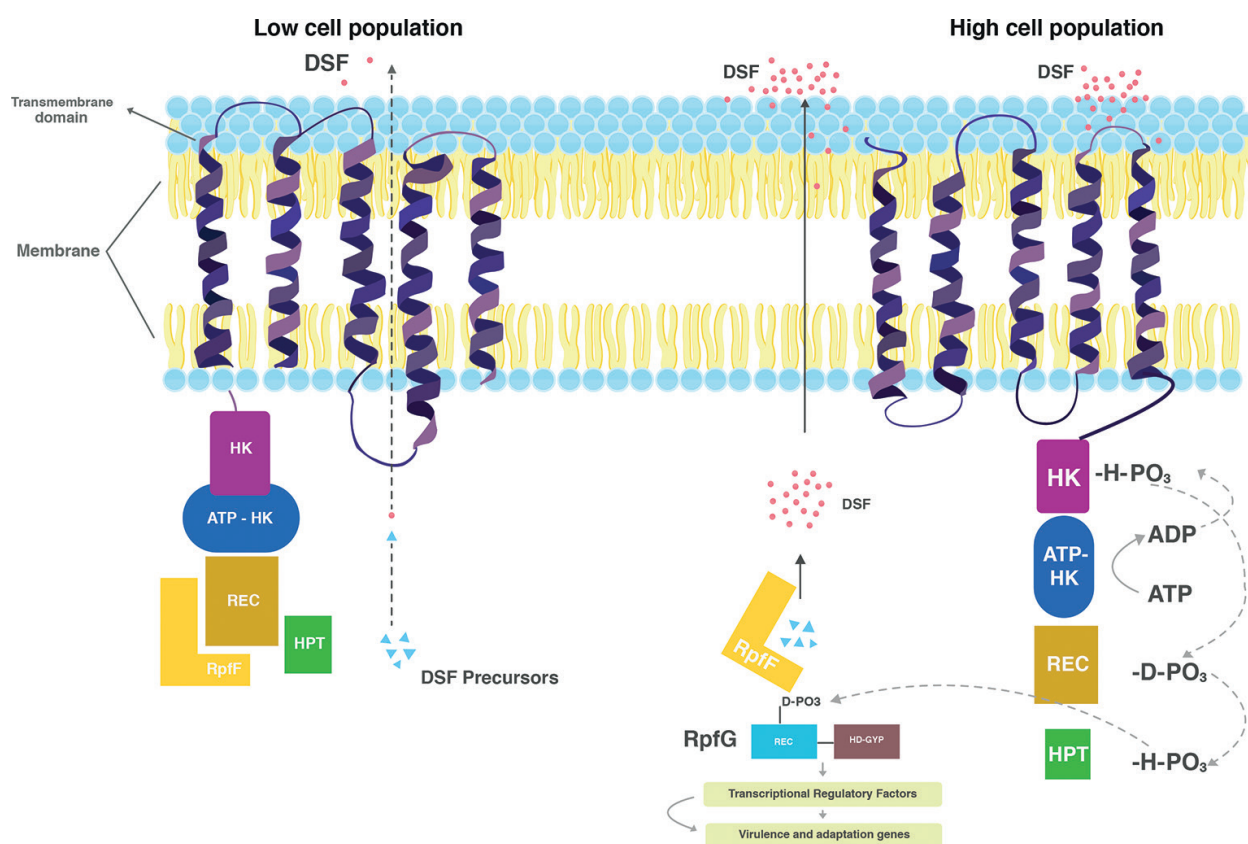


Figure 1. At low cell density, the sensor RpfC maintains a compact conformation, forming a complex with DSF synthase RpfF. At high cell density, DSF binds to RpfC, inducing a conformational change, which initiate the autophosphorylation and phosphorelay to RpfG and release RpfC.

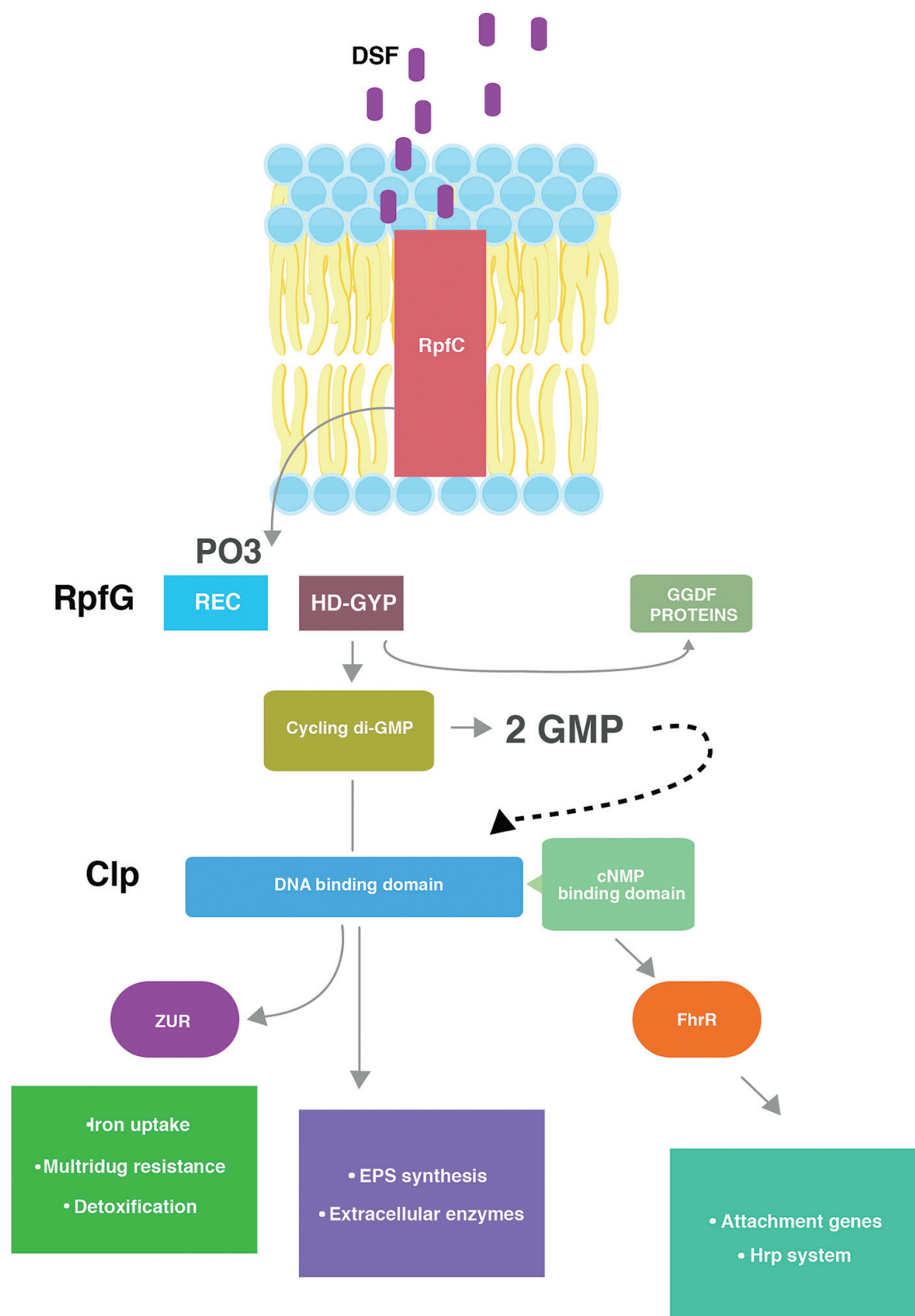


Figure 2. Perception of DSF by RpfC leads the phosphorylation of RpfG, it triggers the activation of RpfG as a cyclic di-GMP phosphodiesterase reducing the level of cyclic di-GMP and releasing Clp that promotes the synthesis of extracellular enzymes and EPS.

RpfG regulator [59]. The phosphorylation of RpfG enables its activation as a cyclic di-GMP phosphodiesterase, resulting in modifications in the level of cyclic di-GMP in the cell affecting the synthesis of virulence factors such as extracellular enzymes, EPS, biofilm dispersal, and motility [60].

Mechanistic detailed studies have been revealed that RpfG the regulator of DSF signaling cascade, it has Che-Y like receiver domain (REC) connected to a HD-GYP domain, which has phosphodiesterase activity, which acts on the degradation of the second messenger cyclic di-GMP (**Figure 2**). As outlined above, the perception of DSF in a RpfC Xcc is bound to the phosphorylation of HD-GYP domain in the RpfG regulator, and the consequent changes in intracellular second messenger cyclic di-GMP trigger major changes in bacterial phenotype [61].

Diverse pathways subsequently act to control different subgroups of virulence functions under the regulation of Rpf. The physical interaction of RpfG with two proteins with a diguanylate cyclase (GGDEF) domain acts to control motility but does not influence extracellular enzyme synthesis or biofilm formation; these two events require the conserved GYP motif in the HD-GYP domain of RpfG and are determined by DSF signaling [59]. The effect of RpfG in the synthesis of extracellular enzymes and biofilm formation could be carried on through the cyclic di-GMP influences on the global transcriptional activator Clp (cAMP receptor-like protein), which contains nucleotide- and DNA-binding domains [62]. At physiologically relevant levels of cyclic di-GMP, the global transcriptional activator Clp remains bound to cyclic di-GMP by nucleotide, avoiding the binding of Clp to the gene promoters that encode for several virulence and adaptation factors. However, when the HD-GYP domain is phosphorylated as a consequence of DSF perception, the HD-GYP domain starts its phosphodiesterase activity over cyclic di-GMP and relieves the Clp inhibition. Therefore, Clp binds to the gene promoters of several virulence factors, that is, extracellular enzymes, iron uptake, and adhesion synthesis [57]. Two transcription factors were identified to be directly regulated by Clp, that is, FhrR which regulates the expression of genes that encode flagellar, Hrp, and ribosomal proteins, the second transcription factor identified was Zur (Zinc uptake regulator), which is implicating in the regulation of multidrug resistance, iron uptake, and detoxification [63]. DSF also regulates the expression of *clp* gene; this suggests a more complex regulatory network of DSF regulon.

5. Virulence factors affected by quorum quenching in Xcc

Quorum sensing helps to coordinate bacterial behavior based on community, but it is not essential for the survival of bacteria. Therefore, the inhibition of QS only disrupts phenotypic traits that could be targets for the control of diseases, such as virulence, biofilm formation, and bacterial resistance to several antibiotics. QS interference could involve signal degradation (quorum quenching) or signal overproduction (pathogen confusion) [64, 65]. Quorum quenching is a mechanism adopted by a number of bacteria to disrupt QS signaling of competitors, affording these organisms an advantage within a particular habitat. A recent study has shown that bacterial members of the autochthonous microbiota of citrus leaves displayed a great ability to disrupt quorum sensing in Xcc, thus drastically reducing the symptoms and severity disease of citrus canker in susceptible host [66].

The bacterium Xcc has evolved a regulatory system to adapt the expression of virulence factors. As mentioned above, the *rpf* gene cluster, which encodes components of cell-cell communication system termed QS, rules a complex and hierarchic regulatory network, which enables the bacteria to express virulence and adaptation genes in a coordinated fashion in accordance with a population density. DSF/Rpf system regulates the expression of almost 180 genes. The biological functions performed by the gene products include chemotaxis and motility, adhesion, stress tolerance, transport, and detoxification [67]. DSF/Rpf system plays a major role in the initial attachment and fitness of Xcc to leaf surface. The leaf surface is considered a restrictive and hostile habitat for the bacterial colonist. Nutrient limitations, sudden temperature changes, and relative humidity are some of the factors that determine the leaf microbiota [68]. Attachment to the leaf surface and colonization is critical aspects of the early stage of pathogenesis [49]. Intriguingly, DSF signaling in Xcc positively regulates five genes encoding cell surface attachment structures such as adhesins (hmsHR) and fimbria (pilM); it plays a crucial role in a biofilm formation [67]. In our recent work, we have shown that the *rpfF* deletion mutant strain of Xcc 306 exhibited an inability to form a well-established biofilm in abiotic surfaces. Moreover, the scanning electron microscopy (SEM) showed direct evidence about the impaired bacterial attachment ability and lack of biofilm formation of Xcc 306 to the leaf surface. Because of this, a drastic reduction of citrus canker symptoms was displayed in susceptible hosts [66]. SEM assay similarly showed that more wild-type cells are taken the depressions between epidermal cells and around stomata than the QS mutants.

The DSF/Rpf QS system is also prerequisite for the full virulence of Xcc after entering the host, at the mesophyll tissue. Deletion mutants *rpfF*, *rpfC*, and *rpfG* genes displayed reduction in the symptom development in Duncan grapefruit leaves, when these strains were inoculated by infiltration pressure at a final concentration of 10^4 CFU/ml. The QS mutants displayed also impairment in motility, and extracellular protease production [67]. DSF/Rpf QS system physically interacts with proteins enclosing a GGDEF domain [69]; this domain possesses diguanylate cyclase activity associated to the synthesis of bacterial second messenger cyclic-di-GMP [70]. GGDEF domain controls motility but not other DSF-mediated phenotypes in *X. campestris* pv. *campestris* [59]. Finally, DSF/Rpf QS system radically regulates the expression of several components of protein secretion systems T2SS and T3SS through direct effect in the cellular concentration of cyclic-di-GMP and its consequent bind/release of the global transcriptional activator Clp [63].

6. Conclusions

Inhibition of quorum sensing can lead to certain phenotypic alterations, such as virulence reduction, reduced biofilm formation, and increased bacterial sensitivity to treatments. All these traits have implication in a severe symptomatology reduction. Interruption of DSF signaling in *X. citri* subsp. *citri* may lead to a downregulation of genes that encode cell surface attachment structures such as adhesins and fimbria, disturbing biofilm formation and epiphytic fitness, factors that are critical in the early stages of pathogenicity. Besides, other bacterial traits essential for disease development are directly or indirectly regulated by DSF/Rpf QS system in Xcc, and such traits are the production of extracellular enzymes and effectors.

Because QS or cell-cell signaling control processes associated with virulence of many pathogens, interference with these processes may afford a route toward disease control. Such interference could involve signal degradation (quorum quenching) or signal overproduction (pathogen confusion). Design and implementation of strategies to disrupt QS in Xcc may represent a highly valuable tool in the process of biological control and offer an alternative to the traditional copper treatment currently used for the treatment of citrus canker disease, with significant environmental, economic, and health implications worldwide.

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