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Motility-mediated regulation of virulence in *Pseudomonas syringae*☆

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ABSTRACT

We have investigated *Pseudomonas syringae* pv. *tabaci*—plant interactions using a large variety of virulence-related mutants. A flagellin-defective mutant, Δ*fliC*, lost flagellar motility and the ability to produce *N*-acyl homoserine lactones; it had reduced ability to cause disease symptoms, but the expression of genes encoding a multidrug efflux pump transporter, *mexEFoprN*, was activated. A type IV pili (T4P)-defective mutant, Δ*pilA*, lost swarming motility, had reduced expression of *hrp*-related genes and virulence toward the host tobacco plant, but expression of the genes encoding another multidrug efflux pump transporter, *mexABoprM*, was activated. These results suggest that the genes regulating flagella- and T4P-mediated motilities also regulate expression of other virulence-related genes.

1. Introduction

Reverse genetics is a powerful method to study plant-microbe interactions. We have investigated the functions of particular genes of *Pseudomonas syringae* pv. *tabaci* 6605 and their interactions with host and nonhost plants using a large variety of virulence-related mutants [10]. These mutants include not only flagella- and type IV pili-related mutants such as Δ*fliC*, a mutant defective for flagellin protein [9,27], Δ*fliD*, a mutant lacking HAP2 capping protein [9,27], Δ*motABCD*, a mutant with motor protein defects [11], Δ*fgt1*, a mutant lacking flagellin-glycosyltransferase 1 [36,39], Δ*pilA*, a mutant lacking the major type IV pilin, PilA [21,28], and Δ*tfpO*, a mutant lacking pilin glycosyltransferase TfpO [21], but also the mutants with defects in some regulatory proteins such as Δ*psyI*, a mutant lacking PsyI (also called AhlI), an *N*-acyl homoserine lactone (AHL) synthase used in quorum sensing [31], Δ*gacA*, a mutant lacking response regulator GacA of the Gac two-component system [17], Δ*aefR*, a mutant lacking AefR, AHL and an epiphytic fitness regulator [12], Δ*mexT*, a mutant lacking MexT, a putative transcriptional activator for *mexEFOprN* [12], and Δ*vfr*, a mutant lacking Vfr, virulence factor regulator [29]. Investigation of the phenotypes of these mutant strains revealed that the respective mutations have altered their gene expression profiles, and the existence of a network that regulates expression of these genes. Loss of bacterial motility might resemble the condition of adherence of bacteria on the plant cell surface. Furthermore, loss of a regulatory protein might reveal the target proteins or genes. In these studies, we attempted to understand the regulation of stage-specific expression of virulence factors during bacterial pathogenesis using these mutant strains.

2. Flagellar motility and virulence

2.1. Flagellar motility and quorum sensing

Motilities are virulence factors that allow phytopathogenic bacteria to enter a plant apoplast [10]. Flagella-motility-defective mutants such as Δ*fliC*, Δ*fliD*, Δ*motABCD*, and Δ*fgt1* have reduced ability to cause disease [9,11,37], indicating the importance of motility for bacterial virulence. However, during the phenotypic investigation of these mutants, we unexpectedly found they also had remarkably reduced ability to produce acyl homoserine lactones (AHL) [11,36,37]. These results indicate that flagellar motility is required for AHL production, and the causes of reduced virulence

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of these mutants are both impaired flagellar motility and loss of AHL production. Among these results, we found a gene, *orf3*, that encodes putative 3-oxoacyl-(acyl carrier protein) synthase III, an enzyme required for production of an AHL precursor, between flagellin glycosyltransferase 2 gene, *fgt2* and a flagellin gene, *fliC* [31,36,39]. This evidence also indicates the existence of a relationship between flagellar motility and quorum sensing.

2.2. Flagellin glycosylation is required for flagella motility

We previously thought that the flagellins of *P. syringae* pvs. *glycinea* and *tabaci* were modified post-translationally because the flagellin of *P. syringae* pv. *glycinea* induces hypersensitive cell death in its non-host tobacco plants, whereas that of pv. *tabaci* remarkably does not induce it, although the deduced amino acid sequences of both flagellins are identical [34]. Furthermore, the flagellins of *P. syringae* pvs. *tabaci*, *tomato*, and *glycinea* are positively detected by glycostaining as 32 kDa proteins. However, the molecular masses of deglycosylated flagellins were decreased about 2–3 kDa by anhydrous trifluoromethanesulfonic acid [33]. These results clearly indicate that flagellins are glycoproteins [33]. Furthermore, we identified glycosylated amino acid residues at positions 143, 164, 176, 183, 193, and 201 in a surface-exposed region of the flagellin filament [36]. Flagellin glycans are composed of common unique tri- or tetrasaccharides consisting of two or three rhamnoses and one modified 4-amino-4,6-dideoxyglucose (viosamine) [14,38]. Furthermore, we identified three flagellin glycosyltransferase genes, *fgt1*, *fgt2*, and *vioT* [22,36,39]. The Δ*fgt1* mutant produced nonglycosylated flagellin, whereas Δ*fgt2* and Δ*vioT* mutant strains produced partially glycosylated flagellins [22,36,39,44]. Thus, Δ*fgt1*, Δ*fgt2*, and Δ*vioT* mutant strains showed reduced flagella motility and virulence [22,36,39,44]. Flagellin mutants defective in glycosylation, such as Δ*fgt1*, produced abnormally shaped flagella filaments, had reduced flagellar filament stability and reduced motility [32]. Because one well-studied microbe-associated molecular pattern, flg22, is localized inside the flagellum, the flagellum must be dissociated for recognition of flg22 by plant receptors. Thus, the glycosylated flagellin might have evolved to evade plant detection and activation of a defence response by stabilizing the flagella filament and suppressing its dissociation [35].

2.3. Gene expression profile of flagella motility- and AHL-production-defective mutants

All flagella-motility-defective mutants had remarkably reduced ability to produce AHL. To understand the gene expression profiles of these mutant strains, we performed microarray analyses of the wild-type strain and Δ*fliC*, Δ*fliD*, Δ*motABCD*, Δ*fgt1*, Δ*psyI*, Δ*aefR*, and Δ*gacA* mutant strains using a Roche NimbleGen microarray system [30] (Table 1). We also carried out microarray analysis of Δ*mexT*, Δ*pilA*, Δ*tfpO*, and Δ*vfr* mutant strains as described below [12,28,29] (Table 1). Each bacterial strain was grown in LB medium with 10 mM $MgCl_2$ at 27 °C to an OD600 of 0.3. The cells were collected by centrifugation and further incubated in MMMF medium (50 mM potassium phosphate buffer, 7.6 mM $(NH_4)_2SO_4$, 1.7 mM $MgCl_2$, and 1.7 mM NaCl, pH 5.7, supplemented with 10 mM each of mannitol and fructose) for an additional 1 h. Then, total RNA was extracted using a High Pure RNA Isolation kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

As shown in Table 1, we found a remarkable reduction of *psyI* and *psyR* gene expression in all mutants with defective flagellar motility, including Δ*fliC*, Δ*fliD*, Δ*motABCD*, Δ*fgt1*, and in other mutants, Δ*psyI*, Δ*aefR* and Δ*gacA*, but not in Δ*mexT*, Δ*pilA*, Δ*tfpO*, or Δ*vfr* mutants. The *psyR* gene encodes the putative autoinducer transcriptional regulator PsyR (also called AhlR). Because *psyI* encodes AHL synthase, loss of AHL production in Δ*psyI* is an expected and logical result [31]. The *gacA* and *aefR* genes were also reported to be required for AHL production [5,7,12,17,25]. Thus, the loss of AHL production is consistent with the remarkable reduction of *psyI* gene expression. Furthermore, loss of AHL production accompanies a remarkable reduction of gene expression for an operon consisting of *orf1* to *orf5* (orthologues of Pspph1613 to Pspph1609) and a moderate reduction of *psyR* (Pspph1615 orthologue) and *marR* (Pspph1617 orthologue). Thus, these genes seem to be a target of the quorum sensing system of this pathogen [30].

Interestingly, most of these AHL production-defective mutants had activated multidrug efflux pump transporter genes *mexEFoprN* (orthologues of Pspph2273 to Pspph2271). In particular, expression of *mexEFoprN* in Δ*fliD* and Δ*aefR* is extremely activated. Although the reason for and mechanism of *mexEFoprN* activation (especially in Δ*fliD* and Δ*aefR*) are not clear yet, there seems to be a closed regulatory network between the AHL-mediated quorum sensing system and the activation of *mexEFoprN*. Because Δ*psyI* also activated the expression of *mexEFoprN*, a quorum sensing system might repress expression of *mexEFoprN*. However, one mutant defective in AHL production, Δ*gacA*, did not have changed *mexEFoprN* expression, as described below. These results suggest that the expression of *mexEFoprN* is not simply repressed by the quorum sensing system. In relation to this, we generated a Δ*mexT* mutant in *P. syringae* pv. *tabaci* 6605, because MexT is known as a LysR-type transcriptional activator for *mexEFoprN* in *Pseudomonas aeruginosa* [8,13,18]. However, we found that expression of *mexEFoprN* and production of AHL were not affected in the Δ*mexT* mutant [12]. The expression level of *mexEFoprN* of the wild-type and Δ*mexT* strains is very low in LB with 10 mM $MgCl_2$ and in MMMF medium [12]. Therefore, we expect that 1) the mutation of *mexT* did not affect the silenced level of *mexEFoprN* expression, 2) MexT is not required for AHL production, and 3) MexEFOprN is also probably not required for AHL production. Recently, we attempted to reveal the mechanism of loss of AHL production in the Δ*fliC* mutant by transposon mutagenesis and found that disruption of *mexEFoprN* genes restored AHL production in the Δ*fliC* mutant, indicating that the activation of *mexEFoprN* interfered with accumulation of AHL (unpublished).

Among the AHL production-defective mutant strains, *mexEFoprN* expression was unaffected only in the Δ*gacA* mutant. This indicates that GacA positively regulates AHL production independent of regulation of *mexEFoprN* and is a higher regulatory factor in AHL production. Furthermore, regulation of AHL production by GacA is independent of both AefR and the flagella motility-related regulatory system.

2.4. Role of quorum sensing in bacterial virulence

As shown in Table 1, all AHL production-defective mutant strains showed remarkably reduced expression of orthologues of Pspph1609 to Pspph1614 and moderately reduced expression of Pspph1615 and Pspph1617. However, we do not know the functions of most of these genes. We know only that orthologues of Pspph1614 and Pspph1615 encode PsyI and PsyR, respectively, although the function of PsyR is not clear yet. The orthologues of Pspph1609, Pspph1610, Pspph1611, Pspph1612, Pspph1613, and Pspph1617 are annotated as encoding a Rieske (2Fe–2S) domain-containing protein (Orf5), a hypothetical protein (Orf4), a dihydrodipicolinate synthetase family protein (Orf3), a hypothetical protein (Orf2), a hypothetical protein (Orf1), and a MarR-family transcriptional regulator (MarR), respectively. To reveal the role of the quorum sensing system, we recently focused on the function of the orthologues of Pspph1609 and Pspph1617 [30]. The deletion mutants of the Pspph1617 orthologue (which we named Δ*marR*)