Genome-Wide Analysis of Gene Expression in *Ralstonia solanacearum* Reveals That the *hrpB* Gene Acts as a Regulatory Switch Controlling Multiple Virulence Pathways

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Submitted 31 March 2005. Accepted 7 May 2005.

A 70-mer oligonucleotide-based DNA microarray covering 5,074 of the 5,120 predicted genes from *Ralstonia solanacearum* has been generated and used to investigate the repertoire of genes that are under the control of the transcription activator HrpB, which governs pathogenicity in this plant pathogenic bacterium. This study identified 143 *hrpB* up-regulated genes and 50 *hrpB* down-regulated genes. In addition to extending the repertoire of type III effector proteins with 26 new candidates, this work demonstrates that the *hrpB* regulon extends beyond type III secretion system-related functions to include a number of genes governing chemotaxy, biosynthesis or catabolism of various low-molecular-weight chemical compounds, and siderophore production and uptake. The presence of several transcriptional regulators and a cluster of genes predicted to encode the synthesis of an acyl-homoserine lactone together with the absence of a consensus *hrpB* box in the promoter of a significant proportion of the *hrpB*-regulated genes suggest that, for some genes, *hrpB* regulation might be indirect. Altogether, the data indicate that *hrpB* acts as a master regulatory gene governing a physiological swing associated with the shift from saprophytic to parasitic life.

Ralstonia solanacearum is a gram-negative β -proteobacterium pathogenic to plants and responsible for the development of bacterial wilt, a severe and devastating plant disease affecting over 200 plant species (Hayward 2000; Prior et al. 1997). Wilting symptoms occur as a consequence of an intensive multiplication of the bacteria within xylem vessels and are associated with the production of copious amounts of exopolysaccharides that act by blocking water traffic in the plant. Molecular and genetic approaches have identified a wide range of genes and functions that play a role in the establishment and multiplication of the bacterium within plant tissues leading to development of the disease (Genin and Boucher 2004; Schell 2000). Such studies have also identified a large set of regulatory

genes governing pathogenicity and have demonstrated that these genes are organized in complex regulatory networks (Schell 2000). Large-scale gene transcription analyses have revealed that many proteins that act in concert are coregulated at the transcriptional level (Grigoriev 2001; Laub et al. 2000). Therefore, genes that are coregulated together with core pathogenicity genes could also be involved in the plant infection process.

Although complete sequences of the genomes of several plant pathogenic bacteria are now available (Plano 2004; Van Sluys et al. 2002), very few studies have sought to make an exhaustive search for the target genes of a transcriptional regulator involved in the control of pathogenicity (Fouts et al. 2002; Noël et al. 2001; Zwiesler-Vollick et al. 2002). In *R. solanacearum*, the widest study toward this goal concerns the definition of the *hrpB* regulon. The *hrpB* gene encodes a positive activator required for the transcription of other *hrp* genes that are involved in the production of a type III secretion system (TTSS), a key pathogenicity determinant, since mutants deficient in the corresponding *hrp* genes are no longer able to cause disease (Alfano and Collmer 2004; Buttner and Bonas 2002). As in many pathogens of plants, animals, or humans, the *R. solanacearum* TTSS allows injection of bacterial proteins, called effectors, into plant cells upon contact of the bacteria with host tissues (Aldon et al. 2000; Cunnac et al. 2004b; Genin and Boucher 2004). The HrpB protein also acts as a positive activator required for the transcription of the effector protein genes (Cunnac et al. 2004b; Guéron et al. 2000), and a recent study identified a consensus motif called the *hrpB* box in the promoters of *hrp* and several effector genes (Cunnac et al. 2004a). A systematic search for this consensus in the promoter region of all *R. solanacearum* genes led to the identification of a set of new candidate effector genes (Cunnac et al. 2004b). On the other hand, other candidate effector genes have been identified as being part of the *hrpB* regulon, although they lack a *hrpB* box in the promoter, indicating that the search for this motif is not sufficient and that additional genes remain to be identified (Cunnac et al. 2004b).

Here, we report the construction of a microarray covering almost all of the genes identified in strain GMI1000 of *R. solanacearum* and on its use for transcriptome analysis, in order to produce an exhaustive view of *hrpB*-regulated genes and functions.

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*The e-Xtra logo stands for “electronic extra” and indicates the HTML abstract available on-line contains a supplemental table that lists the primers used in this study and is not included in the print edition.

RESULTS

Construction of a *R. solanacearum* DNA microarray and experimental design.

A microarray consisting of 5,086 long oligonucleotides deposited in duplicate and representing 5,074 of the 5,120 predicted protein-coding genes identified in the genome of strain GMI1000 of *R. solanacearum* was constructed. The missing oligonucleotides correspond to IS elements already present on the array or to very short putative genes for which an appropriate oligo could not be designed. This microarray also includes three oligonucleotides corresponding to the nontranscribed strand of three genes from *R. solanacearum* GMI1000 (RSp0855, RSp0860, and RSp0861), 10 oligonucleotides corresponding to five *Corynebacterium glutamicum* genes that can be used as possible “spike” controls, and 26 additional controls consisting of oligonucleotides that do not match with any GMI1000 genomic sequence. In addition, the microarray also includes a set of “blank” controls in which buffer without oligonucleotides was spotted.

The *hrpB* gene acts both to activate and repress gene expression.

In order to characterize the *hrpB* regulon, the transcriptome of the wild-type strain GMI1000 was compared with the transcriptome of a *hrpB*-deficient derivative GMI1525 (Table 1). This analysis revealed 111 genes that are down-expressed in the *hrpB* mutant, including 55 of the 70 *brg* genes (*hrpB*-regulated genes) that had previously been identified (Cunnac et al. 2004b; Genin et al. 1992; Guéron et al. 2000). Of the 56 newly identified genes, 44 of the *hrpB* up-regulated genes thus defined belong to 17 putative operons, whereas the remaining genes are most likely monocistronic. A total of 18 of the 56 new genes are preceded by a *hrp_{II}* box. Three other genes might belong to multicistronic *hrp_{II}* box-harboring operons, and 12 are preceded by an imperfect *hrp_{II}* box (TTCGN₁₆TTCG with one mismatch allowed). For the 22 remaining ones, no *hrp_{II}* box is present in the region spanning 500 base pairs upstream of the start codon of the cognate gene or of the first gene of the operon. These experiments also identified one gene, RSp0785, whose transcription was down-regulated by *hrpB*, i.e., at least four times higher in the *hrpB* mutant as compared with the wild-type strain.

Transcriptome of a strain expressing *hrpB* at a high level reveals additional genes whose expression is controlled by HrpB.

Transcription of *hrpB* is increased when bacteria are in contact with plant cells, and this induction results in increased transcription of *hrpB* up-regulated genes (Aldon et al. 2000; Marenda et al. 1998). This plant signaling effect is at least partly mimicked at the transcriptional level by introduction of the plasmid pAM5, which carries a cloned copy of *hrpB*, into strain GMI1000 (Guéron et al. 2000). This strategy has already been used to demonstrate *hrpB*-dependency for certain lowly expressed genes for which this trait was difficult to establish by comparing the *hrpB* mutant to the wild-type strain (Cunnac et al. 2004b). Therefore, we compared gene expression in strain GMI1728, a GMI1000 derivative that carries pAM5, with that of the *hrpB*-deficient mutant GMI1525(pLAFR3), which carries the corresponding empty vector. Columns 4 and 5 of Table 1 show the results of these experiments and confirm the *hrpB*-dependency for 101 of the 111 previously identified genes. In addition, these experiments identified 32 additional *hrpB* up-regulated genes. A total of 16 of these additional genes were detected in the previous experiment but were not selected, since the log₂ value of their induction ratio was lower

than 2 (column 2 of Table 1) when strains GMI1000 and GMI1525 were compared. Moreover, 18 of the 32 genes harbor a *hrp_{II}* box (allowing one mismatch) in their promoter. The 32 additional genes include nine of the 15 known *hrpB*-regulated genes that were not detected when comparing the transcriptomes of strains GMI1000 and GMI1525.

A total of 48 *hrpB* down-regulated genes were identified. For eight of these genes, repression was also observed in the previous experiment with a cutoff *P* value of 0.05. Reciprocally, repression of RSp0785, which was observed when comparing GMI1000 and GMI1525, is also detected in the comparison between the *hrpB*-deficient mutant and the *hrpB*-overexpressing strain with a *P* < 0.05 cutoff threshold. The majority of these HrpB-repressed genes are organized in putative operons.

Finally, four genes, organized in two putative adjacent operons (RSc1804 to RSc1805 and RSc1807 to RSc1808), had a particular behavior, since they were *hrpB* up-regulated in the comparison of GMI1000 with GMI1525 and down-regulated when *hrpB* was overexpressed.

Put together, the data obtained from the two sets of experiments allowed the distinction of four classes of genes based on their regulation patterns and on the structure of their promoters (Table 1). Class I and Class II genes correspond to genes whose transcription is up-regulated by *hrpB* in at least one of the two experimental conditions tested. Class I genes have a *hrp_{II}* box in the promoter, whereas Class II genes do not and could be regulated by *hrpB* in an indirect manner. We were unable to identify a common motif in the promoters of Class II genes. Class III genes include genes whose transcription is directly or indirectly repressed by HrpB, and the four remaining genes were grouped into Class IV. These genes require *hrpB* for induction but are repressed when *hrpB* transcription is increased.

Validation of the *hrpB*-dependency of representative genes.

In order to validate the data from the transcriptome analysis, the *lacZ* reporter gene from *Escherichia coli* was transcriptionally fused with seven genes from classes I and II, encoding candidate type III effectors, as predicted on the basis of the biased amino acid composition of the 50 residues located at the N terminus of the corresponding proteins (Cunnac et al. 2004b). Expression of the resulting gene fusions was compared in wild type and in a *hrpB*-deficient or *hrpB*-overexpressing background of *R. solanacearum*. As shown in Table 2, the resulting data clearly confirmed that transcription of these genes is dependent on the presence of a functional *hrpB* gene.

In a complementary set of experiments, semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) experiments were conducted on mRNA populations obtained from the wild-type strain GMI1000, from the *hrpB*-deficient mutant GMI1525 and from strain GMI1728 (i.e., overexpressing *hrpB*). The data presented in Figure 1 and summarized in column 10 of Table 1 demonstrate the *hrpB*-dependent regulation for 28 of the 32 genes tested and confirm the negative regulatory effect of *hrpB* on expression of genes from Class III. Concerning Class IV genes, although the *hrpB*-dependent transcription of Class IV gene RSc1807 was confirmed, no repression was observed when multiple copies of *hrpB* are present in the strain. However, the fact that two genes from the same bicistronic operon have a common gene expression pattern is a good indication that Class IV actually corresponds to a specific pattern of regulation.

Because the present study is the first one to establish a negative regulatory function for *hrpB* and because it is also the first time we identify *hrpB*-regulated genes that encode proteins not associated with TTSS functions, quantitative RT-PCR was

Table 1. Summary of *hrpB*-regulated genes identified based on transcriptome analysis^a

Gene ID	1000/1525 ratio ^b	P-value	pam5/1525 ratio ^b	P-value	<i>hrpH</i> box ^c	<i>brg</i> genes ^d	Export signal ^e	Corresponding protein or predicted function ^f	RT-PCR validation ^g
Class I									
RSc0041	1.92	0.000	2.19	0.000	++		3	Hypothetical / Unknown	
RSc0245	3.22	0.000	3.59	0.000	+	+	3	TTSS-effector RipB	
RSc0608	7.38	0.000	6.84	0.000	+	+	3	TTSS-effector AvrA	
RSc0609	4.65	0.017	1.56	0.000	in operon?		0	Hypothetical / Unknown	
RSc0616	6.95	0.000	8.05	0.000	in operon?		0	Hypothetical / Unknown	2
RSc0617	6.69	0.000	7.94	0.000	+		1	Hypothetical / Unknown	2
RSc0824	1.73	0.023	2.39	0.000	++		1	Conserved hypothetical protein	
RSc0825	2.16	0.000	3.98	0.000	++		2	Hypothetical / Unknown	
RSc0826	1.73	0.000	3.60	0.000	+		3	TTSS-effector PopP1	
RSc0868	3.39	0.004	4.28	0.000	+	+	3	TTSS-effector PopP2	
RSc0894	7.01	0.000	7.46	0.000	++		1	Hypothetical / Unknown	1
RSc0895	4.18	0.000	3.88	0.000	+		0	Partial homology to <i>Pantoea agglomerans</i> pv. <i>gypsophi</i>	2
RSc1349	2.61	0.001	3.08	0.000	+	+	3	Partial homology to <i>Salmonella</i> TTSS-effector SspH	
RSc1356	4.22	0.001	4.62	0.000	+	+	3	TTSS-effector RipG (LRR protein, GALA subfamily)	
RSc1357	6.06	0.000	6.84	0.000	+	+	3	LRR protein, GALA subfamily	
RSc1386	3.07	0.000	3.72	0.000	++	+	3	Hypothetical / Unknown	1
RSc1474	5.74	0.000	6.60	0.000	+	+	0	Hypothetical / Unknown	
RSc1475	3.21	0.000	4.06	0.000	+	+	3	Hypothetical / Unknown	
RSc1800	5.50	0.000	6.18	0.000	+	+	3	LRR protein, GALA subfamily	
RSc1801	1.43	0.001	2.22	0.000	+	+	3	LRR protein, GALA subfamily	
RSc2101	1.57	0.001	4.00	0.000	++		3	Hypothetical / Unknown	2
RSc2133	2.72	0.002	nda		++		2	Hypothetical / Unknown	
RSc2147	3.04	0.000	2.95	0.000	++		0	Sorbitol dehydrogenase	
RSc2359	4.01	0.000	3.95	0.000	+	+	3	Hypothetical / Unknown	
RSc2661	3.32	0.001	5.15	0.000	++		0	Transcription regulator (repressor, XRE family)	0
RSc2775	7.03	0.000	6.41	0.000	++	+	0	HrpW family harpin	
RSc2897	5.22	0.000	4.49	0.000	+		2	Hypothetical / Unknown	1
RSc3133	2.01	0.001	2.81	0.000	++		0	Acetyl transferase	
RSc3155	3.34	0.000	4.17	0.000	++		3	Streptogramin lyase-related protein	0
RSc3174	nda		2.74	0.000	+	+	1	Hypothetical / Unknown	
RSc3212	3.35	0.002	2.83	0.000	+	+	3	TTSS-effector RipT	
RSc3272	2.70	0.000	2.47	0.000	+	+	0	Hypothetical / Unknown	
RSc3290	4.05	0.000	4.32	0.000	+	+	0	Hypothetical / Unknown	
RSc3369	2.28	0.002	2.63	0.000	+	+	3	AvrPphE family protein	
RSp0028	2.73	0.001	1.09	0.004	+		3	LRR protein, GALA subfamily	
RSp0098	5.01	0.000	2.89	0.000	in operon?		1	Hypothetical / Unknown	
RSp0099	4.53	0.000	3.06	0.000	+	+	3	TTSS-effector RipA	
RSp0160	3.62	0.000	4.13	0.000	+	+	2	Hypothetical / Unknown	
RSp0193	nda		2.25	0.000	++	+	3	PPR repeat containing protein	
RSp0213	6.59	0.000	7.29	0.000	+	+	3	Hypothetical / Unknown	
RSp0215	1.80	0.000	3.53	0.000	+		2	Hypothetical / Unknown	
RSp0218	2.72	0.007	4.68	0.000	+	+	3	Hypothetical / Unknown	
RSp0304	7.30	0.000	7.94	0.000	+	+	3	AvrPphD family protein	
RSp0323	2.47	0.000	3.52	0.000	+	+	1	Hypothetical / Unknown	
RSp0572	4.10	0.000	5.72	0.000	+	+	1	Hypothetical / Unknown	
RSp0693	7.98	0.000	7.44	0.000	+		1	Glyoxalase I (S-lactoylglutathione-lyase)	1
RSp0694	6.81	0.000	6.90	0.000	+		0	Tryptophan-2,3-Dioxygenase	1
RSp0695	6.37	0.000	5.69	0.000	+		0	Dehydrogenase	2
RSp0696	7.32	0.000	6.34	0.000	+		0	Selenocysteine lyase	2
RSp0697	6.97	0.000	7.20	0.000	+		0	Short chain dehydrogenase	2
RSp0698	5.62	0.000	6.25	0.000	+		1	Aspartate/tyrosine/aromatic aminotransferase	2
RSp0672	3.50	0.000	4.43	0.000	+	+	3	LRR protein, GALA subfamily	
RSp0731	nda		2.35	0.000	+		3	Trehalose 6-phosphate synthase	
RSp0732	5.90	0.000	6.74	0.000	+	+	3	Hypothetical / Unknown	
RSp0822	2.15	0.013	3.61	0.000	++		2	AvrPphF family protein	
RSp0837	2.70	0.000	1.00	0.000	++		3	Hypothetical / Unknown	
RSp0839	4.87	0.000	5.74	0.000	+	+	1	HrpY-related protein	
RSp0840	1.35	0.020	2.20	0.000	in operon?		0	Methyl-accepting chemotaxis transducer protein	2
RSp0841	7.08	0.000	7.19	0.000	+	+	1	Lysozyme-related transglycosylase	

Continued on next page

^aDefinition of the different classes is based on regulation pattern and presence or absence of an *hrpH* box in gene promoters.

^bFor each gene and for each experimental condition the ratio (expressed as a log₂ value) of transcription in the *hrpB*-expressing strain versus the *hrpB*-deficient mutant is given together with the corresponding *p* value of the Student *t*-test. Data with absolute values >2 and *p* values <0.01 are highlighted in dark colors (orange for *hrpB* up-regulated gene, blue for *hrpB* down-regulated genes). Data highlighted with light colors either have an absolute value of the ratio between 1 and 2 or have a *p* value between 0.01 and 0.05. A few additional genes showing a low threshold value in the two assay conditions have been included in the table when they form part of an operon that includes other *hrpB*-regulated genes.

^cThe presence of a *hrpH*-box in the gene promoter is indicated by “+” for a canonical motif (9), by “+*” when a single mismatch is present relative to the consensus, “in operon?” indicates that this gene could be part of an operon that harbors a *hrpH* box in its promoter. A border highlights the corresponding operons and the names of the corresponding genes are shown in bold.

^d+ indicates previously identified *brg* genes.

^eN-terminal Type III export signals. This column shows the numbers of the following rules that are fulfilled by protein N-terminus relative to the amino acid bias found for type III secretion system (TTSS)-secreted proteins: i) high content in serine and proline residues (>18%), ii) low content in leucine residues (<6%), and iii) absence of acidic residues in the first 12 amino acids. *hrp* means that the corresponding genes are part of the *hrp* gene cluster that encodes the TTSS.

^fAn updated annotation is available on our annotation webpage.

^gResults of the semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). 2 indicates a qualitative difference was observed (amplified fragment missing in one condition), 1 that difference in band intensity was observed, and 0 indicates same intensity for the band in the three strains.

Table 1. Continued from preceding page

Gene ID	1000/1525 ratio	P-value	pam5/1525 ratio	P-value	hrp _{II} box	brg genes	Export signal	Corresponding protein or predicted function	RT-PCR validation
Class I continued									
RSp0842	3.98	0.000	4.11	0.000	+	+	3	LRR protein, PopC subfamily	
RSp0845	3.07	0.001	5.93	0.000	+	+	3	Hypothetical / Unknown	
RSp0848	4.65	0.000	5.63	0.000	+	+	0	Hypothetical / Unknown	
RSp0853	5.53	0.009	6.91	0.000	+	+	hrp	HrpB	
RSp0854	7.19	0.000	7.55	0.000	+	+	hrp	HrpZ	
RSp0855	7.68	0.030	7.21	0.001	+	+	hrp	HrpY	
RSp0856	6.67	0.000	8.34	0.000	+	+	hrp	HrpX	
RSp0857	4.34	0.000	5.71	0.000	+	+	hrp	HrpW	
RSp0858	4.45	0.000	4.08	0.000	+	+	hrp	HrpV	
RSp0859	7.07	0.000	7.62	0.000	+	+	hrp	HrcS	
RSp0860	6.14	0.330	7.89	0.000	+	+	hrp	HrcR	
RSp0862	5.45	0.001	5.58	0.000	+	+	hrp	HpaP	
RSp0863	2.55	0.000	3.43	0.000	+	+	hrp	HrcV	
RSp0865	8.36	0.000	8.35	0.000	+	+	hrp	HrpK	
RSp0866	7.61	0.000	8.00	0.000	+	+	hrp	HrpJ	
RSp0867	4.76	0.000	6.09	0.000	+	+	hrp	HrcJ	
RSp0868	6.64	0.000	7.37	0.000	+	+	hrp	HrpH	
RSp0869	5.43	0.000	6.28	0.000	+	+	hrp	HrpF	
RSp0870	3.98	0.000	4.60	0.000	+	+	hrp	HrcN	
RSp0871	5.71	0.000	6.03	0.000	+	+	hrp	HrpD	
RSp0872	4.02	0.004	5.88	0.000	+	+	hrp	HrcT	
RSp0875	5.69	0.000	5.59	0.000	+	+	3	PopC	
RSp0876	8.93	0.000	8.89	0.000	+	+	3	PopB	
RSp0877	8.95	0.000	8.14	0.000	+	+	2	PopA	
RSp0879	1.79	0.029	3.20	0.000	+	+	3	Hypothetical / Unknown	
RSp0882	5.23	0.000	6.16	0.000	+	+	0	Hypothetical / Unknown	2
RSp0885	1.42	0.088	2.06	0.002	+	+	1	Hypothetical / Unknown	
RSp0900	6.68	0.000	6.82	0.000	+	+	3	PopF2	
RSp1022	5.12	0.000	6.10	0.000	+	+	2	Hypothetical / Unknown	
RSp1031	1.94	0.007	3.50	0.000	+	+	3	Hypothetical / Unknown	
RSp1130	2.38	0.001	5.24	0.000	+	+	3	Hypothetical / Unknown	
RSp1212	6.40	0.001	4.66	0.000	+	+	3	Hypothetical / Unknown	1
RSp1236	2.76	0.003	4.33	0.000	++	+	3	Hypothetical / Unknown	
RSp1239	3.70	0.000	6.12	0.000	++	+	3	Hypothetical / Unknown	2
RSp1240	2.01	0.000	3.99	0.000	++	+	2	Transglycosylase	
RSp1277	2.57	0.000	4.30	0.000	+	+	2	Hypothetical / Unknown	2
RSp1281	1.46	0.016	4.27	0.000	+	+	3	Hypothetical / Unknown	
RSp1461	3.74	0.000	5.07	0.000	+	+	2	Conserved hypothetical protein	
RSp1462	1.66	0.000	2.30	0.000	in operon?	+	0	Outer membrane efflux protein	
RSp1463	2.06	0.005	2.88	0.000	in operon?	+	0	ABC transporter, permease component	0
RSp1474	4.48	0.002	3.88	0.000	+	+	0	Hypothetical / Unknown	1
RSp1475	3.83	0.000	5.09	0.000	+	+	3	Hypothetical / Unknown	2
RSp1555	8.74	0.000	7.61	0.000	+	+	3	PopF1	
RSp1582	4.16	0.004	4.23	0.000	++	+	3	Hypothetical / Unknown	
Class II									
RSc0096	2.44	0.000	3.20	0.000		+	0	Hypothetical / Unknown	
RSc0257	4.07	0.000	6.58	0.000		+	2	Ankyrin Repeat harboring protein	
RSc0321	2.23	0.003	3.22	0.000		+	3	Hypothetical / Unknown	1
RSc0893	1.35	0.003	3.00	0.000		+	0	Transposase	
RSc1083	2.07	0.000	1.71	0.000		+	0	Aldose 1-epimerase	
RSc1344	1.16	0.002	2.23	0.000		+	1	Esterase	
RSc1517	1.97	0.000	2.62	0.001		+	3	Hypothetical / Unknown	
RSc1723	4.69	0.001	5.19	0.000		+	3	Hypothetical / Unknown	
RSc1815	3.93	0.000	5.24	0.000		+	1	AvrBs3 family protein	
RSc1841	1.80	0.014	2.33	0.000		+	0	Hydrolase	
RSc1900	4.07	0.008	nda				0	Hypothetical / Unknown	
RSc1901	2.01	0.001	1.53	0.000			1	Hypothetical / Unknown	
RSc2109	2.30	0.001	1.98	0.001			1	Hypothetical / Unknown	
RSc2113	2.01	0.001	nda				1	Oxidoreductase	
RSc2200	1.95	0.000	2.19	0.000			3	ABC transporter	
RSc2291	2.30	0.000	nda				2	Lysozyme-related transglycosylase	
RSc2376	2.94	0.000	2.62	0.000			0	4Fe-4S binding electron transporter	
RSc3162	2.41	0.004	1.68	0.004			1	Autotransported hemagglutinin-related protein	
RSp0093	2.62	0.006	3.45	0.000			1	TIS1421-transposase	
RSp0159	3.70	0.003	4.47	0.000			0	Hypothetical / Unknown	
RSp0216	4.03	0.000	4.96	0.000			3	Serine/threonine-protein kinase	
RSp0286	2.63	0.005	3.28	0.000			3	Hemin-binding-related protein	
RSp0587	2.52	0.044	3.95	0.000			1	Conserved hypothetical protein	
RSp0825	5.09	0.000	5.76	0.000			1	Spermidine synthase	1
RSp0834	nda		2.04	0.000			0	Hexuronate transporter	
RSp0852	1.19	0.008	2.26	0.000			hrp	HrpG	
RSp0873	5.10	0.000	7.09	0.000			hrp	HrpB	
RSp0888	1.41	0.000	2.23	0.000			0	Glyoxalase	
RSp0943	1.25	0.021	3.09	0.002			3	Aspartate aminotransferase	
RSp1150	nda		2.41	0.000			1	Hypothetical / Unknown	
RSp1270	3.05	0.000	4.87	0.000			0	Hypothetical / Unknown	
RSp1280	nda		3.82	0.000			0	Carbohydrate kinase	

Continued on next page

conducted on a subset of these genes in order to confirm these observations. These genes included RSp0788, a *hrpB*-repressed gene that encodes a predicted carbamoyl transferase, RSp0840, a Class I gene that encodes a chemotaxy-related protein, and RSp693 and Rsp698, two Class I genes from a single operon and encoding predicted intracellular enzymes. RSp0877 that encodes the PopA protein (Arlat et al. 1994) was also included as a *hrpB*-regulated control, as well as 16S rDNA and RSc2690 (*oxyR*) as constitutive controls, since these two genes behaved as constitutively expressed, based on microarray analysis. These experiments were conducted on the two mRNA populations used for microarray analysis. Data

shown in Figure 2 are the average of the two experiments. They confirm that *hrpB* can act as a negative regulator and that the *hrpB* regulon extends beyond TTSS-related functions.

Biological functions encoded by *hrpB*-regulated genes.

TTSS and TTSS effectors. Apart from the components of the type III secretion machinery and the previously identified candidate effectors, 26 new *hrpB*-activated genes are predicted to encode probable TTSS substrates, since the composition of their first 50 N-terminal amino acid domain complies with at least two of the three rules previously defined for *R. solanacearum* TTSS effectors: i) high content in serine and proline residues

Table 1. Continued from preceding page

Gene ID	1000/1525 ratio	P-value	pam5/1525 ratio	P-value	<i>hrpII</i> box	<i>brg</i> genes	Export signal	Corresponding protein or predicted function	RT-PCR validation
Class II continued									
RSp1386	2.39	0.000	2.47	0.000			0	Hypothetical / Unknown	
RSp1391	nda		2.61	0.000			0	Flagellar protein	
RSp1440	nda		2.94	0.001			1	Hypothetical / Unknown	
RSp1460	2.71	0.006	4.14	0.000		+	3	Hypothetical / Unknown	
RSp1466	4.91	0.000	5.32	0.000			1	Hypothetical / Unknown	2
RSp1467	4.32	0.001	5.05	0.000			1	Hypothetical / Unknown	2
RSp1536	3.06	0.000	2.34	0.000			2	Hemagglutinin-related protein	
RSp1581	nda		2.27	0.000			1	Hydroperoxidase HPII	
Class III									
RSc0447	-1.61	0.162	-2.70	0.000			0	Hypothetical / Unknown	
RSc0594	1.18	0.304	-3.19	0.000			0	Transmembrane protein	
RSc0595	-0.16	0.799	-2.11	0.000			1	Outer membrane TonB-dependent receptor protein	
RSc1810	0.95	0.197	-2.47	0.000			0	Beta-ketoacyl synthase	
RSc1811	1.06	0.016	-2.50	0.000			0	Siderophore synthase	
RSc1812	1.06	0.055	-3.62	0.000			1	Hypothetical / Unknown	
RSc1813	0.97	0.010	-2.02	0.000			0	Transcription activator (AraC family)	
RSc1892	-0.93	0.000	-2.89	0.000			0	Two component response regulator	1
RSc1918	nda		-2.36	0.000			0	Bacteriophage tail protein	
RSc1938	nda		-2.57	0.000			1	Bacteriophage capsid scaffolding protein	
RSc1942	-0.48	0.297	-2.08	0.000			1	Hypothetical / Unknown	
RSc1943	-0.21	0.681	-2.26	0.000			1	Hypothetical / Unknown	
RSc2208	nda		-2.18	0.000			0	Pilin-related protein	
RSc2374	-0.77	0.098	-2.69	0.000			1	Hypothetical / Unknown	
RSc2689	-1.23	0.232	-2.47	0.000			1	Hypothetical / Unknown	
RSc3092	nda		-2.61	0.000			1	Hypothetical / Unknown	
RSc3219	0.54	0.165	-2.08	0.000			0	Hypothetical / Unknown	
RSc3220	nda		-2.29	0.000			1	VGR-related protein	
RSc3222	-0.11	0.877	-2.35	0.000			0	Probable pseudogene	
RSc3248	-0.28	0.818	-2.02	0.000			0	Bacteriophage protein	
RSc3378	-0.29	0.599	-2.03	0.000			0	Phosphatidylserine-synthase related protein	
RSp0081	-0.60	0.028	-2.21	0.000			0	Hypothetical / Unknown	
RSp0100	-0.80	0.026	-2.38	0.000			1	TonB-dependent receptor	
RSp0258	nda		-3.16	0.000			1	Transcription regulator (repressor PadR family)	
RSp0413	0.14	0.544	-2.78	0.000			0	Conserved hypothetical protein	
RSp0414	-0.05	0.804	-2.44	0.000			2	TonB-dependent receptor	
RSp0417	-0.25	0.473	-3.64	0.000			0	O-Acetyl serine sulphydrolase	
RSp0418	-0.23	0.402	-3.87	0.000			0	Ornithine cyclodeaminase	2
RSp0419	-0.54	0.042	-2.86	0.000			0	Siderophore synthetase component	
RSp0420	-0.12	0.513	-3.68	0.000			0	Transporter	
RSp0421	-0.47	0.192	-3.39	0.000			0	Siderophore synthetase component	
RSp0422	-0.14	0.496	-3.96	0.000			0	Siderophore synthetase component	2
RSp0423	0.04	0.900	-3.63	0.000			0	Aldolase	
RSp0424	-0.44	0.208	-3.87	0.000			0	Staphyloferrin B siderophore decarboxylase	
RSp0532	-2.25	0.017	-3.36	0.000			0	Aminopeptidase	
RSp0779	-1.52	0.063	-2.10	0.000			0	Acyl homoserine lactone synthase	1
RSp0780	-1.80	0.015	-2.29	0.000			1	Hypothetical / Partial homology	
RSp0781	-2.42	0.032	-1.92	0.000			0	Cystathionine gamma-synthase	
RSp0782	-2.65	0.021	-2.19	0.000			1	Aminoacyl-tRNA synthetase-related protein	
RSp0783	-2.09	0.049	-1.81	0.001			0	Acyl carrier protein	
RSp0784	-2.58	0.022	-2.36	0.000			0	Fatty acid desaturase	
RSp0785	-2.09	0.010	-1.66	0.000			0	Hypothetical / Unknown	
RSp0786	-2.26	0.015	-2.02	0.000			0	3-oxoacyl-[acyl-carrier-protein] synthase	
RSp0787	-2.51	0.017	-2.24	0.000			0	Acyl-CoA synthetase	
RSp0788	-2.44	0.017	-2.42	0.000			1	Carbamoyl transferase	
RSp0789	-2.25	0.016	-1.27	0.002			0	Hypothetical / Partial homology	
RSp0790	-2.00	0.037	-2.27	0.000			1	Hydrolase	
RSp0792	-2.69	0.029	-2.49	0.001			0	Fatty acid desaturase	1
RSp1377	-0.14	0.365	-2.23	0.000			0	Transcription regulator (AsnC family)	
RSp1378	-0.84	0.058	-2.33	0.000			0	Transposase remnant	
Class IV									
RSc1804	2.90	0.010	-2.05	0.000			0	Non-ribosomal peptide synthase	
RSc1805	2.81	0.015	-2.06	0.000			0	Siderophore-like synthase	
RSc1807	2.17	0.013	-1.11	0.000			0	TonB-dependent siderophore receptor	2*
RSc1808	2.26	0.039	-1.30	0.005			0	ABC transporter	

($\geq 18\%$), ii) low content in leucine residues ($\leq 6\%$), and iii) absence of acidic residues in the first 12 amino acids (Cunnac et al. 2004b). In addition, 18 of 26 of these candidates contain at least one basic amino acid within the seven first residues, another general feature of *R. solanacearum* effectors (Cunnac et al. 2004b). Among these 26 new predicted proteins are some homologs of known TTSS effectors from *R. solanacearum*

(RSp0028, RSp0215) or from other plant pathogens (RSp0822, RSp1239, RSp1277). A large proportion of these candidate effector genes (20 of 26) have no counterpart in the nonpathogenic and taxonomically close species *Ralstonia metallidurans* (Table 3). Among these novel candidate effectors are several proteins with possible enzymatic activity, including RSp0216, a putative protein kinase that is best related to eukaryotic serine/threonine

Table 2. Validation of *hrpB*-regulated genes through gene fusions^a

<i>Ralstonia solanacearum</i> gene	β -galactosidase activity (Miller's units)			Induction ratio wt/ <i>hrpB</i> ⁻ or pAM5/ <i>hrpB</i> ⁻
	Wild-type	<i>hrpB</i> ⁻	+pAM5	
RSp1212	56 (5)	12 (1.4)	ND	4.7
RSp1236	30.7 (1.5)	11.5 (0.4)	114 (5)	9.9
RSp1461	59 (5.7)	11.8 (1.0)	ND	5.0
RSp1475	5.6 (0.2)	3.2 (0.3)	30.6 (5.4)	9.6
RSp1582	19.8 (1.2)	9.8 (0.2)	71 (2.5)	7.2
RSc0041	29.4 (1.4)	10.1 (2.0)	51.9 (1.5)	5.1
RSc2101	21 (2)	13.5 (1)	89 (6)	6.6

^a β -galactosidase activity of strains carrying pCZ367 fusions in different GMI1000 genes and effect of *hrpB* disruption (*hrpB*⁻) or overexpression (+ pAM5). Assays were performed on bacteria grown in *hrp*-inducing minimal medium. Values in brackets are standard variations. ND = not done.

Fig. 1. Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of selected *hrpB*-regulated genes of *Ralstonia solanacearum*. Full gels are only shown for the controls; only the part of the gel showing the appropriate amplicon is shown for the other samples. M = DNA size marker. Lanes 1 and 4, *hrpB*-deficient mutant GMI1525; lanes 2 and 5, GMI1000; lanes 3 and 6, GMI1728; lane 7, water control; lane 8, GMI1000 genomic DNA control. Lanes 4, 5, and 6 correspond to control RNA sample (without reverse transcriptase). Controls include two constitutive genes (RSc2690, 16S rDNA) and the *hrpB*-regulated *popA* gene.

protein kinases, RSc3155, a probable hydrolase, and RSc2291 and RSp1240, which are both related to lysozyme transglycosylases. Another protein encoded by a Class I gene (Rsp0841) and harboring a signal peptide for the general secretion pathway is also predicted to have transglycosylase function, suggesting that

it could play a role in remodeling peptidoglycan during the positioning of the TTSS into the cell envelope.

Metabolic functions. Several *hrpB*-regulated genes encode enzymes most probably involved in intermediate metabolism (such as RSc2147, coding for a predicted intracellular sorbitol-

Fig. 2. Validation of non-type III secretion system-related *hrpB*-regulated genes by reverse transcriptase-polymerase chain reaction (RT-PCR). Expression of different genes in the *hrpB*-deficient mutant GMI1525 (hatched boxes) and in strain GMI1728 overexpressing *hrpB* (dotted boxes), compared with their expression in the wild-type strain GMI1000 by real time RT-PCR. The data are presented on a log scale and correspond to the ratio of the amount of mRNA present in the tested strains relative to the reference strain GMI1000.

Table 3. The 26 novel candidate type III secretion system (TTSS)-effectors identified in this study^a

Gene	Class	Location	Gene product features		CH34 ^b	%GC ^c
			Size (aa)	Description		
RSc0041	I	ACUR ^d	430	No homology	–	49.4
RSc0321	II		642	Partial homology to RSc2132 (E = 1e-08)	–	
RSc1517	II		58	No homology	–	
RSc1723	II		147	No homology, putative nuclear localization signals	–	
RSc2101	I		334	No homology	–	
RSc2133	I		90	Partial homology to RSc3065 (E = 3e-19)	–	
RSc2291	II		198	Homology to <i>Pseudomonas putida</i> PP3422 (E = 4e-33)	E = 2e-51	
RSc2897	I	ACUR border	122	Global homology to <i>R. solanacearum</i> FliT (E = 2e-24)	E = 1.5e-09	52.4
RSp3155	I		451	Hydrolase; homology to <i>Staphylococcus aureus</i> Vgb (E = 8e-21)	–	
RSp0028	I		603	Leucine-rich repeat protein GALA3	–	
RSp0215	I		754	HLK family; homology to <i>Xanthomonas campestris</i> pv. <i>campestris</i> XCC1247 (E = e-43)	–	
RSp0216	II	ACUR	577	Serine/threonine protein kinase; homology to Yeast YKL101W (E = 8e-14)	–	61.9
RSp0286	II		816	Hemin-binding outer-membrane protein; homology to <i>Bordetella bronchiseptica</i> BB1769 (E = e-149)	–	
RSp0731	I	<i>hrp</i> cluster border	557	Alpha, alpha-trehalose-phosphate synthase; homology to RSp1105 (E = e-103)	E = 7e-117	59.2
RSp0822	I		350	Homology to <i>P. syringae</i> pv. <i>tomato</i> type III effector HopPtoF (E = 5e-18)	–	
RSp0837	I		182	No homology	–	
RSp0943	II		402	Aspartate aminotransferase; homology to <i>P. syringae</i> pv. <i>tomato</i> PSPT02925 (E = e-164)	E = 4e-46	
RSp1212	I		296	No homology	–	61.9
RSp1236	I		605	Partial homology to RSp1475 (E = e-110)	–	
RSp1239	I		943	Partial homology to <i>X. campestris</i> pv. <i>vesicatoria</i> type III effector XopC (E = 2e-81)	–	
RSp1240	I		427	Transglycosylase; homology to <i>X. axonopodis</i> pv. <i>citri</i> XACb0007 (E = e-128)	E = 1.6e-14	
RSp1277	I		518	Homology to <i>P. syringae</i> pv. <i>tomato</i> type III effector HopPtoA1 (E = e-33)	–	59.2
RSp1461	I		105	Homology to RSc3204 (E = 6e-16)	–	
RSp1475	I		448	Homology to RSp1236 (E = e-123)	–	
RSp1536	I		846	Hemagglutinin-related protein; homology to <i>X. campestris</i> pv. <i>campestris</i> XCC1794 (E = 3e-31)	E = 4.1e-20	
RSp1582	II		277	No homology	–	

^a Analysis of the N-terminal regions (50 first amino acids) of gene products from Classes I and II indicated that the genes listed in this table encode proteins that display compositional biases in their N-terminal region that are similar to known GMI1000 TTSS-effectors (Cunnac et al. 2004b).

^b The detection of a homologous sequence in the non pathogenic *Ralstonia metallidurans* CH34 is indicated by the E Blast score.

^c The GC content of the gene is indicated when significantly different from the mean of the GMI1000 genome (67%).

^d ACUR = Alternate codon usage region (Salanoubat et al. 2002).

dehydrogenase, RSc3133, coding for an acetyl-transferase, or the RSc1841, RSc2113, and RSp1280 genes [Table 1]). Another example concerns two genes (RSp0888, RSp0893) involved in the degradation of cumate and RSp0834 (*exuT*), a gene involved in the uptake of galacturonic acid, the main degradation product of pectin (Gonzales and Allen 2003). An illustration of functions probably independent of Type III secretion concerns an operon of six genes (RSp0693 through RSp0698) that encodes enzymes putatively involved in the bioconversion of a tryptophan-related molecule (discussed below).

Signal transduction and regulation. Class I genes include a putative transcriptional regulator (Rsc2661) and a gene (RSp0840) that encodes a methyl-accepting protein probably involved in perception and in transduction of a chemotactic signal. Class II includes *hrpG* (RSp0852), the OmpR-related transcriptional activator required for transcription of *hrpB* (Brito et al. 1999). This observation confirms our previous data indicating the existence of a regulatory loop between the *hrpB* and *hrpG* regulatory genes (Genin et al. 1992). Additional regulatory proteins are found in Class III, including three activators (RSc1892, RSc1813, and RSp1377), a repressor (RSp0258), and a long operon (RSp0779-RSp0792) possibly involved in the synthesis of an N-acyl homoserine lactone and of a fatty acid precursor. This latter gene probably plays a role in the production of a molecule governing quorum sensing in *R. solanacearum*.

Acquisition and transport of exogenous compounds. An important outcome from this study is the demonstration that *hrpB* plays a key role in the control of iron acquisition and uptake, since this gene is involved in the repression of two contiguous operons (RSp0413 through RSp0424) that control the production and uptake of the ferrisiderophore staphyloferrin B (Bhatt and Denny 2004), together with an additional siderophore receptor (RSp0100). Moreover, Class IV genes are organized in a cluster of two close operons (Rsc1805 to RSc1808) all probably involved in biosynthesis and uptake of another ferrisiderophore. Several genes (RSp1461-RSp1463, and RSc2200) encode components of additional unknown ABC transporters.

Other functions. Among Class II genes are two hemagglutinin-like proteins (RSc3162 and RSp1536) that could be involved in the attachment of the bacteria to plant cells, since previous studies have established that type IV pili are not the only structures involved in this process (Kang et al. 2002). This class also includes two transposases (RSc0893, RSp0093) and a spermidine synthase (RSp0825), the function of which in pathogenicity remains to be clarified. Class III includes three genes encoding proteins from two different bacteriophages (RSc1918, RSp1938, RSc3248), two operons with unknown functions (RSc1942 to RSc1943 and RSc3219 to RSc3220), a pirin-like protein (RSc2208), and a possible phosphatidyl-synthase (RSc3378).

DISCUSSION

In this work, we have developed a pangenomic microarray for *R. solanacearum* based on 65- to 70-mer oligonucleotides, and we have used it to identify genes whose expression is dependent on HrpB, the AraC-related transcriptional activator that plays a crucial role in the control of pathogenicity of this bacterium. We have also established the reliability of this tool for transcriptome analysis, since under the very highly stringent cutoff conditions that we used (fourfold ratio, $P < 0.01$), we were able to detect 64 of the 70 previously known *hrpB*-regulated genes. This microarray allowed us to identify 133 additional *hrpB*-regulated genes: 79 are *hrpB* up-regulated, 50 are *hrpB* down-regulated, and four have a more complex regulation pattern. These genes have been categorized into four

classes based on expression patterns and promoter structure. Among the new genes identified, 34 of 37 chosen as representative of each of the four classes were validated as being *hrpB*-regulated, using either *lacZ* reporter gene fusions or RT-PCR. It is interesting to note that some of the newly identified up-regulated genes, such as PopP1 or RSp0882, had previously been reported as being independent of *hrpB* for transcription when *lacZ* gene fusions were used to monitor their expression (Cunnac et al. 2004b; Lavie et al. 2002). Similarly, transcriptome analysis clearly confirms the *hrpB*-regulated status of several effectors, such as RSc0608 or RSc1356, for which only a minor differential in expression was observed between wild type and the *hrpB* mutant strains, using gene fusions (Cunnac et al. 2004b), indicating therefore that, for certain genes, transcriptome analysis might be a more sensitive method than the use of transcriptional gene fusions. This could be due to difference in mRNA stability of chimeric transcripts or stability of the chimeric LacZ reporter protein.

Concerning the distribution of *hrpB*-regulated genes over the genome, it is striking that both up- and down-expressed genes are overrepresented on the megaplasmid (120 genes for a total size of 2.1 Mb) as compared with the chromosome (77 genes for a total size of 3.7 Mb) and that this applies to genes encoding both TTSS-related and TTSS-independent functions.

The Hrp regulon extends beyond TTSS-dependent functions.

The amino-acid composition of the N-terminal ends of more than one third of proteins encoded by Class I and Class II genes, including a majority of new proteins with unknown function identified in this study, indicates that these proteins are most likely secreted through the TTSS. These proteins are therefore good candidate effectors or TTSS-accessory proteins involved in the construction or in the functioning of the TTSS. Lysozyme-related transglycosylases are good examples of such potential accessory components. Based on the present experiments, we estimate that 70 to 80 effectors or TTSS-accessory proteins are encoded within the genome of strain GMI1000 and that more than 150 genes (including *hrp* genes) are positively controlled by *hrpB*, whereas more than 50 are negatively affected by this gene.

Most surprisingly, new genes identified in this study also include genes that encode proteins that are not directly involved in the functioning of the TTSS but might be implicated in virulence. An interesting example is the gene cluster RSp0693 to RSp0698 that is located 200 kb away from the *hrp* gene cluster of GMI1000. Interestingly, we found that the six proteins encoded within this *hrpB*-activated operon are highly conserved in another plant pathogen, *Xanthomonas campestris* pv. *campestris* (da Silva et al. 2002). Moreover, these six genes have a similar genetic organization in the two organisms, except that, in *X. campestris* pv. *campestris*, the gene orthologous to RSp0694 is interrupted by an insertion sequence (Fig. 3). The homology is also visible at the DNA level, with a nucleotide conservation of more than 80% over most of the operon. Intriguingly, in *X. campestris* pv. *campestris*, this operon is located within the *hrp* pathogenicity island, 3 kb upstream of the *hrpF* gene, which is a good indication that these genes might play a role in virulence. So far, an analogous operon has not been identified in any of the other completely sequenced genomes, including *X. axonopodis* pv. *citri*. These genes encode enzymes predicted to be involved in the putative bioconversion of a tryptophan-related molecule. One possibility is that these genes could be responsible for the production of a toxin. If so, this situation is reminiscent of that found in *Pseudomonas syringae*, where syringomycin production is coregulated with TTSS activation

through dependence on the alternative sigma factor encoded by the *hrpL* gene (Fouts et al. 2002). Alternatively, these genes could be involved in the production of a small diffusible molecule active on plants, like the *avrD* gene cluster from *Pseudomonas syringae*, which controls the synthesis of C-glycosyl lipids interacting with plants under *hrpL* dependence (Shen and Keen 1993; Smith et al. 1993). Yet another hypothesis is that this operon could be involved in the degradation of a toxic compound produced by the plant, such as camalexin, a tryptophan-derived phytoalexin produced by *Arabidopsis thaliana* (Glawischnig et al. 1993). In conclusion, our study demonstrates that the *hrpB* regulon controls several functions involved in intermediate metabolism and transport of exogenous molecules.

Recently, using a promoter trap strategy to perform a systematic search for *hrpB* up-regulated genes in strain MAFF30103 of *R. solanacearum*, Mukaihara and associates (2004) identified 30 new *hrpB*-activated genes, 20 of which were also detected in the present study. Interestingly, two of these genes classify in Class II in our study, whereas they are reported to be preceded by a *hrpB* box in strain MAFF30103, indicating that, in the two strains, evolution might have led to similar expression patterns using different regulatory pathways.

On the other hand, very little overlap was found between genes identified in our study and a large set of genes identified in an in vivo expression technology (IVET) approach (Brown and Allen 2004) as being required for multiplication of bacteria within the plant. However, it should be noted that this IVET approach identified very few *hrp* and related effector genes, suggesting that this methodology might target functions required at a later stage in the infection process.

hrpB has a dual regulatory role.

We have demonstrated that *hrpB* not only acts to promote expression of genes but that it also negatively affects expression of other genes. This had never been reported before, although it is now established in other organisms that some regulators of the AraC family can act both as activators and repressors (Egan 2002; Plano 2004). The presence of a *hrp_{II}* box in the promoter of genes from Class I is a good indication that the HrpB protein is directly involved in the regulation process, but the situation is more complex for Class II genes which are devoid of the *hrp_{II}* box motif and are still *hrpB*-regulated. Since several transcription factors acting as activators (RSc1892 and RSp1377) or as repressor (RSc2661) have been

identified among *hrpB*-regulated genes, it is possible that these genes could be intermediate regulatory genes mediating *hrpB*-dependent gene expression. Alternatively, it cannot be ruled out that the effect of *hrpB* on gene expression could even result from other indirect effects.

HrpB as a possible switch controlling transition from saprophytic to parasitic life.

Our data indicate that the bacteria undergo a dramatic metabolic shift in the early stage of the infection process, in order to switch from saprophytic life in the soil to parasitic life in the plant. The dual action of *hrpB* to promote up or down expression of different sets of genes, the diversity of biological functions that are controlled by this gene, and the diversity of environmental signals, including plant signals, metabolic status of the bacteria (Aldon et al. 2000; Brito et al. 1999; Marenda et al. 1998), and bacterial cell density integrated in the early stages of plant infection, suggest that this process can be considered a developmental program. It seems likely that Class III of *hrpB*-repressed genes, which includes a large number of genes putatively involved in the production of siderophores, may be not essential for pathogenicity but rather be important for the survival of the bacterium in the soil. The recent characterization of a strain carrying a mutation in the Class III gene RSp0424 and defective for the production of the siderophore staphyloferrin B revealed that, despite being almost incapable of scavenging iron, this mutant remained normally virulent on tomato plants (Bhatt and Denny 2004). A conclusion from the study of Bhatt and Denny (2004) is that *R. solanacearum* does not produce siderophore activity when cultured in tomato xylem sap that is iron replete, implying therefore that siderophore production is certainly more important in the rhizosphere to allow the pathogen to compete with other soil microbes for iron. Here we show that elevated amounts of HrpB in the cell (i.e., early steps of plant infection) lead to a repression of the genes involved in staphyloferrin B production (RSp0416 to RSp0424), which is also consistent with the previous observation.

Taken altogether, our data thus indicate that the regulatory circuits previously described merit further investigation to include regulators acting downstream and upstream of *hrpB* as well as their corresponding target genes. Further transcriptome analysis based on the use of mutants affected in these regulatory genes will help to clarify the status of the different classes of *hrpB*-regulated genes.

Fig. 3. Comparative organization of the RSp0693 to Rsp0698 gene cluster from *Ralstonia solanacearum* and the homologous gene cluster in *Xanthomonas campestris* pv. *campestris* ATCC33913. Identity between the different genes is given at the protein level. The insertion sequence IS1478 is inserted within gene coding for a nonfunctional *X. campestris* pv. *campestris* 1210 gene. Gene nomenclature is according to Salanoubat and associates (2002) and da Silva and associates (2002) for *X. campestris* pv. *campestris*.

MATERIALS AND METHODS

Strains and growth conditions.

The wild-type strain GMI1000 of *R. solanacearum* and its *hrpB*-derivative GMI1525 have been previously described (Boucher et al. 1985; Genin et al. 1992). Strain GMI1525 pLAFR3 was obtained by electroporation of GMI1525 with the empty vector pLAFR3 (Staskawicz et al. 1987). Strain GMI1728 is a derivative of strain GMI1000 that carries the plasmid pAM5, which harbors a cloned copy of *hrpB* (Guéron et al. 1999). Conditions for routine culture of *E. coli* and *R. solanacearum* have been described (Cunnac et al. 2004a). When required, antibiotics were used at the following concentrations ($\mu\text{g/ml}$): ampicillin, 100; tetracycline, 10; and spectinomycin, 40.

Nucleic acid extraction and semiquantitative RT-PCR.

Genomic DNA used as template in PCR reactions was isolated from *R. solanacearum* GMI1000 cells cultured in complete BG liquid medium (Boucher et al. 1985), using a Wizard DNA genomic purification kit (Promega, Madison, WI, U.S.A.).

For RNA extraction, *R. solanacearum* bacteria were grown in logarithmic phase in 1-liter Erlenmeyer flasks on a rotary shaker at 50 rpm in 100 ml of liquid minimal medium MM (Boucher et al. 1985) until they reached an optical density at 600 nm (OD_{600}) = 0.8, with 20 mM glutamate as carbon source. The culture was immediately mixed with 0.05 vol of a cold solution of phenol/ethanol (10% phenol in 95% ethanol) and was harvested by centrifugation ($4,600 \times g$, 20 min). RNA was extracted using the Qiagen RNeasy midi kit (Qiagen, Valencia, CA, U.S.A.), was concentrated by ethanol precipitation, and was treated with RNAase free DNAase I (Ambion, Austin, TX, U.S.A.) according to the manufacturer's recommendations. Purified total RNA was stored at -80°C .

Semiquantitative RT-PCR. Validation of microarray results by semiquantitative RT-PCR was performed on at least two independent biological experiments as described earlier (Cunnac et al. 2004b).

Quantitative RT-PCR. Aliquots of the cDNA samples used for the semiquantitative RT-PCR experiments were used for quantitative reverse transcription-mediated real time PCR. The Light Cycler (Roche, Branchburg, NJ, U.S.A.) with SYBR green chemistry was used to monitor amplification and to quantify the amount of PCR products (Roche). The RSc2690 gene, constitutively expressed (discussed below), was used to normalize expression values for all other genes. The *popA* gene was used as a control for *hrpB*-regulated genes.

Microarray techniques.

Primer design and pangenomic microarray construction. The pattern of the *R. solanacearum* whole-genome oligo arrays was based on the sequenced genome of *R. solanacearum* GMI1000 (Salanoubat et al. 2002). The 5,086 5' aminated oligomers were designed using the ROSO program (Reymond et al. 2004) and were purchased from Sigma Genosys (Saint Quentin Fallavier, France). The length of the gene-specific sequence was a 70 ($n = 3,818$) or a 65 ($n = 1,260$) mer and was chosen to be specific for the coding strand of each individual gene and as close as possible to the 5' end of the gene. However, when some ambiguity remained concerning the possible start codon of a gene, the sequence was chosen downstream of the most distal candidate start codon. In a few cases ($n = 8$), the length was reduced to 60 mers to increase specificity or to prevent the formation of undesirable secondary structures. The sequence of each oligomer, together with its individual best BLAST score on a nonhomologous region of the GMI1000

genome and the free energy of secondary structures, can be provided on request. Each oligonucleotide was adjusted to 25 μM in 150 mM sodium phosphate buffer (Na_2HPO_4 , pH 9.0) and was spotted in duplicate, using a robot Eurogridder (ESI/Eurogentec, Seraing, Belgium) on aldehyde-activated glass slides prepared and treated according to Trévisiol and associates (2003).

mRNA extraction.

Expression profiles were generated using 100 ml of bacteria grown in minimal medium to an $\text{OD}_{600} = 0.8$, conditions that are known to induce expression of *hrpB*-regulated functions (Genin et al. 1992). For each condition, two independent RNA preparations from two independent cultures were used. The bacterial cultures were treated by adding 5 ml of a solution of 5% phenol in ethanol prior to centrifugation to kill the cell and to prevent any further transcription during that step. Lysis and RNA extraction was performed with the RNeasy midi kit from Qiagen according to the manufacturer's instructions.

Probe labeling and microarray slide hybridization. The mRNA were converted to cDNA and labeled with Cy3 or Cy5 fluorescent dyes (Amersham Biosciences, Buckinghamshire, U.K.), using a two step protocol according to Ruberg and associates (2003). For each mRNA, Cy3 and Cy5 labeling were performed in order to allow dye swap for each experiment. Total RNA was first converted to cDNAs in the presence of aminoallyl-dUTP using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.). Total RNA (25 μg) was combined with 6 μg of random hexamers (Invitrogen) and was incubated at 70°C for 10 min. After rapid cooling at 4°C for 30 s, the reaction mixture was brought to a final volume of 30 μl by adding 6 μl of 5 $\times$ Superscript II reverse transcriptase buffer, 3 μl of 0.1 mM dithiothreitol, 0.5 μl of RNasin, 0.6 μl of a 50 $\times$ dNTP-amino allyl dUTP mixture (0.5 M each of dGTP, dATP, and dCTP, 0.16 mM dTTP, 0.34 mM aminoallyl-dUTP), and 2.5 μl of Superscript II reverse transcriptase. After incubation at 25°C for 10 min, the mixture was kept at 45°C for 2 h. After completion of the first-strand synthesis, the RNA was hydrolyzed by adding 15 μl of 0.2 N NaOH and incubating for 10 min at 70°C . Neutralization was performed by adding 15 μl of 0.2 N HCl. The aminoallyl-cDNA was purified from free amines and unincorporated aminoallyl-dUTP, using Qiaquick PCR spin columns (Qiagen). After elution, the aminoallyl-cDNA was dried under vacuum in a SpeedVac and was resuspended in 5 μl of 1 M sodium carbonate (pH 9.0). For fluorochrome labeling, 5 μl of a solution of fluoroLink Cy3 or Cy5 dye, prepared by resuspending one reaction vial into 45 μl of dimethyl sulfoxide, were added and incubated for 1 h at room temperature in the dark. This reaction was quenched by adding 5 μl of 4 M hydroxylamine and incubating for 15 min at room temperature in the dark. Fluorescent-labeled cDNAs were purified using Qiaquick PCR spin columns and were eluted in 30 μl of the manufacturer's EB buffer. The two fluor-labeled cDNAs were then combined and concentrated to a volume of 35 μl , using a SpeedVac.

Hybridizations were conducted using a Lucidea automated slide processor (Amersham Pharmacia Biotech, Piscataway, NY, U.S.A.) under the following conditions. Microarray slides were prehybridized for 1 h at 42°C in Dig Easy buffer (Roche) containing 10 μM of salmon sperm DNA. Hybridizations were done for 13 h under the same conditions, except that the heat-denatured probes were added to the reaction mixture. Following hybridization, slides were washed once in $2\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate for 5 min at 60°C and two times in $0.1\times$ SSC for 1 min at room temperature and then washed in ethanol and dried at 37°C for 5 min.

Hybridized slides were scanned using GenePix 4000B dual-channel confocal laser scanner (Axon Instruments, Union City, CA, U.S.A.).

Data analysis. Signal quantification and data analysis were achieved using ImageJ (version 5.6.1) and GeneSight software (version 4.1) (Biodiscovery Inc., El Segundo, CA, U.S.A.). Following local background subtraction, the signal for each spot was normalized based on the median value of the median intensity of all the spots for each array. Only genes for which the hybridization signal was greater than the average value plus two standard deviations of the negative controls (*Corynebacterium glutamicum* genes and non-GMI1000 DNA) were analyzed. The two signals corresponding to the two replicates present on the same slide were averaged and were used to calculate the ratio of expression between the *hrpB*-expressing strain and the *hrpB*-deficient mutant (*hrp⁺/hrpB⁻*). Each ratio was converted to its log₂ value, and the average log₂ value for each gene of the four independent slides corresponding to each experiment was calculated (Table 1). Statistical significance of the gene expression differential over the course of the replicate experiments was calculated by using a Student's *t* test analysis available in GeneSight software.

Only genes with high levels of significance ($P < 0.01$) and a minimum absolute value of log₂ > 2 were systematically considered in this study, in order to minimize the false positive as up- or down-regulated. This threshold value was chosen as being highly discriminative for a high proportion of the previously known *hrpB*-regulated genes.

All primary data from transcriptome experiments as well as experimental protocols used are available from the ArrayExpress depository (accession numbers A-MEXP-152, E-MEXP-262, and E-MEXP-263).

ACKNOWLEDGMENTS

This work was supported by the Institut National de la Recherche Agronomique (INRA), the Centre National Recherche Scientifique, and Toulouse Genopole. A. Occhialini was supported by a postdoctoral grant from INRA. We are grateful to D. Capela and C. Bruand for useful discussions regarding microarrays technology; H. Charles for the design of oligonucleotides, V. Le Berre-Anton for the spotting of microarrays; S. Sokol for his help on the handling and analysis of transcriptome data; C. Balagué for her help in quantitative RT-PCR and P. Barberis for technical assistance.

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