Pseudomonas syringae pv. *phaseolicola* Mutants Compromised for Type III Secretion System Gene Induction

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Pseudomonas syringae bacteria utilize the type III secretion system (T3SS) to deliver effector proteins into host cells. The T3SS and T3 effector genes (together called the T3 genes hereafter) are repressed in nutrient-rich medium but rapidly induced after the bacteria are transferred into minimal medium or infiltrated into plants. The induction of the T3 genes is mediated by HrpL, an alternative sigma factor that recognizes the conserved hrp box motif in the T3 gene promoters. The induction of *hrpL* is mediated by HrpR and HrpS, two homologous proteins that bind the *hrpL* promoter. To identify additional genes involved in regulation of the T3 genes, we screened for the *P. syringae* pv. *phaseolicola* NPS3121 transposon-tagged mutants with reduced induction of *avrPto-luc* and *hrpL-luc*, reporter genes for promoters of effector gene *avrPto* and *hrpL*, respectively. Determination of the transposon-insertion sites revealed genes with putative functions in signal transduction and transcriptional regulation, protein synthesis, and basic metabolism. A transcriptional regulator (AefR_{NPS3121}) was identified in our screen that is homologous to AefR of *P. syringae* pv. *syringae* strain B728a, a regulator of the quorum-sensing signal and epiphytic traits, but was not known to regulate the T3 genes. AefR_{NPS3121} in *P. syringae* pv. *phaseolicola* NPS3121 and AefR in *P. syringae* pv. *syringae* B728a behave similarly in regulating the quorum-sensing signal in liquid medium but differ in regulating the epiphytic traits, including swarming motility, leaf entry, and epiphytic survival.

Like many pathogenic proteobacteria, pathovars of *Pseudomonas syringae* rely on the type III secretion system (T3SS) for parasitism (Jin et al. 2003). The T3SS is encoded by the hypersensitive response and pathogenicity (*hrp/hrc*) genes that are essential for disease development on host plants and hypersensitive response (HR) on resistant plants and nonhost plants (Collmer et al. 2000). Through the T3SS, bacteria inject an array of virulence proteins termed T3 effectors into the host cells (Galan and Collmer 1999). A number of T3 effectors have been shown to suppress the host basal defense systems (Zhou and Cai 2008). In resistant plants, however, the effectors are recognized by the plant disease resistance proteins, which

trigger the HR and disease resistance (Alfano and Collmer 2004).

The T3SS and T3 effector genes (together called the T3 genes hereafter) of *P. syringae* bacteria are repressed in nutrient-rich medium such as King's B (KB) (King et al. 1954) but are induced in minimal medium (MM) and in the plant (Tang et al. 2006). The induction of the T3 genes is mediated by HrpL, an alternate sigma factor that recognizes the conserved *hrp* box motif in the promoter of T3 genes (Ferreira et al. 2006; Fouts et al. 2002; Lan et al. 2006; Xiao and Hutcheson 1994). The *hrpL* gene itself is induced under the same conditions (Thwaites et al. 2004). *hrpL* induction is mediated by HrpR and HrpS, two DNA-binding proteins that form a heterodimer on the *hrpL* promoter (Hutcheson et al. 2001; Xiao et al. 1994). The HrpR/HrpS heterodimer activates *hrpL* transcription by interacting with the alternate sigma factor RpoN (Chatterjee et al. 2003; Hendrickson et al. 2000). Deletion of either *hrpR* or *hrpS* abolishes *hrpL* induction and bacterial pathogenicity (Xiao et al. 1994). Additional regulators required for maximal *hrpL* induction include CorR, which also regulates coronatine synthesis, and PsrA, which regulates production of the quorum-sensing signal *N*-acyl homoserine lactones (AHL). CorR regulates *hrpL* by binding directly to the *hrpL* promoter (Sreedharan et al. 2006). PsrA directly binds the *rpoS* promoter and *aefR* operator to positively regulate *rpoS* and negatively regulate *aefR*, respectively (Chatterjee et al. 2007). The regulation of *hrpL* by PsrA is likely to be indirect (Chatterjee et al. 2007). Mutation of either *corR* or *psrA* reduces bacterial virulence but does not eliminate pathogenicity altogether (Chatterjee et al. 2007; Sreedharan et al. 2006).

HrpR and HrpS are encoded by the *hrpRS* operon that is moderately expressed in KB medium and further induced two- to fourfold in MM and in the plant (Grimm et al. 1995; Hutcheson et al. 2001; Rahme et al. 1992; Thwaites et al. 2004). The *hrpRS* operon is regulated by two loci encoding the two-component systems GacS/GacA and RhpS/RhpR (Chatterjee et al. 2003; Xiao et al. 2007). GacS is a sensor histidine kinase whereas GacA is the cognate response regulator of GacS. In *P. syringae* pv. *tomato* strain DC3000, *gacA*⁻ mutation significantly attenuates the transcription of *hrpRS*, *rpoN*, and *hrpL* (Chatterjee et al. 2003). GacS is hypothesized to be activated by an as-yet-unidentified signal, which in turn phosphorylates GacA, and the phosphorylated GacA then induces, either directly or indirectly, the *hrpRS* operon (Chatterjee et al. 2003). The role of RhpS/RhpR in regulating the *hrpRS* operon is indicated by mutation of the sensor kinase gene *rhpS*, which abolishes *hrpRS* induction in the plant and in MM (Xiao et al. 2007). However, disruption of the cognate response regulator

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gene *rhpR* in the *rhpS*⁻ mutant or deletion of the *rhpRS* locus completely restores the *hrpRS* induction, suggesting that RhpR is a negative regulator of *hrpRS*. In addition to GacS/GacA and RhpS/RhpR, the *hrpA* gene encoding T3 pilin was also found to affect *hrpRS* induction through an unknown mechanism. Transcription of the *hrpRS* operon, *hrpL*, and T3 genes is severely reduced in the *hrpA*⁻ mutant (Wei et al. 2000).

Another level of control of T3 gene induction involves the stability of HrpR protein, which is regulated by ATP-dependent protease Lon (Bretz et al. 2002). HrpR is stabilized in a *lon*⁻ mutant, leading to elevated expression of *hrpL* and T3 genes in KB medium (Bretz et al. 2002; Lan et al. 2007). The impact of the *lon*⁻ mutation on *hrpL* expression exhibits a dynamic change in MM. *hrpL* is expressed at a higher level in the *lon*⁻ mutant than in the wild-type (WT) strain shortly after induction in MM, but is more abundant in the WT strain at later time points (Lan et al. 2007). HrpS protein stability is not affected by the *lon*⁻ mutation. However, HrpS activity is repressed by HrpV, another negative regulator of the T3 genes that physically interacts with HrpS (Preston et al. 1998; Wei et al. 2005). HrpV-mediated repression can be cleared by HrpG, a protein that interacts with HrpV and liberates HrpS from the HrpV repression (Wei et al. 2005).

The induction of the T3 genes in MM provides an assay system for genetic identification of regulators of the T3 genes. Hendrickson and associates (2000) conducted a screen for *P. syringae* pv. *maculicola* ES4326 mutants that compromised the induction of the T3 genes in MM. From a total of 14,000 colonies, 297 mutants were identified. However, none of these mutant genes was cloned.

To identify additional regulators of the T3 genes, *P. syringae* pv. *phaseolicola* NPS3121 transposon-tagged mutants were screened on the basis of their weak induction of *avrPto-luc* and *hrpL-luc* reporter genes in MM. *avrPto* is a T3 effector gene of *P. syringae* pv. *tomato* DC3000 that carries a typical *hrp* box promoter (Salmeron and Staskawicz 1993). *avrPto-luc* in *P. syringae* pv. *phaseolicola* NPS3121 is induced in MM and in the plant in an *hrpL*- and *hrpRS*-dependent manner (Xiao et al. 2004; Xiao et al. 2007). Here, we report the mutant genes identified in our screen.

RESULTS

Isolation of *P. syringae* pv. *phaseolicola* mutants using the *avrPto-luc* reporter.

P. syringae pv. *phaseolicola* NPS3121 carrying the *avrPto-luc* reporter displayed low luciferase (LUC) activity in KB medium but high LUC activity in MM. This strain was subjected to EZ::TN<Kan-2> transposon insertion mutagenesis. In total, 11,872 mutant clones were screened for reduced LUC activity 6 h after induction in MM. Clones that displayed a LUC activity <20% of the parental strain were selected and named MM-insensitive (*min*) mutants. In all, 84 mutants were recovered from the screening (Table 1; Supplementary Fig. S1).

Transposon insertion sites in these mutants were determined by two-stage semidegenerated polymerase chain reaction (PCR) (Jacobs et al. 2003), and the flanking sequences were searched against the *P. syringae* pv. *phaseolicola* 1448A genomic sequence (Joardar et al. 2005). These mutants were distributed among 45 loci, which can be divided into four groups based on the putative gene function (Table 1). Genes in group A encode proteins with a role in signal perception and transduction, including *hrpS*, *rhpS*, and *PSPPH_3244*_{NPS3121}. *hrpS* and *rhpS* are known regulators of the T3 genes (Xiao et al. 1994, 2007). *PSPPH_3244*_{NPS3121} encodes a transcriptional regulator with 87% amino acid sequence identity to AefR, a regulator of quorum-sensing signal and epiphytic fitness in *P. syringae* pv. *syrin-*

gae B728a strain (Quinones et al. 2004, 2005). Mutants of *hrpL*, *hrpR*, and other known regulatory genes were not identified in the screening. Group B has two genes encoding putative membrane proteins. *PSPPH_4907*_{NPS3121} encodes a porin protein in the OprD family that functions in trafficking small molecules across the outer membrane (Hancock and Brinkman 2002). *PSPPH_5137*_{NPS3121} encodes a putative integral membrane protein of the YeeE/YedE family with an unknown function (Joardar et al. 2005). Group C has only one gene, *trmE*, which encodes a tRNA modification GTPase (Joardar et al. 2005). Mutation of *trmE* increases the rate of misincorporation of amino acids and frame-shifting during the translation process (Cabedo et al. 1999). Mutation of this gene in *Shigella flexneri* reduces bacterial virulence (Durand et al. 1997). Most of the mutant genes belong to group D that encode functions in basic metabolism. Twelve genes are involved in porphyrin metabolism (Table 1). Nineteen genes are involved in the biosynthesis of amino acids arginine, glutamine, glutamic acid, histidine, valine, isoleucine, leucine, methionine, serine, and tryptophan (Table 1). The induction of LUC activity in the amino acid biosynthesis mutants was restored by adding corresponding amino acids to 0.1 mM in the MM. Three genes are in an operon required for fructose uptake and metabolism (Joardar et al. 2005). Two genes encode enzymes that function in purine synthesis. Four additional metabolic genes are *eno-1*, encoding enolase; *PSPPH_2878*_{NPS3121}, encoding a glycosyl hydrolase; *sypA*, encoding a putative peptide/siderophore synthase, and *PSPPH_0569*_{NPS3121}, encoding a putative ATP phosphoribosyltransferase subunit.

All the mutants were examined for growth in MM. Mutants in groups A, B, and C displayed approximately seven- to nine-fold multiplication 36 h after culture in MM, which was similar to the WT strain. However, mutants in group D showed little growth in MM.

Mutant screening using the *hrpL-luc* reporter.

The use of *avrPto-luc* as reporter led to the identification of a large number of metabolic mutants and only three genes with regulatory functions. Many genes with a known function in regulating the T3 genes were not identified in the screening. Because the screening was approximately 2× coverage of the *P. syringae* pv. *phaseolicola* genome, we decided to screen more mutants using *hrpL-luc* as reporter. To avoid metabolic mutants, mutants with low *hrpL-luc* reporter activity were assayed for growth in MM, and those with growth defect were discarded.

Eight mutants were obtained from a total of 16,000 clones. Five mutations were in the *rhpS* gene, one in *PSPPH_3244*_{NPS3121}, one in *trmE*, and one in *miaA* (Table 1). Mutants of *rhpS*, *PSPPH_3244*_{NPS3121}, and *trmE* genes were obtained in the screening with *avrPto-luc* reporter. *miaA* encodes tRNA isopentenyltransferase and, like *trmE*, also has a function in protein translation (Leung et al. 1997). Except for *rhpS* and *PSPPH_3244*_{NPS3121}, no gene with a regulatory function was identified in this screening.

Evaluation of the reporter genes.

It was puzzling that a large number of metabolic mutants and only a few regulatory mutants were recovered from the mutant screening. One possibility was that the metabolic mutants were lethal in MM, as indicated by the lack of bacterial growth in MM. Therefore, we decided to evaluate the reporter genes with the mutant strains to determine whether the reporter gene activities were consistent with the levels of *hrpL* RNA.

We first analyzed the levels of *hrpL* RNA in representative mutants of each functional group that were isolated based on the *avrPto-luc* reporter activity (Fig. 1A). All mutants except *min93* exhibited good correlation between the *avrPto-luc* re-

porter activity and the level of *hrpL* RNA (Fig. 1A). The *min93* mutant had normal induction of *hrpL* RNA but the LUC activity was almost undetectable in this mutant.

We then tested if the *hrpL-luc* reporter activity was consistent with the level of *hrpL* RNA. *min93* and six other mutants (*min24*, *min4*, *min32*, *min42*, *min49*, and *min62*) were cured of the *avrPto-luc* reporter plasmid, the *hrpL-luc* reporter plasmid was introduced into each, and LUC activity derived from *hrpL-luc* was measured (Fig. 1B). The *hrpL-luc* reporter activ-

ity was almost undetectable in the *min93* mutant but the *hrpL-luc* reporter activities in other mutants generally agreed with the levels of *hrpL* RNA.

We further conducted a Western blot analysis of the LUC protein derived from *hrpL-luc* in *min93* and other mutants (Fig. 1C). A high level of LUC protein was detected in the *min93* mutant, suggesting that this mutation impaired the detection of LUC enzymatic activity rather than the induction of the T3 genes. The levels of LUC protein in other mutants were

Table 1. Minimal medium-insensitive (*min*) mutants^a

Genes ^b	Mutants	Gene products	Pathways of biological processes	Disease index
Group A				
<i>rhpS</i>	<i>min12</i> , 80, L1, L2, L3, L5, L7	Sensor histidine kinase	Signal transduction	0
<i>hrpS</i>	<i>min08</i> , 94, 96	Type III transcriptional regulator	Signal transduction	0
<i>aeiR</i>	<i>min16</i> , L4	Transcriptional regulator	Signal transduction	2
Group B				
PSPPH_4907	<i>min79</i> , 93	Outer membrane porin	Membrane protein	2
PSPPH_5137	<i>min107</i>	YeeE/YedE family protein	Putative membrane protein	1
Group C				
<i>trmE</i>	<i>min47</i> , L8	tRNA modification GTPase	Translation	ND
<i>miaA</i>	<i>minL6</i>	tRNA modification isopentenyltransferase	Translation	1
Group D				
<i>cblD</i>	<i>min83</i>	Cobalamin biosynthesis protein	Porphyrin and chlorophyll metabolism	1
<i>cblG</i>	<i>min75</i>	Precorrin-3B C17-methyltransferase	Porphyrin and chlorophyll metabolism	2
<i>cobC</i>	<i>min87</i>	Cobalamin biosynthesis protein	Porphyrin and chlorophyll metabolism	1
<i>cobH</i>	<i>min103</i>	Precorrin-8X methylmutase	Porphyrin and chlorophyll metabolism	1
<i>cobM</i>	<i>min30</i> , 50, 55, 56	Precorrin-4 C11-methyltransferase	Porphyrin and chlorophyll metabolism	1
<i>cobN</i>	<i>min66</i> , 69	CobN/magnesium chelatase family protein	Porphyrin and chlorophyll metabolism	1.5
<i>cobQ</i>	<i>min51</i>	Cobyric acid synthase	Porphyrin and chlorophyll metabolism	1
PSPPH_3701	<i>min104</i> , 106	Cobyric acid a,c-diamide synthase	Porphyrin and chlorophyll metabolism	NA
PSPPH_2598	<i>min64</i> , 95	Tetrapyrrole methylase family protein	Porphyrin and chlorophyll metabolism	2
PSPPH_2227	<i>min77</i>	Magnesium chelatase subunit ChlD	Porphyrin and chlorophyll metabolism	1
<i>bchI</i>	<i>min29</i> , 54	Magnesium chelatase, subunit ChlI, putative	Porphyrin and chlorophyll metabolism	1.5
<i>argH</i>	<i>min10</i> , 13, 45, 68	Argininosuccinate lyase	Arginine biosynthesis	1.5
<i>glbB</i>	<i>min04</i> , 15, 20, 25, 26, 33, 40, 43, 52, 67, 99	Glutamate synthase, large subunit	Glutamate biosynthesis	1.7
<i>glbD</i>	<i>min22</i> , 28	Glutamate synthase, small subunit	Glutamate biosynthesis	2.5
<i>hisA</i>	<i>min38</i>	Phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	Histidine biosynthesis	1
<i>hisB</i>	<i>min59</i> , 74	Imidazoleglycerol-phosphate dehydratase	Histidine biosynthesis	2.5
<i>hisC</i>	<i>min05</i> , 11	Histidinol-phosphate aminotransferase	Histidine biosynthesis	2
<i>hisD</i>	<i>min37</i> , 76	Histidinol dehydrogenase	Histidine biosynthesis	1.5
<i>hisE</i>	<i>min53</i>	Phosphoribosyl-ATP pyrophosphatase	Histidine biosynthesis	3
<i>hisF</i>	<i>min24</i> , 34	Imidazoleglycerol phosphate synthase, cyclase	Histidine biosynthesis	0.5
<i>hisH</i>	<i>min35</i> , 36	Amidotransferase	Histidine biosynthesis	0
<i>ilvD</i>	<i>min32</i> , 86	Dihydroxy-acid dehydratase	Valine and isoleucine biosynthesis	0
<i>leuA</i>	<i>min09</i> , 14, 82	2-Isopropylmalate synthase	Leucine biosynthesis	0.3
<i>leuB</i>	<i>min21</i> , 39, 61	3-Isopropylmalate dehydrogenase	Leucine biosynthesis	0
<i>leuC</i>	<i>min60</i>	3-Isopropylmalate dehydratase, large subunit	Leucine biosynthesis	0
<i>metF</i>	<i>min49</i> , 84	5,10-Methylenetetrahydrofolate reductase	Methionine biosynthesis	1.5
<i>metH</i>	<i>min92</i> , 108	5-Methyltetrahydrofolate homocysteine methyltransferase	Methionine biosynthesis	1.5
<i>serA</i>	<i>min42</i> , 73	D-3-phosphoglycerate dehydrogenase	L-serine biosynthesis	1
<i>trpA</i>	<i>min62</i>	Tryptophan synthase, alpha subunit	Tryptophan biosynthesis	0
<i>trpD</i>	<i>min71</i>	Anthranilate phosphoribosyl transferase	Tryptophan biosynthesis	0
PSPPH_0847	<i>min65</i> , 98	Phosphoenolpyruvate-protein phosphotransferase, EI/HPtr/EIHA components	Fructose and mannose metabolism	2
<i>fruK</i>	<i>min18</i>	1-Phosphofructokinase	Fructose and mannose metabolism	2
PSPPH_0849	<i>min41</i>	Phosphotransferase system, fructose-specific IIBC component	Fructose and mannose metabolism	2
<i>purE</i>	<i>min78</i>	Phosphoribosylaminoimidazole carboxylase, catalytic subunit	Purine metabolism	2
<i>purK</i>	<i>min27</i>	Phosphoribosylaminoimidazole carboxylase, atpase subunit	Purine metabolism	0
<i>eno-1</i>	<i>min23</i>	Enolase	Glycolysis; gluconeogenesis	1
PSPPH_2878	<i>min85</i>	Glycosyl hydrolase, family 15	Polysaccharide metabolism	1
<i>sypA</i>	<i>min63</i>	Putative peptide/siderophore synthetase	...	3
PSPPH_0569	<i>min02</i>	ATP phosphoribosyltransferase subunit, putative	...	2

^a Mutants isolated with the *avrPto-luc* reporter are indicated by *min* followed by a number. Mutants isolated with the *hrpL-luc* reporter are indicated by *min* followed by L and a number. Genes with transposon insertions are named according to their homologs in the *Pseudomonas syringae* pv. *phaseolicola* 1448A strain. ND = not determined.

^b Group A: signal sensor or transcriptional regulator, group B: membrane proteins, group C: protein translation, and group D: metabolic genes.

generally consistent with the *hrpL-luc* reporter activities and the levels of *hrpL* RNA. These results indicated that the activities of *avrPto-luc* and *hrpL-luc* reporter genes generally reflected the level of *hrpL* RNA expression in the mutant bacteria.

Pathogenicity and HR assays of *min* mutants.

Although numerous metabolic mutations severely compromised the induction of T3 genes in MM, many were not reported in previous studies of *P. syringae* mutants that abolished the pathogenicity or HR-inducing activity. To determine whether there is a correlation between the T3 gene expression in MM and pathogenicity in host plants, all the mutant strains at 2×10^4 CFU/ml were infiltrated into the primary leaves of bean plants. The degree of disease symptoms was evaluated according to a disease index (Supplementary Fig. S2). The WT strain caused the most concentrated specks and was assigned a

disease index of 3. The *hrpS* mutants were symptom-free and assigned an index of 0. Mutants with the disease symptoms in between were assigned an index of 1 or 2. A number of metabolic mutants, including *min24*, *min35*, and *min36* for histidine synthesis, *min9*, *min14*, *min21*, *min39*, *min61*, and *min60* for leucine synthesis, *min32* and *min86* for valine and isoleucine synthesis, *min62* and *min71* for tryptophan synthesis, and *min27* for purine synthesis, failed to elicit any visible disease symptoms. Mutants with defects in histidine, leucine, valine, isoleucine, tryptophan, and purine biosynthesis were also identified by Brooks and associates (2004) in a screening of *P. syringae* pv. *tomato* DC3000 mutants that compromised the pathogenicity in *Arabidopsis* plants.

Representative mutants that did not elicit visible symptoms were assayed for bacterial growth 6 days after inoculation into bean plants (Fig. 2). All these mutants displayed a significant

Fig. 1. Assessment of *hrpL* RNA, *hrpL-luc* reporter activity, and HrpL protein in representative minimal medium-insensitive (*min*) mutants. **A**, *hrpL* RNA. Wild-type (WT) *Pseudomonas syringae* pv. *phaseolicola* NPS3121 and *min* mutants were grown in King's B (KB) medium and induced in minimal medium (MM) for 4 h. Total RNA (10 µg) from each sample was subjected to electrophoresis in a denaturing agarose gel. The blot was hybridized with DNA probes derived from the *hrpL* coding region. Loading of RNA samples is indicated by rRNA. **B**, Luciferase (LUC) activity derived from *hrpL-luc* reporter. Bacteria were grown in KB medium and induced in MM for 4 h. LUC activity was measured with a cooled charge-couple device. Each data point represents three replicates. Error bars indicate standard error. **C**, LUC protein derived from *hrpL-luc* reporter. The WT and *min* mutant strains carrying *hrpL-luc* reporter were grown in KB and induced in MM for 4 h. Bacteria were harvested by centrifugation and resuspended in MM to an optical density at 600 nm of 1. Bacteria (30 µl) was boiled in 1× sodium dodecyl sulfate (SDS) sample buffer and loaded to an SDS polyacrylamide gel electrophoresis gel. Western blot was performed using anti-LUC antibodies.

reduction of bacterial growth in the host plants, lower than that of the *rhpS*⁻ mutant (*min12*). With the exception of *min24* (*hisF*⁻), the growth of the remaining metabolic mutants was even lower than that of the *hrpS*⁻ mutant (*min8*). Although *min32*(*ilvD*⁻) displayed a significant level of *hrpL* RNA in MM (Fig. 1A), the growth of this mutant in host bean plants was even lower than that of the *hrpS*⁻ mutant (Fig. 2). In contrast, many metabolic mutants with completely abolished *hrpL* expression in MM were only slightly reduced in pathogenicity (Fig. 1A; Table 1).

Representative mutants of each functional group were also assayed for the HR-inducing activity on tobacco W38 plants (Table 2). A number of mutants that elicited significant disease symptoms on bean plants, such as *min4*(*gltB*⁻), *min18*(*fruK*⁻), *min42*(*serA*⁻), *min49*(*metF*⁻), *min83*(*cibD*⁻), *min85*(*PspH_2818*_{NPS3121}⁻), *min93*(*PspH_4907*_{NPS3121}⁻), and *min47*(*trmE*⁻), also elicited normal HR. On the other hand, mutants such as *min12*(*rhpS*⁻), *min24*(*hisF*⁻), *min27*(*purK*⁻), *min32*(*ilvD*⁻), *min62*(*trpA*⁻), and *min71*(*trpD*⁻) that were significantly reduced in pathogenicity elicited a delayed HR or failed to induce a HR. However, *min14*(*leuA*⁻) and *min21*(*leuB*⁻) mutants, although significantly reduced in pathogenicity on the host bean plants, elicited normal HR, as did the WT strain on tobacco W38 plants.

PSPPH_3244_{NPS3121} is functionally similar to AefR in regulating the *ahlI* gene promoter and its own promoter.

PSPPH_3244_{NPS3121} is 87% identical to AefR that is known to regulate the synthesis of quorum-sensing signal AHL in *P. syringae* pv. *syringae* B728a (Quinones et al. 2004). AefR positively regulates the *ahlI* gene encoding AHL synthase and autoinhibits its own promoter (Quinones et al. 2004). To determine whether PSPPH_3244_{NPS3121} is functionally similar to AefR, we generated a deletion mutant of PSPPH_3244_{NPS3121} by marker exchange and examined how PSPPH_3244_{NPS3121}

regulates the *ahlI* gene promoter and its own promoter. As observed in *P. syringae* pv. *syringae* B728a, *ahlI-luc* exhibited approximately 20% expression in the Δ PSPPH_3244_{NPS3121} mutant relative to the WT strain (Fig. 3A), and overexpression of PSPPH_3244_{NPS3121} using the pNm promoter in pML122 plasmid (Labs et al. 1990) in the deletion mutant severely inhibited the PSPPH_3244_{NPS3121} promoter, as indicated by the

Table 2. Hypersensitive response (HR) assay of representative minimal medium-insensitive (*min*) mutants^a

Strains	Time (h)		
	12	14	18
WT	+	+	+
<i>min4 gltB</i>	+	+	+
<i>min8 hrpS</i>	-	-	-
<i>min12 rhpS</i>	-	+	+
<i>min14 leuA</i>	+	+	+
<i>min16 aefR</i>	+	+	+
<i>min18 fruK</i>	+	+	+
<i>min21 leuB</i>	+	+	+
<i>min23 eno-1</i>	-	+	+
<i>min24 hisF</i>	-	+	+
<i>min27 purK</i>	-	-	-
<i>min32 ilvD</i>	-	+	+
<i>min42 serA</i>	+	+	+
<i>min47 trmE</i>	+	+	+
<i>min62 trpA</i>	-	-	-
<i>min71 trpD</i>	-	-	-
<i>min83 cibD</i>	+	+	+
<i>min85 PSPPH_0569</i>	+	+	+
<i>min93 PSPPH_4907</i>	+	+	+
<i>min107 PPSPPH_5137</i>	-	+	+

^a Wild-type (WT) strain and *min* mutants at 10⁸ CFU/ml were injected into the tobacco W38 leaves. The induction of nonhost HR was documented at 12, 14, and 18 h after inoculation; + indicates positive HR and - indicates lack of HR.

Fig. 2. Growth of minimal medium-insensitive (*min*) mutants in bean plants. Wild-type (WT) *Pseudomonas syringae* pv. *phaseolicola* NPS3121 and *min* mutants that did not elicit visible disease symptoms were inoculated into the primary bean leaves at 2×10^4 CFU/ml. For each data point, three leaf disks (1 cm²) were removed at 0 and 6 days after inoculation and ground separately in sterile water for bacterial count. Error bars represent standard error. The experiment was repeated two times with similar results.

low LUC activity (Fig. 3B). These results suggested that PSPPH_3244_{NPS3121} is functionally similar to AefR of *P. syringae* pv. *syringae* B728a in regulating the AHL signal and, hereafter, is named AefR_{NPS3121}.

Regulation of the T3 genes by AefR_{NPS3121}.

We used the $\Delta aefR_{NPS3121}$ mutant to investigate whether AefR_{NPS3121} regulates the T3 genes. Consistent with the reduced *avrPto-luc* and *hrpL-luc* activities in insertion mutants, *hrpL* RNA was significantly reduced in the $\Delta aefR_{NPS3121}$ mutant, and this was largely complemented by expressing the WT *aefR_{NPS3121}* gene (Fig. 4A). The *hrpR* promoter activity (as indicated by the *hrpR-luc* reporter) in the $\Delta aefR_{NPS3121}$ mutant was 20 to 25% of that in the WT strain (Fig. 4B). Several regulatory genes acting upstream of *hrpRS*, including *lonB*, *rpoS*, *gacA*, and *psrA*, did not show an altered expression pattern in the $\Delta aefR_{NPS3121}$ mutant (Fig. 4A). The activities of the reporter genes were also monitored in the bean plants. Both *avrPto-luc* and *hrpL-luc* displayed lower activities in the $\Delta aefR_{NPS3121}$ mutant than in the WT strain 4 h after the bacteria were infiltrated into the plants (Fig. 4C and D). Consistently, the $\Delta aefR_{NPS3121}$ mutant infiltrated into the bean plants elicited weaker symptoms than did the WT strain (Supplementary Fig. S3). Both insertion and deletion mutants of *aefR_{NPS3121}* showed approximately threefold less bacterial growth than did the WT strain 4 days after infiltration inoculation (Fig. 4E); this difference was observed consistently in four repeat experiments and was statistically significant according to *F* test ($P < 0.05$). The *aefR_{NPS3121}*⁻ mutants and the WT strain showed similar activities in the induction of HR in tobacco W38 plants (Table 2). The expression of *aefR_{NPS3121}* RNA was investigated in mutants corresponding to the *hrpR*, *hrpS*, *rhpS*, *rhpRS*, and *lon* genes (Lan et al. 2007; Xiao et al. 2007) that are known to regulate the T3 genes. None of the mutations affected the *aefR_{NPS3121}* RNA in KB medium, but deletion of the *rhpRS* locus moderately elevated the *aefR_{NPS3121}* RNA in MM (Fig. 4F).

Regulation of swarming motility and epiphytic traits by AefR_{NPS3121}.

AefR regulates epiphytic behaviors in *P. syringae* pv. *syringae* B728a. Mutation of *aefR* enhances swarming motility on semi-

solid agar and invasion into leaves but reduces bacterial survival on the leaf surface (Quinones et al. 2004, 2005). These traits were examined in the $\Delta aefR_{NPS3121}$ mutant.

Unlike *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *tomato* DC3000 that displayed clear swarming motility on semisolid agar plate, *P. syringae* pv. *phaseolicola* NPS3121 strain did not show swarming motility (Fig. 5A). Mutation of *aefR_{NPS3121}* did not enhance swarming motility on a semisolid agar plate (Fig. 5A).

To determine whether AefR_{NPS3121} regulates bacterial invasion into leaves, 2-week-old bean plants were dip-inoculated with a bacterial suspension (10⁶ CFU/ml), each plant was covered with a plastic bag, and the numbers of bacteria inside the leaves and on the leaf surface were measured. Unlike the *aefR* mutant of *P. syringae* pv. *syringae* B728a that showed approximately 1,000-fold increase of the internal population 4 to 7 h after inoculation (Quinones et al. 2005), mutation of *aefR_{NPS3121}* did not alter the number of bacteria inside the leaves within 28 h after inoculation (Fig. 5B).

P. syringae pv. *syringae* B728a and the *aefR*⁻ mutant grew approximately 50-fold epiphytically after the surface-inoculated plants were incubated in moist conditions for 48 h (Quinones et al. 2004). After transfer into a dry environment, epiphytic population of both strains decreased approximately 20-fold in 2 h. The WT strain then stayed stable, but the population of the *aefR*⁻ mutant decreased another five- to sevenfold in 50 h (Quinones et al. 2004). Unlike *P. syringae* pv. *syringae* B728a and the *aefR*⁻ mutant, *P. syringae* pv. *phaseolicola* NPS3121 and the $\Delta aefR_{NPS3121}$ mutant did not exhibit significant epiphytic growth after incubation inside the plastic bags for 48 h (Fig. 5C). After the plants were placed in the greenhouse, both strains displayed approximately twofold reduction of the epiphytic population in the first 6 h, and then the epiphytic populations of *P. syringae* pv. *phaseolicola* NPS3121 and $\Delta aefR_{NPS3121}$ mutant increased approximately four- and sevenfold, respectively, in 48 h (Fig. 5C). These results indicated that AefR_{NPS3121} in *P. syringae* pv. *phaseolicola* NPS3121 and AefR in *P. syringae* pv. *syringae* B728a function differently under the dry environments.

In contrast with what was observed in infiltration inoculation (Fig. 4E), the internal population of the $\Delta aefR_{NPS3121}$

Fig. 3. Regulation of *ahll* and *aefR_{NPS3121}* promoters by AefR_{NPS3121}. The *ahll-luc* and *aefR_{NPS3121-luc}* reporter genes were introduced into wild-type (WT) *Pseudomonas syringae* pv. *phaseolicola* NPS3121, $\Delta aefR_{NPS3121}$ mutant, and $\Delta aefR_{NPS3121}$ mutant carrying the pML122::*aefR_{NPS3121}* plasmid (for *aefR_{NPS3121}* overexpression). The bacteria were grown in King's B (KB) medium and induced in minimal medium (MM) for 0, 3, and 6 h before measurement of luciferase (LUC) activities. **A**, Mutation of *aefR_{NPS3121}* reduces the *ahll* promoter activity in KB (indicated by 0 h) and MM. **B**, Overexpression of *aefR_{NPS3121}* inhibits its own promoter. Error bars represent standard error.

Fig. 4. Regulation of the type III secretion system and type III effector genes and pathogenicity by AefR_{NPS3121}. **A**, Mutation of *aefR*_{NPS3121} reduces the induction of *hrpL*. Wild-type (WT) *Pseudomonas syringae* pv. *phaseolicola* NPS3121, $\Delta aefR$ _{NPS3121} mutant, and $\Delta aefR$ _{NPS3121} mutant carrying the pML122::*aefR*_{NPS3121} plasmid were grown in King's B (KB) medium and induced in minimal medium (MM) for 4 h before being harvested for RNA extraction. Total RNA (10 μ g) from each sample was analyzed by Northern blotting using DNA probes derived from the coding regions of *hrpL*, *lonB*, *rpoS*, *gacA*, and *psrA*. Loading of RNA samples is indicated by rRNA. Lane 1, WT in KB; lane 2, WT in MM; lane 3, $\Delta aefR$ _{NPS3121} mutant in MM; lane 4, the complemented $\Delta aefR$ _{NPS3121} mutant in MM. **B**, Mutation of *aefR*_{NPS3121} reduces the *hrpR* promoter activity in MM. *hrpR-luc* reporter gene was introduced into the WT strain and $\Delta aefR$ _{NPS3121} mutant. Bacteria were grown in KB and induced in MM. Luciferase (LUC) activity was measured at 0, 3, and 6 h after induction. Error bars indicate standard error. **C** and **D**, Mutation of *aefR*_{NPS3121} reduces the *avrPto* and *hrpL* promoter activities in plant. *P. syringae* pv. *phaseolicola* NPS3121 and $\Delta aefR$ _{NPS3121} mutant carrying **C**, *avrPto-luc* and **D**, *hrpL-luc* were resuspended in water to an optical density at 600 nm of 0.5 and infiltrated into the primary leaves of bean plants. Leaves were removed and sprayed with 1 mM luciferin dissolved in 0.01% Tween-20, and the LUC activity was determined using a cooled charge-couple device. The experiments were repeated three times with similar results. **E**, Mutation of *aefR*_{NPS3121} reduces the bacterial growth inside the host plants. Bacteria at 2×10^4 CFU/ml were injected into the primary bean leaves. For each data point, three leaf disks (1 cm²) were removed at 0, 2, and 4 days after inoculation and ground separately in sterile water for bacterial count. Error bars represent standard error. The experiment was repeated four times with similar results. The differences between the WT and mutant strains at 2 and 4 days after inoculation are significant according to *F* test (*P* < 0.05). **F**, Expression of *aefR*_{NPS3121} RNA in *hrpR*⁻, *hrpS*⁻, *rhpS*⁻, *rhpRS*⁻, and *lon*⁻ mutants. WT *P. syringae* pv. *phaseolicola* NPS3121 and the mutant strains were grown in KB medium and induced in MM for 6 h before being harvested for RNA extraction. Total RNA (10 μ g) was analyzed by RNA blotting using probes derived from the coding region of *aefR*_{NPS3121}. Loading of RNA samples is indicated by rRNA.

mutant was approximately two- to fourfold higher than the WT strain 48 h after dip inoculation, and the fold difference remained till the end of experiment (Fig. 5C). The better growth of the $\Delta aefR_{NPS3121}$ mutant on the leaf surface and inside the bean plants was observed consistently in four repeat experiments, including one experiment with an inoculum of 10^8 CFU/ml. The inoculated leaves did not show water-soaked lesions but they senesced earlier than the uninoculated leaves. Leaves dip inoculated with the $\Delta aefR_{NPS3121}$ mutant usually senesced 1 to 2 days earlier than leaves dip inoculated with the WT strain.

DISCUSSION

We screened a total of approximately 28,000 transposon-tagged mutants of *P. syringae* pv. *phaseolicola* NPS3121 based on the compromised induction of *avrPto-luc* and *hrpL-luc* reporter genes in MM, and we isolated 46 mutant genes. The screening was approximately 5 $\times$ coverage of the approximately 6-Mb *P. syringae* pv. *phaseolicola* genome (Joardar et al. 2005), assuming an average bacterial gene length of 1 kb. Some of the mutants may be polar, because the mutant gene is organized in an operon with other genes. Most of the 46 mu-

tant genes encode metabolic enzymes, and only three genes encode regulatory functions. Characterization of the reporter gene activities and *hrpL* RNA expression in the mutant strains indicated that the reporter gene activities generally reflected the T3 gene expression, indicating that the reporting systems are valid.

Many genes that were reported to regulate the T3 genes in other *P. syringae* strains were not identified in our screen. One explanation is that some of these regulatory genes do not have a regulatory function in *P. syringae* pv. *phaseolicola* NPS3121. Another explanation is that the regulatory genes are small in size and missed by the transposon insertion. It is also likely that the mutant screening protocol has some intrinsic defects. We used the protocol for analysis of T3 gene induction in MM for mutant screening (e.g., an individual mutant colony was picked from a KB plate and grown to saturation in KB liquid medium in 96-well plates, the cells were then washed twice with MM, and the reporter genes were subsequently induced with MM). Because it was impossible to pick equal quantities of bacterial cells from each mutant colony, and the growth rates of the mutants differed in KB medium, prolonged growth in KB medium was allowed to achieve saturation prior to induction in MM. However, we later found that an extended stationary phase in KB medium

Fig. 5. Effect of *aefR*_{NPS3121} mutation on bacterial motility and epiphytic fitness. **A**, Swarming motility on semisolid agar. Bacteria were grown in King's B (KB) liquid overnight and resuspended in KB medium to an optical density at 600 nm of 1. Filter disks (6 mm in diameter) were soaked in bacterial suspensions and placed in the center of semisolid KB plates. Plates were photographed after incubation at 28°C for 24 h. **B**, Bacterial invasion of bean leaves. Two-week-old bean plants were dipped into bacterial suspension and covered with plastic bags immediately after inoculation. Surface bacteria were determined by washing the primary leaves with washing buffer in an ultrasonic bath, and the bacteria in washing solution were determined by plating on TSA plates (Xiao et al. 2007). Bacteria inside the leaves were determined by treating the washed leaves with 15% hydrogen peroxide, followed by a treatment with catalase. Leaves were then macerated in sterile potassium phosphate buffer for bacterial count. Population sizes of wild-type (WT) and *aefR*_{NPS3121} mutant are not significantly different according to *F* test. **C**, Epiphytic fitness assay. Two-week-old bean plants were dip inoculated and covered with plastic bags immediately after inoculation. The plants were removed from the plastic bags 48 h after inoculation and placed in the greenhouse. The numbers of bacteria on leaf surface and inside the leaves were determined as described in B. Epiphytic population of the WT and the *aefR*_{NPS3121} mutant are not significantly different at 0, 24, 48, and 54 h postinoculation (hpi) but are significantly different at 72 hpi according to *F* test ($P < 0.05$). The internal bacterial numbers are significantly different after 48 hpi. Error bars indicate standard error. Experiments of B and C were repeated four times with similar results.

reduced the induction of *avrPto-luc* and *hrpL-luc* reporter genes in MM (X. Deng and X. Tang, *unpublished results*). This practice, albeit enabling a uniform number of cells to be compared, narrowed the difference between strains showing WT levels of induction and mutants showing partial induction of the reporter genes. Washing to remove KB medium from the culture before induction of the reporter genes is another step that may affect identification of partial mutants. Contamination of MM with a small amount of KB medium significantly reduced the induction of the reporter genes in *P. syringae* pv. *phaseolicola* NPS3121. However, it was technically difficult to completely remove KB medium from all the microtiter wells, and the residual amount of KB medium could inhibit the induction of the reporter genes. We also noticed bacterial loss when supernatant was discarded from the plate after centrifugation. Because both residual KB medium and bacterial loss caused variations among strains in the plate, we set criteria to select mutants with a LUC activity $\leq 20\%$ of the majority of other strains in the same plate. It is apparent that these intrinsic problems of the screening protocol discriminated against the selection of mutants with partial induction of the reporter genes.

The mutant screening experiments conducted by us and by Hendrickson and associates (2000) indicated that as much as 4 to 5% of *P. syringae* mutants showed reduced induction of the T3 genes in MM. To identify the regulatory genes from the large pool of candidate mutants was a major obstacle. We deployed the two-stage semidegenerated PCR method (Jacobs et al. 2003) to determine the transposon insertion sites in 84 mutants that were identified using the *avrPto-luc* reporter. Most of these mutant genes encode metabolic enzymes. Although these genes provide little insight into the regulatory mechanisms, they do reveal the nature of *P. syringae* mutations that compromise the induction of the T3 genes in MM. These results led us to test whether bacterial growth assays in MM can differentiate metabolic mutants from regulatory mutants. We found that all metabolic mutants showed no or poor growth, whereas all regulatory mutants grew normally in MM. Therefore, we added this procedure to the mutant screening protocol. This procedure eliminated all metabolic mutants from the pool of candidate mutants that were identified using the *hrpL-luc* reporter and significantly enhanced the efficiency of isolation of regulatory genes. However, regulatory genes which are also required for bacterial growth in MM would be excluded by this procedure.

Pathogenicity assays indicated that the level of T3 gene expression in many metabolic mutants in MM was not correlated with their pathogenicity in host plants. In fact, a large number of metabolic mutants were almost as pathogenic as the WT strain, although they displayed no induction of the T3 genes in MM. The pathogenicity of most metabolic mutants was correlated with the abundance of the corresponding metabolites in the apoplastic fluid (Rico and Preston 2008; Ritte et al. 1999; Solomon and Oliver 2002; Tanaka and Tanaka 2007), indicating that nutrient auxotroph plays a critical role in the induction of T3 genes in plants and bacterial pathogenicity. The pathogenicity of most metabolic mutants was correlated with their HR-inducing activity, which is consistent with the fact that apoplastic fluids in different plant species are similar in nutrient compositions (Rico and Preston 2008; Solomon and Oliver 2002).

The only novel regulatory gene identified in our screen was *aefR*_{NPS3121}. AefR in *P. syringae* pv. *syringae* B728a regulates the AHL signal by upregulating the *ahlI* promoter, and it autoinhibits its own promoter (Quinones et al. 2004). Both functions were observed with AefR_{NPS3121}, suggesting that AefR_{NPS3121} is orthologous to AefR in *P. syringae* pv. *syringae* B728a. The role of AefR in regulating the T3 genes was not

reported, although the *aefR*⁻ mutant was found to cause smaller disease lesions than did *P. syringae* pv. *syringae* B728a when injected into bean pods (Quinones et al. 2005). We found that mutation of *aefR*_{NPS3121} reduced the induction of T3 genes and the bacterial pathogenicity when bacteria were injected into the bean plants. Because induction of the T3 genes is essential for bacterial pathogenesis, the reduced T3 gene induction in the *aefR*_{NPS3121}⁻ mutant is probably responsible for the reduced pathogenicity. *P. syringae* pv. *phaseolicola* NPS3121 and the *aefR*_{NPS3121}⁻ mutant did not show a clear difference in the HR. A possible explanation is that HR is sensitive, and a partial induction of the T3 genes is sufficient for the elicitation of a full HR. The finding that AefR_{NPS3121} regulates both the quorum-sensing signal and T3 genes is exciting and suggests that *P. syringae* pv. *phaseolicola* NPS3121 may regulate the T3 genes in response to cell density. Quorum-sensing signals are known to regulate the production of exoenzymes and exopolysaccharides, two major virulence factors, in numerous plant-pathogenic bacteria (Von Bodman et al. 2003) but its role in regulating the T3 genes has not been well documented in *P. syringae*. Our studies showed that AefR_{NPS3121} regulates the T3 genes, possibly by modulating the *hrpR* promoter activity. It remains to be determined whether AefR_{NPS3121} regulates the *hrpR* promoter directly by binding to the promoter or indirectly via the AHL-mediated signaling pathway.

Although AefR of *P. syringae* pv. *syringae* B728a and AefR_{NPS3121} act similarly in regulating AHL synthesis, the respective mutants exhibit significant difference in several epiphytic traits that are proposed to be regulated by AHL in *P. syringae* pv. *syringae* B728a (Quinones et al. 2004, 2005). Compared with *P. syringae* pv. *syringae* B728a, the *aefR*⁻ mutant is hypermotile on semisolid agar and invades leaves more rapidly (Quinones et al. 2005). The hypermotility on semisolid agar was assumed to be related to the more rapid invasion of leaves (Quinones et al. 2005). Unlike *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *tomato* DC3000, *P. syringae* pv. *phaseolicola* NPS3121 did not show visible swarming motility on semisolid agar, and mutation of *aefR*_{NPS3121} did not enhance the swarming motility. Nonetheless, both *P. syringae* pv. *phaseolicola* NPS3121 and the *aefR*_{NPS3121}⁻ mutant were capable of invading bean leaves following dip inoculation. However, the two strains displayed similar rates of invasion, as indicated by the similar numbers of internal bacteria 4 to 28 h after dip inoculation (Fig. 5B and C). This result indicated that AefR_{NPS3121} did not play a major role in regulating *P. syringae* pv. *phaseolicola* NPS3121 invasion into bean leaves.

P. syringae pv. *syringae* B728a and the *aefR*⁻ mutant grew approximately 50-fold on bean leaf surfaces within 2 days under the moist conditions, and a short exposure to a dry environment reduced the epiphytic population by 20- to 50-fold. Extended incubation in the dry conditions further reduced the epiphytic population, and mutation of *aefR* rendered the epiphytic bacteria more susceptible to the stress (Quinones et al. 2004). Different from *P. syringae* pv. *syringae* B728a and the *aefR*⁻ mutant, *P. syringae* pv. *phaseolicola* NPS3121 and the *aefR*_{NPS3121}⁻ mutant did not grow significantly on bean leaf surface under the moist conditions, and a short exposure to the dry conditions reduced the epiphytic population only by approximately twofold. Interestingly, both strains grew slowly on leaf surface after the small reduction of the epiphytic population in the dry environment (Fig. C). These results indicated that AefR_{NPS3121}, like AefR, does not affect bacterial growth under moist environment conditions. However, under the dry conditions, AefR positively regulates the epiphytic survival of *P. syringae* pv. *syringae* B728a, whereas AefR_{NPS3121} negatively regulates the epiphytic growth of *P. syringae* pv. *phaseolicola* NPS3121. The remarkable difference of AefR and

AefR_{NPS3121} in modulating the epiphytic behaviors of their respective bacteria suggested that the epiphytic associations of *P. syringae* pv. *phaseolicola* NPS3121 and *P. syringae* pv. *syringae* B728a with their host plants involve different mechanisms.

Although the *aefR*_{NPS3121}⁻ mutant is less pathogenic than the WT strain after infiltration into bean plants, it is more pathogenic than the WT strain following dip inoculation, as indicated by the larger *aefR*_{NPS3121}⁻ mutant population inside the leaves at the end of experiment (Fig. 5C) and the earlier senescence of the inoculated leaves. Similar results were also reported for the *aefR*⁻ mutant that elicited more disease lesions on bean leaves if the spay-inoculated plants were incubated in moist conditions for more than 48 h before being placed in a dry environment (Quinones et al. 2005). It was proposed that more *aefR*⁻ mutant cells entered the leaves during the incubation, which caused more severe symptoms (Quinones et al. 2005). Indeed, we detected a larger internal population of the *aefR*_{NPS3121}⁻ mutant 48 h after incubation in the moist conditions (Fig. 5C). Two possible routes could lead to the larger internal population. i) Although the *aefR*_{NPS3121}⁻ mutant was not found to enter the leaves more rapidly during the first 24 h of incubation, the mutant might enter the leaves more rapidly during the later hours under the moist conditions. ii) Although infiltrated *P. syringae* pv. *phaseolicola* NPS3121 grew better than did the *aefR*_{NPS3121}⁻ mutant in the greenhouse, the *aefR*_{NPS3121}⁻ mutant may grow better than the WT strain under the wet conditions. In addition to the larger internal population gained in the wet conditions, we noticed that the *aefR*_{NPS3121}⁻ mutant produced a larger epiphytic population in the greenhouse (Fig. 5C), which may allow more bacterial cells to enter the leaves during the prolonged interaction with the plants. Other than these scenarios, it should be pointed out that infiltration-inoculation and surface-inoculation are different biological processes. We cannot rule out the possibility that AefR_{NPS3121} has a positive role in regulating bacterial pathogenicity following infiltration inoculation but a negative role following surface inoculation.

MATERIALS AND METHODS

Plant materials and culture media.

Bean (*Phaseolus vulgaris* cv. Red Kidney) plants and tobacco W38 plants were used for pathogenicity and HR assays, respec-

tively. Plant materials were grown in a greenhouse as described previously (Xiao et al. 2007). *Escherichia coli* strains were cultured in Luria-Bertani medium at 37°C. *Pseudomonas syringae* bacteria were cultured in KB medium (King et al. 1954) at room temperature. Induction of T3SS was performed at room temperature in MM (50 mM KH₂PO₄, 7.6 mM (NH₄)₂SO₄, 1.7 mM MgCl₂, 1.7 mM NaCl, and 10 mM fructose, pH 5.7) (Huynh et al. 1989). Bacteria were plated on TSA plates (Xiao et al. 2007) for counting of CFU. Antibiotics for selection of *P. syringae* strains were rifampicin, 25 mg/liter; kanamycin, 10 mg/liter; spectinomycin, 50 mg/liter; tetracycline, 10 mg/liter; and gentamycin, 10 mg/liter. Antibiotics for selection of *E. coli* were ampicillin, mg/liter 100; kanamycin, 50 mg/liter; spectinomycin, 100 mg/liter; and gentamycin, 20 mg/liter.

Plasmids.

Plasmids and primers are listed in Tables 3 and 4, respectively. To construct the reporter gene for the *aefR*_{NPS3121} promoter, the *luc* gene was released from pHM2::avrPto-*luc* by *Bam*HI and *Xba*I digestion and ligated into pBluescript-SK(+), resulting in pBluescript-SK-*luc*. The promoter of *aefR*_{NPS3121} was PCR amplified using pspph3244-P-F (carrying *Eco*RI) and pspph3244-P-R (carrying *Bam*HI) as primers and *P. syringae* pv. *phaseolicola* NPS3121 genomic DNA as template. The PCR product was digested with *Eco*RI and *Bam*HI and cloned into pBluescript-SK-*luc*, resulting in pBS-*aefR*_{NPS3121}-*luc*. After sequencing, the *Eco*RI and *Xba*I fragment in pBS-*aefR*_{NPS3121}-*luc* was released and then cloned into pHM2, resulting in pHM2::*aefR*_{NPS3121}-*luc* for the *aefR*_{NPS3121} promoter assay. The same strategy was used to generate the reporter gene for the *ahII* (PSPPH_1614) promoter. The *ahII* promoter was PCR amplified using pspph1614-P-F and pspph1614-P-R as primers.

To construct the *hrpR*-*luc* reporter gene, a 1-kb fragment of the *hrpR* promoter was amplified using primers pspH-hrpR-PF and pspH-hrpR-PR. The PCR product was digested with *Eco*RI and *Bam*HI, cloned into pBluescript-SK(+) plasmid, and sequence confirmed. The promoter DNA was then released from the plasmid with *Eco*RI/*Bam*HI digestion and cloned upstream of the *luc* gene in the pPTE6::*luc* plasmid (Xiao et al. 2004) to generate pPTE6::*hrpR*-*luc*. To generate pLT::*hrpR*-*luc*, the *kanR* gene in pPTE6::*hrpR*-*luc* plasmid was knocked out by EZ-Tn5<TET-1> transposon insertion.

Table 3. Plasmids

Plasmids	Description	References
pBluescript-SK(+)	Cloning and sequencing	Stratagene, La Jolla, CA, U.S.A.
pGEM-7Z	Cloning and sequencing	Promega Corp., Madison, WI, U.S.A.
pGEM-T	Cloning and sequencing	Promega Corp., Madison, WI, U.S.A.
pML122	Broad-host plasmid	Labes et al. 1990
pHM2	Broad-host plasmid	Xiao et al. 2007
pHM2::avrPto- <i>luc</i>	avrPto- <i>luc</i> reporter in pHM2	Xiao et al. 2007
pHM2::hrpL- <i>luc</i>	hrpL- <i>luc</i> reporter in pHM2	Xiao et al. 2007
pPTE6:: <i>luc</i>	Firefly luciferase in pPTE6	Xiao et al. 2004
pBS-hrpR	Intermediate construct for pLT-hrpR- <i>luc</i>	This study
pPTE6::hrpR- <i>luc</i>	hrpR- <i>luc</i> in pPTE6	This study
pLT::hrpR- <i>luc</i>	Derived from pPTE6::hrpR- <i>luc</i> by EZ-Tn5<TET-1> insertion	This study
pGEM-T-PSPPH_3244	Intermediate construct for pML122::PSPPH_3244	This study
pML122::PSPPH_3244	PSPPH_3244 in pML122 plasmid, under pNm promoter	This study
pBluescript-SK- <i>luc</i>	Firefly luciferase in pBluescript-SK(+)	This study
pBS-PSPPH_3244- <i>luc</i>	Intermediate construct for pHM2-PSPPH_3244- <i>luc</i>	This study
pBS-PSPPH_1614- <i>luc</i>	Intermediate construct for pHM2-PSPPH_1614- <i>luc</i>	This study
pHM2::PSPPH_3244- <i>luc</i>	PSPPH_3244- <i>luc</i> reporter in pHM2	This study
pHM2::PSPPH_1614- <i>luc</i>	PSPPH_1614- <i>luc</i> reporter in pHM2	This study
p7Z::PHPPH_3244FR	Intermediate construct for marker exchange	This study
p7Z::FKanR	Intermediate construct for marker exchange	This study
pHM1::PSPPH_3244-FKanR	For marker exchange	This study

To construct pML122::*aeFR*_{NSP3121}, the coding region of *aeFR*_{NSP3121} was PCR amplified using primers pspph3244-O-F (carrying *Xho*I) and pspph3244-O-R (carrying *Cl*aI). The PCR fragment was cloned into pGEM-T and confirmed by sequencing. The fragment was then released by *Xho*I and *Cl*aI digestion and cloned into pML122, resulting in pML122::*aeFR*_{NSP3121} for *aeFR*_{NSP3121}⁻ mutant complementation and *aeFR*_{NSP3121} overexpression.

Mutant screen.

The transposon insertion mutant library was constructed in *P. syringae* pv. *phaseolicola* NPS3121 strain carrying the pHM2::*avrPto-luc* and pHM2::*hrpL-luc* reporter plasmids as described previously (Xiao et al. 2007). Mutant colonies grown on KB plates containing rifampicin, kanamycin, and spectinomycin were picked with sterile toothpicks into 100 µl of liquid KB medium containing the same antibiotics in 96-well plates and cultured for 36 h until complete saturation. The 96-well plates were centrifuged, and the bacteria were washed twice with MM and resuspended in 500 µl of MM. After induction in MM for 6 h, 100 µl of cell suspension was transferred from each sample to a new 96-well plate and mixed with 10 µl of 0.1 mM luciferin. LUC activity was measured using a cooled charge-couple device (CCD, Roper Scientific, Trenton, NJ, U.S.A.). Mutants with approximately 20% LUC activity relative to the other clones in the same plate were selected as putative mutants. These mutants were confirmed for the induction of reporter genes in MM as described previously (Xiao et al. 2007).

Table 4. Primers

Primer names	Sequences
pspsh3244-O-F	TTCTCGAGTGGCAACAAGTAACTGCTGA
pspsh3244-O-R	TTATCGATGGGGCGTAGTACTCGACGTA
pspsh3244-P-F	TTGAATTCGGTTTCCATCAGTGTCAG
pspsh3244-P-R	TTGGATCCCATAGGTTTCGCTTCAAACGGA
pspsh1614-P-F	TTGAATTCGCAAGTATCTGGCCACTT
pspsh1614-P-R	TTGGATCCCATATAAACTCCACTC
p3244LF	TTTCTAGAACGCAGGTATAGGACGCAGT
p3244LR	TTGGATCCGATGGATTTCGGTTTCTGAT
p3244RF	TTGGATCCGAACATGTTTCTGGGCTGGT
p3244RR	TTGAGCTCGCCAATCCACGTGATTTTCT
Kan-BF	TTGGATCCCATCGATGAATTGTGTCT
Kan-BR	TTGGATCCGGTGGACCAAGTTGGTGAT
Pspsh-hrpR-PF	GAATTCGTTTAAAGCCGGATGTATAG
Pspsh-hrpR-PR	TTGGATCCGTCATATCCAGAAACGC
psph-hrpL-NF	GACTCTTCGTCTGCCGGTAT
psph-hrpL-NR	GGGTCAATCTGCTGCTTCAA
psph-gacA-NF	CATAGACGGTCTGCAGGTTG
psph-gacA-NR	GTGACGTACAGCGAGCAAAG
psph-rpoS-NF	AAGGAAGCGTCAAACGAGAA
psph-rpoS-NR	AGCCCGTTCTTTTCAAGGAT
psph-rpoN-NF	CGCCTTACTCCAGCTTTCCAC
psph-rpoN-NR	GTCGCCGTACTCAAGAAAGC
psph-psrA-NF	CGTTGAACGCATTCTTGATG
psph-psrA-NR	GATCATGGTCGGGTCACTG
psph-ahII-NF	GAGCGGGTTTAAAGGTTTCAGT
psph-ahII-NR	AGCAGGTATCCGTGACAG
psph-lonB-NF	GATTCGTGGCCCTGTACTGT
psph-lonB-NR	TGGATATGCGTGTGCTGTTT
Kan2-SP1	GATAGATTGTGCGACCTGATTG
Kan2-SP2	AAGACGTTTCCCGTTGAATATG
Kan2-SP3	GCAATGTAACATCAGAGATTTTGAG
CEKG 2A	GGCCACGCGTCGACTAGTACNNNNNNNNNA GAG
CEKG 2B	GGCCACGCGTCGACTAGTACNNNNNNNNNA CGCC
CEKG 2C	GGCCACGCGTCGACTAGTACNNNNNNNNNG ATAT
CEKG 4	GGCCACGCGTCGACTAGTAC
HrpL-FLAG-F	TTCTCGAGGTGCTGTGGTCAGCCCGTG
HrpL-FLAG-R	CCTTCGAAGCGCAACGGGTCAATCTG

Measurement of reporter gene activities in MM and in the plant.

Bacteria were grown in liquid KB medium containing rifampicin and spectinomycin to an optical density at 600 nm (OD₆₀₀) of 2.0 to 2.5. To induce the reporter genes in MM, bacteria were washed twice with MM, resuspended in MM to OD₆₀₀ = 0.1, and incubated for 4 h for the induction of *hrpL-luc* and 6 h for the induction of *avrPto-luc*. Cell suspension (100 µl) was mixed with 10 µl of 0.1 mM luciferin, and the LUC activity was measured using a cooled CCD (Roper Scientific). After LUC measurement, the bacteria were diluted and plated on TSA plates for counting of CFU. The relative LUC activity was normalized to the numbers of bacteria in MM. To induce the reporter genes in the plant, bacteria were washed twice with sterile water, resuspended in sterile water to OD₆₀₀ = 0.5, and infiltrated into the primary leaves of 2-week-old bean plants. The inoculated leaves were excised 4 and 6 h after inoculation for measurement of *hrpL-luc* and *avrPto-luc* reporter activities, respectively. The excised leaves were sprayed with 1 mM luciferin dissolved in 0.01% Tween-20, and the LUC activity was determined using a cooled CCD (Roper Scientific). After LUC measurement, the number of bacteria inside the inoculated leaves was measured as described previously (Xiao et al. 2007) and the relative LUC activity was normalized to the bacterial number inside the leaves. Each data point represents an average of three to four replicates. Each experiment was repeated three to five times with similar results.

Mapping of transposon insertion sites.

The transposon insertion sites were determined by a two-stage semidegenerated PCR according to Jacobs and associates (2003) using two transposon-specific primers (Kan2-SP1 and Kan2-SP2) and four degenerated primers (CEKG 2A, CEGK 2B, CEGK 2C, and CEGK). The PCR product was sequenced using the third transposon-specific primer Kan2-SP3. Sequences flanking the transposon DNA were searched against the *P. syringae* pv. *phaseolicola* 1448A genome sequence using Blastn. The mutant genes of *P. syringae* pv. *phaseolicola* NSP3121 were distinguished from the *P. syringae* pv. *phaseolicola* 1448A genes by adding a subscript “_{NSP3121}”.

Assay of bacterial growth in MM.

Mutant colonies were grown in KB medium to OD₆₀₀ = 2.5. Bacterial cells were collected by centrifugation, washed twice with MM, and resuspended in MM. The bacteria were diluted in MM to OD₆₀₀ = 0.1 and grown at room temperature for 36 h with constant shaking at 250 rpm. The bacterial density was determined using a spectrophotometer.

Infiltration inoculation and HR assay.

Preparation of bacterial inoculum for plant inoculation was as described previously (Shan et al. 2000). Bacteria at 2 × 10⁴ CFU/ml were hand injected into the primary leaves of 2-week-old bean plants for pathogenicity assays. Disease symptoms on bean leaves were documented 5 days after inoculation. For bacterial growth assays, leaf disks (1 cm²) were removed at 0, 4, or 6 days after inoculation and ground in sterile water. Bacteria were diluted to proper concentration and plated on a TSA plate containing rifampicin at 30 mg/liter (Xiao et al. 2007) for bacterial count. For HR assay, bacteria at 10⁸ CFU/ml were injected into the fully expanded tobacco W38 leaves. At 8 h after injection, death of the inoculated area was visually examined hourly.

Motility assay.

Swarming motility was assessed on semisolid KB plates containing 0.4% agar, as described by Quinones and associates

(2005). Cells were grown in KB liquid overnight and resuspended in KB to $OD_{600} = 1$. Filter disks (6 mm in diameter) were soaked in bacterial suspensions and placed in the center of the plate. Plates were then incubated at 28°C for 24 h before photography.

The procedures described by Quinones and associates (2005) were used to determine the ability of bacteria to enter the interior of leaves. Two-week-old bean plants were dipped into a bacterial suspension of 1×10^6 CFU/ml in 10 mM potassium phosphate buffer (PB), pH 7.0, plus Silwet at 50 μ l/liter for 1 min. The plants were covered with plastic bags immediately after dip inoculation to maintain humidity. Primary leaves were excised at various times and surface sterilized with 15% hydrogen peroxide for 10 min, followed by a treatment with catalase at 7.5 μ g/ml for 10 min. Leaves were then macerated in sterile PB and the released bacteria were plated on a TSA plate containing rifampicin at 30 mg/liter for counting of CFU. The number of bacteria was normalized according to the surface area of leaves. Each experiment was repeated at least three times with similar results.

Epiphytic fitness assay.

Two-week-old bean plants were dipped into a bacterial suspension (1×10^6 CFU/ml in PB plus Silwet at 50 μ l/liter) for 1 min. Plants were covered with plastic bags immediately after dip inoculation, removed from the plastic bag 48 h after inoculation, and placed in the greenhouse. Primary leaves were excised at various times after inoculation and immersed individually into 50 ml of washing buffer (100 mM potassium phosphate buffer containing 0.1% Bacto-peptone) in a plastic tube. The plastic tubes were sonicated in an ultrasonic bath for 7 min and vortexed briefly to wash off the surface bacteria. Bacteria in washing solution were plated on a TSA plate containing rifampicin at 30 mg/liter for bacterial count. To measure the bacteria inside the leaves, the leaves were further treated with 15% hydrogen peroxide for 10 min, followed by a treatment with catalase at 7.5 μ g/ml for 10 min. Leaves were then macerated in sterile PB, and the released bacteria were plated on a TSA plate containing rifampicin at 30 mg/liter for bacterial count. The numbers of bacteria were normalized according to the surface area of leaves. Each experiment was repeated at least three times with similar results.

RNA extraction and Northern blotting.

Bacterial RNA was extracted using a modified hot phenol method (Lan et al. 2006). DNase I (Promega Corp., Madison, WI, U.S.A.) treatment was used to remove the contaminating DNA in RNA samples. Total RNA (10 μ g) was used for Northern blotting. The *hrpL*, *rpoS*, *psrA*, *lonB*, *gacA*, and *aefR_{NPS3121}* coding regions were PCR amplified using primers listed in Table 4 and radiolabeled with 32 P dCTP using the Random Primed DNA Labeling kit (Ambion, Austin, TX, U.S.A.) as probes. Procedures described by Tang and associates (1999) were followed for hybridization and washing.

Construction of $\Delta aefR_{NPS3121}$ mutant.

A 1.8-kb DNA fragment upstream of *aefR_{NPS3121}* was PCR amplified using primers P3244LF and P3244LR (Table 4, *Xba*I and *Bam*HI sites are underlined). A 1.8-kb DNA fragment downstream of *aefR_{NPS3121}* was PCR amplified using primers P3244RF and P3244RR (Table 4, *Bam*HI and *Sac*I sites are underlined). The PCR products were digested with *Xba*I/*Bam*HI and *Bam*HI/*Sac*I, respectively, and cloned into the *Xba*I and *Sac*I sites of pGEM-7Z, resulting in p7Z-3244FR. A DNA fragment containing the kanamycin resistance gene was PCR amplified from EZ::Tn<KAN-2> (Epicentre Technologies, Madison, WI, U.S.A.), using primers

Kan-BF and Kan-BR (Table 4, *Bam*HI sites underlined), digested with *Bam*HI, and cloned into the *Bam*HI site of p7Z-3244FR, resulting in p7Z-3244FkanR. The *Xba*I and *Sac*I fragment in p7Z-3244FkanR was cloned into pHM1, and the resulting pHM1::3244FkanR plasmid was introduced into *P. syringae* pv. *phaseolicola* NPS3121 for marker exchange. Colonies sensitive to spectinomycin but resistant to kanamycin were further verified by PCR and Southern blotting using DNA probes derived from the *aefR_{NPS3121}* coding region.

Western blot analysis.

Bacteria grown in KB and MM media were adjusted with corresponding medium to $OD_{600} = 1$. Bacteria (30 μ l) was boiled in 1 $\times$ sodium dodecyl sulfate (SDS) sample buffer and loaded to an SDS polyacrylamide gel electrophoresis gel. Western blot was performed as described (Shan et al. 2000) with the anti-LUC antibodies (NeoMarkers, Fremont, CA, U.S.A.).

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