Genome-Wide Gene Expression Analysis of *Pseudomonas syringae* pv. *tomato* DC3000 Reveals Overlapping and Distinct Pathways Regulated by *hrpL* and *hrpRS*

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Pseudomonas syringae pv. *tomato* DC3000 is a model pathogen infecting tomato and *Arabidopsis* plants. Genes encoding the type III secretion system and substrate proteins (collectively called TTSS genes) of this bacterium are induced in plants and in minimal medium (MM). The induction of TTSS genes is mediated by HrpL, an alternative sigma factor recognizing the *hrp* box in the promoter of TTSS genes. The transcription of *hrpL* is activated by HrpR and HrpS, two homologous DNA-binding proteins encoded by the *hrpRS* operon. Microarray analysis was conducted to evaluate the DC3000 genes regulated by *hrpL* and *hrpRS* in MM. The analysis identified a number of novel *hrpL*-activated genes with a putative TTSS-independent function. Genes regulated by *hrpL* were mostly regulated by *hrpRS* in the same manner, but a large number of genes regulated by *hrpRS* were *hrpL*-independent, indicating that *hrpL* represents one branch of the regulatory pathways downstream of *hrpRS*. The induction of the TTSS genes was associated with downregulation of the housekeeping genes, indicating that the activation of the TTSS has a cost on the basic cellular activities. The novel genes and pathways identified by the microarray provide new insight into the bacterial functions coordinating with the TTSS.

Pseudomonas syringae pv. *tomato* DC3000 is a model pathogen that causes bacterial speck diseases in tomato and *Arabidopsis* (Whalen et al. 1991). The ability of DC3000 to infect its host plants depends on a cluster of genes, termed hypersensitive response and pathogenicity (*hrp*) genes, that are also essential for the induction of hypersensitive response (HR) and disease resistance on resistant plants (Collmer et al. 2000). *hrp* genes encode the type III secretion system (TTSS) through which the bacteria deliver a suite of proteins, named Hops (*hrp* out proteins), into the plant cells or into the plant intercellular spaces (Alfano and Collmer 2004). *hrp* genes are organized into five operons in the *hrp* cluster (Alfano et al. 2000). The complete genome sequence of DC3000 combined with various functional genomics studies enabled the identification of nearly forty Hops in DC3000 (Buell et al. 2003; Chang et al.

2005; Fouts et al. 2002; Guttman et al. 2002; Lindeberg et al. 2005; Petnicki-Ocwieja et al. 2002; Schechter et al. 2004; Zwiesler-Vollick et al. 2002).

In *P. syringae* strains, the expression of *hrp* and *hop* genes (together called TTSS genes) is regulated by environmental and host factors (Huynh et al. 1989; Rahme et al. 1992; Xiao et al. 1992, 2004). These genes are suppressed when bacteria grow in nutrient-rich medium such as KB (King et al. 1954) but are rapidly induced after the bacteria are infiltrated into plants or cultured in minimal medium (MM) (Huynh et al. 1989). The induction of TTSS genes is mediated by the “*hrp* box” promoters that carry a consensus motif of GGAACC-N15/16-CCACNNA (Xiao and Hutcheson 1994). The *hrp* box is recognized by the transcription activator HrpL, an alternate sigma factor of the ECF family (Xiao and Hutcheson 1994). Activation of *hrpL* requires HrpR and HrpS, two homologous DNA-binding proteins similar to the NtrC family regulators but lacking the modulating receiver domain (Xiao et al. 1994). HrpR and HrpS are encoded by the *hrpRS* operon in DC3000, and the two proteins form a heterodimer that binds the *hrpL* promoter to activate *hrpL* transcription (Hutcheson et al. 2001).

The identification of the complete gene set regulated by *hrpL* and *hrpRS* is critical for our understanding of the biological processes associated with bacterial parasitism. Two experimental approaches have been used to identify the *hrpL*-induced genes in DC3000 on the genome scale. Fouts and associates (2002) used the mini-Tn5gus transposon insertion approach to identify the DC3000 promoters that activate the reporter *gus* gene inserted in the DC3000 genome upon overexpression of *hrpL*. Chang and associates (2005) constructed a genomic library of DC3000 in a plasmid containing the promoterless green fluorescent protein (GFP) gene to identify genomic DNA fragments that activate the expression of GFP in cells overexpressing HrpL. Both approaches rely on the activation of reporter genes by HrpL overexpression. However, whether genes identified by these approaches display the *hrpL*-dependent expression in the wild-type DC3000 strain is unknown. In addition, it is unclear if the genes identified by these experimental approaches represent the complete list of the *hrpL*-activated genes in DC3000. Furthermore, although previous studies indicated that *hrpRS* and *hrpL* function in the same transcriptional cascade, it remains to be determined if *hrpL* and *hrpRS* regulate the same downstream genes. Finally, although the TTSS genes are known to be induced by *hrpL* and *hrpRS*, whether other cellular functions are also regulated by *hrpL* and *hrpRS*

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remains largely unknown. Therefore, we conducted whole-genome microarray analyses of DC3000 to determine genes regulated by *hrpL* and *hrpRS* in MM and genes that are differentially expressed in MM as compared with KB. This study identified a number of novel *hrpL*-dependent genes. Comparison of *hrpL*- and *hrpRS*-dependent genes revealed that *hrpRS* has a much broader regulation profile than does *hrpL*. *hrpL* appears to represent only one of the *hrpRS*-regulated pathways.

RESULTS AND DISCUSSION

Microarray hybridization, reproducibility, and data analysis.

To identify the *hrpL*- and *hrpRS*-dependent genes and genes that are differentially expressed in KB and MM on the genome scale, total RNA was prepared from wild-type (WT) DC3000 grown in KB at the log phase and from DC3000, *hrpL*⁻ mutants, and *hrpRS*⁻ mutants incubated in MM for 6 h; these RNA samples were named WT-KB, WT-MM, *hrpL*⁻-MM, and *hrpRS*⁻-MM, respectively. Each RNA sample was fluorescently labeled and was hybridized to an oligonucleotide array representing 5,608 predicted open reading frames (ORF) (97.3% of 5,763 ORF predicted in the DC3000 genome). To control the biological variation that might interfere with data interpretation, a minimum of two biological replicates were prepared for each sample. In addition, two analytical replicates (RNA derived from the same RNA pool) were prepared for WT-MM and *hrpL*⁻-MM. Microarray hybridizations of replicate samples showed a high correlation in log₂ relative fluores-

cence intensities, demonstrating a good reproducibility of the microarray data.

The transcription levels of the 5,608 predicted ORF in *hrpL*⁻-MM, *hrpRS*⁻-MM, and WT-KB were compared with those in WT-MM. The cutoff threshold was set to include most of the *hrp* and *hop* genes known to be regulated by *hrpRS*, *hrpL*, and MM (Chang et al. 2005; Fouts et al. 2002) and also to allow the novel genes to be selected stringently. For comparison of *hrpRS*⁻-MM and WT-MM, ORF that showed more than three-fold ($\log_2 3 \approx 1.585$) difference or more than twofold ($\log_2 2 = 1$) difference but with significance values of $P < 0.05$ (t -test) were considered to be differentially expressed. For comparisons of *hrpL*⁻-MM and WT-MM and WT-KB and WT-MM, a >3-fold difference or a >1.5-fold ($\log_2 1.5 \approx 0.585$) difference but with significance values of $P < 0.05$ (t -test) was used to determine the differentially expressed ORF. Globally, 122, 266, and 240 genes are regarded as differentially expressed in *hrpL*⁻-MM, *hrpRS*⁻-MM, and WT-KB when compared with expression in WT-MM, respectively.

Validation of the microarray data by RNA blotting.

To validate the microarray data, 14 genes representing a wide range of relative fluorescence signal intensity (from 884 to 18,853 in WT-MM) and different gene expression profiles were subject to RNA blot analysis. Of 23 data points that were indicated as differentially expressed by the microarray analyses, three data points (PSPTO1951, *hrpRS*⁻-MM; PSPTO0065, *hrpRS*⁻-MM; PSPTO1925, *hrpRS*⁻-MM) were inconsistent with the RNA blot assay (Fig. 1), indicating that the differential ex-

Fig. 1. Verification of the microarray data by Northern blotting. The coding region of each gene was polymerase chain reaction–amplified and radiolabeled with ³²P-dCTP as probes. * indicates genes that meet the cutoff criteria in microarray analysis. ** indicates genes that nearly meet the cutoff criteria. The numbers underneath each blot represent fold of difference relative to wild type in minimal medium (WT-MM) (the value is set at 1), based on microarray data. For instance, for PSPTO4001, 0.08 under WT-KB (wild type in King's B medium) means that the PSPTO4001 RNA in WT-KB is 8% of that in WT-MM. The ethidium bromide–gel pictures showing the loading of RNA samples are placed beneath images from the corresponding Northern blot analysis. The inconsistency of RNA loading was considered in comparison of gene expression. PSPTO1951, PSPTOA0065, and PSPTO1925 did not show significant differential expression between *hrpRS*⁻-MM (*hrpRS*⁻ in MM) and WT-MM and are inconsistent with the microarray data. Data points that showed significant differential expression in Northern blotting but not indicated by microarray as differentially expressed are not considered in the verification analysis, because these data points showed significant variations among biological replicates.

pression data obtained by microarray analysis are approximately 87% reliable.

HrpL upregulated genes in MM.

Comparison of *hrpL*-MM and WT-MM identified 76 *hrpL* upregulated genes in MM (excluding *hrpL*). Of these genes, 58 are known *hrp*, *hop*, and *shc* (specific hop chaperone) genes. Of the remaining 18 genes (Table 1), seven were previously identified as *hrpL*-induced genes (Boch et al. 2002; Chang et al. 2005; Fouts et al. 2002). Among the 11 genes newly identified in this study, three (PSPTO0835, PSPTO0836, PSPTO0874) were reported to be induced in plant but not regulated by *hrpL* when tested in *Escherichia coli* (Boch et al. 2002).

The 11 genes identified in this study were first screened for the presence of an hrp box motif in their promoter regions. Seven genes have a putative hrp box in their promoters (PSPTO0873, PSPTO2691, PSPTO4733, PSPTOB0005) or are closely located downstream (intergenic region <100 bp) of a gene with an hrp box promoter (PSPTO0874, PSPTO0875, PSPTO2679). These genes are likely to be regulated by the hrp box promoter. Three other genes (PSPTO5355, PSPTO1409, and PSPTO0851) are 125, 184, and 262 bp, respectively, downstream of ORF that are associated with an hrp box promoter. Promoter analysis of PSPTO1409 indicated that this gene is coregulated with the upstream PSPTO1408 by the hrp box promoter (Fig. 2). Similarly, another *hrpL*-regulated gene,

PSPTO0018 (*hopPtoSI*), which is separated from its upstream gene by 253 bp, is also coregulated with the upstream gene by the hrp box promoter (Fig. 2). Based on these results, we propose that PSPTO5355 and PSPTO0851, like PSPTO1409, are also controlled by the hrp box promoters. PSPTO2128 encodes a response regulator immediately downstream of the gene encoding a sensory box histidine kinase and response regulator protein. At 3,208 to 3,180 bp upstream of PSPTO2128 is a putative hrp box motif, located in the middle of PSPTO2131, encoding a sensor histidine kinase. Whether PSPTO2128 is regulated by this remote hrp box motif remains to be determined. Hence, of 76 *hrpL* upregulated genes identified by microarray, 75 genes are probably controlled by the hrp box promoter.

Eight genes identified by Chang and associates (2005) as *hrpL*-activated genes were excluded by our cutoff criteria. Six of these genes (PSPTO1372, PSPTO1378, PSPTO1568, PSPTO0502, PSPTO0044, and PSPTO0901) displayed low levels of *hrpL*- and *hrpRS*-dependent induction in MM and repression by KB, the same pattern as that of the other *hrpL* upregulated genes. These six genes are also preceded by the hrp box promoters. The other two genes (PSPTO4595 and PSPTO4680) showing an expression pattern different from the *hrpL*-activated genes in our microarray analysis do not appear to have a functional hrp box promoter. Four pseudogenes (PSPTO1407, PSPTO4705, PSPTO4724, and PSPTO4726) identified by Chang and associates (2005) as *hrpL*-activated

Table 1. Twenty-one *hrpL*-induced genes unrelated to known *hrp*, *hop* and *shc* genes^a

Synonym	Function	<i>hrpL</i> -MM/ WT-MM	<i>P</i> value (<i>t</i> -test)	Putative hrp box	Feature ^b	Start ^c	End ^c	<i>hrpL</i> activation ^d
PSPTO0371	Indoleacetate-lysine ligase	-1.5817	0.0178	GGAACCGATCTCGCCATCGAGG TAACCTCA	0	-113	-85	Chang, Fouts
PSPTO0834	Alcohol dehydrogenase, zinc-containing	-1.3868	0.0006	GGAACCAAACTGGAAAAACAT CCACTCA	2	-235	-207	Chang
PSPTO0835	ribD C-terminal domain protein	-2.0019	0.0087	PSPTO0834	2			Chang
PSPTO0836	Candidate type III effector Hop protein	-0.7286	0.0468	PSPTO0834	0			Chang
PSPTO0851	Conserved hypothetical protein	-0.7185	0.0178	PSPTO0852	0			
PSPTO0873	Amidinotransferase family protein	-2.0905	0.0064	GGAACCGATAACGACTTTTTGG CCACTTA	0	-918	-890	
PSPTO0874	Nikkomycin biosynthesis domain protein	-1.3070	0.0074	PSPTO0873	2,3			
PSPTO0875	Homolog of PSPTO0874	-0.6026	0.0459	PSPTO0873	0			
PSPTO1369	Conserved effector locus protein	-3.7755	0.0068	GGAACCGCATCACGCTTGAAC CACAGA	2	-61	-34	Fouts Boch
PSPTO1408	Hypothetical protein	-1.8815	0.0079	GGAACCGATTTCGATGAGTCGC CACACA	2,3	-529	-502	
PSPTO1409	Conserved hypothetical protein	-1.3117	0.0141	PSPTO1408	3			Fouts
PSPTO2105	Thiamine biosynthesis lipoprotein, putative	-0.6137	0.0183	GGAACCTGCGCAGGTCATTGA CCACTCA	0	-142	-114	Chang, Fouts, and Boch
PSPTO2128	Response regulator	-0.6695	0.0127	GGAACCTGAGTGACGGAAACGTT TCACTAA	0	-3208	-3180	
PSPTO2679	Hypothetical protein	-1.2016	0.0127	PSPTO2678	0			
PSPTO2691	Membrane protein, TerC family	-0.7233	0.0130	GGAACCTTTCGGTACAGGAGCTA CCACAAA	2	-104	-76	
PSPTO4733	Hypothetical protein	-1.8163	0.0147	GGAACCAATCCGCCTCAAAGT CCACTCA	0	-84	-56	
PSPTO5355	Hypothetical protein	-0.7060	0.0038	PSPTO5353	2			
PSPTOB0005	Phosphoesterase family protein	-2.2719	0.0053	GGAACCGATTCCGCTATCTGACC ACCTA	0	-818	-791	

^a The ratio of *hrpL*-MM to WT-MM was log transformed (base 2). *P* value (*t*-test) was calculated from three repeat experiments.

^b Features of the type III secretion signal pattern. 1 = >10% of amino acids are serine in the N-terminal 50 amino acids; 2 = position 3 or 4 contains Ile, Leu, Val, or Pro; 3 = no acidic amino acids in the first 12 positions; and 0 = none of 1, 2, or 3 as described above.

^c The start and ending sites of the putative hrp box relative to the translation start codon. To the genes that are not immediately downstream of a hrp box promoter, the upstream open reading frame preceded by the hrp box promoter are indicated in the column of "Putative hrp box".

^d References are provided to the genes known to be activated by *hrpL*. Chang = Chang et al. 2005, Fouts = Fouts et al. 2002, and Boch = Boch et al. 2002.

genes are not present in our microarray. The data from our microarray and Chang and associates (2005) clearly indicated the *hrp* box-dependent regulatory function of HrpL.

To evaluate if any of the 18 genes might encode a Hop candidate, proteins encoded by these genes were analyzed for the type III secretion-associated patterns. Alignment of the N-terminal 50 amino acids of known Hops has identified three features: i) >10% of amino acids are serine; ii) position 3 or 4 contains Ile, Leu, Val, or Pro; and iii) no acidic amino acids in the first 12 positions (Guttman et al. 2002; Petnicki-Ocwieja et al. 2002). None of these ORF have all three features; two ORF have two of the features, and six ORF have one of the features (Table 1).

BlastP analysis indicated that some of the *hrpL*-activated genes probably do not encode a Hop. PSPTO0371, the indole-acetate-lysine ligase gene (*iaaL*), was identified as an *hrpL*-dependent gene in DC3000 overexpressing *hrpL* (Chang et al. 2005; Fouts et al. 2002) and was implicated with a function in producing indole 3-acetic acid conjugates, the inactive form of the phytohormone auxin (Glass and Kosuge 1988). DC3000 is capable of producing auxin (Glickmann et al. 1998) that may interfere with the plant physiology. But why the bacterium produces IaaL to inactivate auxin is not immediately understood. PSPTO0805 encodes a protein of the phosphoesterase family and is secreted from bacterial cells by the twin-arginine translocation pathway (Bronstein et al. 2005). A phospholipase activity was detected for this protein in the culture medium (Bronstein et al. 2005). This protein may play a role in degradation of the plant cell membrane. PSPTO0873, PSPTO0874, and PSPTO0875 are likely to be organized in an operon regulated by the *hrp* box promoter upstream of PSPTO0873. PSPTO0873 encodes an amidinotransferase family protein; enzymes in the family include those involved in creatine and streptomycin biosynthesis (Humm et al. 1997; Pissowotzki et al. 1991). Proteins encoded by PSPTO0874 and PSPTO0875 are 54% identical, and both have a domain weakly homologous to D-alanine D-alanine ligase domain and similar to nikkomycin biosynthesis domain proteins (Buell et al. 2003). These enzymes may act together to catalyze the synthesis of certain secondary metabolites. The *Erwinia amylovora* locus corresponding to PSPTO0873-PSPTO0874-PSPTO0875 is near the *hrp* cluster and is required for systemic infection (Oh et al. 2005). PSPTO0834, PSPTO0835, and PSPTO0836 are also tightly clustered and probably belong to one transcription unit. PSPTO0834 encodes a zinc-containing alcohol dehydrogenase; PSPTO0835 encodes a riboflavin deaminase (RibD) C-terminal domain protein; PSPTO0836 encodes a protein with a haloacid dehalogenase-like hydrolase domain. These three proteins may act together to catalyze certain metabolic reactions. PSPTO2691 encodes a membrane protein of the TerC family. TerC proteins were implicated in resistance to tellurium, possibly by exporting tellurium ions that are highly toxic to most microorganisms (Taylor 1999). The DC3000 genome carries three TerC homologs. Whether PSPTO2691 has a role in tellurium resistance remains to be determined. The identification of these genes by microarrays indicated that the TTSS is coordinately activated with other protective and invasive mechanisms by HrpL. It will be interesting to determine the roles of the non-TTSS genes to the pathogenicity of DC3000 and other *P. syringae* strains. Assessment of these genes to the bacterial virulence will provide new insight into the parasitism of these bacteria.

hrpL downregulated genes in MM.

Based on our cutoff criteria, 46 genes were considered as *hrpL* downregulated genes in MM. Strikingly, most of these genes are involved in protein synthesis, including 21 ribosomal

proteins, one translation elongation factor, one 16S rRNA processing protein, and one DNA-directed RNA polymerase alpha subunit. The rest are involved in energy generation, metabolism, and stress responses. This result indicates that the DC3000 cell reduces its basic cellular activities during the activation of the TTSS. This is consistent with what was observed in *Yersinia* spp., that the activation of the TTSS was associated with growth arrest (Ramamurthi and Schneewind 2002). None of the *hrpL* downregulated genes carries an *hrp* box promoter, indicating that the elevated expression of the housekeeping genes in *hrpL* mutants in MM probably results from an indirect effect of the *hrpL* mutation. The molecular mechanisms coordinating the TTSS and the basic cellular activities are not known.

hrpRS upregulated genes in MM.

A total of 129 genes were identified as *hrpRS* upregulated genes in MM. Among these genes, 69 were also upregulated by *hrpL*. For the remaining 60 genes (Table 2), we investigated their possible regulation by *hrpL* by searching for the *hrp* box motif in their promoters.

hrpE and *hopA1* are likely to be activated by *hrpL*, although they were excluded by our cutoff criteria from the *hrpL* upregu-

Fig. 2. Assay of PSPTOA0018 and PSPTO1409 promoters. **A**, Schematic diagram of promoter regions of PSPTOA0018 and PSPTO1409 fused to the *luc* reporter gene. The *hrp*-box motifs are in the promoters of PSPTOA0017 and PSPTO1408, which are 253 and 184 bp upstream of PSPTOA0018 and PSPTO1409, respectively. The numbers in the parentheses following 18F1, 18F2, 09F1, and 09F2 indicate the beginnings and ends of the promoter regions relative to the translation initiation sites of PSPTOA0018 and PSPTO1409 that were analyzed in the promoter fusion constructs. For example, -1,063/+23 means that the promoter region covers 1,063 bp upstream and 23 bp downstream of the PSPTOA0018 translation initiation site. The diagrams are not in scale. **B**, Promoter activity assay. Bacteria were grown in King's B medium and subsequently induced in minimal medium (MM) for 6 h. Aliquots containing 1×10^7 bacterial cells were measured for luciferase activities using cooled CCD. Each data point consisted of three replicates. Error bars indicate standard errors. The experiments were repeated three times with similar results. *hrpL*-MM indicated promoter activity in *hrpL* mutant after a 6-h induction in MM. pHM2 lacking a cloned promoter did not show detectable LUC activity.

lated gene list. Both genes are in operons regulated by an *hrp* box promoter. *hrpE* and *hopA1* displayed 1.86- and 1.43-fold of *hrpL*-dependent induction, respectively, in the microarray analysis. *corR* was not identified by our microarray analysis as an *hrpL*-activated gene, but it was reported as an *hrpL*-induced gene in the DC3000 strain overexpressing *HrpL* (Fouts et al. 2002). *corR* is separated from the upstream *hopAQ1* by 151 bp, and the intergenic region does not bear a potential *hrp* box motif. However, there is a functional *hrp* box motif in the *hopAQ1* promoter that may drive the expression of *corR*, if the two genes belong to one operon. Consistent with the *hrpRS*-dependent induction of *corR* in MM, *cfa3* (PSPTO4683), a gene regulated by *corR* and involved in the biosynthesis of coronatine (Penfold et al. 1996), also displayed 2.34-fold of *hrpRS*-dependent induction. A putative *hrp* box motif was also found upstream of PSPTO1378, PSPTO1478, and PSPTO5546 genes.

The remaining *hrpRS* upregulated genes are considered to be *hrpL*-independent because of the lack of *hrpL*-dependent induction and the absence of an *hrp* box motif in their promoters.

Among them, five genes besides *corR* encode transcriptional regulators (Table 2). The identification of these transcriptional regulators indicated that *hrpRS* activates multiple pathways in addition to *hrpL*. It is possible that some of the *hrpRS*-regulated genes in Table 2 are mediated by these transcriptional regulators.

Twelve genes encode transporter proteins or a function directly associated with transport (Table 2). The transporter proteins are involved in transport of carbohydrates, iron, polyamine, sulfate, phosphate, and potassium. PSPTO2135 encodes the pyoverdine chromophore precursor synthetase. Pyoverdine is a siderophore that chelates iron in the extracellular medium and shuttles it into the cells via TonB-dependent iron transporter (Ravel and Cornelis 2003). Thus, PSPTO2135 is likely involved in iron transport. Coordination of the transport-associated genes with the TTSS genes may facilitate nutrient uptake by bacteria during the parasitic interactions with plants.

Four genes (PSPTO1240, PSPTO1236, PSPTO5002, and PSPTO3175) may be involved in the synthesis and degradation of exopolysaccharides. PSPTO1240 encodes a protein in algi-

Table. 2. Sixty *hrpRS*-induced genes in minimal medium (MM)^a

Synonym	Function	<i>hrpL</i> ⁻ -MM vs WT-MM	<i>P</i> value(<i>t</i> -test)	<i>hrpRS</i> ⁻ -MM vs WT-MM	<i>P</i> value(<i>t</i> -test)	WT-KB vs. WT-MM	<i>P</i> value(<i>t</i> -test)
TTSS genes (2)							
PSPTO1372*	Type III effector HopPtoA1	-0.5138	0.0828	-1.0886	0.0344	-0.5332	0.1876
PSPTO1386*	Type III secretion protein HrpE	-0.8936	0.0866	-2.0837	0.0387	-0.9392	0.0728
Transport and binding proteins (12)							
PSPTO0309	Sulfate ABC transporter, permease protein CysT	-0.0594	0.7169	-1.0227	0.0281	-0.177	0.3855
PSPTO0674	tonB domain protein	-0.0323	0.8478	-1.2096	0.0472	-0.1634	0.6555
PSPTO2242	Potassium-transporting ATPase, A subunit	-0.0732	0.4718	-1.0715	0.0056	-0.3605	0.1519
PSPTO2369	Ribose ABC transporter, permease protein	-0.3522	0.3086	-1.5179	0.0259	0.1113	0.5294
PSPTO2463	TonB-dependent siderophore receptor, putative	-0.1779	0.53	-1.2233	0.0165	-0.1472	0.3952
PSPTO3294	TonB-dependent siderophore receptor, putative	-0.2576	0.3153	-1.0257	0.0104	-0.2436	0.1939
PSPTO3716	ABC transporter, periplasmic substrate-binding protein	0.0362	0.77	-1.0408	0.0203	-0.212	0.5291
PSPTO3881	Polyamine ABC transporter, permease protein, putative	-0.2053	0.4567	-1.0826	0.0467	-0.2131	0.341
PSPTO4548	MFS transporter, phthalate permease family	-0.166	0.4405	-1.2574	0.0276	-0.1466	0.5114
PSPTO5486	Phosphate ABC transporter, permease protein, putative	-0.2385	0.2502	-1.0117	0.0235	-0.129	0.4473
PSPTO5561	Iron-compound ABC transporter, ATP-binding protein, putative	-0.1712	0.1979	-1.0469	0.0188	-0.1506	0.2871
PSPTO5563	Haemin ABC transporter, permease protein, putative	0.0434	0.7817	-1.1308	0.0254	-0.0186	0.9372
Cell envelope (8)							
PSPTO0120	Membrane protein, putative	-0.1036	0.4656	-1.0792	0.0136	-0.1901	0.3606
PSPTO1236	Alginate lyase	-0.0548	0.753	-1.0065	0.0341	-0.1197	0.5746
PSPTO1240	Alginate biosynthesis protein AlgK	0.0297	0.8398	-1.39	0.0113	-0.2096	0.4329
PSPTO1378*	Membrane-bound lytic murein transglycosylase D, putative	-0.346	0.1622	-1.1244	0.0275	-0.3401	0.2124
PSPTO3715	Membrane-bound lytic murein transglycosylase D	0.0115	0.9266	-1.0516	0.0096	-0.1064	0.6048
PSPTO4471	Rod shape-determining protein MreC	-0.2209	0.2902	-1.0459	0.013	0.0636	0.5739
PSPTO5002	Lipopolysaccharide heptosyltransferase	-0.0164	0.9284	-1.2214	0.0449	0.0458	0.8476
PSPTO5102	Membrane protein, putative	-0.0619	0.7688	-1.0065	0.0184	-0.0816	0.4868
Regulatory functions (6)							
PSPTO0781	Transcriptional regulator, LysR family	-0.0357	0.8645	-1.1543	0.029	-0.0642	0.7082
PSPTO1042	Transcriptional activator ChrR	-0.0974	0.6474	-1.1679	0.0179	-0.3518	0.0883
PSPTO1578	Transcriptional regulator, LysR family	-0.1758	0.2401	-1.0896	0.011	-0.246	0.2249
PSPTO2594	Transcriptional regulatory protein NfxB	0.0231	0.922	-1.004	0.018	0.0018	0.9911

(Continued on the next page)

^a For each pair-wise sample comparison, the ratio was log-transformed (base 2). A 5-kb upstream sequence of the 60 genes were searched for a potential *hrp* box. Genes with a potential *hrp* box are:

PSPTO1372 (cagtGGAACCGTCAACCGATCCGGGACCACAGCCttcg, -193 to -162)
 PSPTO1386 (cgctGGAACCGATTTCGAGGCTGCTGCCACCTAGTGagta, -3365 to -3334)
 PSPTO1378 (aaaaGGGAACCGTAACGGCGAGCGTGCCACGTAGGGtgga, -58 to -27)
 PSPTO4704 (gctaGGAAGCTGCAAGCTGGTGCGGCTCCACTAAAAAcctc, -515 to -484)
 PSPTO1478 (cgccTGAAACTTGCTGCCGTCGATGCCACCAATTCaccg, -592 to -561)
 PSPTO5546 (cggtGAAACCGAGCTGAGCGCCAACACCACATGGAGcgac, -817 to -786).

nate polymer formation and is in the same operon with PSPTO1236, encoding the alginate lyase (Jain and Ohman 1998). Alginate lyase has a role in clearing away the alginate polymers accumulated in the periplasm (Bakkevig et al. 2005). Both genes are important for cell-wall development in *P. aeruginosa* (Jain and Ohman 2005). PSPTO5002 encodes the lipopolysaccharide heptosyltransferase that is involved in the lipopolysaccharide synthesis (Kanipes et al. 2003). PSPTO3175 encodes the membrane-bound lytic murein transglycosylase D protein and is homologous to PSPTO1378, another *hrpRS*-induced gene with an *hrp* box promoter. But the two genes share <70% homology at the nucleotide levels; thus, cross-hybridization is unlikely to be the cause of the similar expression patterns of the two genes. Deletion of PSPTO1378 in DC3000 reduces the bacterial virulence in tomato plants (Boch et al. 2002). Induction of these genes by *hrpRS* may be important to the dynamics of the bacterial cell-wall structure.

PSPTO2408 and PSPTO2411 are in the same operon and encode two subunits of urease with a function converting urea

to ammonia and CO₂. A few other discrete genes of diverse functions were also identified as the *hrpRS*-induced genes in MM. Nineteen genes encode proteins of unknown function.

The analyses showed that nearly all of the *hrpL* upregulated genes also are up-regulated by *hrpRS*. But a large number of genes of diverse functions upregulated by *hrpRS* are *hrpL*-independent. This result is consistent with the upstream position of *hrpRS* in the *hrpRS*-*hrpL* regulation pathway and indicates that *hrpRS* regulates additional genes other than *hrpL*.

***hrpRS* downregulated genes.**

It is striking that 54 transposase genes displayed more than twofold (*P* value < 0.05, *t*-test) induction in *hrpRS*⁻ mutants compared with the WT strain grown in MM. Because multiple copies of transposase genes exist in the DC3000 genome, we have not determined which genes are responsible for the increased transcripts in the *hrpRS*⁻ mutant. Nevertheless, this observation suggested that certain transposase genes are negatively regulated by *hrpRS* in MM.

Table 2. (Continued from preceding page)

Synonym	Function	<i>hrpL</i> ⁻ -MM vs WT-MM	<i>P</i> value(<i>t</i> -test)	<i>hrpRS</i> ⁻ -MM vs WT-MM	<i>P</i> value(<i>t</i> -test)	WT-KB vs. WT-MM	<i>P</i> value(<i>t</i> -test)
Regulatory functions (6) (Continued)							
PSPTO4080	DNA-binding response regulator, LuxR family	-0.2873	0.1674	-1.1353	0.0142	-0.3569	0.0963
PSPTO4704*	DNA-binding response regulator CorR	-0.272	0.1908	-1.4473	0.0052	-0.2676	0.0963
Protein fate (2)							
PSPTO1683	Peptidase, SprT family	0.0246	0.8276	-1.0915	0.0196	-0.1841	0.5269
PSPTO3634	Heme exporter protein CcmB	-0.0859	0.6851	-1.0991	0.044	0.0945	0.7466
Cellular processes (1)							
PSPTO4683	Coronafacic acid beta-ketoacyl synthetase component	0.1443	0.4811	-1.2276	0.0302	0.1077	0.7099
Central intermediary (2)							
PSPTO2408	Urease accessory protein UreD	0.0807	0.6383	-1.2492	0.0459	-0.0876	0.7099
PSPTO2411	Urease, beta/gamma subunit	-0.1904	0.357	-1.1063	0.0072	-0.3806	0.2738
Energy metabolism (2)							
PSPTO1357	Aliphatic amidase	-0.2787	0.2235	-1.0132	0.0222	-0.1029	0.5956
PSPTO2911	Mandelate racemase/muconate lactonizing enzyme family protein	-0.1432	0.3405	-1.0972	0.0158	-0.2562	0.1947
Fatty acid and phospholipid metabolism (2)							
PSPTO4660	Acyl-CoA thioesterase II	-0.1286	0.4969	-1.102	0.037	-0.2281	0.2569
PSPTO5184	Acyl-CoA dehydrogenase family protein	0.064	0.6962	-1.0983	0.0272	0.102	0.7045
Protein synthesis (1)							
PSPTO4183	Ribosomal protein L31	-0.005	0.9673	-1.1552	0.0096	0.033	0.8376
Amino acid biosynthesis (1)							
PSPTO2732	5-Methyltetrahydrofolate--homocysteine methyltransferase	-0.0541	0.7215	-1.4488	0.0057	-0.01	0.9391
DNA metabolism (1)							
PSPTO1478*	Integrase/recombinase XerD	-0.1763	0.3011	-1.2653	0.0111	-0.1647	0.3629
Biosynthesis of cofactors, prosthetic groups, and carriers (1)							
PSPTO2135	Pyoverdine chromophore precursor synthetase	-0.1902	0.445	-1.061	0.0206	-0.3961	0.0938
Unknown function (19)							
PSPTO0561	Hypothetical protein	-0.0331	0.7906	-1.0857	0.0173	-0.0895	0.7887
PSPTO2067	Hypothetical protein	-0.2492	0.1847	-1.105	0.0285	0.0247	0.934
PSPTO2271	Hypothetical protein	-0.1658	0.3045	-1.201	0.0045	0.6025	0.4922
PSPTO3622	Hypothetical protein	0.0331	0.9498	-1.7093	0.1609	-0.0183	0.9813
PSPTO3697	Hypothetical protein	-0.048	0.7673	-1.2992	0.0165	-0.204	0.5721
PSPTO4050	Hypothetical protein	-0.095	0.6066	-1.0641	0.0352	-0.1128	0.6636
PSPTO4053	Hypothetical protein	-0.1802	0.3519	-1.0668	0.0459	0.022	0.9364
PSPTO4993	Hypothetical protein	-0.2753	0.4024	-1.1334	0.0385	-0.2307	0.3809
PSPTO5088	Hypothetical protein	-0.3417	0.3281	-1.9839	0.0399	0.1421	0.8058
PSPTO5362	Hypothetical protein	-0.1632	0.4632	-1.6589	0.0074	-0.2424	0.0966
PSPTO5386	Hypothetical protein	-0.1991	0.5173	-1.3638	0.0356	-0.0412	0.8802
PSPTO5546*	Hypothetical protein	-0.1797	0.2644	-1.104	0.006	-0.0762	0.7646
PSPTO0290	Conserved hypothetical protein	0.1072	0.4667	-1.1377	0.0208	0.1078	0.5416
PSPTO0479	Conserved domain protein	-0.3332	0.3236	-1.0715	0.0405	-0.3811	0.1192
PSPTO2982	Conserved hypothetical protein	0.0086	0.9471	-1.171	0.0098	-0.0052	0.9648
PSPTO3597	Conserved hypothetical protein	0.0053	0.9822	-1.1978	0.0248	-0.1124	0.5294
PSPTO3858	Conserved hypothetical protein	-0.1716	0.2723	-1.0634	0.0018	-0.2183	0.0345
PSPTO0996	HD domain protein	0.0009	0.9959	-1.0552	0.0308	-0.1599	0.4876
PSPTO2231	YD repeat protein	-0.0295	0.7314	-1.0712	0.0001	-0.3972	0.1389

A total of 83 genes other than transposase genes displayed more than twofold induction in the *hrpRS*⁻ mutant compared with the WT strain in MM and are considered to be *hrpRS* downregulated genes. A total of 20 are listed as *hrpL* downregulated genes, including 18 genes involved in protein synthesis. Of the remaining 63 genes (Table 3), a significant number displayed a low level of induction in *hrpL*⁻ mutants; many of these genes are also housekeeping genes. The result further supported the opposite regulation of the TTSS and basic cellular activities. A number of genes induced in *hrpRS*⁻ MM are *hrpL*-independent in MM, including those related to flagella function, chemotaxis, membrane transport, leucine synthesis, and transcriptional regulation (Table 3). Most of these genes are induced in WT-MM as compared with WT-KB.

Eight *hrpRS* downregulated genes are involved in transcriptional regulation, including two DNA-binding proteins of the HU family (PSPTO1266 and PSPTO5472). HU family proteins bind DNA and dynamically modulate the nucleoid architecture in response to environmental changes, thus dictating the change of global transcription profiles (Kar et al. 2005). The identification of the multiple regulatory genes suggested that *hrpRS* negatively regulates diverse pathways. Some of the *hrpRS*-repressed genes in Table 3 are probably regulated directly by these regulatory genes. For example, FlgM, the negative regulator of flagella (Sorenson et al. 2004), is likely the regula-

tor of another five flagella genes. Suppression of the flagella genes by *hrpRS* under the *hrp*-inducing condition is probably beneficial to bacterial infection, because the flagella structural protein flagellin is a known pathogenicity-associated molecular pattern that triggers the host defense response (Zipfel et al. 2004). Flagella and chemotaxis genes are often coordinately regulated (Scharf and Schmitt 2002), and this is consistent with the similar expression pattern of these genes in the *hrpRS*⁻ mutant.

Promoter motif analysis of *hrpRS*-regulated genes.

The microarray data suggested that *hrpRS* regulates genes of multiple pathways. In an attempt to find the *hrpRS*-regulated promoter motifs, we searched for the over-represented patterns in the promoters of *hrpRS*-regulated genes using the MEME and Regulatory Sequence Analysis Tools (RSAT). No over-represented patterns could be identified if all *hrpRS* upregulated genes (excluding *hrpL*-activated genes) or all *hrpRS* downregulated genes were included in the pattern search. However, when we narrowed the search to only the regulatory genes, four over-represented motifs were found. These motifs are present in the *hrpL* promoter region and the region upstream of *hrpS* that was reported to bind HrpR (Grimm et al. 1995). Whether these motifs play a role in *hrpRS*-mediated gene regulation remains to be determined.

Table 3. Sixty-three *hrpRS*-downregulated genes in minimal medium (MM)^a

Synonym	Function	<i>hrpRS</i> ⁻ -MM vs. WT-MM	<i>P</i> value (<i>t</i> -test)	<i>hrpL</i> ⁻ -MM vs. WT-MM	<i>P</i> value (<i>t</i> -test)	WT-KB vs. WT-MM	<i>P</i> value (<i>t</i> -test)
Cellular processes (11)							
PSPTO1274	Cold shock domain family protein	1.4027	0.0103	0.3693	0.1185	-0.906	0.0244
PSPTO1927	Chemotaxis protein CheV, putative	1.1615	0.0438	-0.0758	0.761	-0.8976	0.056
PSPTO1933	Flagellar basal-body rod protein FlgB	1.1837	0.0043	0.0503	0.794	-0.7786	0.0366
PSPTO1934	Flagellar basal-body rod protein FlgC	1.0869	0.0375	-0.1481	0.6533	-1.0733	0.0659
PSPTO1936	Flagellar hook protein FlgE	1.2889	0.0235	0.1236	0.479	-0.8911	0.0359
PSPTO1950	Flagellin FlaG, putative	1.4313	0.0285	0.0567	0.9217	-1.1523	0.1751
PSPTO1951	Flagellar hook-associated protein FlhD	1.6899	0.0076	0.34	0.3656	-1.1424	0.1111
PSPTO1988	Chemotaxis protein CheW	1.4077	0.0134	0.3217	0.3007	-0.6675	0.1287
PSPTO3237	Methyl-accepting chemotaxis protein	1.0129	0.0068	0.0543	0.7912	-0.4381	0.064
PSPTO3524	Chemotaxis protein CheV, putative	1.1524	0.0007	0.418	0.11	-0.6856	0.0524
PSPTO3925	Gas vesicle protein, putative	1.3157	0.0122	0.2828	0.4751	-1.2401	0.0547
Protein synthesis (7)							
PSPTO0622	Ribosomal protein S7	1.121	0.0173	0.575	0.0217	2.0685	0.0735
PSPTO0623	Translation elongation factor G	1.254	0.0095	0.5934	0.0537	2.1702	0.0589
PSPTO0630	Ribosomal protein S19	1.139	0.0363	0.6952	0.0662	2.686	0.0434
PSPTO1205	Ribosomal subunit interface protein, putative	1.6128	0.0114	0.3307	0.2248	1.3392	0.144
PSPTO2379	Translation initiation factor IF-3	1.2766	0.0413	0.3643	0.1676	-0.0509	0.8244
PSPTO2381	Ribosomal protein L20	1.2853	0.0097	0.2695	0.3491	0.6496	0.2341
PSPTO4454	Ribosomal subunit interface protein	1.4235	0.0323	0.3157	0.3673	-0.4371	0.451
Regulatory functions (8)							
PSPTO1925	Negative regulator of flagellin synthesis FlgM, putative	1.3269	0.0248	0.0935	0.8341	-0.818	0.2088
PSPTO0217	Nitrogen regulatory protein P-II	1.4077	0.017	0.5746	0.0321	0.037	0.8753
PSPTO0430	RNA polymerase sigma-32 factor	1.2211	0.0492	0.1494	0.5191	0.0609	0.8432
PSPTO0465	Transcriptional regulator, AraC family	1.0796	0.0012	0.1661	0.1685	-0.5572	0.0364
PSPTO1847	Transcriptional repressor, putative	1.0551	0.0281	0.4119	0.0737	0.413	0.2342
PSPTO3103	Transcriptional regulator, putative	1.1244	0.0089	0.178	0.2894	-0.537	0.1664
PSPTO5472	DNA-binding protein HU family	1.5284	0.0116	0.2141	0.575	-3.0917	0.0257
PSPTO1266	DNA-binding protein HU family	1.1465	0.0122	1.0018	0.0741	1.9765	0.0228
Energy metabolism (5)							
PSPTO0728	Aldehyde dehydrogenase family protein	1.3084	0.0058	0.3904	0.1222	-0.5644	0.0836
PSPTO2200	2-Oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succinyltransferase	1.11	0.0352	0.4889	0.0154	0.8113	0.0314
PSPTO2201	2-Oxoglutarate dehydrogenase, E3 component, lipoamide dehydrogenase	1.2589	0.0287	0.3969	0.1351	0.9871	0.0979
PSPTO2202	Succinyl-CoA synthase, beta subunit	1.4749	0.0059	0.4852	0.0936	1.1666	0.1526
PSPTO5243	Thioredoxin	1.0157	0.0113	0.4699	0.0551	0.7043	0.0324

(Continued on next page)

^a For each pair-wise sample comparison, the ratio was log-transformed (base 2). *P* value (*t*-test) was calculated from three repeat experiments.

Genes differentially expressed in KB and MM.

Comparison of gene-expression profiles in DC3000 grown in KB and MM identified 132 genes induced by MM. Of these genes, 66 are *hrpL*-dependent. This is consistent with the induction of *hrpL* by MM. Of the *hrpL*-independent genes, PSPTO5472, encoding the HU family DNA-binding protein, is induced in MM more than eightfold. This gene may play an important role in changing the global gene-expression profiles when DC3000 cells were transferred from KB to MM. Another *hrpL*-independent gene is PSPTO2310 (ribosome modulation factor-related protein). PSPTO2310 was induced more than 30-fold in MM. The function of this protein is to inactivate ribosome by covering the peptidyl transferase center and entrance of the peptide exit tunnel (Yoshida et al. 2004). Inactivation of ribosome is directly linked to the reduced protein synthesis, a response that is often observed in bacteria under nutrient starvation (Givskov et al. 1994). Five genes were implicated with stress-related functions, including PSPTO3355 (cold-shock domain family protein), PSPTO1565 (RpoS), PSPTO4530 (catalase/oxidase HPI), PSPTO1274 (cold-shock domain family protein), and PSPTO5317 (antioxidant, AhpC/Tsa family). RpoS is an alternate sigma factor regulating various stress-related genes and is required for the virulence of *P. syringae* bacteria in plants (Chatterjee et al. 2003; Miller et al. 2001). The induction of stress-related proteins is also ob-

served in bacteria in response to nutrient starvation (Givskov et al. 1994). The induction of genes encoding the ribosome-inactivating protein and stress-related proteins in MM reflects the nutrient-poor condition of MM. Multiple genes involved in chemotaxis and flagella function are also induced in MM. Other genes induced in WT-MM are involved in diverse functions, such as transport, metabolisms, protein degradation, and transcriptional regulation. Half (33 of 66) of the *hrpL*-independent genes induced in MM encode hypothetical proteins.

A total of 108 genes were considered to be downregulated genes in MM. The majority (76 of 108) of the genes are involved in protein synthesis (including the ribosomal proteins, translation elongation factors, and RNA polymerase subunits) and energy metabolism. Whereas the reduced expression of the housekeeping genes is associated with bacterial response to nutrient starvation (Givskov et al. 1994), the opposite expression pattern of the housekeeping genes and the TTSS genes in MM was also observed in the *hrpL*⁻ and *hrpRS*⁻ mutants grown in MM. Five chaperonin proteins involved in maintaining stability of other proteins are also reduced in MM. Two DNA-binding proteins of the HU family (PSPTO1266 and PSPTO3723) are repressed in MM. These genes may similarly play a role in regulating the global gene regulation but in a way opposite to PSPTO5472, which is induced in MM. Also downregulated in MM are the operon-encoding tellurium resis-

Table 3. (Continued from preceding page)

Synonym	Function	<i>hrpRS</i> ⁻ -MM vs. WT-MM	<i>P</i> value (<i>t</i> -test)	<i>hrpL</i> ⁻ -MM vs. WT-MM	<i>P</i> value (<i>t</i> -test)	WT-KB vs. WT-MM	<i>P</i> value (<i>t</i> -test)
Transport and binding proteins (4)							
PSPTO1255	Amino acid ABC transporter, periplasmic amino acid-binding protein	1.1037	0.0312	0.1873	0.4344	-0.897	0.0781
PSPTO1296	Porin B	1.5568	0.0184	0.5069	0.2508	-1.3266	0.086
PSPTO3987	Porin D	1.27	0.0444	0.0343	0.9455	-2.1365	0.0773
PSPTOB0010	Major facilitator family transporter	1.1711	0.0006	0.2475	0.0827	-0.290	0.3788
Mobile and extrachromosomal element functions (3)							
PSPTOA0008	Partition protein, ParB family	1.5585	0.0045	ND	-	-0.3718	0.373
PSPTOA0009	ATPase, ParA family	1.5621	0.0301	ND	-	0.1171	0.8269
PSPTOA0065	Exclusion-determining protein, putative	1.7512	0.0142	0.1724	0.6052	-0.3819	0.3952
Cell envelope (3)							
PSPTO0927	Type IV pilus biogenesis protein	1.1874	0.0341	0.2433	0.2047	0.1752	0.6265
PSPTO2272	Outer membrane lipoprotein OprI	1.3188	0.0147	0.6961	0.0888	0.4574	0.4954
PSPTOA0007	Membrane protein, putative	1.499	0.0421	ND	-	-0.9022	0.0543
Amino acid biosynthesis (3)							
PSPTO2173	3-Isopropylmalate dehydratase, large subunit	1.4194	0.0399	0.1398	0.6837	-0.8355	0.1764
PSPTO2174	3-Isopropylmalate dehydratase, small subunit	1.1922	0.0245	0.1475	0.6203	-0.9403	0.0961
PSPTO2175	3-Isopropylmalate dehydrogenase	1.0411	0.0068	0.2146	0.1766	-0.3916	0.1147
Central intermediary (1)							
PSPTO5147	Polyhydroxyalkanoate granule-associated protein PhaF	1.4271	0.009	0.2699	0.3829	-1.0738	0.055
Unknown function (18)							
PSPTO0231	Conserved hypothetical protein	1.4879	0.0088	0.524	0.1791	-1.9001	0.0341
PSPTO1246	PhoH-like protein	1.9199	0.1721	0.1078	0.7378	-1.3635	0.0505
PSPTO1725	Conserved hypothetical protein	1.0198	0.0182	0.2897	0.3217	-0.3927	0.2543
PSPTO1840	Hypothetical protein	1.5214	0.0061	0.4685	0.1027	-0.76	0.1262
PSPTO1953	Conserved hypothetical protein	1.1986	0.0044	0.1709	0.3742	-0.5485	0.0611
PSPTO2025	Conserved hypothetical protein	1.8779	0.1882	0.07	0.6905	-0.3248	0.0851
PSPTO2049	Conserved hypothetical protein	1.6191	0.3365	0.2947	0.3847	-0.9615	0.0052
PSPTO2057	Conserved hypothetical protein	2.4604	0.1182	0.411	0.1401	-0.8584	0.1063
PSPTO2700	Hypothetical protein	2.0002	0.0105	0.5836	0.0536	-0.8391	0.041
PSPTO3123	Hypothetical protein	1.9221	0.0071	0.3179	0.4928	-2.2167	0.0601
PSPTO3544	Conserved hypothetical protein	1.657	0.0095	0.6822	0.0554	-1.6697	0.0346
PSPTO3645	Conserved hypothetical protein	1.1849	0.0034	0.2975	0.0789	0.5668	0.0697
PSPTO4484	Conserved hypothetical protein	1.7698	0.2401	0.6316	0.1229	-2.1029	0.0001
PSPTO4529	Hypothetical protein	1.0109	0.0136	0.5889	0.0625	1.2425	0.1256
PSPTO4900	Phosphate starvation-inducible protein PsiF	1.2765	0.031	0.3597	0.074	-0.5577	0.0518
PSPTOA0058	Conserved hypothetical protein	1.625	0.0072	0.0462	0.7487	0.2861	0.5449
PSPTOB0003	Conserved hypothetical protein	1.0838	0.034	-0.3248	0.4262	-0.3527	0.5231
PSPTOB0021	Conserved hypothetical protein	1.0088	0.0078	0.1959	0.3811	1.4988	0.2349

tance proteins (PSPTO0942, PSPTO0944, and PSPTO0945) and a few discrete genes of diverse functions.

Cluster analysis of genes regulated by *hrpL*, *hrpRS*, and KB.

hrpL-, *hrpRS*-, and KB-regulated genes (excluding the transposase genes) were subject to cluster analysis using the average linkage clustering algorithm. These genes fall into four major clusters (Fig. 3). There are a total of 80 genes in cluster I, with the pattern of upregulation in WT-KB, *hrpRS*⁻-MM, *hrpL*⁻-MM when compared with WT-MM. Of these, 63 genes are involved in basic cellular functions such as protein synthesis and energy metabolism. The upregulation of these genes in WT-KB, *hrpRS*⁻-MM, and *hrpL*⁻-MM indicated elevated basic cellular activities when the TTSS is silenced.

Cluster II contains 79 genes that were down-regulated in WT-KB, *hrpRS*⁻-MM, and *hrpL*⁻-MM compared with WT-MM. All these genes, except PSPTO2128 and *hrpL*, are likely to be controlled by *hrp* box promoters. Most genes in this cluster are TTSS genes. However, a number of genes apparently have a function different from that of the TTSS.

Fig. 3. Cluster analysis of the 365 differentially expressed gene. The microarray expression levels are hierarchically clustered using the average linkage clustering methods. The scale of gene activities is represented from green (downregulated) to red (upregulated).

A total of 57 genes were classified into cluster III by the average linkage clustering algorithm, although a few genes in this cluster (for example, PSPTO1372, PSPTO1378, and *corR*) may actually belong to cluster II. Nevertheless, the majority of genes in this cluster seems to be induced specifically by *hrpRS* but is independent of *hrpL* and medium conditions. Genes in cluster III are functionally diverse. Consistently, at least five transcriptional regulator genes belong to this cluster. It is possible that *hrpRS* regulates the expression of these regulatory genes, which in turn regulate downstream genes of diverse functions. An outstanding group of genes in this cluster is the group of 13 transport-related genes that may play an important role in nutrient uptake. At least four genes in this cluster are involved in exopolysaccharide metabolism.

The 83 genes in cluster IV are down-regulated in WT-KB and up-regulated in *hrpRS*⁻-MM but did not show a significant change in *hrpL*⁻-MM as compared with WT-MM. Nearly half of the genes in this cluster (40 of 83) have unknown functions, and the rest of the genes have diverse functions in cellular processes, transport, transcriptional regulation, metabolism, amino acid biosynthesis, and protein degradation.

To determine if genes in each cluster share a conserved promoter motif, the upstream sequence (–1 to –400 bp) of each of the genes was retrieved, and the sense strands were searched for over-represented patterns, using the MEME program with default parameter and the maximum width of 10 bp. This search identified the *hrp* box motifs in cluster II and another motif (CTGCTGG) apparently over-represented in clusters II (24 of 79 upstream sequences or 30.4%) and III (14 of 57 upstream sequences or 24.6%) but not so in clusters I (10%) and IV (6%). This motif is found in the upstream region of 801 genes of the 5,608 ORF (14.3%) in the DC3000 genome. The common feature of genes in clusters II and III is the *hrpRS*-dependent induction in MM. However, genes in cluster II are mostly regulated by *hrpL*, while genes in cluster III are mostly *hrpL*-independent. This result raises a question if some genes activated by the *hrp* box motif can be induced by a second motif directly under the control of *hrpRS*.

MATERIALS AND METHODS

Bacterial strains.

Bacterial strains used in this study are *P. syringae* pv. *tomato* DC3000 (Buell et al. 2003) and the *hrpL*⁻ and *hrpRS*⁻ mutant strains derived from DC3000. The *hrpL*⁻ mutant was provided by B. Kunkel (Zwiesler-Vollick et al. 2002). *hrpRS*⁻ mutant was isolated from the DC3000 transposon-insertion mutant library. The mutant carries the EZ-Tn5<TET-1> transposon inserted 173 bp downstream of the *hrpR* translation initiation site. Southern blot analysis indicated that the mutant carries a single-copy transposon insertion. This mutant was called *hrpRS*⁻ mutant because its HR and disease-inducing activity could be complemented by the *hrpRS* locus but not by *hrpR* alone, indicating polar mutation. Northern blotting with *hrpR* and *hrpS* as probes did not detect clear hybridization signal of the mutant strain (data not shown). DC3000 and the *hrpL*⁻ and *hrpRS*⁻ mutant strains were grown in KB broth (King et al. 1954) to approximately 7×10^8 CFU/ml. The bacteria were centrifuged, were washed twice with MM (Huynh et al. 1989), and were resuspended in MM to 3×10^8 CFU/ml. The bacteria were cultured in MM for 6 h before being harvested for RNA extraction. The wild-type DC3000 strain was grown in KB broth to 5×10^8 to 7×10^8 CFU/ml and was harvested for gene expression analysis in KB.

RNA isolation and Northern blotting.

RNA was extracted using a modified hot phenol procedure (Aiba et al. 1981). Briefly, 15 ml of bacterial culture was pel-

leted by centrifugation. The pellet was mixed with 2 ml of extraction buffer (20 mM sodium acetate at pH 5.5, 0.5% sodium dodecyl sulfate, 1 mM EDTA) and an equal volume of phenol preheated to 60°C. After vortexing, the bacterial suspension was incubated at 60°C for 5 to 10 min, chilled on ice for 3 to 5 min, and was then centrifuged. The supernatant was extracted by acid phenol-chloroform twice and was precipitated with 2.5 vol of 100% ethanol. RNA was pelleted, washed with 75% ethanol, and resuspended in RNase-free water. Contaminating DNA was digested with RQ1 DNase (Promega, Madison, WI, U.S.A.). Total RNA (10 µg) was used for Northern blot analysis, following the procedures described by Tang and associates (1999). The coding regions of the tested genes were PCR-amplified and radiolabeled with ³²P dCTP as probes. For microarray hybridization, the RNA was purified by using an RNeasy midi kit, following the manufacture's instructions (Qiagen, Valencia, CA, U.S.A.).

High-density oligonucleotide microarray.

A high-density oligonucleotide array was designed to cover 5,608 predicted ORF of DC3000. Each probe pair has a perfect match oligonucleotide of 24 nt and a mismatch oligonucleotide of the same sequence with substitutions at positions 6 and 12. The substitutions are A to T, C to G, G to C, and T to A. Seventeen distinct high-quality probe pairs were designed for each ORF.

First-strand cDNA synthesis, labeling, and microarray hybridization.

First-strand cDNA was synthesized by mixing 6 µl of 200 U/µl SuperScript II (Invitrogen, Carlsbad, CA, U.S.A.) with 10 µg of total RNA template in a 60-µl reaction mixture. The reaction was incubated at 42°C for 55 min and then at 72°C for 15 min. To degrade RNA, 1 µl of RNase H (Invitrogen) and 1 µl RNase A (Promega) was added to the reaction mixture. After a 10-min incubation at 37°C, the mixture was extracted with phenol, the cDNA was precipitated by ethanol, and the yield of the cDNA was determined. The cDNA was partially digested to 50- to 200-bp fragments by DNaseI treatment (Promega). The digested cDNA was collected and the 3' end was labeled with biotin-6-ddATP and deoxynucleotidyl terminal transferase (Promega). Array hybridization, washing, and staining were performed according to the standard protocol provided by NimbleGen Systems Inc. (Madison, WI, U.S.A.). Arrays were stained with Cy3-streptavidin and then antistreptavidin antibody before scanning with an Axon Genepix 4000B scanner at 532 nm and a resolution of 5 µm.

Microarray data analysis and motif identification.

For each probe on the array, the median signal intensity was calculated using the NimbleGen extraction software. The data were normalized by using quantile normalization (Bolstad et al. 2003), and gene calls were generated by using the RMA algorithm (robust multichip average) (Irizarry et al. 2003). The relative fluorescent intensities of each ORF were scaled by assuming a constant mean signal intensity of 1,000 signal units for each array, and the intensity values of replicate measurements were averaged. For each pair-wise sample comparison, *P* values were calculated for each ORF using the two-sided *t*-test, and the ratio was log-transformed (base 2).

Motif search was restricted to the region 400-nt upstream of the translation start codon, because most known prokaryotic transcription factor binding sites can be found in this region (McGuire et al. 2000; Mironov et al. 1999). RSAT were used for upstream sequence retrieving and motif search. The MEME program (Bailey and Elkan 1994) was used to search the over-represented patterns in the sense strand with the default

parameter. The logos of motifs were generated using Weblogo. Hierarchical clustering was performed using Cluster and Tree-View as described by Eisen and associates (1998).

Analysis of PSPTO1409 and PSPTOA0018 promoters.

Reporter genes were constructed for analysis of the PSPTO1409 and PSPTOA0018 promoters. To clone the region containing the PSPTO1409 promoter, a 1,084-bp fragment (−1,061 to +23 relative to the putative translation start codon) and a 214-bp fragment (−191 to +23 relative to the putative translation start codon) were PCR-amplified, using the DC3000 genomic DNA as template and the primer pairs of P1409-F1 and P1409-R and P1409-F2 and P1409-R, respectively. To clone the region containing the PSPTOA0018 promoter, a 1,034-bp fragment (−1,009 to +25 relative to the putative translation start codon) and a 294-bp fragment (−268 to +25 relative to the putative translation start codon) were PCR-amplified, using the DC3000 genomic DNA as template and the primer pairs of PA0018-F1 and PA0018-R and PA0018-F2 and PA0018-R, respectively. The PCR products were digested with *Eco*R1 and *Bam*H1, cloned into pBluscript-KS (Stratagene, La Jolla, CA, U.S.A.), sequence-verified, and subsequently cloned into the broad host pHM2 plasmid carrying the *luc* reporter gene (X. Tang, *unpublished data*). The resulting plasmids were introduced into wild type DC3000 and the *hrpL*[−] mutant.

To determine the promoter activity, bacteria containing the reporter genes were grown in KB medium to an optical density at 600 nm (OD₆₀₀) = 2, pelleted and washed twice with MM, resuspended in MM to OD₆₀₀ = 0.1, and cultured at 28°C with constant shaking. Two 100-µl aliquots of bacteria culture at 0 and 6 h were mixed with 2 µl of 1 mM luciferin in a 96-well plate, and the luciferase activity was determined using a cooled CCD camera (Roper Scientific, Trenton, NJ, U.S.A.).

Accession codes.

Microarray design and experimental data are available in the National Center for Biotechnology Information Gene Expression Omnibus under series GSE4848.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

National Center for Biotechnology Information Gene Expression Omnibus website: www.ncbi.nlm.nih.gov/projects/geo/index.cgi

MEME (Multiple Em for Motif Elicitation) website: meme.nbcr.net/meme/meme.html

Regulatory Sequence Analysis Tools (RSAT) web servers: rsat.scmbb.ulb.ac.be/rsat

NimbleGen Systems, Inc. website: www.nimblegen.com/products/exp/microb.html

Weblogo website: weblogo.berkeley.edu.