Transcriptional Regulation of Components of the Type III Secretion System and Effectors in *Pseudomonas syringae* pv. *phaseolicola*

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Quantitative real-time polymerase chain reaction was used with specific TaqMan probes to examine transcription of selected *hrp* and effector genes in *Pseudomonas syringae* pv. *phaseolicola* strains 1448A (race 6) and 1449B (race 7). Transcripts examined were from genes encoding the regulators *hrpR* and *hrpL*, core structural components of the type III secretion system (TTSS) *hrcC*, *hrcJ*, *hrcN*, *hrcU*, and *hrpA*; the first open-reading frame of each *hrp* operon, including *hrpF*, *hrpJ*, *hrpP*, and *hrpY*; and also secreted effectors *hrpZ*, *avrPphE*, *avrPphF*, and *virPphA*. All genes were induced by incubation in a minimal medium and showed patterns of expression indicating regulation by HrpRS and HrpL. Basal mRNA levels and the timing of accumulation of transcripts after induction differed significantly, suggesting the operation of additional regulatory elements. However, no clear transcriptional hierarchy emerged to explain the ordered construction of the TTSS. Quantitative analysis confirmed that the rates and levels of transcript accumulation within the first 2 h after inoculation were considerably higher in planta than in vitro, and indicated that plant cell wall contact may enhance transcription of TTSS and effector genes in *P. syringae* pv. *phaseolicola*. The low-abundance *hrcU* mRNA had a half-life of 16.5 min, whereas other transcripts had half-lives between 3 and 8 min.

Additional keywords: *hrc*, pathogenicity, RNA stability.

Many bacterial pathogens of animals and plants construct a remarkable piece of cellular architecture, the type III secretion system (TTSS), which is exquisitely designed to deliver proteins into host cells (Blocker et al. 2003; Cornelis and Van Gijsegem 2000; Jin et al. 2003). In *Pseudomonas syringae*, as in other plant pathogens, genes encoding the TTSS are clustered into a pathogenicity island. Genetic dissection of the TTSS in *P. syringae* began with the discovery of so called *hrp* mutants of the bean pathogen *P. syringae* pv. *phaseolicola* which lost the ability to elicit a hypersensitive resistant reaction (HR) in nonhost plants such as tobacco and also lost patho-

genicity to bean (Lindgren 1997; Lindgren et al. 1986; Rahme et al. 1992). Further research revealed clusters of *hrp* genes, some of which encoded proteins conserved among plant and animal pathogens and therefore designated *hrc* (*hrp*-conserved). Nomenclature of the conserved components of the TTSS in plant pathogens is based on the identity of their functional homologues in *Yersinia* spp. (Bogdanove et al. 1996). An additional and exceptional component of the injectosome in plant pathogens, including species of *Pseudomonas*, *Xanthomonas*, *Erwinia*, and *Ralstonia*, is the Hrp pilus which is an extension of the extracellular domain of the TTSS (Cornelis and Van Gijsegem 2000). In pathovars of *P. syringae*, the Hrp pilus is composed of subunits of HrpA protein. Polar extension of the appendage through the distal addition of HrpA subunits to the filament tip allows the pilus to penetrate the plant cell wall (Jin and He 2001; Li et al. 2002). The *hrp* genes encode not only structural components of the TTSS but also secreted proteins such as HrpZ which may be similar in function to the translocon components YopB/IpaB/EspD and YopD/IpaC/EspB of the animal pathogens *Yersinia* spp., *Salmonella* spp., and enteropathogenic *Escherichia coli* (Blocker et al. 2003; Lee et al. 2001).

DNA sequence analysis and northern hybridization experiments have shown that genes within the *hrp* cluster of pathovars of *P. syringae* are organized into at least six operons (Lindgren 1997). Comparison between *P. syringae* pv. *phaseolicola* and related pathovars such as *syringae* and *tomato* have indicated some differences in operon structure. For example, in *P. syringae* pv. *phaseolicola*, *hrpA* and *hrpZ* are members of separate transcriptional units, but they are both within a single operon in *P. syringae* pv. *syringae* (Lee et al. 2001; Preston et al. 1995). Reporter gene constructs have been used to reveal regulatory networks controlling expression of genes encoding the TTSS in plant pathogens (Huynh et al. 1989; Rahme et al. 1992). In *P. syringae*, an important regulatory cascade has been identified involving three positive regulators: HrpR and HrpS (transcribed as a single operon) and HrpL. HrpR and HrpS interact directly and, with the sigma factor σ^{54} , activate expression of *hrpL* (Grimm et al. 1995; Xiao and Hutcheson 1994). Other *hrp* and *hrc* genes are thought to be regulated primarily by HrpL. A characteristic promoter motif, the “*hrp* box”, has been identified upstream of genes regulated by HrpL which include not only TTSS components but also genes encoding effector proteins which are delivered into the plant cell cytoplasm (Collmer et al. 2002). Basal levels of *hrp* and effector gene expression have been reported to be very low in rich media but transcript accu-

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mulation is induced in minimal medium which is thought to replicate conditions within the intercellular spaces of plant hosts (Huynh et al. 1989; van Gijsegem 1997). Contact with host cells is recognized as an essential activator of the TTSS in animal pathogens, including species of *Yersinia*, *Salmonella*, and *Shigella* (Blocker et al. 2003). Experiments using plant cell suspension cultures have also demonstrated an important role for contact in the expression of *hrp* genes in the vascular wilt pathogen *Ralstonia Solanacearum* (Aldon et al. 2000). However, unlike *P. syringae*, *R. solanacearum* utilizes an AraC-based regulation system (Marenda et al. 1998).

Building the TTSS and the coordination of secretion of effector proteins must require a remarkable degree of fine-tuned expression of approximately 25 proteins which may contribute to the injectosome and approximately 30 effectors predicted to have key roles in virulence in *P. syringae* (Collmer et al. 2002). The structural and functional similarity between the Hrp-based TTSS and the flagellum has been described often (Blocker et al. 2003; He 1997). The regulation of flagellum biosynthesis is governed by a highly complex network, linking synthesis and assembly of the flagellar apparatus and employing both transcriptional and post-transcriptional regulation (Soutourina and Bertin 2003).

We have examined the transcription of a selection of *hrc* and *hrp* genes, including the first members of each *hrp* operon in *P. syringae* pv. *phaseolicola*, and also three effectors, *avrPphE*, *avrPphF*, and *virPphA* (Jackson et al. 1999; Mansfield et al. 1994; Tsiamis et al. 2000). Genes examined here include those encoding key structural components *hrcC* (the outer membrane "secretin"), *hrcU* (located to the inner membrane), *hrcJ* (spanning the inner and outer membrane), and *hrcN* (inner membrane associated ATPase), and the secreted proteins the pilin subunit *hrpA* and putative translocon component *hrpZ*. Previous analysis of gene expression in *P. syringae* has used reporter genes or microarrays, neither of which allows unambiguous quantitative measurement of transcript levels (Huynh et al. 1989; Rahme et al. 1992; Zwiesler-Vollick et al. 2002). We have developed methods to quantify RNA levels in vitro and in the plant, in comparison with the constitutively expressed 16S RNA gene. We chose to use TaqMan real-time polymerase chain reaction (PCR) assays which utilize the exonuclease activity of *Taq* polymerase to release a fluorophore from a specific nucleotide probe. The high level of sensitivity and specificity afforded by the TaqMan probe provides the most accurate method of mRNA quantification (Bustin 2002). Our hypothesis was that the direct, quantitative analysis of transcript abundance might reveal a transcriptional hierarchy similar to that observed during construction of flagella.

RESULTS

Expression in vitro.

The locations of the genes examined within the *hrp* cluster are shown in Figure 1. Preliminary experiments compared

expression levels in race 6 and race 7 using template cDNA generated either with primers designed to be specific for each gene or with random oligonucleotides. No differences were observed and, therefore, random oligonucleotides were used for synthesis of all cDNAs in the experiments described. Thus, we were able to examine all transcripts in the same cDNA preparation.

Basal levels of transcription determined directly from bacteria grown in Luria-Bertani (LB) broth or after the initial washing in 10 mM MgCl₂ (Table 1) were considerably higher for *hrpA* and *virPphA* (approximately 10⁻⁷ per 16S transcript in washed cells) compared with *hrpR* (10⁻⁸) and other genes (basal levels 10⁻⁹ to 10⁻¹⁰ per 16S transcript). Transfer of the wild-type strains to minimal medium led to rapid induction of expression of all genes. Detailed time course data obtained with wild-type race 6 for the regulators *hrpR* and *hrpL*, the highly expressed pilus subunit-encoding *hrpA*, and *hrpP* as an example of a typical transcript, are given in Figure 2. There were significant differences in the rates of accumulation of individual RNAs. Most striking was the increase in *hrpR* mRNA within 5 min of induction and, by 20 min, this transcript had increased 10-fold. Other transcripts accumulated more gradually, significant increases being recorded after 10 min for *hrpL*, *hrpA*, and *hrpZ*, and 20 min for *hrcC*, *hrcJ*, *hrpF*, *hrpP*, *hrpY*, and the effector *avrPphE*. Despite the higher constitutive levels of *virPphA* RNA, clear induction of this transcript was apparent only after 40 min, when increases in *hrpJ* and *hrcN* RNAs also were recorded. The *hrcU* transcript increased significantly only after 80 min. Differences in transcript abundance were apparent after induction for 120 min;

Table 1. Induction of transcripts of *Pseudomonas syringae* pv. *phaseolicola* race 6 in vitro^a

Transcript	No. of transcripts relative to 16S ^b		Fold increase
	Basal (time 0) ^c	120 min	
<i>virPphA</i>	3.7 × 10 ⁻⁷	6.4 × 10 ⁻⁶	17
<i>hrpA</i>	1.0 × 10 ⁻⁷	4.6 × 10 ⁻⁵	459
<i>hrpR</i>	1.5 × 10 ⁻⁸	1.8 × 10 ⁻⁷	12
<i>hrcN</i>	8.6 × 10 ⁻⁹	1.1 × 10 ⁻⁶	125
<i>hrpF</i>	1.9 × 10 ⁻⁹	1.4 × 10 ⁻⁶	740
<i>hrpL</i>	1.1 × 10 ⁻⁹	6.2 × 10 ⁻⁷	587
<i>hrpZ</i>	1.0 × 10 ⁻⁹	3.2 × 10 ⁻⁶	3,074
<i>hrpJ</i>	7.9 × 10 ⁻¹⁰	1.5 × 10 ⁻⁷	188
<i>hrpY</i>	6.9 × 10 ⁻¹⁰	1.3 × 10 ⁻⁶	1,857
<i>hrpP</i>	5.8 × 10 ⁻¹⁰	1.1 × 10 ⁻⁶	1,905
<i>avrPphE</i>	5.7 × 10 ⁻¹⁰	5.2 × 10 ⁻⁷	921
<i>hrcU</i>	2.7 × 10 ⁻¹⁰	6.3 × 10 ⁻⁹	23
<i>hrcJ</i>	2.1 × 10 ⁻¹⁰	1.9 × 10 ⁻⁷	922
<i>hrcC</i>	1.9 × 10 ⁻¹⁰	2.4 × 10 ⁻⁷	1,299

^a Levels are compared before and after incubation for 120 min in minimal medium.

^b Data are means compiled from two repeated experiments using duplicate analyses at each time point.

^c Basal levels recorded after washing bacteria in 10 mM MgCl₂ before suspension in minimal medium.

Fig. 1. The *hrp* cluster of *Pseudomonas syringae* pv. *phaseolicola*. Genes are organized in nine operons (indicated by arrows); ♦ indicates an *hrp* box and σ is a σ⁵⁴-dependent promoter. Genes with major regulatory functions are shaded. Open reading frames selected for expression analysis are indicated by bold underlining. DNA sequences were obtained from GenBank, accession numbers U16817, AJ430232, AF043444, AF268940, X77638, AY505348, and AY505349.

the levels of expression relative to 16S RNA and fold induction over basal levels are recorded for all genes in Table 1.

Examples of gene expression data recorded in vitro for an *hrpL* Tn3gus insertion mutant of race 6 are compared with results for the wild-type strain in Figure 2. In the *hrpL* mutant, only *hrpR* was induced during incubation in minimal media. However, abundance of the *hrpR* transcript throughout the time course was approximately half that recorded in wild-type rifampicin-resistant 1448A.

RNA of the effector *avrPphF* was not detected in race 6; however, in race 7, it accumulated in a pattern very similar to *avrPphE*, reaching 5.0×10^{-7} copies relative to 16S after 120 min. The patterns of accumulation of other transcripts in race 7 (data not shown) also were similar to those presented for race 6 (Fig. 2).

Expression in leaves.

The sensitivity of quantification afforded by the TaqMan system allowed gene expression to be examined in small amounts of inoculated leaf tissue. Transcript levels of race 6 (1448A) were examined for up to 24 h after injection of bacterial suspension into the intercellular spaces of unifoliate bean leaves (Fig. 3). In the plant, increases in expression were notably delayed compared with those found in minimal medium. However, after 120 min, transcript levels for all genes examined were greatly increased in leaves and were, on average,

8.6× higher than recorded in vitro. Enhanced accumulation of the regulators *hrpL* and *hrpR* was particularly striking, being over 15× higher in planta than in vitro. The same orders of abundance and onset of accumulation, with induction of the regulator *hrpR* being most rapid, were noted in the plant as in minimal medium. The times after incubation when twofold increases in mRNA over basal levels were recorded are compared in Table 2.

In leaves, transcripts increased for all genes except the regulator *hrpR* between 120 and 240 min. A striking reduction in RNA levels was found after 24 h, as the colonies of the pathogen expanded within the intercellular spaces and bacterial numbers had increased 50-fold as determined from levels of 16S RNA within the susceptible cv. Canadian Wonder.

Extraction procedures were modified in an attempt to examine expression of transcripts in bacteria (race 6) in contact with the plant cell walls after 120 min. Following homogenization, cell wall debris and attached bacteria were recovered on 10-μm filters. Unattached bacteria were released and passed through the filter by repeated washing. Analysis of 16S RNA levels indicated that very few bacteria remained attached to the cell wall debris and consistent, significant detection of RNA was possible only for the most abundant transcripts, *hrpA*, *hrpZ*, and *virPphA*. Expression levels were significantly higher in the bacteria attached to cell wall debris. The mean (±standard error of the mean) ratios for RNA levels in attached/unattached

Fig. 2. Induction of *hrp* genes in *Pseudomonas syringae* pv. *phaseolicola* race 6 strain 1448A (rifampicin-resistant wild-type) and *hrpL*⁻ strains after transfer to minimal medium (in vitro). Inserts with expanded scales are included to show early changes in transcript abundance up to 40 min after incubation began. Data for other genes examined are provided as supplemental material; results are from duplicate analyses.

bacteria recorded from three experiments were 1.7 ± 0.64 , 2.3 ± 0.4 , and 2.4 ± 0.65 for *hrpA*, *hrpZ*, and *virPphA*, respectively. Therefore, in addition to the overall increase in expression detected in the plant, there was an indication of enhanced upregulation in bacteria in contact with plant cell walls.

RNA stability in vitro.

A rifampicin-sensitive strain of race 7 (1449B) was used to examine the possible contribution of RNA stability to expression of TTSS components and effectors. Time course analysis up to 120 min after incubation in minimal media confirmed the established patterns of expression in this experiment as recorded for representative transcripts *hrpA*, *hrcJ*, *hrpR*, and *hrpZ*. After 120 min, rifampicin was added to the incubation medium (final concentration $200 \mu\text{g ml}^{-1}$), and transcript levels of all target genes subsequently were determined for up to 60 min. Data obtained for selected mRNAs are given in Figure 4. An initial increase in signal was observed for most genes after addition of antibiotic, but transcript decay was subsequently evident for all genes by 5 min after addition of the RNA polymerase inhibitor. Levels dropped rapidly and were undetectable for most genes after 20 min, exceptions being the more abundant species (such as *hrpA* and *hrpZ*) and the comparatively long-lived transcript for *hrcU*. Half-lives determined for each transcript were as follows: *hrpJ*, 3 min; *hrcN*, 4 min; *hrcJ* and *avrPphE*, 4.5 min; *hrcC*, *hrpY*, and *avrPphF*, 5.5 min; *hrpP* and *virPphA*, 6 min; *hrpL*, *hrpR*, and *hrpZ*, 6.5 min; *hrpF*, 7.5 min; *hrpA*, 8 min; and *hrcU*, 16.5 min. No signifi-

cant reduction in 16S RNA was recorded during the 60-min incubation.

DISCUSSION

The exceptionally rapid induction of *hrpR* both in vitro and in planta is consistent with its proposed role as a key regulator in *P. syringae* (Xiao and Hutcheson 1994). Interestingly, the intermediate role of HrpL in the regulon could not be inferred directly from the timing of accumulation of *hrpL* RNA because other transcripts, putatively regulated by HrpL, increased at approximately the same time. Nevertheless, the absence of induced expression of effector and *hrp* genes (except *hrpR*) in the *hrpL* mutant confirmed the importance of HrpL in the regulatory cascade. The rapid induction of *hrpR* observed here has not been reported for other pathovars of *P. syringae* or strains of *P. syringae* pv. *phaseolicola* in which promoter fusions were used to assess expression (Bretz et al. 2002; Rahme et al. 1992). We also found that *hrpR* was at least partially regulated by HrpL, indicating the existence of a previously unrecognized autoregulatory loop in what has come to be considered a unidirectional expression cascade from *hrpRS* $\rightarrow$ *hrpL* $\rightarrow$ *hrp* and *effector* genes (Xiao and Hutcheson 1994). However, autoregulation has been proposed in the similar cascade described in *Erwinia herbicola* pv. *gypsophylae* (Nizan-Koren et al. 2003). Regulatory controls, in addition to HrpL, allowing more gene-specific fine tuning of transcription were also indicated by the clear differences between mRNA levels recorded in *P. syringae* pv. *phaseolicola*. In

Fig. 3. Induction of *hrp* genes in *Pseudomonas syringae* pv. *phaseolicola* race 6 strain 1448A (rifampicin-resistant wild-type) in leaves of the susceptible bean cv. Canadian Wonder. Data for other genes examined are provided as supplemental material; results are from duplicate analyses.

particular, basal levels of *hrpA* and *virPphA* transcripts were at least 10-fold higher than those of other genes examined in either wild-type or *hrpL* background. The other gene to reach high mRNA levels was *hrpZ* which encodes the readily secreted “harpin” protein (Lee et al. 2001).

Table 2. Accumulation of transcripts in vitro and in planta by *Pseudomonas syringae* pv. *phaseolicola* 1448A (race 6)^a

Transcript	Position in operon	Time until first twofold increase over basal levels (min) ^b	
		In vitro	In planta
<i>hrpR</i>	1 of 1	<5	5 to 10
<i>hrpL</i>	1 of 1	10	20
<i>hrpA</i>	1 of 1	10	20 to 40
<i>hrpZ</i>	1 of 1	10 to 20	20 to 40
<i>hrcC</i>	3 of 5	20	40 to 80
<i>hrpF</i>	1 of 5	20	20 to 40
<i>hrcJ</i>	1 of 3	20	20
<i>hrpP</i>	1 of 7	20	20 to 40
<i>hrpY</i>	1 of 1	20	20 to 40
<i>avrPphE</i>	1 of 1	20	20 to 40
<i>hrpJ</i>	1 of 5	40	40 to 80
<i>hrcN</i>	4 of 5	40	80 to 120
<i>virPphA</i>	1 of 1	40 to 80	40 to 80
<i>hrcU</i>	7 of 7	80	80 to 120

^a Basal level is defined as the level of transcription after washing bacteria in 10 mM MgCl₂ before inoculation.

^b Data compiled from two repeated experiments using duplicate analyses at each time point.

Regulation by HrpL has been linked to the presence of the *hrp* box motif upstream of *hrp* and effector genes (Fouts et al. 2002). There are variations in the region among the genes examined here. In particular, the motif found upstream of *avrPphE* is quite divergent from the general consensus but the effector is clearly regulated by HrpL. For *virPphA*, the second conserved motif is CCAA rather than the consensus CCAC. There is, however, no obvious link between patterns of gene expression observed and *hrp* box sequence; for example, through the presence of 13, 15, or 16 nucleotides between the conserved GGAACC and CCAC motifs as listed in Table 3.

Two additional regulators of *hrp* gene expression reported in *P. syringae* are HrpV and Lon protease (Bretz et al. 2002; Preston et al. 1998). It is difficult to attribute the different patterns of expression we have observed to the action of either of these proteins. The Lon protease has been reported to act directly on HrpR protein and thereby provide a more general downregulation of HrpL and downstream gene expression. HrpV is thought to operate by reducing transcription of *hrpR* and also would be expected to have a more general role. The involvement of HrpV in the fine tuning of early transcript accumulation also seems unlikely because it is the fifth gene of the *hrpF* operon and *hrpF* mRNA itself follows the most common pattern of *hrp* transcript accumulation (Table 2).

The patterns of induction observed were significantly different in timing and quantity in the plant compared with in vitro. In particular, more rapid onset of transcript accumulation was found in vitro, but levels reached after 2 h typically were sev-

Fig. 4. Decay of mRNA in *Pseudomonas syringae* pv. *phaseolicola* race 7 strain 1449B after the inhibition of transcription with rifampicin (200 µg ml⁻¹), following a 2-h incubation in minimal medium. Half-lives were estimated to the nearest half-minute from the time that mRNA levels began to decay. Data for other genes examined are provided as supplemental material; results are from duplicate analyses.

eral-fold greater in planta. In vitro, bacteria are exposed immediately to a stable inducing environment; whereas, in the plant, conditions are more dynamic. The 10-mM MgCl₂ solution used to suspend bacteria for injection would be expected immediately to solubilize material from the apoplastic face of the plant cell wall. The solution is taken up by the transpiration stream as the inoculum effectively evaporates, so that no water-soaking is observed after approximately 30 min. Bacteria are, therefore, deposited on the cell wall as the aqueous suspension dries out (Brown and Mansfield 1988). The conditions on the plant cell wall surface would appear to be highly inductive. Our results also suggest that that contact with the cell wall may itself enhance transcription of *hrpA*, *hrpZ*, and *virPphA*. Whether or not specific plant factors are required for the extremely rapid mRNA synthesis observed between 80 and 240 min in the plant (Fig. 3) remains to be determined. However, in a critical analysis of such factors it would be extremely difficult to mimic the in planta micro-environment, not only regarding nutritional status but also the purely physical conditions of life on the apoplastic face of the plant cell wall. The drop in transcript levels recorded between 4 and 24 h after inoculation into leaves indicates that neither the TTSS nor effectors are required for colonies to expand following the initial establishment of parasitism. The nutritional conditions established within colonized tissue may be equivalent to those found in suppressive rich media.

Transcription of flagellar genes is regulated in a strictly hierarchical fashion involving the sequential expression of three classes of transcript encoding activator, basal body, and filament components (Karlinsey et al. 2000; Kurtsukake et al. 1990; Soutourina and Bertin, 2003). Contrary to our original hypothesis, a similar synthetic hierarchy has not emerged from our experiments with the TTSS in *P. syringae* pv. *phaseolicola*. In the plant pathogen, it appears that genes encoding effector proteins secreted through the TTSS are expressed alongside (or before) those for core components of the secretion. Intuitively, one would expect levels of secreted proteins to be higher than those of membrane-anchored TTSS components. For example, numerous copies of extracellular HrpA are required to construct the Hrp pilus which must traverse the plant cell wall extending from a single-basal TTSS unit. The transcript levels recorded largely reflect the differential protein levels that would be expected despite all genes being regulated primarily by HrpL. For example, the abundance of RNA from *hrpA*, *hrpZ*, and *virPphA* is much greater than that of the core components *hrcC*, *hrcJ*, and also *hrcU*, for which the slowest induction and lowest abundance was recorded. *hrcU* encodes an inner membrane component of the TTSS and is the seventh open reading frame (ORF) within the *hrpP* operon (Fig. 1). Irrespective of proposed function, mRNA from the first ORF of each *hrp* operon typically accumulated more rapidly than those from downstream ORFs, as summarized in Table 2.

Protein expression may be regulated at the level of transcription or translation, and also by the rapidity with which mRNAs

are degraded by endogenous RNases. The *hrpA* transcript of *P. syringae* pv. *tomato* strain DC3000, for example, appears to be stabilized by its 5' untranslated RNA, without which production of the HrpA protein is greatly reduced (Hienonen et al. 2002). We tested the relative stability of *hrp* and effector gene transcripts in *P. syringae* pv. *phaseolicola* race 7 by measuring their abundance after RNA synthesis was arrested by rifampicin. Most previous studies on RNA stability in bacteria have been carried out on *E. coli*, where 80% of mRNAs can be expected to have a half-life of between 3 and 8 min (Bernstein et al. 2002). Almost all of the RNAs examined in this study had half-lives within this range, the only exception being *hrcU*, which had a half-life of 16.5 min. The *hrcU* coding region is located at the 3' end of the *hrpP* operon (Fig. 1). Studies on *E. coli* (Selinger et al. 2003) and *Acinetobacter calcoaceticus* (Schirmer and Hillen 1998) have shown that RNA degradation is most likely to occur in the 5' to 3' direction. Therefore, the relatively long-lived *hrcU* transcript may be well protected by RNA further toward the 5' end of the operon. The stability of *hrcU* RNA might compensate for its low abundance.

It is possible that high levels of expression of proteins such as HrcU, which are targeted to membranes, might be harmful to bacteria. For example, overexpression of the *hrcC* homologue in *Xanthomonas* spp. induces transcription of phage-shock (*psp*) genes when expressed in *E. coli* (Wengelnik et al. 1996). Expression of the *psp* operon of *Y. enterocolitica* is also activated when a functional TTSS is present (Darwin and Miller 2001), perhaps to limit potential damage caused by TTSS membrane-spanning proteins.

Organization of the TTSS (like the flagellum) provides an excellent model for understanding regulation of cellular architecture within gram-negative bacteria. Real-time PCR has proved to be a sensitive means to examine gene expression within the host plant as well as in vitro. Application of the approach of quantitative analysis of gene expression that we have developed here should help to unravel the puzzle of how 30+ effector proteins might be delivered in a strict hierarchy through the Hrp pilus and into plant cells (Fouts et al. 2002). Regulation of effector delivery has been proposed to operate via protein chaperones (Guttman et al. 2002; Thomas and Finlay 2003), but the subtle transcriptional controls revealed by our experiments indicate that transcript abundance and stability may also have a key role in the establishment of parasitism by allowing the sequential and selective accumulation of the effector proteins that suppress plant defenses.

MATERIALS AND METHODS

Preparation of inoculum.

Rifampicin-resistant strains of *P. syringae* pv. *phaseolicola* isolates 1488A (race 6) and 1449B (race 7) were grown at 25°C overnight in LB medium (10 g liter⁻¹ of tryptone, 5 g liter⁻¹ of yeast extract, 10 g liter⁻¹ of NaCl, pH 7.0) containing rifampicin at 80 µg ml⁻¹. Strain 1448A::143, a race 6 strain carrying

Table 3. *Pseudomonas syringae* pv. *phaseolicola* races 6 and 7 *hrp* box sequences, showing spacing between 5' and 3' conserved regions^a

Gene	<i>hrp</i> Box sequence	Spacing (nt)
<i>hrpA</i>	GGAACCGATT TAAGGGTCGTTACCA CCTA	16
<i>hrpF</i>	GGAACCGCTC CAAGCCGTTTGCTCCACT CA	16
<i>hrcJ</i>	GGAAC TTGGGCAGGATGTGCCACA AG	13
<i>hrpJ</i>	GGAACCGAAT CTTGCCCAAGGCCCACT CA	16
<i>hrpP</i>	GGAAC TGAAATCGATGCTCGACCACT TA	15
<i>hrpY</i>	GGAACCAACT CGCACGCAAAACCA CACA	15
<i>avrPphF</i>	GGAAC CAGTACATAGGTATGACCACT GA	16
<i>virPphA</i>	GGAACCACTT ATGTCCGATCGCCAAC TCA	15
<i>avrPphE</i>	GCAAC GCCGGTTATCGGCTGGGCCAT CGA	16

^a Conserved regions, according to Fouts et al. (2002), are marked in bold; nt = nucleotides.

an insertional inactivation of *hrpL* (Mansfield et al. 1994) was grown overnight in LB supplemented with rifampicin at 80 µg ml⁻¹ and kanamycin at 40 µg ml⁻¹. Cells were washed twice in 10 mM MgCl₂ and adjusted to an optical density at 600 nm of 0.6 (corresponding to an approximate concentration of 5 × 10⁸ CFU ml⁻¹).

In vitro induction.

After quantification, 100 ml of washed cell suspension was centrifuged and resuspended in 100 ml of minimal medium, pH 7.0 (Huyhn et al. 1989) containing 10 mM fructose, pre-warmed to 25°C. Minimal medium cultures were shaken at 25°C and 0.5-ml aliquots removed, usually after 5, 10, 20, 40, 80, and 120 min. Samples were also taken from LB cultures before washing and from the second MgCl₂ wash. Samples were mixed immediately with 1 ml of RNA protect reagent (Qiagen, Crawley, U.K.) to arrest RNA synthesis and degradation, incubated at room temperature for 5 min, and pelleted at 5,000 × g for 10 min. Pellets were frozen at -70°C until RNA extraction was performed. Prior to RNA extraction, pellets were resuspended in 100 µl of Tris-EDTA (TE) containing 80 µg of lysozyme (Sigma, Poole, U.K.) and incubated for 10 min at room temperature.

Bean inoculations.

French bean plants (*Phaseolus vulgaris* L. cv. Canadian Wonder) were inoculated with bacterial suspensions (prepared as above) as previously described (Harper et al. 1987), taking care to remove any bacterial suspension adhering to the outer surfaces of the leaves. Plants were maintained at 25°C throughout the course of the experiment and samples were usually taken at intervals of 5, 10, 20, 40, 80, 120, 240, and 1,440 min after inoculation.

Extraction of total bacterial RNA from inoculated leaves.

Discs, approximately 9 mm in diameter, were cut from inoculation sites in leaves and frozen immediately in liquid N₂. Two discs were macerated in 500 µl of 1× RNA protect reagent (Qiagen), incubated at room temperature, and centrifuged at 5,000 × g for 10 min. Pellets were resuspended in 100 µl TE containing 100 µg of lysozyme (Sigma) and incubated at room temperature for 10 min. RNA was extracted immediately from lysates as described below.

Fractionation of attached and unattached bacteria from inoculated leaves.

Discs were cut from leaves, frozen, macerated, and incubated in RNA protect reagent as described above. The resulting

suspensions then were spun through 10-µm filters (VectaSpin Micro, Whatman, Maidstone, U.K.) for 30 s at 1,000 × g. The filtrate (containing bacteria not attached to leaf tissue) was spun for a further 10 min at 5,000 × g and the resulting pellet resuspended in 100 µl of TE containing 100 µg of lysozyme (Sigma) and incubated at room temperature for 10 min. The filter residue (containing plant debris and attached bacteria) was mixed with 100 µl of TE-lysozyme mix as above, incubated for 10 min at room temperature, and spun again through the filter for 30 s at 1,000 × g. The resulting filtrate contained predominately RNA released from bacteria attached to plant material. RNA was extracted immediately from lysates as described below.

Stability of mRNAs in vitro.

The rifampicin-sensitive wild-type race 7 strain 1449B was grown overnight in LB, washed, and transferred to minimal medium, pH 7.0, containing 10 mM fructose as described above. Induction of TTSS genes was allowed to proceed for 2 h, during which samples were taken as described above for in vitro induction. RNA transcription then was arrested by addition of rifampicin to a final concentration of 200 µg ml⁻¹ and, after a further 1, 2, 5, 10, 15, 20, 30, 45, and 60 min, 0.5-ml aliquots were mixed with 1 ml of RNA protect reagent and prepared for RNA extraction as described above.

Isolation of nucleic acids.

Genomic DNA was isolated from strains of *P. syringae* pv. *phaseolicola* as previously described (Thwaites et al. 1999). Plasmid DNA was isolated from *E. coli* strains using the Qiagen plasmid mini kit (Qiagen). RNA was isolated from cell lysates using the RNeasy bacteria mini kit, using the on-column DNase kit (Qiagen) to remove contaminating DNA. RNAs were eluted in either 100 µl (in vitro inductions) or 30 µl (in planta inductions) of sterile water and were treated a second time with DNase (Promega, Southampton, U.K.) in order to ensure complete removal of genomic DNA.

Synthesis and testing of cDNAs.

RNA from cells induced in vitro was quantified by spectrophotometry and 2 µg of RNA was used for each cDNA synthesis. RNA levels obtained from in planta samples were too low to be reliably measured by this method; therefore, in these cases, the whole RNA extract was used for cDNA synthesis. cDNAs were synthesized using the Omniscript reverse transcriptase system (Qiagen) with either a mixture of oligonucleotide primers specific to the target genes (reverse primers used for cloning targets listed in Table 4) or random hexanu-

Table 4. Primers for real-time polymerase chain reaction (PCR) experiments: sequences are quoted 5' to 3'

Target	Primers for cloning targets		Primers and probes for TaqMan real-time PCR		
	Forward	Reverse	Forward primer	Reverse primer	Probe
<i>hrpA</i>	tcatgattctctgactaacgcg	actggacgaccgagttccgg	cagggtatcaacacgcgtcaaga	gctaccctgtttttggtcagt	cagcgccgaccgcaaca
<i>hrpC</i>	cgccctgatcggtgactcaggtca	gagcagtgatggccgactctct	gcgaacactgccatttgaaa	acaacgcgaacacccaat	cccgcgcccggatcacaaa
<i>hrpF</i>	cgcagacgactgtaatcgc	gcggatcaatggcggaagg	agggtggcgacgcgacattt	cgatgacatttttggctattt	tcaacatcgctgaccagtgcga
<i>hrpJ</i>	gtcgcagatagacggtgtcatc	gcgactccgcgccgccaac	cgcgccgctgattcatca	tttctcgattctgccgattg	cactcggtcgctggacc
<i>hrpJ</i>	cacgtgtgtctgcaagcgcc	agcaggtgttcacaaccgcgc	gctcaccacaacgctgaaac	cgccctggcagttatattgc	cgcaagttcggaccacacgcc
<i>hrpL</i>	gtaatgaacccggatgatctcg	cccgtgccggggcagctcg	gcgcaacgagcacaagttt	ggttcaacgcaataccacaaa	acatgccagcaagccgcagacc
<i>hrpN</i>	ggaagacgcccacgcagcagc	gcgaagcaccgagggcaatcac	gccaagggtggcgatctt	attctgagtgaaaccgagatt	ctgcctgcgcttgcctcct
<i>hrpP</i>	acacagccttctgcggccgc	tcagaacgtcgcgacggcgga	tgatacctcaggtcggtagaag	gggagggcgatgctgc	tcagcaaggtttcggcgcc
<i>hrpR</i>	ggttaagacacctggccaatcg	tgttccaccagttcatccagagg	cccgaatcgttggcagaa	cgagcgcggcagaca	tgcgccattgacgacaccgaataa
<i>hrpU</i>	cagtcggaaggcgatcctcatg	ctcaacctgaatcacttcatcg	ggccttgaagctggacatca	ttctgcccggaaaacatctg	aagatcaacccttctcgatgccaa
<i>hrpY</i>	cgcttcgaacttcagcagggg	ggacgctcgcgacccatc	gagttctctgcaaacgcagataa	acttcacgctcaccattaacga	atcagcggcgacgacgcttcat
<i>hrpZ</i>	tggcgagtcacagaatgcagc	cagcagcatgttgaccagcagc	atccgcttatcgacgctaacc	gtctatcagctcaccgatcaactg	cccgcagcaacagcaattcca
<i>virPphA</i>	cgaatacgcgttgcgtagactg	caagccttctgtgttcagcgg	tcacaattttagtcaattgcgtaca	cgcatctctgaaacccaactc	tctcgaaagcagatgctgaatctga
<i>avrPphE</i>	cgcgcgagcccttagcgt	cggcgtgcttcacgcaacgc	cctagcgtggaaaccactgaa	cggtctgtttgtgaactgcaaga	atcatcgccgcagaaacccgc
<i>avrPphF</i>	ggaacacgactacatagtatgac	ggatcacatggctgtcagaccg	ggggactcctcaggaattcg	aggaaaaccaggcaccata	cgcttattcgatgggctcgca
<i>16S</i>	cgggtactgtacctggtggc	cttgccagttttgtagcagttc	caatggcggaagcctgat	agtgtttacatccgaagacctt	agccatgccgctgtgtga

cleotides (Promega). After synthesis, cDNAs were diluted 1:20 in TE and tested by PCR using a selection of primers listed in Table 4. An aliquot of RNA was also tested by PCR to ensure that no trace of contaminating genomic DNA was detectable.

PCR reactions.

All PCR reactions were carried out in 15- μ l volumes containing 1 $\times$ PCR buffer (16 mM (NH₄)₂SO₄, 67 mM Tris-HCl [pH 8.8], 0.01% Tween-20), 1.5 mM MgSO₄, 200 μ M each dNTP, 1 μ M each primer, and 1.5 units of *Taq* DNA polymerase. PCR cycling was performed in a Perkin-Elmer 9700 thermocycler using the following program: 95°C for 30 s followed by 30 cycles of 94, 55, and 72°C for 30 s each. Reaction products were visualized on ethidium bromide-stained agarose gels.

DNA sequencing.

Sequences upstream of a selection of target genes were amplified with the following primers (forward/reverse primers, 5' to 3'): *hrpA* (tggagttggacatgccacgc/ctcttgacgtgttgatcacctgc), *hrpF* (gcctgcgtctgagcgatgcc/caagcacgtatccacgtcgg), *hrcJ* (ctgctgcctgactgataccc/gcaccgcttgaaacattatccg), *hrpJ* (ctatagcgataac cgtgccagcc/gcgaatcggcgtgaccgtgtgg), *hrpP* (cactggaagacgatcc gcacgg/gccatcggtcgatcgatcttcgg), *hrpY* (ggatcggcgcaacacgctagg c/ctcagcagcagttcgccagagg), *hrpZ* (gaccgcaacattgactgacc/acg accgagttccggatcttcg), *avrPphE* (cattgattgacagtacgagagcg/ctg ttgtgaactgcaagaagcg), and *virPphA* (catatgcagctgagtgaaacag c/gatggctctgctcgttgatacc). PCR products were purified from agarose gels with the min-elute gel purification kit (Qiagen) and cloned using the pGEM-T easy PCR cloning system (Promega). Plasmid clones were sequenced using the BigDye cycle sequencing kit and an ABI310 sequence analyzer (ABI, Warrington, U.K.).

TaqMan real-time PCR.

All target genes for real-time PCR analysis were amplified using the primers given in Table 4. Reaction products were purified using the min-elute PCR purification kit (Qiagen) and cloned using the pGEM-T Easy PCR cloning system (Promega). Plasmid clones of all targets were quantified by spectrophotometry for use as quantitative standards in real-time PCR.

TaqMan PCR reactions were performed in 25- μ l volumes containing 12.5 μ l of qPCR Mastermix Plus solution (consisting of thermostable polymerase, buffers, and reference dye) (Eurogentec, Romsey, U.K.), 100 nM forward and reverse primers, 300 nM double-labeled oligonucleotide probe, and 5 μ l of cDNA template. Oligonucleotide probes were labeled with FAM and ELLE dyes at the 5' and 3' ends, respectively. Sequences of TaqMan primers and probes (purchased from Oswel/Eurogentec, Romsey, U.K.) are given in Table 4. Primers were designed to yield reaction products of between 70 and 120 bp in length. PCR amplification was as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 95°C (15 s) and 60°C (1 min). Reactions were run in an ABI PRISM 7700 sequence detection system and data were collected at all isothermal stages. Following PCR amplification, threshold detection parameters (baseline and cycle threshold limit) were adjusted manually to optimize the results relative to standard curves which were linear over the concentrations tested, obtained by amplification of four 10-fold dilutions of clones of all target sequences. Duplicate samples were quantified for each transcript and mean values used to express mRNA abundance relative to 16S RNA.

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