ANNUAL REVIEWS

Annual Review of Microbiology

The *phc* Quorum-Sensing System in *Ralstonia solanacearum* Species Complex

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Annu. Rev. Microbiol. 2023. 77:213–31

First published as a Review in Advance on April 26, 2023

The *Annual Review of Microbiology* is online at micro.annualreviews.org

https://doi.org/10.1146/annurev-micro-032521-030537

Keywords

Ralstonia solanacearum, *Ralstonia pseudosolanacearum*, cell–cell communication, quorum sensing, pathogenicity, virulence

Abstract

Ralstonia solanacearum species complex (RSSC) strains are devastating plant pathogens distributed worldwide. The primary cell density–dependent gene expression system in RSSC strains is *phc* quorum sensing (QS). It regulates the expression of about 30% of all genes, including those related to cellular activity, primary and secondary metabolism, pathogenicity, and more. The *phc* regulatory elements encoded by the *phcBSRQ* operon and *phcA* gene play vital roles. RSSC strains use methyl 3-hydroxymyristate (3-OH MAME) or methyl 3-hydroxypalmitate (3-OH PAME) as the QS signal. Each type of RSSC strain has specificity in generating and receiving its QS signal, but their signaling pathways might not differ significantly. In this review, I describe the genetic and biochemical factors involved in QS signal input and the regulatory network and summarize control of the *phc* QS system, new cell–cell communications, and QS-dependent interactions with soil fungi.

Contents

INTRODUCTION

Quorum sensing (QS) regulates bacterial gene expression in response to fluctuations in cell population density (59, 63, 96, 97). Diffusible molecules exchanged between bacterial cells convey cell density information. The most common QS signals used by gram-negative bacteria are *N*-acyl-homoserine lactone (AHL) molecules (27). Fatty acid–derived molecules such as diffusible signaling molecules (DSFs) and *Vibrio cholera* autoinducer 1 (CAI-1) are known to be employed as QS signals by some bacteria (38, 94). However, these two signal types differ in their perception and signaling mechanisms. AHLs are received by the LuxR-type transcriptional regulators, which directly regulate expression of target genes. In contrast, DSF and CAI-1 families are recognized by a two-component system consisting of histidine kinase (HK) and a response regulator (RR) that regulate target gene expression either directly or through downstream signaling (9). Both regulated gene groups include many involved in the pathogenic/symbiotic interactions with hosts (59, 63, 96, 97). In particular, they include cellular motility, extracellular substance production, biofilm formation, and secondary metabolism.

The betaproteobacterium *Ralstonia solanacearum* is a causative agent of bacterial wilt disease. It enters the host roots through wounds, invades the xylem vessels, and proliferates (11, 29, 37). The bacterial cells and extracellular matrix, including extracellular polysaccharide I (EPS I), inhibit water flow there, causing plant death (29, 57). The pathogen is widespread in tropical, subtropical, and warm temperate regions, where it is a major hindrance to the production of diverse crop species, both monocots and dicots (29). The host range is vast for a plant pathogen: over 250 species in 50 botanical families. This pathogen has a diverse genetic background and has thus been referred to as *R. solanacearum* species complex (RSSC) since 2005 (3). Recently, based on the phylogenetic clades known as phylotypes I, II, III, and IV, taxonomists have divided the RSSC strains into three species: *R. solanacearum* (phylotype II), *R. pseudosolanacearum* (phylotypes I and III), and *R. syzygii* (phylotype IV) (70). Some of the strains discussed in this review have not been categorized into a species. For simplicity, I use RSSC and the strain's name. RSSC strains' host

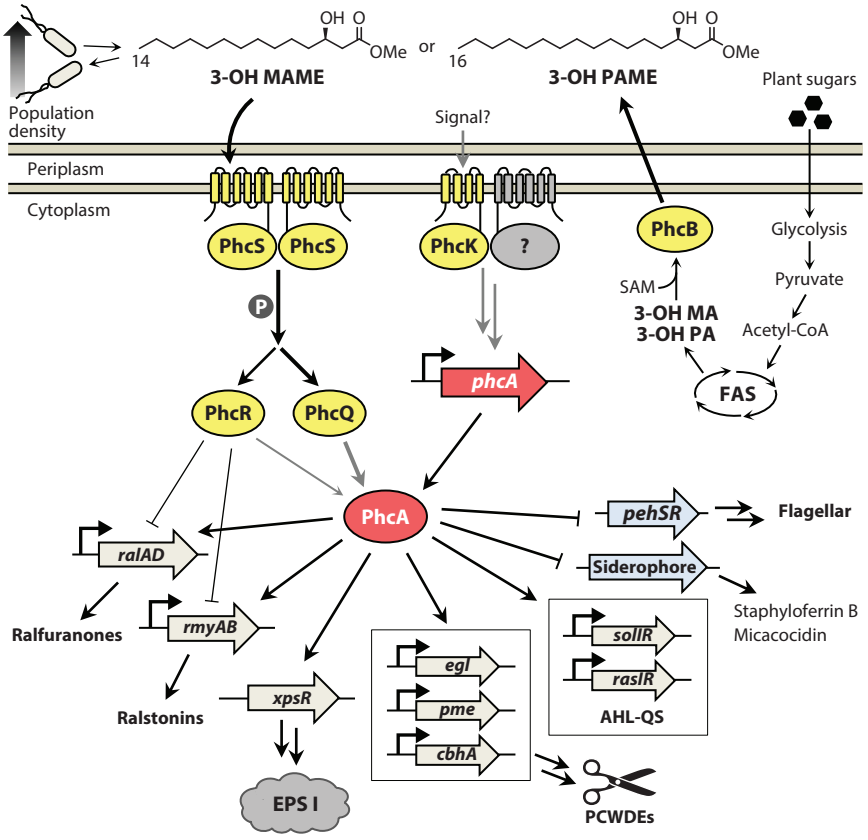

Figure 1

The major regulatory circuits of the *phc* QS system in RSSC strains. The model organized, the Phc proteins, and the key traits are directly controlled by the PhcA global virulence regulator. As bacterial density increases, the concentration of 3-OH MAME or 3-OH PAME, synthesized by PhcB, increases, and the molecules are received by the PhcS–PhcRQ two-component system. Then, PhcA is functionalized and induces expression of virulence-related genes. Gray lines show the presumed signal transduction. Lines ending with a crossbar indicate repression, whereas lines with an arrowhead indicate activation. Line thickness shows the strength of signal input. Abbreviations: 3-OH MA, 3-hydroxymyristic acid; 3-OH MAME, methyl 3-hydroxymyristate; 3-OH PA, 3-hydroxyplamitic acid; 3-OH PAME, methyl 3-hydroxypalmitate; AHL, *N*-acyl-homoserine lactone; EPS I, extracellular polysaccharide I; FAS, fatty acid synthesis; PCWDE, plant cell wall–degrading enzyme; SAM, *S*-adenosylmethionine.

range expands continuously, and the agricultural damage increases annually (28, 29). Therefore, there is an urgent need to develop appropriate control methods.

RSSC strains use the QS system at each infection step to successfully move from the soil to the stem (29, 72). The QS system consists of a group of proteins named Phc (**Figure 1**). The QS signals mediating this cell–cell communication are methyl 3-hydroxymyristate (3-OH MAME) and methyl 3-hydroxypalmitate (3-OH PAME), and each RSSC strain utilizes only one of these molecules (46, 87). Both molecules are biosynthesized by PhcB synthase. The two-component system PhcS–PhcRQ, which detects 3-OH MAME or 3-OH PAME, is involved in signaling that activates the PhcA regulator. Conversely, the expression of PhcA involves an enigmatic two-component system that includes the orphan HK PhcK (75). This RSSC-specific QS system has

been referred to by several names (e.g., Phc QS, PhcA QS, PhcB QS, and PhcBSR QS) (48, 50, 54), but here I propose "*phc* QS system."

This review focuses on the progress made over the last 25 years in research and understanding of the *phc* QS system of RSSC strains. This includes identification and functional analysis of genetic and biochemical factors, biosynthesis and response specificity of the QS signals, RNA-seq-based transcriptome, and QS inhibition. New cell–cell communications and endoparasitic interactions with fungi that have recently been reported are also discussed. I have chosen to rely on previous review articles (29, 72) for the important virulence determinants for which there has not been great progress in the last 25 years.

DISCOVERY OF THE *phc* QUORUM-SENSING SYSTEM AND IDENTIFICATION OF ITS QUORUM-SENSING SIGNALS

The global regulator PhcA, a LysR-type transcriptional regulator, controls numerous traits associated with the pathogenicity/virulence and metabolism of RSSC strains in response to cell density (**Figure 1**). *phcA* was first seen as the causative gene of a mutant strain of phenotype conversion (mucoid → nonmucoid) (10). Later, it was observed that a volatile factor produced by the *phcB*-encoded enzyme is involved in the activation of PhcA (17). In 1997, the factor was partially purified from the spent culture broth of strain AW1 and identified as 3-OH PAME by gas chromatography and mass spectroscopy (GC/MS) analysis (25). Incidentally, 3-OH PAME is not as volatile as initially expected. 3-OH PAME rescued EPS I and endoglucanase production in the *phcB* mutant. Furthermore, Clough et al. (16) proposed a possible mechanism for suppressing PhcA function by PhcS–PhcR and activating PhcA by canceling this suppression in response to QS status. Generally, identifying QS signals accelerates the development of the field (59, 63, 96, 97). However, the RSSC strains that later became well used (such as GMI1000 and OE1-1) did not produce 3-OH PAME (46), and the research on the *phc* QS system stalled. Since the type III secretion system (T3SS) was discovered in RSSC somewhat late (6, 18, 30), researchers may shift their attention from the *phc* QS system to molecular RSSC–plant interactions.

In 2015, a new type of QS signal from strain OE1-1 was discovered by Kai and coworkers (46). Using recovery of EPS I production in a Δ*phcB* mutant as an indicator, the QS signal molecule was isolated from the wild-type strain culture using various chromatographic techniques. Its chemical structure was unequivocally determined to be 3-OH MAME by nuclear magnetic resonance and MS analyses and comparison with a synthetic standard. The absolute configuration of the C-3 position in 3-OH PAME and 3-OH MAME was revealed to be *R*. The QS activity tests of the compounds with similar structures confirmed the importance of the (3*R*)-hydroxy group, methyl ester, and acyl chain length in inducing QS responses in Δ*phcB* (46). Phylogenetic analysis of PhcB and PhcS in some strains showed that RSSC strains are divided into two clades, 3-OH MAME and 3-OH PAME types (46). The signal production selectivity based on the clade of the phylogenetic tree of PhcB was correctly assessed by expressing the corresponding *phcB* gene in *Escherichia coli* BL21(DE3) and examining the products by GC/MS (46, 87). Meanwhile, the phylogenetic tree of PhcR exhibited the clades reflecting the phylotype of RSSC strains (46). Thus, the RSSC strains have two types of the *phc* QS system, 3-OH MAME and 3-OH PAME, which were independent of their phylotypes. It is unknown whether this is because the QS system was acquired before the current phylotype system was established or whether it came about through convergent evolution. The model strain GMI1000 was confirmed to employ the 3-OH MAME–type QS system (46).

BIOSYNTHESIS OF 3-OH MAME AND 3-OH PAME

3-OH MAME and 3-OH PAME are synthesized by the PhcB enzymes, which belong to the class I *S*-adenosylmethionine (SAM)-dependent *O*-methyltransferases (87) (**Figure 1**). The schematic

diagrams showing membrane localization of PhcB are presented in several review articles, but so far there is no experimental or bioinformatic evidence to support such a prediction (66, 72). Biochemical analysis of PhcB (from strain OE1-1) by Ujita et al. (87) confirmed that 3-OH MAME is synthesized from 3-hydroxymyristic acid (3-OH MA) and SAM. Although the *E. coli* recombinant expressing *phcB* produced significant amounts of the QS signal (46, 87), the enzyme may be changed to a holo form during the purification process because the purified enzyme alone was not active (87). A cofactor or activation may be needed for PhcB to exhibit enzymatic activities in vitro, but its essence is still unknown. The 3-OH MAME–type PhcB enzymes showed a high substrate selectivity to 3-OH MA, and the 3-OH PAME–type ones exhibited a high substrate selectivity to 3-hydroxyplamitic acid (3-OH PA) (87). On the other hand, the PhcB enzymes were ambiguous in their stereochemical (*R* or *S*) recognition of the C-3 position of the precursor 3-hydroxy fatty acids (87). For example, the PhcB from strain OE1-1 was somewhat more efficient at methylating (*R*)-3-OH MA than (*S*)-3-OH MA. However, the absolute configuration of the 3-hydroxy fatty acids supplied was restricted to *R*. A feeding study with deuterium-labeled precursors confirmed that SAM-dependent methylation occurred on 3-OH MA/3-OH PA in RSSC cells.

In bacteria, the (*R*)-3-hydroxy fatty acids are most likely obtained from the acyl carrier protein (ACP) complexes, which exist as intermediates in fatty acid synthesis (90). Since the inhibition of β-oxidation by an inhibitor resulted in the elevation of 3-OH MAME or 3-OH PAME levels, the involvement of the β-oxidation pathway in QS signal production was ruled out (87). A thioesterase may release 3-OH MA and 3-OH PA precursors from their ACP conjugates. However, there are at least eight thioesterase genes for fatty acid production in the RSSC genome, and it is unknown whether one of these enzymes is specific for the biosynthesis of the QS signals. As with other QS signals, such as AHLs (27), the precursors of 3-OH MAME/3-OH PAME are provided by a fatty acid synthesis pathway (**Figure 1**).

When ^{13}C-labeled glucose or galactose was fed to strains OE1-1 and K60, active uptake of the ^{13}C label into the carbon skeletons of 3-OH MAME/3-OH PAME was noted (87). This result suggested that the monosaccharides taken into the bacterial cells are efficiently utilized for QS signal production through the glycolysis and fatty acid synthesis systems. There might be a mechanism by which the QS signals are preferentially produced (102). In tomato plants (the most representative host of RSSC), sugars were considered abundant in the intercellular spaces and limited in the xylems (24, 57, 103). However, a recent study confirmed that monosaccharides are abundant even within tomato xylems (8). Thus, in both intracellular spaces and xylems, RSSC strains can produce QS signals using plant-derived sugars, activating the *phc* QS circuit when required. There is a sugar influx transporter whose expression is induced by the QS system (67). The QS system functions in the xylem of the plant, as confirmed by the active production of EPS I and secondary metabolites by RSSC cells (48, 58). RSSC researchers have seen large amounts of bacterial fluids (containing EPS I) coming out of the cut stems of infected plants.

The *phcB* gene has been found in some bacterial species other than RSSC strains (29). The only one that has been shown to function is the QS system of *Cupriavidus taiwanensis* LMG19424 (93). Because this strain was a rhizobial symbiont unique to *Mimosa putida*, its genome was analyzed earlier (4). Expression of the *phcB* gene in *E. coli* resulted in production of 3-OH MAME, and 3-OH MAME was detected in the culture extract of LMG19424 (93). In addition, *phcB*, *phcSRQ*, and *phcA* are also present in the genome of LMG19424 (4). The *phc* QS system was involved in the swimming and production of secondary metabolites but not in nodule formation. It is not known how many genes are regulated by the QS system in *C. taiwanensis*. A phylogenetic tree of PhcB showed that the enzymes from *Cupriavidus* spp. belong to a different clade than those of RSSC, and several other clades exist (93). Given the absence of *phcA*, the *phcB* gene of bacterial strains other than the RSSC and *Cupriavidus* clades were expected to result from incomplete acquisition

by horizontal gene transfer from an ancient RSSC strain (29). In bacteria with an incomplete form of the *phc* genes, *phcB* and its product 3-OH MAME or 3-OH PAME should not have an important function, but perhaps they have ecological significance.

SIGNALING PATHWAYS TO FUNCTIONAL ACTIVATION OF *phcA*

Although it became clear earlier that PhcA functions as a central regulator of the *phc* QS system (10, 25, 72), how the signal is input to result in PhcA activation is still a mystery to this day (**Figure 1**). The perception of the QS signals (3-OH MAME and 3-OH PAME) by PhcS was supported primarily by mutant analyses (16, 46). Deletion of *phcS* resulted in QS dysfunction, which was not restored by the addition of 3-OH MAME but was restored by *phcS* complementation. Biochemical data on how PhcS receives 3-OH MAME or 3-OH PAME and transfers the signal are limited. PhcS is a transmembrane sensor HK composed of N-terminal sensor, HK, and C-terminal ATPase domains and may function as a homodimer. PhcS is expected to have four to six transmembrane regions at its N terminus, but different prediction results have been obtained depending on the program used (16, 46). The sequence differences between the 3-OH MAME– and 3-OH PAME–type PhcS proteins are concentrated in the N-terminal, cytoplasmic sensor, and transmembrane 1–3 regions (46). Thus, it is likely that these regions are involved in ligand recognition. As is often the case with other HKs (13, 62, 83), the sensor domain directly recognizing the ligand may cause a conformational change of PhcS, activating the HK domain and transferring the phosphate group of ATP via its own histidine residue to a downstream RR. A mutation in the phosphorylation site (His-230) of PhcS did cause a QS signaling defect. We have attempted to heterologously express PhcS and use it for biochemical analysis, but we have not obtained it as an active form. Hopefully, progress will be made in the biochemistry and structural biology of PhcS to know how 3-OH MAME or 3-OH PAME is received by the receptor.

phcR, which is located directly under the *phcS* gene in RSSC genomes, had been considered the central RR that outputs the "QS active" information to downstream factors (46) (**Figure 1**). PhcR is an intracellular soluble RR composed of N-terminal RR receiver, HK, and C-terminal ATPase domains. At the time of its discovery, the hypothesis that PhcR function is regulated by PhcS in an inhibitory manner was proposed (16, 72). However, the detailed analysis of Δ*phcS* suggested that the PhcS rather activates QS signaling when it receives 3-OH MAME (46). It was later determined that this conflict was due to the fact that PhcQ, not PhcR, was the central RR of the *phc* QS system (53, 84, 85) (**Figure 1**). PhcQ has an RR receiver domain at the C terminus, but the function of the rest of the sequence is enigmatic. Takemura et al. (84) performed RNA-seq-based transcriptome analysis of the wild-type OE1-1 and its QS-related mutants and found that the deletion of *phcQ* was more similar in expression profile to Δ*phcA* (**Figure 2**). Δ*phcR* was characterized by less variation in the expression of QS-related genes and more active production of secondary metabolites (ralstonins and ralfuranones) (84) (**Figure 1**). Recently, it was reported that PhcR binds to the promoter region of *rmyA* and represses *rmyA/rmyB* expression (53). That is, the 3-OH MAME signal input through PhcS may branch at the RRs (PhcR or PhcQ), and PhcQ is mainly involved in the functionalization of PhcA. Tang et al. (85) reached a similar conclusion from different experimental systems using strain GMI1000. Unfortunately, it is still unclear which protein(s) interacts directly with PhcQ.

A further new HK, PhcK, is likely to be involved in the transcriptional activation of PhcA (75) (**Figure 1**). PhcK is a putative sensor HK with N-terminal transmembrane sensor, HK, and C-terminal ATPase domains and without a DNA-binding domain. In general, HK and RR are contiguously arranged in bacterial genomes and function as a pair in a two-component system (13, 62, 83), but PhcK and the downstream RR RSc1352 may not function as a pair (75). RNA-seq

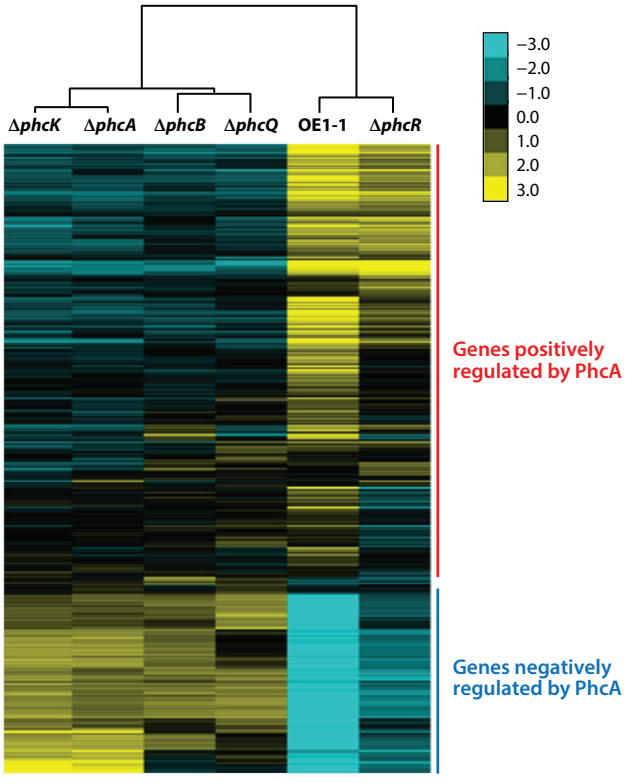

Figure 2

RNA-seq transcriptome analyses of QS-dependent genes of RSSC strain OE1-1. Hierarchical clustering of relative expression levels of PhcA-regulated genes in strain OE1-1 and its *phc* gene mutants (Δ*phcK*, Δ*phcA*, Δ*phcB*, Δ*phcQ*, and Δ*phcR*). Fragments per kilobase of exon per million fragments mapped values were normalized before the analysis of differentially expressed genes.

transcriptome analysis by Senuma et al. (75) indicated that PhcK induces *phcA* gene expression independently of the PhcS–PhcRQ system. Thus, Δ*phcK* exhibited a gene expression profile similar to that of Δ*phcA* (**Figure 2**). They proposed that PhcK alone (as a homodimer) or with PhcS (as a heterodimer) receives a diffusible ligand and activates *phcA* expression in strain OE1-1 (75). The possibility that this ligand is 3-OH MAME was not ruled out. There might be two systems: one that receives QS signal input and transmits it to PhcA, and another that regulates *phcA* expression. This model is built solely from transcriptome comparison, and biochemical validation would be essential. It is unclear how PhcK regulates the expression of *phcA*. *phcK* is not conserved in all RSSC strains, and many strains from phylotypes II and III did not have this gene (75). Perhaps there is some diversity in the regulation of *phcA* expression among RSSC strains.

GENES REGULATED BY THE *phc* QUORUM-SENSING SYSTEM

Next-generation sequencers are being used in RSSC QS research. To analyze the gene sets regulated by the *phc* QS system, *phcA*-deficient mutants have been used exclusively for comparison with wild-type strains (48, 67, 75, 84). This is because, as mentioned above, PhcA is the central transcriptional regulator of the *phc* QS system in RSSC strains (29, 72). In this section, I use data from

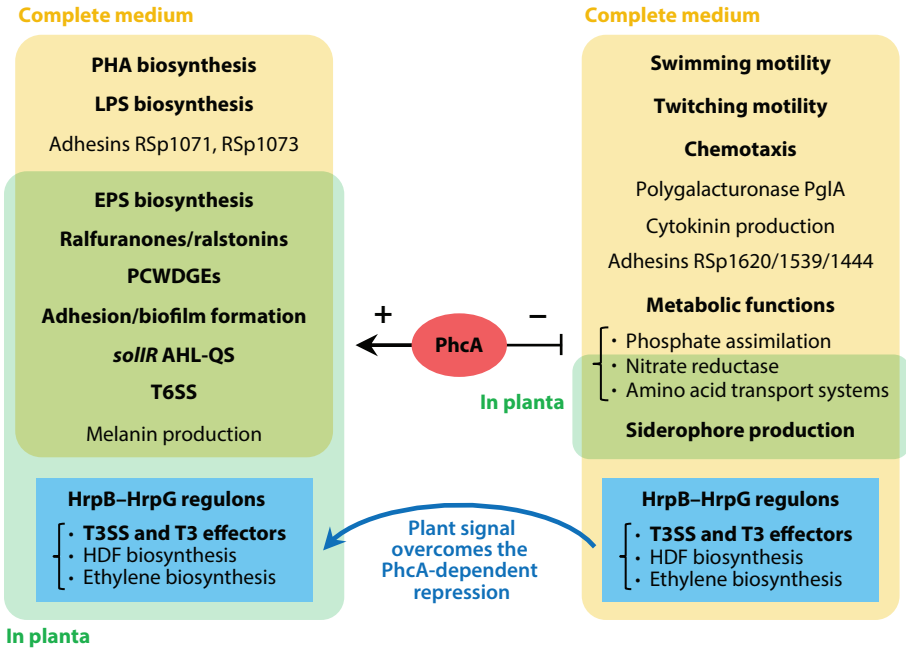

Figure 3

Overview of the key PhcA-dependent traits in RSSC strain GMI1000. The genes and associated functions are either positively regulated (*left*) or negatively regulated (*right*) by PhcA. The gold boxes highlight the traits controlled in complete medium, while green boxes show in planta–regulated traits. Traits showing similar regulatory patterns in complete medium and in planta appear in overlapping boxes. For example, the HrpB and HrpG regulons (*blue boxes*) are repressed by PhcA in complete medium but are specifically activated in planta (*blue arrow*). Abbreviations: AHL-QS, *N*-acyl-homoserine lactone quorum-sensing system; EPS, extracellular polysaccharide; HDF, HrpB-dependent diffusible factor (3-hydroxy-indolin-2-one); LPS, lipopolysaccharide; PCWDGE, plant cell wall–degrading enzyme; PHA, poly-β-hydroxybutyrate; T6SS, type VI secretion system. Adapted from Reference 67.

strain GMI1000 (67) as an example to show characteristics of the QS-dependent gene expression profile rather than an exhaustive list of QS-dependent genes.

RNA-seq-based comparison by Perrier et al. between GMI1000 and its *phcA* mutant in rich media showed that PhcA is involved in the transcriptional regulation of about 30% of RSSC genes (787 upregulated and 794 downregulated genes, more than twofold differentially expressed) (67) (**Figure 3**). Many of those positively regulated by PhcA function in virulence, and many of those negatively regulated function during saprophytic growth. PhcA controlled the expression of 166 transcription regulators. Forty-four transcription regulators were controlled positively and 122 negatively. Many of these target transcription regulator genes were well-known regulators of cellular functions: *hrpB/hrpG* (T3SS), *oxyR* (antioxidant response), *flbC* (flagellar motility), *cheY* (chemotaxis), *ntrC* (nitrogen assimilation), and *phoB* (phosphate assimilation) were negatively regulated by PhcA; and *epsR/xpsR* (EPS biosynthesis) and *solIR* (AHL system) were positively regulated by PhcA. PhcA also regulated the cellular processes: negative regulation of siderophore production (staphyloferrin B and micacocidin) and *tzs* (cytokinin biosynthesis) and positive regulation of T6SS, twitching motility, adhesins, lectins (*rsl*, *lecM*, and *RSp0569*), Flp pili (biofilm formation), plant cell wall–degrading enzymes (*pglA*, *pehC*, and *pme*), poly-β-hydroxybutyrate storage genes, ralstonins (*rmyAB*), and ralfuranones (*ralAD*).

Among the 199 genes annotated to be part of ABC transporters, only 15 were negatively regulated by PhcA, and the majority were positively regulated (67). This supports Peyraud et al.'s (68) finding that the enlarged metabolic versatility of a *phcA* mutant compared to the wild-type strain at high cell density is due to a resource allocation trade-off between growth and virulence instead of transcriptional control of substrate usage. This dramatic change in central metabolism was not part of the early conception of bacterial QS systems (59, 96, 97). However, recently it has become clear that the QS systems cause drastic changes in the central metabolic system in other gram-negative bacteria (20, 31). Some of the transporters negatively regulated by PhcA were involved in phosphate utilization (67). Except in a few instances, the functions of the transporters regulated by QS are not properly understood.

DIFFERENT EXPRESSION OF QUORUM SENSING–RELATED GENES BETWEEN RICH MEDIA AND IN PLANTA

Nutrient composition in plant tissues (e.g., xylem) is greatly reduced compared with that in a rich medium. The QS-dependent gene expression profile differed in some aspects between the complete medium and in planta (41, 67). In the GMI1000 strain, the 383 PhcA-dependent genes were expressed in both conditions, with 100 genes upregulated and 283 genes downregulated (67). Among these 383 genes, 326 (85%) had a similar PhcA-dependent regulation pattern in complete medium and in planta. The remaining 57 genes (15%) displayed an opposite PhcA regulation pattern. In the *phcA* mutant, 30 genes were downregulated in planta but upregulated in the complete medium, and 27 genes were upregulated in planta but downregulated in the complete medium. A majority of the 27 genes were ABC-type transporters for unidentified metabolites; one was annotated as a sugar transporter (*RSc0547*). This class of transporters specifically induced in planta in the *phcA* mutant highlights a probable metabolic adaptation of the *phcA* mutant in the plant xylem environment.

Most of the genes repressed by PhcA in complete medium but activated in planta belong to the HrpG–HrpB regulon, representing a set of key genes required for RSSC pathogenesis (88, 89, 101) (**Figure 3**). Thus, the genes regulated by HrpG–HrpB appear to have a unique expression pattern in which they are activated by PhcA as essential factors of the virulence program in the plant but are repressed under nutrient-rich conditions, such as during the late infection period when the plant is dying. Expression of T3SS genes is still effective when bacteria colonize xylem tissues, and PhcA plays a vital role in fine-tuning T3SS expression. In plant xylems, a certain plant-derived signal might be perceived by RSSC to relieve the PhcA-dependent repression, exerted on *hrpG–hrpB*, or to specifically convert PhcA from a repressor to an activator of this class of genes (21, 39, 67). It remains unclear whether this signal is independent of the plant cell contact activation pathway known to activate HrpG–HrpB (2). The plant-derived signal probably significantly impacts the QS regulation in planta.

Several other transcriptome studies have also reported differences in QS-regulated gene expression in complete media and in planta (21, 41). There is a trend in unmeaningful expression profiles (wild type versus *phcA* mutant) in rich media. However, we expect that there may be a situation (e.g., interactions with other organisms) that requires activation of the *phc* QS system, as when RSSC was grown in rich media.

RESPONSE SPECIFICITY TO 3-OH MAME OR 3-OH PAME

To what extent do RSSC strains respond to the QS signals that differ from those they produce? Using strains OE1-1 (3-OH MAME type) and K60 (3-OH PAME type), Ujita et al. (87) evaluated the

signal specificity (3-OH MAME versus 3-OH PAME) in biofilm formation, secondary metabolism induction, and EPS I production. The *phcB* deletion mutants showed a tenfold stronger response to their molecules in these assays.

Ujita et al. (87) also conducted transcriptome analysis by RNA-seq to investigate differences in gene expression responses of Δ*phcB* (derived from OE1-1) to 3-OH MAME and 3-OH PAME. The 688 genes were expressed at significantly lower levels in Δ*phcB* than in strain OE1-1, indicating they were positively regulated by the *phc* QS system. The supplementation of 3-OH MAME or 3-OH PAME (1 μM) rescued the expression of 480 or 70 genes, respectively. Among them were genes involved in EPS I biosynthesis (*epsR*, *xpsR*, and *epsB*), ralfuranone biosynthesis (*ralA* and *ralD*), and ralstonin biosynthesis (*rmyA* and *rmyB*) and those encoding lectins (*rsl*, *lecM*, and *RSp0569*) and AHL-QS (*solI* and *solR*). Some plant cell wall–degrading enzymes (*pme*, *egl*, *cbhA*, and *pehC*) were included. There were 298 genes negatively regulated by *phcB*. 3-OH MAME and 3-OH PAME significantly reduced the expression of 227 and 147 genes, respectively. These included many vital genes (e.g., those for cell motility and chemotaxis and *hrp*). The transcriptional response of strain OE1-1 to 3-OH MAME or 3-OH PAME was different, and strain OE1-1 preferentially responded to its own QS signal, 3-OH MAME.

Furthermore, the virulence of OE1-1, Δ*phcB*, and $phcB_{OE1\text{-}1}$/$phcB_{8107}$-complemented Δ*phcB* strains ($phcB_{OE1\text{-}1}$-comp and $phcB_{8107}$-comp, respectively) on tomato plants was investigated (87). Strain 8107 produced 3-OH PAME as a QS signal (46). The virulence of Δ*phcB* was markedly reduced compared with that of strain OE1-1 because the *phc* QS system was not driven. $phcB_{OE1\text{-}1}$-comp showed similar virulence to strain OE1-1; however, the virulence of $phcB_{8107}$-comp was not rescued and was almost identical to that of Δ*phcB*. This result showed that the QS activation by strain-specific *phcB* genes (or strain-specific QS signals) is important for the full virulence of strain OE1-1 on tomato plants.

FEEDBACK REGULATION OF THE *phc* QUORUM-SENSING SYSTEM

Ralfuranones A and B, aryl-furanone-type secondary metabolites, were first found as the *phc* QS-dependent metabolites from strain GMI1000 (73). Later, in addition to these compounds, ralfuranones J–M were also identified from strain OE1-1 (45). Ralfuranone I (a biosynthetic intermediate) is biosynthesized by the combination of RalA (NRPS-like furanone synthase) and RalD (amino transferase), and the gene cluster involved in these enzymes were positively regulated by the *phc* QS system (65, 92) (**Figure 4**). The conversion from ralfuranone I onward will likely proceed in nonenzymatic and enzymatic manners (44, 64). The deletion of *ralA* not only stopped the production of ralfuranones but also significantly reduced their virulence on tomato plants (45). Further analysis of Δ*ralA* revealed that the abilities of EPS I production and biofilm formation, both involved in virulence, were reduced to the same levels as in the QS-deficient mutants (60). Additionally, the production level of 3-OH MAME in Δ*ralA* was significantly reduced compared to the wild-type strain. Most of these reduced abilities were restored with *ralA* gene complementation, but only some were restored by adding ralfuranones (45, 60). The RNA-seq-based transcriptome analysis also supported the fact that Δ*ralA* is almost QS deficient. These results indicated the existence of a feedback control mechanism of the *phc* QS system via ralfuranones (**Figure 4**). Interestingly, the analogs of ralfuranones were reported to promote *Vibrio cholerae* biofilm formation (95).

The QS dysfunction, similar to Δ*ralA*, was observed in the mutants deficient in producing EPS I or LecM (35, 36). Δ*epsB* and Δ*lecM* reduced the production of biofilm, EPS I, and secondary metabolites and showed higher cell motility. These mutants showed virtually no virulence on tomato plants. As reported in *Pseudomonas aeruginosa* (19), EPS and lectin may comprise the extracellular matrix of RSSC cells (**Figure 4**). The matrix may contribute to the diffusion and stability

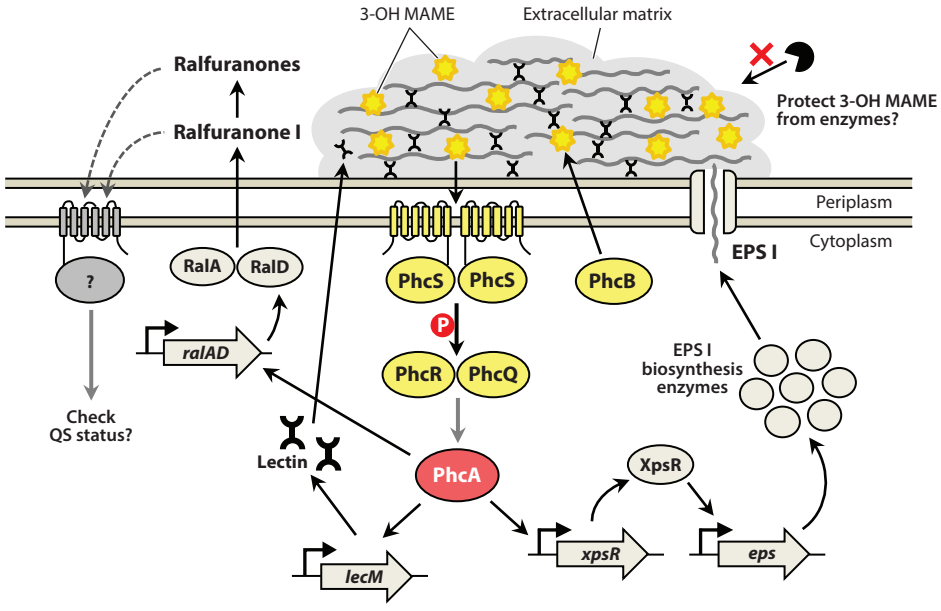

Figure 4

Feedback regulation of the *phc* QS system in RSSC strain OE1-1. The QS system activates the production of ralfuranones, the LecM lectin, and EPS I. Ralfuranones produced by RalA and RalD are secreted out of the cell. The concentration of ralfuranones in the external environment may be monitored by an unidentified receptor that serves as a surveillance system for the QS status. EPS I molecules may associate with each other via lectins and stick to the cell surface to form an extracellular matrix. 3-OH MAME diffuses through this matrix and may remain stable via protection from degrading enzymes. This model largely omits the pathway from QS signal input to PhcA activation. The control from components other than the *phc* QS system is not shown. Abbreviations: 3-OH MAME, methyl 3-hydroxymyristate; EPS I, extracellular polysaccharide I.

of the QS signals (such as protection from degrading enzymes). Extracellular 3-OH MAME levels were significantly reduced in Δ*epsB* and Δ*lecM* (35, 36). In recent years, lack of adequate resources has been demonstrated to suppress the activation of the QS circuit (15, 79, 99). Therefore, there must be avoidance systems to suppress the production of high-energy-requiring virulence factors in inappropriate cellular states. Regarding the old reports that loss of EPS I production decreases virulence, I strongly argue that the contribution of QS dysfunction needs to be examined (5, 71). The feedback control to the QS circuit by EPS I–LecM and that by ralfuranones may differ.

CONTROL OF THE *phc* QUORUM-SENSING SYSTEM TO ATTENUATE VIRULENCE

Inhibition of the *phc* QS system is an attractive approach to control the pathogenicity and virulence of RSSC strains. Particularly, the pesticides that exert strong survival selection pressure on microbes, such as antibiotics and fungicides, will be increasingly difficult to use in the field (14, 55, 82). In studies of the AHL-QS systems, enzymatic degradation of signaling molecules was shown to dramatically affect bacterial virulence (22, 23, 32). Later, several synthetic antagonists were developed, and some have been reported to be effective in vitro and in vivo (40, 43). Similarly, for the *phc* QS system, an effort began with developing a degrading enzyme for 3-OH MAME or 3-OH PAME.

Shinohara et al. (77) discovered *Ideonella* sp. 0-0013, which has strong esterase activity, from a screening of microorganisms that can grow on a medium with 3-OH PAME as the sole carbon

source. The esterase cloned from that strain and heterologously expressed in *E. coli* showed activity to degrade 3-OH PAME to 3-OH PA. As a result of the addition of the esterase, RSSC strains AW1-3 and MAFF 301487 stopped EPS production on a TZC agar plate. Unfortunately, the effects of the esterase on the production of other virulence factors and the virulence on plants were not studied. Several similar approaches by other groups have been reported (1, 52). We look forward to future reports of research results supporting the usefulness of esterase.

From the structure–activity relationship studies on 3-OH MAME (46), it was suggested that there are compounds in the structural analogs that specifically inhibit the *phc* QS system. A small set of 3-OH MAME analogs was prepared to study their effects on the QS system of strain OE1-1 (100). Consequently, methyl 3-hydroxy-8-phenyloctanoate, named PQI-1 (*phc* QS inhibitor-1), inhibited biofilm formation by strain OE1-1. PQI-2–5 evolved from PQI-1 inhibited not only biofilm formation but also the production of ralfuranones and EPS I. RNA-seq analysis revealed that the PQIs effectively inhibited QS-dependent gene expression and repression in strain OE1-1. Application of the PQIs to the wild-type strain nearly recapitulated the gene expression profiles of Δ*phcA* and Δ*phcB*. The PQIs did not affect the canonical QS systems (AHL-QS) of the representative reporter bacteria (69, 76). These antagonists, especially PQI-5, reduced wilting symptoms of the tomato plants infected with strain OE1-1 (100). This inhibitory effect was canceled by the simultaneous application of 3-OH MAME. Therefore, targeting the *phc* QS system has the potential to develop chemicals that protect crops from bacterial wilt disease.

Unfortunately, the PQIs were not active enough to be effective at the field level. Finding more potent QS inhibitors will require extensive structural modification of PQIs. For example, a method of large-scale library construction using click chemistry would be suitable (33, 49). Advances in the structural biology of the interaction between PhcS and PQI should allow for the rational designs and the in silico discovery of potent inhibitors. The effectiveness of the PQIs for a 3-OH PAME–type QS system has not been fully tested, and this may also be an issue. Inhibition of 3-OH MAME or 3-OH PAME biosynthesis by chemicals is also expected to be investigated in the future.

OTHER CELL–CELL COMMUNICATION

In addition to the *phc* QS system, there is an AHL-type QS system encoded by *solI*/*solR* in RSSC strains (26) (**Figure 1**). SolI produces C_6- and C_8-HSLs. The *solI*- or *solR*-deficient mutants did not show significant changes in virulence and QS-regulated behavior and only regulated the expression of a limited number of factors, such as *aidA*. The biological significance of this finding is unknown. Coordinated with major virulence factors, expression of *solIR* is activated over 20-fold by PhcA (26). The *phc* QS system targets are activated when density exceeds ~10^7 cells/mL, while *solIR*-regulated targets are not activated until density exceeds ~10^8 cells/mL. Thus, hierarchical QS mechanisms, as seen in some bacterial species (51, 56), were present in RSSC strains.

Very recently, a new AHL-type QS system in GMI1000 was revealed. The genomes of some RSSC strains are known to have genes with high identity to *lasIR* (the *P. aeruginosa* AHL-QS system) apart from *solIR* (74). Yan et al. reported that these genes (newly named *rasIR*) are an essential second QS system that functions downstream of the *phc* QS system (98). They demonstrated chemically and biologically that the ligand is 3-OH-C_{12}-HSL, and the *rasIR* system controls cellulase activity, swimming, and biofilms and also contributes to the virulence of tomato plants. A small amount of 3-OH-C_{14}-HSL was also produced and seemed to function as a minor ligand. This system may regulate key virulence factors more directly. I would like to investigate in detail three QS systems (*phc*, *sol*, and *ras*) when they are activated and how they contribute to the physiology and virulence of RSSC strains.

It was also reported that anthranilic acid is involved in the physiology and pathogenicity of RSSC (78, 79). Anthranilic acid controls some biological functions and virulence of strain GMI1000 through the receptor protein RaaR, which contains helix-turn-helix and LysR substrate-binding domains. Anthranilic acid–deficient mutant phenotypes were rescued by the complementation of *raaR*. Anthranilic acid binds to the LysR substrate-binding domains of RaaR, induces allosteric conformational changes, and then enhances the binding of RaaR to the promoter DNA regions of target genes. Song et al. (79) argued that this signaling system might be a new type of cell–cell communication in RSSC. Similar modulators regulated by amino acids and related substances have recently been reported (15, 99). These studies also mentioned the possibility that the systems are new cell–cell communications. Rather, I expect them to be the important systems that prevent QS activation, which requires large amounts of energy, from overworking when certain amino acids and other substances are lacking. This would explain why the ligand concentration required for activation is higher than that for the typical bacterial QS system (59, 63, 96, 97). Therefore, referring to these systems as a new type of cell–cell communication in RSSC strains may be inappropriate.

INVOLVEMENT OF THE *phc* QUORUM-SENSING SYSTEM IN INTERACTIONS WITH SOIL FUNGI

The recent discovery that RSSC interacts with soil fungi has attracted significant attention (47, 80, 81, 91). In 2016, strain GMI1000 was found to be parasitic on the chlamydospores of many fungi (80). Ralstonins (aka ralsolamycins) exhibit chlamydospore-inducing activity on a diverse range of fungi and are thought to be key molecules in RSSC parasitism in fungi (7, 61, 80). Although it is a preprint article at this time, Tsumori et al. (86) reported that the *phc* QS system is important for this phenomenon. Δ*phcB* lost the ability both to produce ralstonins and to invade *Fusarium oxysporum* chlamydospores. These disabilities were rescued by 3-OH MAME. Meanwhile, the exogenous ralstonin A did not rescue the invasion ability of Δ*phcB* into *F. oxysporum* chlamydospores. Gene-deletion and -complementation experiments revealed that the QS-dependent production of EPS I is crucial for this invasion. RSSC cells adhered to *F. oxysporum* hyphae and formed biofilms before inducing chlamydospores. This biofilm formation was not observed in the EPS I– or ralstonin-deficient mutant. Microscopic observation revealed that RSSC infection caused the death of *F. oxysporum* chlamydospores. Altogether, the *phc* QS system is important for this lethal endoparasitism. Among the factors regulated by the QS system, ralstonins, EPS I, and biofilm are important parasitic factors.

We do not know whether the *phc* QS system works in interactions with other fungi. Endoparasitic interactions with fungi may provide clues to the mystery of how RSSC can survive for so long in soil (80, 91). There are also several glycolytic enzymes that are not important in demonstrating virulence in plants. It is possible that those enzymes act on fungal cell walls. Unbeknownst to us, RSSC may be interacting closely with other hosts (such as nematodes and insects), plants, and fungi (37).

PERSPECTIVE AND FUTURE DIRECTIONS

In the last 25 years, research of QS in RSSC strains has greatly developed. The impetus for this research begins with identifying the QS signals (3-OH MAME and 3-OH PAME) and understanding biosynthesis and possible receptor systems. Comprehensive expression analyses of QS-dependent gene expression profiles by RNA-seq have differentiated the *phc* genes that tend to appear similarly regulated and contributed to more specific functional analogies. However, after writing this review, I feel that the detailed functions of each gene and protein on RSSC

physiology and virulence are mostly unexplored. Particularly, the biochemistry of the process that begins with QS signal perception by PhcS and ends with the functionalization of PhcA is virtually untouched. Is the proposed model, from the perception of the QS signals to the activation of PhcA, biochemically correct?

The next task is to observe QS status in host plants. The *phc* QS system should have different degrees of activation at each stage of plant infection: until it enters the plant, in the intercellular spaces, at xylem entry, at the explosive growth in the xylems, and in the late stages of infection. I am also wondering whether the *phc* QS system is involved in the switching on and off of the *hrp* system as we now expect. We want to obtain temporal and spatial information of those events. This will require the development of imaging techniques with a confocal laser microscope or MS revealing the relationship between RSSC behavior and QS in a tissue or smaller sections of host plants. The same is true in interactions with new hosts such as fungi. Such imaging techniques would be easier to apply to the interactions with fungi.

The next goal is to understand the behavior of RSSC cells and the role of the *phc* QS system in the plant rhizosphere and soil. Recently, studies on plant microbiomes have been tremendous (12, 34, 42), but we do not think much valuable information has been obtained on the physiology and ecology of RSSC strains in the soil environment. Furthermore, RSSC cells might affect the surrounding microbial flora and prepare it in advance to infect host plants. I expect that a comparative analysis using a wild-type strain and *phcA*-deficient mutant would provide valuable information on what they are doing there. If the *phc* QS system plays an important role even before RSSC encounters the host plant, it may be easier to control QS compared to when the bacteria are in the plant. Hopefully, by successfully controlling QS, it will possible to develop technology to prevent bacterial wilt disease.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

I apologize to colleagues and researchers whose work was not included because of space limitations. This work was supported by JSPS KAKENHI (17K19244, 19H02899, 20K21286, 22H04888, and 22H02275) and a grant from the Institute for Fermentation, Osaka (L-2023-2-004).

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