Genetic Characterization of *Pseudomonas fluorescens* SBW25 *rsp* Gene Expression in the Phytosphere and In Vitro

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The plant-colonizing *Pseudomonas fluorescens* strain SBW25 harbors a gene cluster (*rsp*) whose products show similarity to type III protein secretion systems found in plant and animal pathogens. Here we report a detailed analysis of the expression and regulation of the *P. fluorescens* *rsp* pathway, both in the phytosphere and in vitro. A combination of chromosomally integrated transcriptional reporter fusions, overexpressed regulatory genes, and specific mutants reveal that promoters controlling expression of *rsp* are actively transcribed in the plant rhizosphere but not (with the exception of the *rspC* promoter) in the phyllosphere. In synthetic medium, regulatory (*rspL* and *rspR*) and structural (*rspU*, plus the putative effector *ropE*) genes are poorly expressed; the *rspC* promoter is subject to an additional level of regulatory control. Ectopic expression of regulatory genes in wild-type and mutant backgrounds showed that RspR controls transcription of the alternate sigma factor, *rspL*, and that RspL controls expression of gene clusters encoding structural genes. Mutation of *rspV* did not affect RspR-mediated expression of *rspU*. A search for additional regulators revealed two candidates—one with a role in the conversion of alanine to pyruvate—suggesting that expression of *rsp* is partly dependent upon the metabolic status of the cell. Mutations in *rsp* regulators resulted in a significant reduction in competitive colonization of the root tips of sugar beet seedlings but also caused a marked increase in the lag phase of laboratory-grown cultures, indicating that *rsp* regulatory genes play a more significant general role in the function of *P. fluorescens* SBW25 than previously appreciated.

The plant rhizosphere is a complex environment comprising organic matter and a plethora of microbes, some of which can affect plant health. Pathogens, such as *Pythium ultimum*, can hinder plant growth, whereas strains of *Pseudomonas fluorescens*, including *P. fluorescens* SBW25, can promote plant growth. The mechanism of plant growth promotion involves a combination of competition, antagonism of pathogens via the production of antimicrobial compounds, and induction of systemic resistance (15, 16, 31, 34).

To understand the interaction between *P. fluorescens* SBW25 and plants, SBW25 genes specifically activated in the plant environment were identified via a promoter trapping strategy (11, 36). Approximately 100 rhizosphere-induced (*rhi*) genes have been identified and categorized into six groups: nutrient acquisition, stress response, attachment and surface colonization, antibiotic production, secretion, and unknown. One *rhi* gene (*rhi-18*) is *hrcC* (redesignated *rscC*), whose product is a component of a type III protein secretion system (TTSS) termed Rsp (33, 36) (Fig. 1).

The *P. fluorescens* SBW25 *rsp* cluster exhibits a high level of synteny to the group I TTSS clusters of *Pseudomonas syringae* pv. tomato DC3000 and *Erwinia amylovora* and is genetically most similar to the *P. syringae* cluster. The cluster is organized into three putative transcriptional units (the *rspU*, *rspC*, and *rspJ* gene clusters) and contains at least two genes encoding regulatory proteins: a putative enhancer binding protein (EBP) gene, *rspR*, and a putative alternate sigma factor gene, *rspL*.

However, the SBW25 cluster lacks the *P. syringae* enhancer binding protein gene *hrpS*, the harpin gene *hrpZ*, and four genes of the *hrpJ* gene cluster (*hrpJ*, *hrcV*, *hrpQ*, and *hrcN*). Despite this, Preston et al. (33) demonstrated that at high inoculum levels, SBW25 expressing RspL and AvrB (from *P. syringae* pv. *glycinea*) could elicit a Rsp-dependent gene-for-gene resistance reaction in *Arabidopsis thaliana* Col-O.

Adjacent to the *rsp* cluster is *ropE*, a homologue of *avrE* (23, 28) and *dspA* (also called *dspE*) (3, 12). This region is similar to the minimal exchangeable effector loci and conserved effector loci of some *P. syringae* TTSS pathogenicity islands (PAIs) (1, 5, 9), although the gene arrangement is inverted, since a tRNA gene resides between the *rsp* cluster and *ropE*; in *P. syringae* the tRNA gene lies beyond the effector locus. Unlike its counterparts in pathogens, there is no chaperone gene, *avrF* or *dspB/F*, next to *ropE*, and *ropE* is located on the opposite side of the *rsp* cluster from *avrE*, i.e., *ropE* is located next to *rspL*, whereas *avrE* is adjacent to *hrpRS*.

In *P. syringae*, the “downstream” section of the regulatory cascade leading to TTSS gene expression is known (13, 14, 18, 19, 27, 35, 41). In *P. syringae*, a pair of enhancer binding proteins, HrpR and HrpS, control expression of the *hrp/hrc* cluster. Expression of the *hrpRS* operon is constitutive in *P. syringae* pv. *syringae* 61 (41) but inducible in *P. syringae* pv. *phaseolicola* NPS3121 (35). In *P. syringae* pv. *syringae* and *P. syringae* pv. *tomato*, HrpR and HrpS interact to form a heterodimer that activates the alternative sigma factor gene, *hrpL* (19), in an RpoN-dependent manner (17). HrpL controls a regulon consisting of *hrp/hrc* structural genes and effector genes by recognition of a specific promoter sequence upstream of the regulated genes (10, 21, 42, 43). The *hrpV* gene, within the *hrcC* operon, encodes a negative regulator of *hrp* gene

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FIG. 1. The *P. fluorescens* SBW25 *rsp* gene cluster. In silico analysis of the *P. fluorescens* SBW25 *rsp* gene cluster (A) identified six potential transcriptional units: *rspR*, *rspL*, *ropE*, *rspU* gene cluster, *rspC* gene cluster, and *rspJ* gene cluster. (B) *ropE* promoter region, highlighting three *rspL* binding site (promoter)-like sequences (bold), the probable *rspL* promoter sequence (bold, underlined), start codon (bold italics), and potential Shine-Dalgarno sequence (double underlined). (C) Putative RspL promoters, which show homology to *P. syringae* *hrp* promoters, were identified upstream of the three structural gene clusters and *ropE*. Divergent nucleotides are indicated in bold; the *ropE* promoter has a shorter 15N spacer region between the -10 and -35 sites. Two potential promoters were identified upstream of the *rspU* gene cluster, one with an imperfect promoter and 18-nucleotide spacer region (GGGAACCCCTTTTGCACTGGCTGTGCCAAAGA), which overlaps the RpoN promoter of *rspL* (conserved nucleotides are shown in bold and reverse complement RpoN sites underlined).

expression that is predicted to act above the level of *hrpL* expression, possibly binding to and affecting HrpS activity (20, 32). Recently, Lon protease has been shown to negatively regulate *hrp* gene expression (4) by affecting the level of HrpR in bacterial cells and thus imposing stringent negative regulation. It was found that *hrp*-inducing medium diminished Lon-dependent repression of HrpR, allowing HrpR levels to increase along with expression of the TTSS structural genes. In *Pseudomonas aeruginosa*, new research has shown that metabolic imbalance appears to be an underlying cause of TTSS gene expression (38).

The TTSS of *P. syringae* has an important role both in pathogenesis and in the induction of plant defense responses. In contrast, *P. fluorescens* SBW25 does not naturally elicit a *rsp*-dependent resistance reaction in plants, as observed with pathogens on resistant hosts or nonhosts, and no change in this phenotype is observed when *P. fluorescens* SBW25 *rsp* mutants

are inoculated into plant leaves (33). Moreover, in *P. fluorescens* SBW25, mutations in *rsp* structural genes and *ropE* do not affect ability to colonize or grow in the rhizosphere of sugar beet plants, at least over a 2-week period (33).

The fact that the *rsp* cluster of *P. fluorescens* SBW25 does not have a clear phenotype in standard assays for plant-bacterium interactions suggests several possibilities: that it does not normally use the secretion system; that it does not use *rsp* for plant interactions; that it does not secrete proteins that are recognized by plants; that *rsp* has a subtle or conditional phenotype; or that the system has an as yet unidentified role in the interaction with plants or even with other soil organisms. Central to defining the role of these genes is an understanding of the expression of the gene cluster in the plant environment, the regulatory processes controlling expression, and the importance of the regulators. Here we report an analysis of the expression and regulation of the *P. fluorescens* *rsp* pathway,

TABLE 1. Principal bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
<i>Pseudomonas fluorescens</i>		
SBW25	Wild-type strain isolated from phyllosphere of sugar beet plant	37
SBW25 Δ <i>dapB</i>	Unmarked deletion of <i>dapB</i> ; DAP and lysine auxotroph	11
SBW25 <i>rspL</i> ::Tn#10	Marker exchange mutant of <i>rspL</i> gene with GPS1 transposon insertion; Km ^r	This work
SBW25 <i>rspR</i> ::Tn#42	Marker exchange mutant of <i>rspR</i> gene with GPS1 transposon insertion; Km ^r	This work
SBW25 <i>rspV</i> ::Tn#112	Marker exchange mutant of <i>rspV</i> gene with GPS1 transposon insertion; Km ^r	This work
SBW25 <i>ropE</i> ::Tn#2	Marker exchange mutant of <i>ropE</i> gene with GPS1 transposon insertion; Km ^r	This work
SBW25 <i>rscR</i> ::Tn#19	Marker exchange mutant of <i>rscR</i> gene with GPS1 transposon insertion; Km ^r	This work
SBW25 Δ <i>dapB</i> <i>rspL</i> ::Tn#10	Marker exchange mutant of <i>rspL</i> gene with GPS1 transposon insertion; Km ^r ; Δ <i>dapB</i> and lysine auxotroph	This work
PBR391	Constitutively expressed promoter-' <i>dapB</i> fusion	44
PBR393	Null expression promoter-' <i>dapB</i> fusion	44
SG116	SBW25 containing Km ^r gene in noncoding region of chromosome	S. Gehrig
<i>Pseudomonas syringae</i> pv. <i>tomato</i>		
DC3000	Tomato and <i>Arabidopsis thaliana</i> pathogen	6
<i>Escherichia coli</i>		
DH5 α	F ⁻ <i>recA</i> , Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15), <i>endA</i> <i>hsdR</i> <i>gvrA</i>	Gibco-BRL
S17-1 λ pir	Tp ^r Sm ^r <i>recA</i> <i>thi</i> <i>hsdR</i> ⁻ M ⁺ RP4::2-Tc::Mu::Km::Tn7, λ pir lysogen	40
Plasmids		
pUIC3	Tc ^r Tra ⁻ Mob ⁺ , R6K replicon	36
pIVETD	Tc ^r Tra ⁻ Mob ⁺ , promoterless <i>dapB</i> <i>lacZY</i>	11
pGFPGUS	Tc ^r Tra ⁻ Mob ⁺ , promoterless <i>gfp</i> <i>uidA</i>	N. Bertrand
pML122/3	Gm ^r Tra ⁻ Mob ⁺ IncQ, <i>npII</i> promoter	24
pRspL	Coding region of SBW25 <i>rspL</i> in pML122, Gm ^r	33
pRspR	Coding region of SBW25 <i>rspR</i> in pML122, Gm ^r	33
pGMP2	pIJ3200-based cosmid carrying SBW25 <i>rsp</i> cluster, Tc ^r	33
pGMP2::Tn#2	GPS1 transposon insertion in <i>ropE</i> ; Tc ^r Km ^r	33
pGMP2::Tn#10	GPS1 transposon insertion in <i>rspL</i> ; Tc ^r Km ^r	This work
pGMP2::Tn#19	GPS1 transposon insertion in <i>rscR</i> ; Tc ^r Km ^r	33
pGMP2::Tn#42	GPS1 transposon insertion in <i>rspR</i> ; Tc ^r Km ^r	This work
pGMP2::Tn#112	GPS1 transposon insertion in <i>rspV</i> ; Tc ^r Km ^r	This work
pCM639	IS <i>phoA</i> /hah plasmid, Mob ⁺ /Km ^r	29

both in the rhizosphere and in vitro. We use a combination of reporter fusions, mutants, and strains carrying overexpressed regulatory genes to test the hypothesis that the *P. fluorescens* SBW25 *rsp* gene cluster is expressed in the plant environment, that the *P. fluorescens* SBW25 *rsp* gene cluster is regulated in a manner analogous to that found in the TTSS of *P. syringae*, and that the gene cluster is functional. Our data show that the cluster is expressed specifically in the plant root environment, that regulation differs from that found in *P. syringae*, and that *rsp* mutants are impaired in growth in both laboratory and plant environments.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids are listed in Table 1. *Escherichia coli* strains were grown at 37°C overnight on Luria-Bertani (LB); (Difco) (39) agar or broth. *P. fluorescens* SBW25 and *P. syringae* pv. *tomato* strain DC3000 were grown at 28°C on LB agar or King's medium B (KB) (22) agar or in LB broth. M9 and *hrp*-inducing media (HIM) were used for testing gene expression (30, 39). *P. fluorescens* SBW25 Δ *dapB* was grown on medium supplemented with diaminopimelate (DAP) and lysine to final concentrations of 800 μ g ml⁻¹ and 80 μ g ml⁻¹, respectively (11). Antibiotics and supplements were used to the following final concentrations: ampicillin, 50 μ g ml⁻¹; gentamicin, 10 μ g ml⁻¹; kanamycin, 25 μ g ml⁻¹; tetracycline, 10 μ g ml⁻¹; 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (XGlcA), and isopropyl β -D-1-thiogalactopyranoside, 40 μ g ml⁻¹ (each). All plasmids were maintained in *E. coli* strains DH5 α or S17-1 λ pir. Biparental matings between *P. fluorescens* strain SBW25 or SBW25 Δ *dapB* and plasmid-bearing strains of S17-1 λ pir were conducted using broth cultures grown over-

night by mixing 500 μ l of *E. coli* cells with 1 ml of heat-shocked (45°C for 20 min) *P. fluorescens* cells. The cells were pelleted, and the pellet was placed on a KB plate (supplemented with DAP and lysine, where appropriate) and incubated at 28°C for 16 h. Transconjugants were obtained by plating on selective media with 0.5 $\times$ CFC (cetrimide, fucidin, cephalosporin; Oxoid) or nitrofurantoin (100 μ g ml⁻¹; Sigma) to counterselect *E. coli*. The transposon IS-Omegon-Km/hah was developed by replacing the chloramphenicol gene of IS-*phoA*/hah (29) with a kanamycin gene (S. R. Giddens, C. D. Moon, R. W. Jackson, and P. B. Rainey, unpublished). Transposon mutants of chromosomal *rspR*, *rspL*, *ropE*, and *rspV* were generated by marker exchange using pGMP2 cosmids carrying GPS1 transposon insertions in the genes (33) and confirmed by PCR analysis. Assessment of bacterial growth in vitro was done by measuring the optical density at 600 nm (OD₆₀₀) of overnight cultures and inoculating three fresh 10-ml LB broths (containing kanamycin for mutants) to a final optical density of 0.01. Broth samples were removed, and the OD₆₀₀ was measured over 24 h.

Molecular biology techniques. Plasmid DNA was extracted using either a midiprep or a miniprep extraction kit (QIAGEN) and DNA cleaned up from agarose gels or enzymatic reactions using QIAGEN's QIAquick purification kits. Restriction endonucleases and buffers (New England Biolabs), shrimp alkaline phosphatase (USB), and DNA ligase (Invitrogen) were used according to the manufacturer's instructions. Standard PCR and arbitrary primed PCRs were performed with *Taq* polymerase and buffer (QIAGEN), and high-fidelity PCR was performed with Super*Taq* Plus (HT Biotechnology) using either a PTC-100 thermal cycler (MJ Research, Inc.) or a GeneAmp 2400 (Perkin-Elmer) instrument. All PCR-amplified DNA fragments were sequenced to confirm fidelity. Arbitrarily primed PCR protocol followed that of Manoil (29), and primer sequences are available on request. PCR products were examined on agarose (Melford) gels and cloned into pCR2.1 with an Original TA cloning kit (Invitrogen) for sequencing and subcloning. DNA sequencing was done using BigDye v3.1 (ABI) according to the manufacturer's instructions, and sequencing reactions were run on an ABI Prism 310 genetic analyzer automated sequencer. Sequence

traces were analyzed using Chromas 1.45 (Technelysium Pty. Ltd.), and database comparisons of the sequences were made via the BLASTN, BLASTP, and BLASTX algorithms (2).

Assessment of gene expression. Putative RspL-binding sites (promoters) were identified in the *P. fluorescens* SBW25 *rsp* gene cluster (Fig. 1A to C). Promoter activity was measured by transcriptionally fusing PCR-amplified promoter fragments from *P. fluorescens* SBW25 to either '*dapB*-'*lacZ*' or '*uidA*'. This was achieved using the integration vector pIVETD and derivatives thereof (11), which contain promoterless copies of *dapB-lacZ* and *uidA*. Both are R6K-based plasmids and do not replicate in *P. fluorescens* SBW25, so transconjugants arise by integration of the constructs into the chromosome by homologous recombination (Campbell integration) between promoters on the chromosome and the plasmid. DNA fragments were amplified using either pGMP2 DNA or total DNA as a template with primers (primer sequences available on request) designed from the *P. fluorescens* SBW25 genome or *rsp* gene sequence deposit (GenBank accession number AF292566). The DC3000 *hrpA* promoter was amplified using primers (primer sequences available on request) designed from sequence AF232004. Correct integration was confirmed by PCR, using a reverse primer from the vector *dapB* or *gfp* genes (primer sequences available on request) and a forward primer from the *P. fluorescens* SBW25 chromosome 200 to 500 bp upstream of the insert site (primers available on request). Promoter expression in the plant rhizosphere was tested using the pIVETD plasmid in *P. fluorescens* SBW25 Δ *dapB*, using the same principle as that described by Rainey (36). In vitro expression was tested using strains containing *uidA* fusions and measuring the activity of β -glucuronidase produced by *uidA*, except for testing of the pIVETD-*rspU* fusion suppressor mutants, when *lacZ* activity was tested.

For in vitro experiments testing expression in LB or HIM broth, single colonies of *P. fluorescens* transconjugants containing the fusions were initially grown overnight at 25°C in LB broth supplemented with tetracycline (LB+Tet). A 50- μ l aliquot of cell suspension was used to inoculate fresh LB+Tet broths (three replicates), while a 200- μ l aliquot was used for HIM broths (three replicates). For DC3000 pGFPGUS-*hrpA*, the strain was grown overnight in LB broth and a 50- μ l aliquot used to inoculate fresh LB+Tet broths. The remaining cells were harvested by centrifugation, washed once with sterile distilled water, and then resuspended in HIM to the initial volume. For experiments involving ectopic expression of regulatory genes, single colonies (three independent replicates) of each test strain were inoculated into LB broths, supplemented with the appropriate antibiotics, and incubated at 28°C for 16 h (or 40 h for experiments including strains overexpressing *rspL* due to slow growth of these strains).

The culture optical density at 600 nm was determined, and a 100- μ l aliquot of cells was harvested and resuspended in 800 μ l of sonication buffer (50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 100 mM NaCl, pH 8, plus 1 Complete proteinase inhibitor tablet [Roche] per 50 ml). Cells were sonicated (Soniprep 150; MSE) at an amplitude of 5 for 4 min and cell debris pelleted. The supernatant was aspirated and mixed with 200 μ l of protein dye (Bio-Rad) and total protein estimated by comparison to bovine serum albumin standards (1 to 15 μ g ml⁻¹). One microliter of each of the overnight cell suspensions was mixed in 40 μ l extraction buffer (50 mM NaH₂PO₄, pH 7.0, 10 mM 2-mercaptoethanol, 10 mM Na₂EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100) and 50 μ l of GUS assay buffer (2 mM 4-methylumbelliferone- β -D-glucuronide [4MUG] dissolved in extraction buffer) in individual wells of a 96-well microtiter plate (Falcon 3072). The plate was incubated in the dark at 37°C for 60 min, and 10 μ l of each reaction was stopped by addition to 190 μ l of stop buffer (0.2 M sodium carbonate). β -Glucuronidase activity (as a measure of promoter activity) was measured by assessing the amount of fluorescent 4-methylumbelliferone released from hydrolysis of 4MUG (nonfluorescent). Fluorescence was measured using a Polarstar plate reader (BMG Laboratories) with an excitation wavelength of 370 nm, emission wavelength of 460 nm, and gain of 10. Enzyme activity was calculated using the Fluostar computer program (BMG Laboratories) and expressed as units of 7-hydroxy-4-methylcoumarin (4MU) min⁻¹ μ g⁻¹ protein, except where protein samples were not obtained and expression was calculated relative to optical density of the culture medium. This ensured there were no differences due to cell growth variation and allowed direct comparison between strains.

Measurement of *lacZ* activity was done as described by Rainey (36) except that 4-methylumbelliferyl- β -D-galactoside was used as the substrate, and the product, 4MU, was detected using a fluorometer as described above.

Statistical analyses of the data including analysis of variance (ANOVA) and comparison of means were carried out using JMP software, version 5 (SAS Institute). All gene expression data represent observations from at least two independent experiments. Where possible, details of statistical analyses are presented in the figure legends.

Arabidopsis thaliana seedlings inoculated with reporter gene strains were visualized by using a light microscope.

Plant growth conditions and experiments. For experiments testing expression of in vivo expression technology (IVET) fusions, sugar beet seedlings were grown in vermiculite, as described by Rainey (36). Vermiculite was sieved (2-mm aperture) through a brass laboratory test sieve (Gallenkamp) and used to fill scintillation vials (three-quarter fill); the vermiculite was moistened with distilled water. Bacterial suspensions from overnight broth cultures were washed in sterile distilled water and adjusted to an optical density (600 nm) of 0.05. *Beta vulgaris* (sugar beet) var. Amethyst seeds were soaked in the bacterial suspension for 5 min and laid on top of vermiculite in the vials; the seeds were covered with 1 to 2 cm of vermiculite. The vials were incubated in a LEEC plant growth chamber (16:8 h, day:night; 21°C) for 16 days and bacteria recovered from roots and shoot as described previously (36). For root tip fitness experiments, sugar beet seeds were sterilized by soaking in 70% ethanol (3 min), sterile water (twice rinsed), 5% sodium hypochlorite, and 0.025% Triton X-100 (20 min), with eight washes in sterile water. Seeds were aseptically placed on 1% water agar plates, which were stood vertically in the dark for 2 to 3 days to allow seed germination and development of the seedling. A hole was melted in the lid of sterile 1.5-ml Eppendorf tubes and filled with a 1:1 bacterial suspension (*P. fluorescens* SBW25 wild type and an SBW25 *rsp* mutant; each suspension was made by dilution of an overnight broth in sterile water to an OD₆₀₀ of 0.1). The roots of germinated seeds were placed into the cell suspension such that the hypocotyls sat on the Eppendorf lid, and the tubes were incubated for 24 h at 25°C. A 5-mm section of root from the root tip upwards was removed and placed in an Eppendorf tube containing sterile glass balls, and bacteria were washed from the root tip by vortexing. Serial dilutions were plated on selective media, and cell counts were done to determine the selection rate constant (26). *Arabidopsis thaliana* plants were grown from seed; seed was sterilized by soaking in 70% ethanol (7 min), ethanol drained, and soaking in a 30% sodium hypochlorite, 0.025% Triton X-100 solution for 15 min. Seeds were washed five times in sterile distilled water and suspended in sterile 0.1% agar. Seeds were placed onto 0.5 $\times$ Murashige and Skoog agar plates and germinated in a LEEC plant growth chamber (8:16 h, day:night; 20°C). Ten-day-old seedlings were used for *uidA* expression experiments by transplanting seedlings onto M9 agar plates (containing XGlcA and appropriate antibiotics) with a lawn of test bacterium (spread at an OD₆₀₀ of 0.3, dried for 30 min).

RESULTS

Rsp promoters are expressed in the rhizosphere. Previous work has shown that the *rspC* gene cluster is plant inducible (36; P. Rainey, unpublished work); however, the responsiveness of the other *rsp* promoters was (at the start of this work) unknown. We took advantage of a *dapB*-based promoter trap (IVET [11]) to study the activity of the promoters controlling expression of the putative structural gene clusters (*rspU*, *rspC*, and *rspJ*), the alternative sigma factor (*rspL*), and a putative effector (*ropE*) in situ, that is, in the complex sugar beet phytosphere environment within which the bacterium is found naturally. To do this, the promoter region of each gene/gene cluster (hereafter referred to collectively as *rsp* promoters) was transcriptionally fused to an otherwise promoterless copy of *dapB* (which was itself transcriptionally fused to a promoterless *lacZ* reporter gene) and integrated into the genome of SBW25 Δ *dapB* by homologous recombination. Because SBW25 Δ *dapB* is unable to grow in the absence of an exogenous source of DAP (and there is no DAP in the phytosphere), growth of each Δ *dapB* *rsp*-'*dapB* fusion strain reflects the activity of the specific promoter fused to '*dapB*' (11).

Each *rsp*-'*dapB* fusion strain was inoculated onto replicate sugar beet seeds, and population densities (harvested from seedlings) were determined after 16 days. A negative control (strain PBR393) was included in which the DNA fragment fused to '*dapB*' did not contain a rhizosphere-active promoter (Fig. 2) (44). The density of the negative control strain after 16 days was typically below the level of detection (see Fig. 2). A positive control (strain PBR391) was also included that con-

FIG. 2. Analysis of SBW25 *rsp* gene expression in the rhizosphere. Rhizosphere growth of *P. fluorescens* SBW25 Δ *dapB* strains containing chromosomally integrated fusions between various *rsp* promoters and a promoterless copy of *dapB* (growth reports the activity of each promoter; see the text). Figure shows growth of *rspU*-*dapB*, *ropE*-*dapB*, *rspL*-*dapB*, *rspJ*-*dapB*, and *rspC*-*dapB* fusion strains in the rhizosphere of sugar beet seedlings. The figure also shows growth of the *P. fluorescens* SBW25 Δ *dapB* *rspC*-*dapB* fusion strain in the presence and absence of *rspL* (*rspL*:Tn#10). 391 is a positive control strain (PBR391) that contains a fusion between a constitutive promoter and *dapB*; the solid line at 10³ CFU indicates the maximum population density of the negative control (strain PBR393), which contains a fusion between a DNA fragment with no promoter activity and *dapB*. Data are means and standard errors from triplicate plants, and population densities were determined after 16 days. Bacterial recovery was significantly different by one-way ANOVA ($P < 0.0001$; $df = 6$), with a specific effect due to the *rspL* mutation ($P = 0.0025$; $df = 1$). Significantly different values determined by comparison of means by Tukey-Kramer honestly significant difference (HSD) ($P = 0.05$) are indicated by letters above the columns.

tained a fusion between a constitutively active promoter and *dapB* and typically reached population densities of $\sim 10^7$ CFU per rhizosphere.

The number of CFU recovered with the *rspC*-*dapB* fusion was approximately 100-fold higher than that from the other *rsp*-*dapB* fusion strains although significantly lower than that from the constitutive positive control strain, PBR391 (Fig. 2). The *rspJ*-*dapB*, *rspU*-*dapB*, *rspL*-*dapB*, and *ropE*-*dapB* strains were statistically comparable. These data demonstrate that all the *rsp* promoters are expressed in the rhizosphere.

We next examined the activities of the *rsp* promoters in the phyllosphere: with the exception of the *rspC*-*dapB* fusion strain, which reached 1.7×10^4 cells per phyllosphere, no bacteria were recovered from the *rspU*-*dapB*, *rspJ*-*dapB*, *rspL*-*dapB*, or *ropE*-*dapB* fusion strains, indicating that these promoters are essentially inactive in this environment.

The high population densities achieved by the *rspC*-*dapB* fusion strain, particularly in the rhizosphere, suggest that a second promoter or an additional transcription factor might control the activity of *rspC*. To test this, a *P. fluorescens* SBW25 Δ *dapB* strain was made with a mutation in *rspL* (SBW25 Δ *dapB* *rspL*:Tn#10), and the *rspC*-*dapB* fusion was integrated into the genome of this strain. The population densities of the *rspC*-*dapB* fusion strain (with and without the *rspL* mutation) from sugar beet seedlings were then determined in an experiment that included both the positive control strain PBR391 and the *ropE*-*dapB* fusion strain. The *rspL* mutant

FIG. 3. Expression of the *rspC* gene cluster is controlled by an *rspL*-independent regulator. Strains of *P. fluorescens* SBW25, carrying integrated *uidA* reporter gene fusions to *rspU*-*uidA*, *rspC*-*uidA*, *ropE*-*uidA*, or *rspJ*-*uidA*, were grown in LB broth for 16 h at 28°C. One-way ANOVA revealed a significant difference ($P = 0.0003$; $df = 3$); significant differences among means were revealed by Tukey-Kramer HSD ($P = 0.05$) and are indicated by letters above the columns. The concentration of 4MU product generated from 4MUG substrate by 1 μ l of cells was measured relative to optical density of the cell suspension, and values shown are the means and standard error of three replicates.

rspC-*dapB* fusion strain was significantly impaired in its ability to colonize the rhizosphere (Fig. 2), indicating a regulatory role for RspL in *rspC* expression in the rhizosphere. However, the observation that it did colonize the rhizosphere to a limited extent suggests that RspL-independent expression of *rspC* did occur.

We next asked whether there is a basal level of *rspC* expression in vitro and, if so, whether this was *rspL* dependent. To this end, promoterless *uidA* fusions to *rspC*, *rspU*, *ropE*, and *rspJ* were constructed (producing *rspC*-*uidA*, *rspU*-*uidA*, *ropE*-*uidA*, and *rspJ*-*uidA*, respectively) in a manner analogous to—and with identical fusion joint-points—the fusions described above. The activity of each promoter was then determined in vitro (LB broth) by assaying β -glucuronidase. All four promoters showed a low level of activity; however, the basal level of expression of *rspC* was significantly greater than that of the other *rsp* promoters (Fig. 3). Inactivation of *rspL* in the *rspC*-*dapB* fusion strain had no significant effect on expression of *rspC* (by one-tailed Student's *t* test). These data were further corroborated by examining the growth of SBW25 Δ *dapB* *rspL*:Tn#10 *rspC*-*dapB* and SBW25 Δ *dapB* *rspL*-*dapB*, *rspU*-*dapB*, *rspC*-*dapB*, and *ropE*-*dapB* fusion strains on minimal agar lacking DAP and lysine. After 2 days of incubation, only the two *rspC*-*dapB* fusion strains and the positive control strain, PBR391, grew: growth of the *rspC*-*dapB* fusion strain was *rspL* independent. Together these data confirm a basal level of expression of *rspC* and show that this basal level of expression is independent of *rspL*.

Visualization of *rsp* expression in the plant hypocotyl and root environment. *P. fluorescens* SBW25 *rsp* genes appear to be expressed in the rhizosphere but poorly or not at all in the phyllosphere, the exception being the *rspC* gene cluster. To obtain further evidence of rhizosphere induction, we took advantage of the range of promoterless *uidA* reporter fusion strains and asked whether, when grown alongside seedlings on agar plates containing the substrate (XGlcA) for β -glucuronidase, there was direct visual evidence of gene activation and,

FIG. 4. RspL-dependent expression of *P. fluorescens* SBW25 *rsp* genes in the hypocotyl and root environments of *Arabidopsis* seedlings. Strains of SBW25 and SBW25 *rspL*:Tn#10 carrying a transcriptional fusion of *uidA* to *rspU*, *rspC*, *ropE*, or *rspL* were grown alongside 10-day-old seedlings of *Arabidopsis thaliana*. *P. syringae* pv. tomato DC3000 with a *hrpA*-*uidA* fusion was a positive control. Bacteria were inoculated onto agar plates, and 10-day *Arabidopsis thaliana* ecotype Col-0 seedlings were then transferred and placed over the dried bacterial suspension. Plates were incubated at 22°C in the dark. Images were taken after 6 days and modified using Adobe Photoshop 7.0 to increase resolution and improve contrast. Arrows indicate the areas of blue color, indicative of *rsp* gene expression; note the lack of blue color in the plate surrounding the seedling. Asterisks indicate bacterial biofilm surrounding the plant.

if so, whether activation was specific to different regions of the seedling. Each fusion strain was inoculated onto minimal M9 agar plates (containing XGlcA) alongside sterile *Arabidopsis thaliana* seedlings. *P. syringae* strain DC3000 containing a *hrpA*-*uidA* reporter fusion was included as a control. After 4 days, β -glucuronidase activity was clearly evident from the *rspU*-*uidA*, *rspC*-*uidA*, and *ropE*-*uidA* fusion strains but not from the *rspL*-*uidA* reporter strain (Fig. 4). Little growth or β -glucuronidase activity was seen for bacteria growing on the shoots or leaves of seedlings. Each *uidA* fusion strain was also created in a *rspL*-mutant background: activity of the *ropE*-*uidA* and *rspU*-*uidA* fusions was completely dependent upon *rspL*, and as expected, expression of *rspC*-*uidA* showed *rspL*-independent expression, particularly in the hypocotyl region.

DC3000 harboring the *hrpA*-*uidA* fusion showed strong gene expression on both the root and the shoot.

Low expression of the *P. fluorescens* *rsp* genes in *hrp*-inducing medium. The TTSS genes of *P. syringae* are induced in HIM (30), a medium which mimics the nutrient environment of the plant apoplast. Since the *P. syringae* *hrpA* promoter was induced by both plant leaves and plant roots in the seedling assay described above, we tested whether HIM would induce expression of *rsp* genes in *P. fluorescens*. *P. fluorescens* SBW25 strains carrying the *rspR*-*uidA*, *rspL*-*uidA*, *ropE*-*uidA*, or *rspU*-*uidA* promoter fusion were grown in HIM, and the level of promoter activity was determined by assaying for β -glucuronidase: DC3000 carrying a *hrpA*-*uidA* fusion was employed as a positive control. In LB medium all genes exhibited a basal

FIG. 5. Expression of *P. fluorescens* SBW25 *rsp* genes in synthetic media. Strains of *P. fluorescens* SBW25 carrying a transcriptional fusion of *uidA* to *rspU*, *ropE*, *rspL*, or *rspR* were grown in LB broth (white columns) or *hrp*-inducing medium (gray columns) at 28°C for 16 h. *P. syringae* pv. tomato DC3000 with the *hrpA*-*uidA* fusion was a positive control. One-way ANOVA revealed significant effects of both medium (HIM versus LB) and strain (*P. fluorescens* versus *P. syringae*; $P < 0.0001$ and $df = 1$ in both instances). The concentration of 4MU product generated from 4MUG substrate by 1 μ l of cells was measured relative to cellular protein, and values shown are the means and standard error for three replicates.

level of expression, but there was an approximately two- to fivefold increase in expression of the SBW25 *rsp* genes, including *rspR*-*uidA*, in HIM (Fig. 5) and M9 (data not shown). In contrast, we observed a 23-fold increase of *hrpA*-*uidA* expression in DC3000 when grown in HIM compared to LB broth. ANOVA revealed significant effects of both medium (HIM versus LB) and strain (*P. fluorescens* versus *P. syringae*), which demonstrates that the *P. fluorescens* SBW25 *rsp* cluster is HIM responsive, although the magnitude of induction was less than that observed with *P. syringae*.

We considered the possibility that expression may have been limited because the medium or growth conditions used did not provide the appropriate signal(s) required for full activation, and therefore, we examined the responsiveness of *rsp* genes to a range of additional stimuli. No significant change in *rsp* expression was elicited by changes in alternative carbon source (0.2% glycerol), osmolarity (sorbitol, 150 mM), iron availability (0.45 mM FeSO_4 [high iron]; 100 μ M 2,2-dipyridyl [low iron]), pH (10 mM citrate), calcium (none or 0.1 mM), sodium nitrite (2.5 mM), sodium nitrate (20 mM), or histidine (15 mM) (data not shown).

Elevated expression of *rsp* genes occurs in strains expressing RspR and RspL. Given that SBW25 contains only a single putative enhancer binding protein, that the *rspC* gene cluster displays constitutive expression, and that the *P. syringae* TTSS induction medium is a poor inducer of the SBW25 *rsp* genes, we reasoned that the regulatory pathway controlling *rsp* expression might either be defective or function differently from that found in *P. syringae*.

When either *rspL* or *rspR* was expressed ectopically on plasmid pML122 under control of the *npfII* promoter, we observed a pronounced increase in transcription of *ropE* and *rspU* (assayed using *uidA* reporter fusions to both genes) (Fig. 6). ANOVA indicated a significant difference in promoter activity between *ropE*-*uidA* and *rspU*-*uidA*. We also observed a highly

FIG. 6. The *P. fluorescens* SBW25 *rsp* gene regulators, RspL and RspR, activate expression of structural and effector genes. Strains of *P. fluorescens* SBW25 carrying a transcriptional fusion of *uidA* to *ropE* (gray bars) or *rspU* (white bars) were grown in LB broth at 28°C with or without the addition of plasmids expressing (A) RspL and (B) RspR. The concentration of 4MU product generated from 4MUG substrate by 1 μ l of cells was measured relative to cellular protein, and values shown are the means and standard error for three replicates. One-way ANOVA indicated there was a significant difference in promoter activities between *ropE*-*uidA* and *rspU*-*uidA* for both experiments (RspL experiment, $P = 0.0002$ and $DF = 1$; RspR experiment, $P = 0.0014$ and $DF = 1$) and in expression of the individual promoters in strains carrying *rspL* or *rspR*, compared to controls ($P < 0.0001$; $DF = 2$).

significant difference in expression of the individual promoters in strains carrying *rspL* or *rspR* from that of controls (Fig. 6). A comparison of means indicated that *rspU*-*uidA* expression was significantly higher than that of *ropE*-*uidA* in both experiments. In separate experiments, we observed that RspL activated both the *rspJ*-*uidA* (mean expression levels in nM 4MU $\text{min}^{-1} \mu\text{g protein}^{-1} \pm$ standard errors, 161.1 ± 44.6 units of 4MU $\mu\text{g protein}^{-1} \text{min}^{-1}$) and *rspC*-*uidA* (587.7 ± 20.5 units of 4MU $\mu\text{g protein}^{-1} \text{min}^{-1}$) fusions relative to strains carrying vector alone (*rspJ*-*uidA*, 9.5 ± 1.0 units of 4MU $\mu\text{g protein}^{-1} \text{min}^{-1}$; *rspC*-*uidA*, 7.9 ± 1.4 units of 4MU $\mu\text{g protein}^{-1} \text{min}^{-1}$). These data demonstrate that the *rsp* regulators RspR and RspL positively regulate both *P. fluorescens* structural *rsp* genes and the putative effector gene *ropE*.

RspR, the putative enhancer binding protein, activates the alternative sigma factor gene, *rspL*, leading to activation of the structural genes. In *P. syringae*, the enhancer binding proteins HrpR and HrpS are thought to bind to a *cis*-acting enhancer

FIG. 7. The regulatory hierarchy of *rsp* gene expression in *P. fluorescens* SBW25. (A) The effect of ectopic expression of Rsp regulators RspR and RspL in *P. fluorescens* SBW25 carrying an *rspL-uidA* reporter gene fusion. One-way ANOVA ($P < 0.0001$; $DF = 2$) and subsequent comparison of means by Tukey-Kramer HSD ($P = 0.05$; different letters above the columns specify significant differences) indicated a significant effect of RspR expression on *rspL*. (B) The effect of ectopic expression of Rsp regulators RspR in *P. fluorescens* SBW25 (white bars) and RspR and RspL in *P. fluorescens* SBW25 *rspL*:Tn#10 (gray bars) carrying a *rspU-uidA* fusion. One-way ANOVA confirmed that the *rspL* mutation had a significant negative effect on expression of *rspU-uidA* by RspR ($P < 0.0001$; $df = 1$). Strains were grown in LB broth at 28°C with or without expression plasmids for 40 h. The concentration of 4MU product generated from 4MUG substrate by 1 µl of cells was measured relative to cellular protein, and values shown are the means and standard error for three replicates.

sequence as a heterodimer, allowing RpoN-dependent expression of the alternative sigma factor gene *hrpL*, which subsequently activates transcription of *hrp* genes (14, 19). To determine whether the same hierarchy exists in SBW25, we examined expression of the SBW25 *rspL* promoter using the *rspL-uidA* reporter strain in which RspR was overexpressed on plasmid pML122. Expression of RspR resulted in a three-fold increase in expression of the *rspL-uidA* promoter compared to that for the control strain (Fig. 7A). We tested the effect of ectopic expression of RspL from pML122 on expression of the *rspL-uidA* gene to determine if RspL plays any autoregulatory role but observed no significant difference in expression (Fig. 7A). Since we knew that overexpression of RspR leads to increased expression of the structural *rsp* gene fusion *rspU-uidA* and the alternative sigma factor gene fusion

rspL-uidA, it was logical to assume, based on the *P. syringae* model, that RspR activates *rspL* and that RspL activates *rspU*. Consistent with this hypothesis, no expression of *rspU-uidA* was detected in a strain overexpressing RspR but carrying a mutation in *rspL* (Fig. 7B). Ectopic expression of RspL from pML122 in the *rspL* mutant strain complemented the *rspL* mutation. These data confirm that genes controlling expression of structural genes are regulated by RspR acting via *rspL*.

RspV mutation does not affect *rspU* expression. HrpV negatively regulates TTSS gene expression in *P. syringae*, possibly by negatively regulating HrpS (20). In rich growth medium, mutation of *hrpV* in *P. syringae* pv. *syringae* leads to an increase in expression of the HrcJ protein (provided that HrpR and HrpS are ectopically expressed) (32). To test whether RspV acts to negatively regulate expression of *rspR* or *rspL* or their protein products, *rspV* was mutated and its effect on *rspU* transcription determined using a *uidA* reporter. As observed previously, overexpression of RspR significantly increased *rspU-uidA* expression in the wild-type strain (mean expression levels in nM 4MU min⁻¹ OD₆₀₀⁻¹ with standard errors: 148.41 ± 18.45 4MU min⁻¹ OD₆₀₀⁻¹ compared to basal level 2.83 ± 1.21 4MU min⁻¹ OD₆₀₀⁻¹). In the *rspV* mutant strain (SBW25 *rspV*:Tn#1112), overexpression of RspR did not result in a further increase in *rspU-uidA* expression (69.83 ± 26.32 4MU min⁻¹ OD₆₀₀⁻¹ compared to basal level, 2.50 ± 0.28 4MU min⁻¹ OD₆₀₀⁻¹) compared to the wild type overexpressing RspR. ANOVA indicated that the *rspV* mutation did not have a significant effect on the basal level of *rspU-uidA* expression ($P = 0.1074$; $df = 1$) or RspR-mediated expression of this (*rspU*) gene cluster ($P = 0.1180$; $df = 1$).

Searching for additional regulators of *rsp*. Low transcriptional activity of the SBW25 *rsp* gene cluster could have numerous causes: one possibility is that additional regulators exist but that high expression requires either activation of a positive activator or inactivation of a negative regulator. To identify such components, SBW25Δ*dapB* *rspU-uidA* was mutagenized using (separately) two transposons: one a modified IS*phoA*/hah transposon, IS-Omegon-Km/hah, which contains an outward-facing *npt* promoter (to identify potential positive regulators), and the other mini-Tn5-Km (to identify repressors). Transconjugants were screened for ability to grow on minimal M9 medium (activation of a positive regulator or inactivation of a repressor would allow transcription of *dapB*, thus restoring SBW25Δ*dapB* *rspU-uidA* to prototrophy).

Seven IS-Omegon-Km/hah mutants and two mini-Tn5-Km mutants were identified from a screen of ~10⁵ mutants (~16-fold coverage) for each transposon (assuming 6,130 genes and insertions distributed according to a Poisson distribution, then a screen of 10⁵ mutants means that the probability of sampling every gene at least once is marginally less than 1.0; the probability of not sampling a gene is less than 0.000008), and all nine mutants grew on minimal M9 plates. No spontaneous prototrophic mutants were detected. Quantitative analysis of promoter activity using the promoterless *lacZ* reporter (which is fused to *rspU*: *rspU-dapB-lacZ*) showed that *rspU* expression in all mutants increased significantly compared to that of the control strain ($P = 0.0033$ and $df = 2$ by one-way ANOVA), with the seven IS-Omegon-Km/hah mutants showing ~10-fold-greater expression than the other two.

FIG. 8. Mutations in *rsp* genes cause changes in bacterial fitness in the rhizosphere and growth in vitro. Competitive colonization of sugar beet root tips (A) by marker exchange mutants containing transposon insertions in *ropE*, *rspL*, *rspR*, *rscR*, *rscT*, or *rspV*. Data are means and 95% confidence intervals for three replicates and indicate the fitness of each mutant relative to the wild type, *P. fluorescens* SBW25, after 24 h. Values less than 0 indicate that the mutant is competitively inferior to the wild type. One-way ANOVA ($P = 0.0005$; $df = 6$) reveals a significant difference among means; significantly different values are indicated by different letters above the columns. (B) Growth dynamics of *ropE*, *rspL*, *rspR*, *rscR*, *rscT*, and *rspV* mutants in LB broth over 24 h. Broth media were seeded from starter cultures to a starting OD₆₀₀ of 0.01 and the OD₆₀₀ of each broth measured over time. Values are the means for three replicates and standard errors. Solid lines are *P. fluorescens* SBW25 (filled diamond) and SG116 (positive control; open triangle), dotted lines are structural mutants *rscT* (open square), *rscR* (filled circle), and *ropE* (filled square), and dashed lines are regulator mutants *rspL* (filled triangle), *rspR* (open diamond), and *rspV* (open circle). Note that the *rspR* and *rscT* mutant growth curves are extremely similar on this graph.

Arbitrarily primed PCR was used to identify the genomic location of the transposon in each mutant. Interrogation of the SBW25 genome sequence showed that the seven IS-Oregon-Km/hah insertions were located immediately upstream of the *rspL* gene in the predicted promoter region between *rspL* and the *rspU* gene cluster (leading to greatly enhanced expression of *rspU* or *rspL*). Of the two mini-Tn5-Km insertions, one was located in a putative beta-alanine pyruvate aminotransferase gene (PFLU0675) and the second in a hypothetical gene (PFLU2224) whose protein product is predicted to have an alpha/beta hydrolase fold. The protein product of this gene

showed greatest similarity (33% identity, 47% similarity) to animal proteins and is the last of a five-gene cluster with characteristics of an ABC transporter locus.

Fitness effects associated with *rsp* mutations indicate a general effect on bacterial growth. Previous work showed that over a 2-week period, *rscR* and *rscT* mutants of SBW25 were uncompromised in their ability to colonize the rhizosphere of the sugar beet (33). During the course of this study, several more mutants were generated (including *rspR* and *rspL* regulatory mutants), which provided additional opportunity to examine the contribution of specific *rsp* genes to ecological performance.

Strains containing (separately) mutations in *rspR*, *rspL*, *rscR*, *rscT*, *rspV*, and *ropE* were mixed 1:1 with wild-type SBW25 and introduced into sterile buffer contained within a 1.5-ml culture vial. The fitness of each mutant was then determined (relative to that of the wild type) by a simple 24-h colonization assay in which the ability of the mutant to competitively colonize the root tip (5 mm) of a 3-day-old sugar beet seedling was determined.

All the mutants, except the *rscR* and *rscT* mutants, showed a drop in fitness relative to the wild type (Fig. 8A). One-way ANOVA confirmed that there was significant variation in the experiment, with the *rspL* and *rspV* mutants being significantly less fit than the positive control strain, SG116.

To check whether the decrease in fitness of the regulator mutants in the rhizosphere was also evident in vitro, we analyzed growth of the mutants in LB broth over 24 h. All mutants, including the *rscT* and *rscR* mutants, displayed altered growth in the initial 15 to 20 h compared to wild-type SBW25, although all strains reached equivalent cell densities after 24 h (Fig. 8B). Growth of the *rspL* mutant was the least impaired in vitro, but it was one of the worst-affected strains in the rhizosphere.

DISCUSSION

The challenges associated with understanding the functions of bacteria in complex environments are considerable—and all the more so when phenotypes associated with genes of interest do not manifest in vitro. The *P. fluorescens* SBW25 *rsp* cluster, while showing many similarities to TTSS gene clusters of pathogenic bacteria, lacks genes that encode components of the pathway essential for functionality as a type III secretion system (33). Despite this, Preston et al. (33) were able to demonstrate an Rsp-dependent hypersensitive reaction in *Arabidopsis thaliana*; however, such a response was dependent upon infiltration of high numbers of bacteria into leaves (33). The requirement for nonphysiological levels of bacteria, plus defects in the *rspI* gene cluster, calls into question the role of *rsp* in a dedicated protein secretion pathway. If *rsp* has no role in protein secretion, then what purpose might it serve? A range of possibilities exist: it may have no biological role—it may be an “evolutionary relic” and a reflection of a previous eukaryote interaction-based lifestyle; alternatively, it may have a new function as a consequence of cooption, for example, it is possible to envisage a role for the pilus (RspA) in cell adhesion or cell-cell interactions.

If an evolutionary relic, then evidence of mutational decay ought to be apparent and evident at the level of regulation:

selection will favor regulatory mutants because such mutants no longer pay the cost of expressing components that are surplus to requirement. We found little evidence of mutational decay. For a start, putative (but clearly defined) *rspL* (*hrpL*) promoter sequences are present upstream of each structural operon and upstream of the gene encoding the putative secreted *ropE* protein (Fig. 1). Second, all components of the *rsp* cluster show increased levels of expression in the rhizosphere environment (and are induced, albeit poorly, in HIM). Third, components predicted to be involved in the regulation of *rsp* are operable. Fourth, *rsp* mutants (particularly regulatory mutants) are compromised in their ability to grow in vitro and in their ability to colonize the plant rhizosphere. Together, these findings suggest that the *rsp* cluster has functional significance, although its function remains obscure.

In terms of regulation, the *rsp* pathway of *P. fluorescens* SBW25 shows both similarities and differences from the model of regulation developed for TTSS in *P. syringae*. The most significant difference is the responsiveness to environmental stimuli: the *rsp* pathway responds primarily to signals present in the rhizosphere and with the exception of the *rspC* promoter (which has a basal level of expression independent of RspL) is insensitive to phyllosphere-derived signals. In contrast, the TTSS of *P. syringae* is induced in the leaf apoplast; interestingly, the *hrpA-uidA* fusion of DC3000 used here was induced by both the root and the shoot of *Arabidopsis thaliana* seedlings, thus showing little evidence of plant tissue-specific expression. The most striking evidence of difference in inducing signals came from the analysis of gene expression in Hrp-inducing medium (HIM), a medium that mimics the plant apoplast environment. In DC3000, HIM caused an approximately 28-fold increase in expression, whereas in *P. fluorescens* SBW25, the increase in *rsp* expression was, while statistically significant, modest (two- to fivefold). Despite poor induction in HIM, ectopic expression of the alternate sigma factor (RspL) showed that *rsp* gene expression has the potential to increase ~100-fold. A further distinction concerns RspV: our studies here failed (under the conditions tested) to provide evidence of a negative regulatory role; in fact, a slight decrease in *rspU* expression was observed following inactivation of *rspV*.

Similarities in regulation were also noted. For example, the regulatory cascade controlling expression of *rsp* in *P. fluorescens* mirrors that found in *P. syringae*. In *P. fluorescens* the HrpRS homolog, RspR, controls expression of the *rspL* gene, and RspL activates transcription of the structural genes and putative effector, *ropE*. This indicates that RspR sits higher in the regulatory hierarchy than RspL. It also indicates that in the absence of a HrpS homologue, the single regulatory component (RspR) is sufficient to activate *rspL*. This suggests that RspR functions as a homodimer: in *P. syringae*, there is a heterodimeric association between HrpR and HrpS (19).

Motivated by the need to understand further the reasons for low induction of *rsp* in laboratory media, we considered the possibility of additional regulators and sought their existence (and identity) using a mutagenesis strategy. Following an intensive mutant screen (16-fold coverage of the genome) based upon conversion to prototrophy, nine "regulator" mutants were found. Seven were caused by IS-Omegon-Km/hah: in each instance the transposon was located immediately upstream of *rspL*. Given that IS-Omegon-Km/hah carries an in-

ternal kanamycin promoter capable of activating downstream genes, this set of mutants implicates RspL as a positive activator of *rsp* transcription—a fact already established (see above) but one that nicely demonstrates the utility of this approach. No additional positive regulators were found, and no insertions were found upstream of *rspR* (failure to detect *rspR*-activating insertions may reflect the complexity of EBP-dependent transcription). Mutagenesis with mini-Tn5-Km, a transposon with strong polar effects (8), resulted in two prototrophic mutants but none with obvious regulatory ties to *rspR* or *rspL*. However, one mutation was located in a beta-alanine pyruvate aminotransferase gene (PFLU0675), whose protein product is predicted to be involved in the conversion of alanine to pyruvate. Previously, Dacheux et al. (7) showed that mutation of the *P. aeruginosa aceAB* genes (which encode pyruvate dehydrogenase and convert pyruvate to acetyl-coenzyme A) leads to a decrease of TTSS-dependent cytotoxicity in human polymorphonuclear cells. Furthermore, transcription of the *exsCBA* (TTSS) operon is not inducible in the *aceAB* mutants. It is possible that our discovery mirrors that described for *P. aeruginosa*, where it appears that pyruvate metabolism plays a role in TTSS gene expression; it is also consistent with the more general suggestion that the metabolic status of the cell is a "sensor" for induction of TTSS—at least in *P. aeruginosa* (38)—and of *rsp* in *P. fluorescens*.

In previous work (33), using a 2-week rhizosphere colonization assay, we failed to detect a fitness effect due to mutations in *rsp* structural genes. Armed with a new set of mutants, we devised a root colonization assay where colonization was dependent upon bacteria actively adhering to and spreading along the root surface from a liquid reservoir. To our surprise we found significant differences in fitness due to mutations in *rsp*, primarily in the regulators of *rsp*. Such pronounced defects prompted an examination of the growth of mutants in laboratory medium. Contrary to expectation, some mutants displayed significantly altered growth dynamics (e.g., the *rspR*, *rspV*, *rscT*, and *rscR* mutants).

While the rhizosphere colonization data support a role for *rsp* in colonization, the discovery of more general effects in laboratory medium suggests otherwise. These data are difficult to reconcile: on one hand we found low to negligible expression of all *rsp* components (except *rspC*) in vitro, and yet mutants show significant growth defects in laboratory medium. This suggests that defects in the *rsp* pathway and in *rsp* regulators impair growth of laboratory-grown cells. It is particularly puzzling that the *rspV* and *rspR* regulatory mutants show strong defects in vitro, but mutations in the more specific regulator *rspL* do not. Without additional work we can but speculate, but these data strongly suggest a more significant and general effect of *rsp* mutations on the cellular functioning of SBW25 than is currently appreciated. Most analyses of TTSS mutants have focused on host interactions rather than fitness in vitro, and it may be that this finding is not unique to mutants of the *rsp* pathway of SBW25. Research into this phenomenon is further complicated by the fact that plasmids and cosmids expressing *rsp* structural genes and *rsp* regulators also impair growth of *P. fluorescens* SBW25 and *rsp* mutants (33; R. Jackson, G. Preston, and P. Rainey, unpublished), indicating that the stoichiometry and expression levels of *rsp*-encoded proteins

are tightly regulated in SBW25. This effect has also been noted for *Erwinia carotovora* (25).

Elucidation of the biological function and ecological significance of the *P. fluorescens* SBW25 *rsp* pathway remains a significant challenge. Here we have shown that *rsp* is an integral component of the SBW25 cell, that its expression is controlled by an intact regulatory network, and that the network is responsive to signals from the plant rhizosphere. Despite the challenges ahead, the analysis of pathways such as that of *rsp* that do not conform to the expected paradigms offers, in the long term, potentially new insights into the biology of bacteria and, importantly, insights into the biology of bacteria in their natural environment.

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