Mutation of Lon Protease Differentially Affects the Expression of *Pseudomonas syringae* Type III Secretion System Genes in Rich and Minimal Media and Reduces Pathogenicity

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The bacterial Lon protease participates in a variety of biological processes. In *Pseudomonas syringae*, mutation of *lon* is known to activate *hrpL* and a few *hrpL*-regulated genes in rich medium. The elevated expression of *hrpL* and *hrpL*-regulated genes results from increased stability of HrpR, the transcriptional activator of *hrpL*, in the *lon* mutant. Here, we conducted a microarray analysis to identify genes that are differentially expressed in a *lon*[−] mutant of *P. syringae* pv. *tomato* DC3000 grown in the rich medium King's B (KB). Most genes induced in the *lon*[−] mutant belong to the HrpL regulon or are related to transcription, protein synthesis, and energy metabolism. A major group of genes reduced in the *lon*[−] mutant are related to cell wall biogenesis. The HrpL-regulated genes exhibit different induction patterns in the *lon*[−] mutant, suggesting that additional regulators other than HrpL are likely to be involved in regulation of these genes. Compared with the wild-type bacteria, *lon*[−] mutants of *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *phaseolicola* NPS3121 strains exhibit elevated *hrpL* expression in KB medium, but reduced *hrpL* expression in minimal medium (MM). The reduced *hrpL* RNA is correlated with reduced *hrpR* and *hrpS* RNAs, suggesting that the Lon-mediated regulation of *hrpL* involves different mechanisms in KB and MM. The *lon*[−] mutation also reduced bacterial pathogenicity.

The bacterial Lon protein is an ATP-dependent protease participating in the degradation of a variety of abnormal or denatured proteins and is believed to play an important role in amino acid recycling under stringent conditions (Kuroda 2006; Kuroda et al. 2001). Lon also is able to recognize and specifically degrade a number of regulatory proteins (Tsilibaris et al. 2006). Mutation of *lon* was reported to affect a number of biological processes in various bacteria, including cell division (Schoemaker et al. 1984), capsule synthesis (Torres-Cabassa and Gottesman 1987), flagella biosynthesis (Stewart et al. 1997), UV tolerance (Howard-Flanders et al. 1964; Whistler et al. 2000), and antibiotic production (Whistler et al. 2000). The biological pathways affected by the *lon*[−] mutation vary in dif-

ferent bacterial species. For example, the *lon*[−] mutant of *Escherichia coli* exhibits increased sensitivity to UV irradiation (Howard-Flanders et al. 1964), a defect in cell division resulting in filamentation (Howard-Flanders et al. 1964), and increased exopolysaccharide synthesis leading to mucoidy (Markovitz 1964). However, mutation of *lon* in *Agrobacterium tumefaciens* does not affect sensitivity to UV irradiation or exopolysaccharide synthesis, and the mutant cells do not form filaments (Su et al. 2006). Lon also was reported to regulate the type III secretion system (TTSS) negatively in *Salmonella* spp. and positively in *Yersinia* spp. In *Salmonella* spp., Lon was found to degrade HilC and HilD, two positive transcriptional regulators of the TTSS genes in pathogenicity island 1 (Takaya et al. 2005). In *Yersinia* spp., Lon was reported to degrade YmoA, a histone-like protein that represses the expression of plasmid-encoded TTSS genes and, thus, allows elevated expression of the TTSS genes (Jackson et al. 2004).

Lon was first detected in *Pseudomonas syringae* as a negative regulator of *hrpA* and *hrpL* genes in the rich medium King's B (KB) (Bretz et al. 2002). *hrpA* encodes pilin, a structural protein of the *P. syringae* type III pilus (Roine et al. 1997). *hrpL* encodes an alternate sigma factor essential for the induction of *P. syringae* genes carrying *hrp* box promoters, including *hrpA* (Xiao et al. 1994). More than 110 genes were shown to be regulated by HrpL and *hrp* box promoters in *P. syringae* pv. *tomato* DC3000 using various experimental methods (Chang et al. 2005; Ferreira et al. 2006; Fouts et al. 2002; Lan et al. 2006; Zwiesler-Vollick et al. 2002). Most of the HrpL-regulated genes encode structural proteins of the TTSS and type III effector proteins, but some apparently have a function independent of TTSS (Chang et al. 2005; Ferreira et al. 2006; Lan et al. 2006; Lindeberg et al. 2006; Petnicki-Ocwieja et al. 2002; Schechter et al. 2006). The HrpL-regulated genes, together with *hrpL*, are repressed in rich media such as KB but induced when the bacteria are cultured in minimal medium (MM) or infiltrated into the plant (Tang et al. 2006; Xiao and Hutcheson 1994). The *hrpL* induction is mediated by HrpR and HrpS together with the alternate sigma factor RpoN (Hendrickson et al. 2000; Hutcheson et al. 2001; Xiao et al. 1994). RpoN recognizes the σ^{54} -dependent DNA element in the *hrpL* promoter and interacts with the RNA polymerase holoenzyme (Hendrickson et al. 2000). HrpR and HrpS are similar in amino acid sequences, and both have the RpoN-interaction domains (Hutcheson et al. 2001). The two proteins form a heterodimer on the *hrpL* promoter and, together with RpoN, activate *hrpL* transcription (Hutcheson et al. 2001). The *hrpR*

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and *hrpS* genes are organized in an operon controlled by the *hrpR* promoter, but *hrpS* also can be transcribed independently under the control of a weak promoter immediately upstream of *hrpS* (Grimm et al. 1995; Hutcheson et al. 2001). The *hrpRS* operon is expressed at a relatively high level in rich medium and further induced up to two- to fourfold in MM or in the plant (Rahme et al. 1992; Thwaites et al. 2004; Xiao et al. 2007). In a search for genes required for repression of *hrpA* in rich medium, *lon*⁻ mutants were isolated that allowed elevated expression of *hrpA* and *hrpL* (Bretz et al. 2002). Further studies indicated that mutation of *lon* stabilizes HrpR in rich medium, resulting in the constitutive activation of *hrpL* and downstream TTSS genes (Bretz et al. 2002). In addition to HrpR, Lon also was found to degrade various type III effector proteins (Losada and Hutcheson 2005).

Lon is known to regulate multiple regulatory proteins in other bacterial species, and many of these regulatory proteins have their counterparts in *P. syringae* (Buell et al. 2003); therefore, the *lon*⁻ mutation in *P. syringae* may affect other biological processes in addition to the TTSS. Indeed, the *P. syringae lon*⁻ mutant displays hypersensitivity to UV irradiation, abnormal cell morphology, and filamentation (Bretz et al. 2002). To gain a global view of Lon protease function in *P. syringae*, we conducted a microarray analysis to compare gene expression profiles in the *P. syringae* pv. *tomato* DC3000 wild-type (WT) and *lon*⁻ mutant strains grown in the rich KB medium. The assay identified 155 genes induced and 45 genes (excluding *lon*) reduced in the *lon*⁻ mutant. Some of these differentially expressed genes probably are regulated directly by substrate proteins of the Lon protease, whereas others probably are due to an indirect effect of the *lon*⁻ mutation. We also investigated how mutation of *lon* affected the expression of TTSS regulatory genes in MM. We found that the *lon*⁻ mutation reduced expression of the TTSS regulatory genes in MM. The *lon*⁻ mutants displayed reduced pathogenicity.

RESULTS AND DISCUSSION

Microarray data analysis and validation by RNA blotting.

Microarray design and analysis were as described by Lan and associates (2006). Of the 5,763 open reading frames (ORF) predicted in the DC3000 genome, 5,608 were represented by the array. To identify genes differentially expressed in the *lon*⁻ mutant, total RNA samples were prepared from WT DC3000 and the *lon*⁻ mutant growing in KB liquid medium at log phase. The RNA sample was fluorescently labeled and hybridized to the array. Three biological replicates were performed for each sample. The replicate samples showed a high correlation in log₂ relative fluorescence intensities (Table 1), demonstrating good reproducibility of the microarray data.

The RNA levels of the 5,608 predicted ORF were compared between the *lon*⁻ mutant and the WT strain. The following cut-off criteria were combined for the identification of differentially expressed genes: 1, >2-fold difference; 2, >1.32-fold difference but with significance values of *P* < 0.1 (*t* test); and 3, >1.32-fold difference with *P* > 0.1 but clustering with genes of the first two groups. In all, 155 genes (Table 2) were considered to be induced and 46 genes (Table 3) reduced in the *lon*⁻ mutant.

To validate the microarray data, seven genes representing a wide range of relative fluorescence signal intensities and different gene expression profiles were subjected to RNA blot analysis. All the genes displayed the expected differential expression pattern (Fig. 1). In addition, *hrpL* and three HrpL-regulated genes that were indicated by microarray as differentially expressed also displayed the expected patterns in Northern analysis (Fig. 2A and B). These results indicated that the

differential expression data obtained by microarray analysis are largely reliable.

Genes upregulated in the *lon*⁻ mutant.

Of the 155 genes upregulated in the *lon*⁻ mutant, 39 genes belonged to the HrpL-regulon (Ferreira et al. 2006; Lan et al. 2006). Consistent with a previous report (Bretz et al. 2002), *hrpL* also was induced in the *lon*⁻ mutant (Table 2; Fig. 2). The induction of *hrpL* likely plays a central role in the induction of these genes in the *lon*⁻ mutant.

Seven genes had putative functions in transcription and global transcriptional regulation. These included an RNA polymerase alpha subunit (PSPTO0651), an RNA polymerase sigma factor sigW (PSPTO2298), a transcription elongation factor (PSPTO4491), a transcription antitermination protein

Table 1. Pearson correlations of the replicate (Rep) microarray hybridizations^a

Samples ^b	Rep 1 vs. Rep 2	Rep 1 vs. Rep 3	Rep 2 vs. Rep 3
WT-KB	0.91	0.84	0.88
<i>lon</i> ⁻ -KB	0.95	0.94	0.94

^a The numbers indicate the correlation of the log₂ relative fluorescence intensities of all open reading frames between the replicate samples.

^b WT-KB = WT strain in KB, *lon*⁻-KB = *lon*⁻ mutant treated with KB.

Fig. 1. Verification of the microarray data by Northern blotting. The coding region of each gene was polymerase chain reaction (PCR) amplified and radiolabeled with ³²P-dCTP as probes. All genes were tested with the same RNA samples. Equal loading of RNA samples is indicated by rRNA fragments in ethidium bromide-stained gels. The composite bands for each probe were from the same Northern hybridization and autoradiography.

Table 2. Genes induced in the DC3000 *lon*⁻ mutant

PSPTO no. and description	<i>lon</i> ⁻ -KB vs. WT-KB (fold) ^a	<i>P</i> value
HrpL regulon and HrpL (40)		
PSPTO1404 HrpL	1.380	0.216
PSPTO0044 HopK1	1.581	0.053
PSPTO0061 HopY1	1.459	0.075
PSPTO0371 indoleacetate-lysine ligase	1.483	0.269
PSPTO0503 SchF	2.461	0.145
PSPTO0588 HopH1	5.123	0.105
PSPTO0589 HopC1	6.566	0.094
PSPTO0834 alcohol dehydrogenase zinc-containing	4.141	0.115
PSPTO0835 ribD C-terminal domain protein	3.084	0.058
PSPTO0836 conserved domain protein	2.228	0.118
PSPTO0837 conserved hypothetical	2.176	0.062
PSPTO0873 amidinotransferase family protein	7.906	0.138
PSPTO0874 nikkomycin biosynthesis domain protein	2.270	0.111
PSPTO0875 Homolog of PSPTO0874	1.519	0.067
PSPTO1022 HopAM1-1	10.070	0.107
PSPTO1369 ShcN	3.154	0.073
PSPTO1373 HrpW	2.333	0.130
PSPTO1381 HrpA	5.271	0.117
PSPTO1382 HrpZ	3.281	0.160
PSPTO1383 HrpB	1.895	0.180
PSPTO1384 HrcJ	1.862	0.047
PSPTO1387 HrpF	2.648	0.131
PSPTO1389 HrcC	1.604	0.118
PSPTO1390 HrpT	1.326	0.028
PSPTO1391 HrpV	1.422	0.126
PSPTO1399 HrpO	1.563	0.242
PSPTO1405 HrpK	1.502	0.256
PSPTO1408 hypothetical protein	2.088	0.114
PSPTO1409 conserved hypothetical protein	1.581	0.031
PSPTO3087 AvrPtoB	1.971	0.176
PSPTO4001 AvrPto	7.819	0.081
PSPTO4101 HopAK1	1.360	0.141
PSPTO4331 HopE1	6.864	0.082
PSPTO4599 ShcS1	1.393	0.206
PSPTO4703 hopAQ1	2.385	0.193
PSPTO4776 HopPmaI	1.955	0.096
PSPTO5353 SchA	1.524	0.051
PSPTOA0005 HopAM1-1	9.241	0.100
PSPTOA0017 ShcO1	3.063	0.045
PSPTOA0018 HopO1-1	4.053	0.205
Transcription and transcriptional regulation (7)		
PSPTO0614 transcription antitermination protein NusG	3.025	0.056
PSPTO0651 DNA-directed RNA polymerase α subunit	1.676	0.113
PSPTO1274 cold shock domain family protein	1.401	0.079
PSPTO2298 RNA polymerase sigma factor-70 sigW, putative	1.597	0.029
PSPTO3723 DNA-binding protein HU- β	1.477	0.003
PSPTO4145 cold shock protein CapB	2.178	0.118
PSPTO4491 transcription elongation factor NusA	1.808	0.060
Protein synthesis (51)		
PSPTO0089 ribosomal protein L28	1.833	0.065
PSPTO0090 ribosomal protein L33	1.523	0.212
PSPTO0539 ribosomal protein S21	3.593	0.016
PSPTO0616 ribosomal protein L1	1.646	0.216
PSPTO0617 ribosomal protein L10	1.936	0.058
PSPTO0618 ribosomal protein L7/L12	1.750	0.041
PSPTO0621 ribosomal protein S12	1.618	0.091
PSPTO0622 ribosomal protein S7	1.421	0.345
PSPTO0625 ribosomal protein S10	1.723	0.100
PSPTO0626 ribosomal protein L3	1.536	0.361
PSPTO0627 ribosomal protein L4	1.583	0.135
PSPTO0629 ribosomal protein L2	1.449	0.292
PSPTO0640 ribosomal protein S8	1.957	0.032
PSPTO0641 ribosomal protein L6	1.648	0.079
PSPTO0642 ribosomal protein L18	1.514	0.311
PSPTO0643 ribosomal protein S5	1.427	0.346
PSPTO0644 ribosomal protein L30	1.331	0.310
PSPTO0647 ribosomal protein L36	1.816	0.092
PSPTO0648 ribosomal protein S13	2.073	0.043
PSPTO0649 ribosomal protein S11	1.657	0.151

(continued on next page)

^a The *lon*⁻ mutant treated with the rich medium King' B (*lon*⁻-KB) versus the wild type treated with KB (WT-KB); numbers indicate the fold of induction in *lon*⁻-KB.

Table 2. (continued from preceding page)

PSPTO no. and description	lon ⁻ -KB vs. WT-KB (fold) ^a	P value
Protein synthesis (51) (<i>continued</i>)	Protein synthesis (51)	Protein synthesis (51)
PSPTO0650 ribosomal protein S4	1.631	0.148
PSPTO0652 ribosomal protein L17	1.640	0.213
PSPTO0797 ribosomal protein L21	2.509	0.027
PSPTO0798 ribosomal protein L27	1.587	0.065
PSPTO0802 ribosomal protein S20	2.914	0.002
PSPTO1103 50S ribosomal protein L25	1.879	0.049
PSPTO1473 ribosomal protein S16	2.583	0.031
PSPTO1474 16S rRNA processing protein RimM	2.772	0.014
PSPTO1475 tRNA (guanine-N1)-methyltransferase	3.901	0.120
PSPTO1476 ribosomal protein L19	1.803	0.101
PSPTO1533 methionine aminopeptidase type I	1.375	0.059
PSPTO1534 ribosomal protein S2	1.674	0.185
PSPTO1535 translation elongation factor Ts	1.747	0.063
PSPTO1765 translation elongation factor P	1.763	0.040
PSPTO2378 threonyl-tRNA synthetase	1.399	0.093
PSPTO2379 translation initiation factor IF-3	1.843	0.075
PSPTO2380 ribosomal protein L35	1.863	0.111
PSPTO2381 ribosomal protein L20	2.061	0.078
PSPTO3352 translation initiation factor IF-1	3.114	0.013
PSPTO3727 ribosome-binding	1.618	0.091
PSPTO3835 ribosomal protein L32	3.387	0.071
PSPTO4426 ribosomal protein L13	2.370	0.037
PSPTO4487 ribosomal protein S15	1.587	0.180
PSPTO4489 ribosome-binding factor A	1.382	0.031
PSPTO4490 translation initiation factor IF-2	1.331	0.477
PSPTO4930 ribosomal protein L9	2.415	0.151
PSPTO4932 ribosomal protein S18	2.151	0.184
PSPTO4933 ribosomal protein S6	2.124	0.042
PSPTO5136 ribosomal protein L31	2.405	0.054
PSPTO5614 ribonuclease P protein component	2.089	0.120
PSPTO5615 ribosomal protein L34	2.102	0.065
Protein fate (8)		
PSPTO0613 preprotein translocase SecE subunit	2.721	0.008
PSPTO0646 preprotein translocase SecY subunit	1.795	0.115
PSPTO1414 preprotein translocase YajC subunit	1.462	0.003
PSPTO4376 GroEL chaperonin 60 kDa	1.524	0.008
PSPTO4377 co-chaperoninGroES protein nin 10 kDa	1.555	0.035
PSPTO4493 preprotein translocase SecG subunit	2.719	0.046
PSPTO4506 heat shock protein GrpE	1.829	0.093
PSPTO5612 inner membrane protein 60 kDa	1.492	0.256
Energy metabolism (22)		
PSPTO1422 rrf2 family protein	1.378	0.115
PSPTO1423 cysteine desulfurase	1.576	0.062
PSPTO1424 iron-binding protein IscU	1.643	0.098
PSPTO1425 iron-binding protein IscA	1.532	0.186
PSPTO1428 ferredoxin 2Fe-2S	1.383	0.333
PSPTO1429 assembly of Fe-S clusters	1.458	0.038
PSPTO1536 uridylyate kinase	1.576	0.039
PSPTO1844 carbon storage regulator	1.563	0.099
PSPTO2211 3-hydroxydecanoyl-ACP dehydratase	1.439	0.049
PSPTO2288 2-methylcitrate synthase	1.500	0.069
PSPTO3566 carbon storage regulator	2.725	0.083
PSPTO3831 acyl carrier protein	1.932	0.047
PSPTO3832 3-oxoacyl-(acyl-carrier-protein) reductase	1.393	0.261
PSPTO4161 glutaredoxin-related protein	1.496	0.027
PSPTO4494 triosephosphate isomerase	1.971	0.034
PSPTO4508 ferric uptake regulation protein	1.481	0.035
PSPTO5600 ATP synthase gamma chain (EC 3.6.3.14)	1.825	0.032
PSPTO5602 ATP synthase delta chain (EC 3.6.3.14)	1.532	0.053
PSPTO5603 ATP synthase B chain (EC 3.6.3.14)	1.691	0.054
PSPTO5604 ATP synthase C chain (EC 3.6.3.14)	1.535	0.138
PSPTO5605 ATP synthase A chain (EC 3.6.3.14)	1.527	0.048
PSPTO5606 ATP synthase protein I	1.455	0.091
Other functions (11)		
PSPTO0433 thiamine biosynthesis protein ThiS	2.105	0.040
PSPTO0513 coenzyme PQQ synthesis protein A	1.490	0.086
PSPTO1104 ribose-phosphate pyrophosphokinase	1.459	0.170
PSPTO1256 amino acid ABC transporter permease protein	1.360	0.033
PSPTO1529 arsC family protein	1.983	0.054

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(PSPTO0614), a global transcriptional regulator (PSPTO3723), and two homologous genes (PSPTO1274 and PSPTO4145) that may function as transcription antiterminators. PSPTO3723 belongs to the DNABII family proteins that bind and bend DNA and function in many cellular processes, including transcriptional regulation (Swinger and Rice 2004). Proteins encoded by PSPTO1274 and PSPTO4145 contain the cold shock DNA-binding domain and are highly homologous to the *E. coli* CspA protein. CspA is an RNA chaperone that facilitates translation at low temperature by destabilizing mRNA secondary structures. CspA and the homologous RNA chaperones CspE and CspC are transcription antiterminators in *E. coli* (Bae et al. 2000).

A total of 51 genes may be involved in protein synthesis. Of these, 38 genes encoded ribosomal proteins. The remaining 13 genes may be involved in translation initiation and elongation (PSPTO2379, PSPTO3352, PSPTO4490, PSPTO1535, and PSPTO1765), ribosome-binding (PSPTO1103 and PSPTO3727), tRNA processing and maturation (PSPTO1475, PSPTO2378, and PSPTO5614), 16S RNA processing (PSPTO1474 and PSPTO 4489), and the removal of N-terminal amino acids from peptides (PSPTO1533). It is well known that the Lon protease can degrade ribosomal proteins, and this proteolytic activity plays a critical role in regulating the abundance of ribosomal proteins and, therefore, basic cellular activities (Kuroda 2006). Here, we demonstrated the upregulation of ribosomal protein genes in a *lon*⁻ mutant, suggesting another mechanism through which Lon regulates the abundance of ribosomal proteins.

Additional induced genes appear to be associated with the elevated protein synthesis. Three of these genes encode putative chaperonin proteins that are involved in protein folding and stability (PSPTO4376, PSPTO4377, and PSPTO4506). Another gene (PSPTO5612) has a homologue in *E. coli* that functions to insert inner membrane proteins into their destination. The induction of this gene was expected because protein synthesis was elevated in the *lon*⁻ mutant, and a significant portion of these proteins were targeted to cell membranes. Interestingly, four genes with a function in preprotein translocation (PSPTO0613, PSPTO0646, PSPTO1414, and PSPTO4493) also were induced in the *lon*⁻ mutant. The induction of these genes suggests an increase in protein secretion through the general secretion pathway.

Twenty-two genes may be involved in energy metabolism. Six of these genes (PSPTO0560, PSPTO0562, PSPTO0563, PSPTO0564, PSPTO0565, and PSPTO0566) are clustered and encode different subunits of ATP synthase. PSPTO1536 encodes a putative uridylate kinase catalyzing formation of UDP from UMP. Two other genes with putative metabolic functions were PSPTO2288 and PSPTO4494, which encode methyl citrate synthase and triosephosphate isomerase, respectively. PSPTO1844 and PSPTO3566 encode putative carbon storage regulators (CsrA-2 and CsrA-3), which are RNA-binding regulatory proteins that globally regulate carbon metabolism and a variety of other processes (Romeo 1998). Three genes (PSPTO2211, PSPTO3831, and PSPTO3832) are involved in fatty acid and phospholipid metabolism, a process related to energy metabolism as well. A cluster of six genes (PSPTO1422, PSPTO1423, PSPTO1424, PSPTO1425, PSPTO1428, and PSPTO1429) appear to be involved in the synthesis and assembly of Fe-S clusters (Johnson et al. 2005). The Fe-S cluster containing proteins plays an important role in electron transfer processes and in energy production and conversion. PSPTO4508 encodes a ferric-uptake regulatory protein. PSPTO4161 encodes a glutaredoxin-related protein that functions in an Fe-S cluster-dependent manner.

The induction of genes involved in transcription, translation, and energy metabolism suggests elevation of basic cellular activities in the *lon*⁻ mutant. Despite these changes in basic cellular processes, the *lon*⁻ mutants displayed growth similar to the WT strain during log phase in KB liquid culture (data not shown).

In addition to the above three major functional groups, a few genes of various other functions also were induced in the *lon*⁻ mutant. Among them were four genes (PSPTO0433, PSPTO0513, PSPTO1104, and PSPTO4859) encoding putative catalytic enzymes that are involved in biosynthesis of co-factors, amino acids, and nucleotides. Two putative ABC transporters (PSPTO1256 and PSPTO4588) are involved in transport of amino acids and dipeptides. Two genes (PSPTO1529 and PSPTO4442) appear to encode proteins with detoxification functions. TolR encoded by PSPTO3974, together with the TolA, TolB, TolQ, and Pal proteins, may be involved in the maintenance of outer membrane stability (Cascales and Lloubes 2004). MreB, encoded by PSPTO4722, is a putative

Table 2. (continued from preceding page)

PSPTO no. and description	<i>lon</i> ⁻ -KB vs. WT-KB (fold) ^a	<i>P</i> value
Other functions (11) (continued)	Protein synthesis (51)	Protein synthesis (51)
PSPTO3729 lipoprotein, putative	1.689	0.079
PSPTO3974 tolR protein	1.334	0.078
PSPTO4442 toluene tolerance protein, putative	2.377	0.033
PSPTO4472 rod shape-determining protein MreB	1.552	0.104
PSPTO4558 dipeptide ABC transporter, putative	1.489	0.080
PSPTO4859 3-dehydroquinase dehydratase (EC 4.2.1.10)	1.425	0.059
Hypothetical and unknown function (17)		
PSPTO0219 hypothetical protein	1.478	0.150
PSPTO0220 conserved hypothetical protein	1.385	0.060
PSPTO0367 conserved hypothetical protein	2.493	0.168
PSPTO0605 HesB/YadR/YfhF family protein	1.375	0.075
PSPTO1840 identified by Glimmer2; putative	1.784	0.102
PSPTO3387 conserved hypothetical protein	2.387	0.048
PSPTO3477 identified by Glimmer2; putative	1.706	0.079
PSPTO3836 hypothetical protein	4.554	0.004
PSPTO3980 hypothetical protein	1.402	0.020
PSPTO4417 hypothetical protein	1.433	0.078
PSPTO4492 hypothetical protein	2.049	0.169
PSPTO4816 conserved hypothetical protein	1.967	0.040
PSPTO4931 hypothetical protein	2.654	0.102
PSPTO5228 hypothetical protein	2.533	0.019
PSPTO5365 conserved domain protein	1.999	0.033
PSPTO5613 conserved hypothetical protein	2.025	0.002

bacterial actin homologue required for the maintenance of rod-shaped cells (Carballido-Lopez et al. 2006); the induction of this gene may contribute to the elongation of *lon*⁻ mutant cells (Bretz et al. 2002). Seventeen genes encoded hypothetical proteins or proteins of unknown function; some of these genes were clustered with genes of the above functional categories.

Genes downregulated in the *lon*⁻ mutant.

A group of genes downregulated in the *lon*⁻ mutant were related to cell membrane, including eight genes with putative functions in polysaccharide biogenesis (PSPTO0110, PSPTO0170, PSPTO0171, PSPTO0172, PSPTO1453, PSPTO2952, PSPTOA0030 and PSPTOA0032), three lipoproteins (PSPTO1507, PSPTO1634, and PSPTOA0031), and two inner-membrane and two outer-membrane proteins involved in transport (PSPTO0679, PSPTO1506, PSPTO2707, PSPTO5112). The reduction in polysaccharide biogenesis and membrane proteins may weaken the *lon*⁻ mutant cell wall and, together

with the elevated basic metabolism, this may contribute to the increased size and reduced fitness of *lon*⁻ mutant cells under stress conditions (Bretz et al. 2002). Alginate is the major exopolysaccharide of *P. syringae* bacteria; however, genes involved in alginate biosynthesis are not affected by *lon*⁻ mutation. This is consistent with the notion that alginate biosynthesis is not affected in the *lon*⁻ mutant (Bretz et al. 2002).

Seven genes encoding putative metabolic enzymes were downregulated, in striking contrast to the large group of genes involved in energy metabolism that were induced in the *lon*⁻ mutant. Similarly, only two genes (PSPTO2310 and PSPTO4951) with a putative function in modulating protein synthesis were downregulated in the *lon*⁻ mutant. These two proteins may negatively regulate the protein synthesis in *P. syringae*. Four genes (PSPTO0231, PSPTO1910, PSPTO3021, and PSPTO0853) with a potential transcriptional regulatory function were reduced in the *lon*⁻ mutant. The target genes regulated by these regulatory proteins are not known. In addition, a cluster of four genes en-

Table 3. Genes downregulated in the DC3000 *lon*⁻ mutant

PSPTO no. and description	WT-KB vs. <i>lon</i> ⁻ -KB (fold) ^a	P value
PSPTO3724 ATP-dependent protease Lon	1.975	0.055
Cell wall-related (15)		
PSPTO0110 metallo-β-lactamase superfamily protein	1.463	0.072
PSPTO1070 lipopolysaccharide biosynthesis protein	1.370	0.191
PSPTO1071 glycosyl transferase group 2 family protein	1.376	0.076
PSPTO1072 aminotransferase DegT/DnrJ/EryC1/StrS family	1.540	0.038
PSPTO1453 Levansucrase (EC 2.4.1.10)	2.055	0.032
PSPTO2952 glycosidase, putative	1.340	0.027
PSPTOA0030 glycosyl hydrolase family 25	1.475	0.154
PSPTOA0032 Levansucrase (EC 2.4.1.10)	2.144	0.050
PSPTO1507 putative lipoprotein	2.104	0.177
PSPTO1634 putative lipoprotein	1.508	0.097
PSPTOA0031 putative lipoprotein	1.571	0.012
PSPTO0679 outer membrane protein OmpW	1.607	0.060
PSPTO1506 ompA family protein	1.481	0.305
PSPTO2707 periplasmic mannitol-binding protein	1.328	0.013
PSPTO5112 major facilitator superfamily protein	1.325	0.025
Metabolism (7)		
PSPTO0506 acyl-CoA dehydrogenase family protein	1.380	0.132
PSPTO1126 amine oxidase flavin-containing	1.436	0.021
PSPTO4168 glycerol kinase	1.324	0.012
PSPTO4285 alcohol dehydrogenase II	1.912	0.097
PSPTO5383 phosphoglycerate mutase family protein	1.324	0.233
PSPTO4284 acetyltransferase GNAT family	1.382	0.206
PSPTO5540 acyltransferase, putative	1.320	0.102
Others (14)		
PSPTO2310 ribosome modulation factor-related protein	1.989	0.135
PSPTO4951 ribosome-associated GTPase	1.336	0.043
PSPTO0231 DNA-binding protein inhibitor Id-2-related protein	1.522	0.086
PSPTO1910 SH3 domain protein	1.442	0.087
PSPTO3021 transcriptional regulator LysR family	1.328	0.034
PSPTO0853 DksA, DNA repair and repress rRNA transcription	1.970	0.107
PSPTO0576 Mu-like phage protein gp38	1.392	0.096
PSPTO0577 tail sheath protein, putative	1.349	0.114
PSPTO0578 phage tail tube protein	1.364	0.061
PSPTO0579 prophage PSPPH01, putative tail protein	1.358	0.312
PSPTO4770 UvrA domain	1.328	0.040
Hypothetical and unknown functions (12)		
PSPTO0221 HAD-superfamily hydrolase	1.366	0.043
PSPTO0156 hypothetical protein	2.095	0.213
PSPTO0507 Salt stress induced hydrophobic peptide	2.539	0.341
PSPTO0854 hypothetical protein	1.783	0.275
PSPTO1037 conserved hypothetical protein	1.360	0.065
PSPTO1038 hypothetical protein	1.459	0.153
PSPTO1635 ligand binding	1.499	0.121
PSPTO4762 von Willebrand factor type A domain protein	1.410	0.387
PSPTO4763 hypothetical protein	1.326	0.029
PSPTO2057 hypothetical protein	2.031	0.156
PSPTO5384 hypothetical protein	1.482	0.020
PSPTOB0071 surface exclusion protein, putative	1.396	0.069

^a The wild type (WT) treated with the rich medium King's B (KB) versus the *lon*⁻ mutant treated with KB; numbers indicate the fold of induction in WT-KB.

coding phage proteins also were repressed in the *lon*⁻ mutant. The DNA fragment harboring these genes likely is derived from a phage insertion.

Although the *P. syringae lon*⁻ mutant displayed increased sensitivity to UV irradiation, only two genes (PSPTO4770 and PSPTO0853) with an implicated function in DNA repair were downregulated in *lon*⁻ mutant. PSPTO4770 encodes a protein with the excision repair protein UvrA domain I. UvrA acts together with UvrB and UvrC proteins in nucleotide excision repair (Croteau et al. 2006). PSPTO0853 encodes the putative dnaK suppressor protein DksA. DksA is a stringent response regulator that plays a critical role in regulating bacterial gene expression. This gene also plays a critical role in transcription-coupled DNA repair (Meddows et al. 2005). Whether the reduced expression of these two genes is related to the increased UV sensitivity of the *lon*⁻ mutant is not known. Nevertheless,

other genes with known functions in DNA repair were not downregulated in the *lon*⁻ mutant. Perhaps genes involved in DNA repair are induced upon UV irradiation in the WT strain and, here, the bacteria analyzed by the microarray were not exposed to UV irradiation.

Twelve genes were classified into the functionally unknown group, although sequence alignment identified conserved domains in several of these genes. A few of these genes were clustered with genes of the other functional categories and, thus, may have related functions. In addition, two pairs of functionally unknown genes were clustered in two genomic loci.

Expression of the HrpL-regulated genes in the *lon*⁻ mutant.

Previous microarray analysis identified 112 DC3000 genes controlled by hrp box promoters and activated by *hrpL* (Ferreira et al. 2006; Lan et al. 2006); 5 of them were not

Table 4. Expression of HrpL-regulated genes in *lon*⁻ mutant

PSPTO no. and description	<i>lon</i> ⁻ -KB vs. WT-KB ^a	<i>P</i> value	WT-MM vs. <i>lon</i> ⁻ -KB ^a	<i>P</i> value	Category ^b
PSPTO1404 HrpL	1.380	0.216	13.718	0.002	#
PSPTOA0005 HopAM1-1	9.241	0.100	16.223	0.024	1
PSPTO1373 HrpW	2.333	0.130	15.274	0.013	1
PSPTO1022 HopAM1-1	10.070	0.107	13.804	0.029	1
PSPTO1382 HrpZ	3.281	0.160	8.486	0.009	1
PSPTO4776 HopPmaI	1.955	0.096	6.413	0.007	1
PSPTO1381 HrpA	5.271	0.117	6.238	0.004	1
PSPTO0589 HopC1	6.566	0.094	6.054	0.030	1
PSPTO4599 ShcS1	1.393	0.206	5.942	0.034	1*
PSPTO1383 HrpB	1.895	0.180	5.483	0.068	1
PSPTO1399 HrpO	1.563	0.242	5.032	0.015	1
PSPTO1387 HrpF	2.648	0.131	4.925	0.008	1
PSPTO1369 ShcN	3.154	0.073	4.595	0.015	1
PSPTO5353 SchA	1.524	0.051	4.359	0.004	1
PSPTO0503 SchF	2.461	0.145	4.344	0.086	1
PSPTOA0017 ShcO1	3.063	0.045	4.167	0.038	1
PSPTO1405 HrpK	1.502	0.256	4.135	0.017	1
PSPTOA0018 HopO1-1	4.053	0.205	4.110	0.071	1
PSPTO0044 HopPtoK	1.581	0.053	0.818	0.275	2
PSPTO0834 alcohol dehydrogenase zinc-containing	4.141	0.115	0.563	0.311	2
PSPTO0835 ribD C-terminal domain protein	3.084	0.058	1.060	0.852	2
PSPTO0836 conserved domain protein	2.228	0.118	0.681	0.312	2
PSPTO0837 conserved hypothetical protein	2.176	0.062	0.585	0.130	2
PSPTO0873 amidinotransferase family protein	7.906	0.138	0.369	0.255	2*
PSPTO0874 nikkomycin biosynthesis domain protein	2.270	0.111	0.787	0.450	2
PSPTO0875 Homolog of PSPTO0874	1.519	0.067	0.914	0.575	2
PSPTO4703 hopAQ1	2.385	0.193	0.522	0.267	2
PSPTO4101 HopAK1	1.360	0.141	3.727	0.003	3
PSPTO1384 HrcJ	1.862	0.047	3.256	0.007	3
PSPTO3087 AvrPtoB	1.971	0.176	3.187	0.019	3
PSPTO1389 HrcC	1.604	0.118	3.040	0.010	3
PSPTO0588 HopH1	5.123	0.105	2.828	0.134	3
PSPTO1390 HrpT	1.326	0.028	2.789	0.012	3
PSPTO4331 HopE1	6.864	0.082	2.757	0.053	3
PSPTO0061 HopY1	1.459	0.075	2.504	0.050	3
PSPTO1391 HrpV	1.422	0.126	2.382	0.005	3
PSPTO1408 hypothetical protein	2.088	0.114	1.919	0.074	3
PSPTO0371 indoleacetate-lysine ligase	1.483	0.269	1.819	0.094	3
PSPTO4001 AvrPto	7.819	0.081	1.620	0.235	3*
PSPTO1409 conserved hypothetical protein	1.581	0.031	1.397	0.124	3
PSPTO0370 MATE efflux family protein	0.909	0.772	0.979	0.937	4
PSPTO0473 hypothetical protein	0.802	0.302	1.426	0.031	4
PSPTO0474 hypothetical protein	0.821	0.266	1.338	0.171	4
PSPTO0501 type III effector HopPtoS2	0.898	0.680	1.070	0.768	4
PSPTO0502 type III effector HopPtoF	0.948	0.628	1.247	0.092	4
PSPTO0524 peptidase, M20/M25/M40 family	0.933	0.821	0.990	0.975	4
PSPTO0838 major facilitator family transporter	1.129	0.456	0.841	0.170	4
PSPTO0851 conserved hypothetical protein	0.920	0.509	1.388	0.082	4
PSPTO0852 HopAJ1	1.142	0.317	2.553	0.001	4

(continued on next page)

^a The *lon*⁻ mutant treated with the rich medium King's B (KB) versus the wild type (WT) treated with KB, and WT treated with minimal medium (MM) versus *lon*⁻-KB; numbers indicate the fold of induction in the two comparisons.

^b Genes indicated by * were examined by Northern analysis; # *hrpL* transcript level served as a reference. The last five genes were not present in the microarray.

present in this microarray analysis. Of these genes, 39 were considered to be induced in the *lon*⁻ mutant, and the remaining 68 genes displayed minor or no induction (Table 4). Microarray and Northern blot analyses indicated that the *hrpL* RNA, even though elevated in the *lon*⁻ mutant compared with the WT strain in KB (WT-KB), still was much less abundant than seen in WT treated with MM (WT-MM) (Fig. 2A). This observation triggered us to compare the RNA levels of the HrpL-regulated genes in WT-MM and in the *lon*⁻ mutant treated with KB (*lon*⁻-KB) to determine whether there is a correlation between *hrpL* RNA abundance and the levels of hrp box gene expression.

Comparison of the microarray hybridization signals between WT-MM and *lon*⁻-KB indicated that the *hrpL* RNA was 13.7-fold higher in WT-MM than in *lon*⁻-KB, but the HrpL-regulated genes displayed a range of 0.37- to 16.2-fold difference

in RNA abundance (Table 4). The HrpL-regulated genes could be divided into four groups according to their gene expression patterns (Table 4).

Genes in the first group were induced in *lon*⁻-KB versus WT-KB, and their RNA abundance in WT-MM and in *lon*⁻-KB correlated well with the abundance of *hrpL* RNA (4- to 16.2-fold difference versus 13.7-fold for *hrpL*). Compared with the hrp box genes that are not induced or poorly induced in *lon*⁻-KB, these genes probably have a stronger promoter with high HrpL-binding affinity. Thus, a low level of HrpL in *lon*⁻-KB is sufficient to activate these genes. These genes can be further induced with the increasing level of HrpL in WT-MM. This is consistent with our observation that HrpL, when overexpressed in *P. syringae*, strongly induces the hrp box genes (data not shown). It is worthwhile to note that HrpL may not be the only factor controlling the high levels of expression of the group 1 genes in WT-

Table 4. (continued from preceding page)

PSPTO no. and description	<i>lon</i> ⁻ -KB vs. WT-KB ^a		WT-MM vs. <i>lon</i> ⁻ -KB ^a		Category ^b
		<i>P</i> value		<i>P</i> value	
PSPTO0876 HopD1	1.170	0.208	3.382	0.005	4
PSPTO0877 HopQ1-1	1.288	0.128	6.105	0.041	4
PSPTO0883 HopR1	1.084	0.824	1.463	0.139	4
PSPTO0901	0.962	0.827	1.078	0.628	4
PSPTO1370 HopN1	1.305	0.320	4.557	0.004	4
PSPTO1372 HopPtoA1	0.837	0.554	1.729	0.023	4
PSPTO1374 ShcM	1.093	0.427	5.700	0.022	4
PSPTO1375 HopM1	1.098	0.610	4.528	0.002	4
PSPTO1376 type III chaperone candidate protein	0.895	0.311	1.590	0.007	4
PSPTO1377 AvrE	0.939	0.485	1.858	0.000	4
PSPTO1378 membrane-bound lytic murein transglycosylase D, putative	0.881	0.540	1.436	0.104	4
PSPTO1385 HrpD	1.198	0.293	2.113	0.003	4
PSPTO1386 HrpE	0.917	0.789	2.090	0.059	4
PSPTO1388 HrpG	1.123	0.498	2.708	0.045	4
PSPTO1392 HrcU	0.863	0.611	1.311	0.308	4
PSPTO1393 HrcT	0.911	0.814	1.208	0.561	4
PSPTO1394 HrcS	0.865	0.751	1.123	0.756	4
PSPTO1395 HrcR	1.020	0.928	1.860	0.045	4
PSPTO1396 HrcQb	1.123	0.657	2.218	0.003	4
PSPTO1397 HrcQa	0.984	0.904	2.051	0.007	4
PSPTO1398 HrpP	0.925	0.289	1.805	0.000	4
PSPTO1400 HrcN	0.841	0.650	1.866	0.042	4
PSPTO1401 HrpQ	1.031	0.822	2.121	0.001	4
PSPTO1402 HrcV	1.028	0.949	1.686	0.124	4
PSPTO1403 HrpJ	0.865	0.589	2.721	0.001	4
PSPTO1406 HopB1	1.319	0.163	5.752	0.010	4
PSPTO1568 HopPtoJ	1.210	0.206	0.941	0.671	4
PSPTO1645 transcriptional regulator, MarR family	0.867	0.066	1.426	0.004	4
PSPTO2105 thiamine biosynthesis lipoprotein	1.009	0.949	1.407	0.034	4
PSPTO2678 HopP1	1.197	0.357	3.173	0.002	4
PSPTO2679 hypothetical protein	0.953	0.393	2.299	0.013	4
PSPTO2691 membrane protein TerC family	1.056	0.766	1.727	0.010	4
PSPTO4588 HopS2	1.011	0.934	2.512	0.003	4
PSPTO4589 ShcS2	0.973	0.815	11.959	0.022	4
PSPTO4597 HopS1	1.224	0.111	5.491	0.004	4
PSPTO4691 HopAD1	0.914	0.439	1.809	0.005	4
PSPTO4718 type III effector HopPtoA2	0.966	0.942	1.522	0.364	4
PSPTO4720 HopV1	1.119	0.217	3.986	0.002	4
PSPTO4721 ShcV	1.188	0.385	6.080	0.021	4
PSPTO4722 HopAO1	1.018	0.851	1.716	0.012	4
PSPTO4727 HopG1	0.891	0.524	6.998	0.104	4
PSPTO4732 HopQ1-2	1.045	0.726	1.586	0.023	4
PSPTO4733 hypothetical protein	1.195	0.310	2.003	0.051	4
PSPTO5354 HopA1	1.118	0.297	5.901	0.029	4
PSPTO5355 hypothetical protein	1.009	0.947	1.433	0.023	4
PSPTOA0012 HopX1	0.931	0.691	3.149	0.004	4
PSPTOA0019 HopT1-1	1.311	0.117	3.403	0.017	4
PSPTOB0005 phosphoesterase family protein	1.182	0.227	4.061	0.007	4
PSPTO5616	...	...	...	...	...
PSPTO5617	...	...	...	...	...
PSPTO5619	...	...	...	...	...
PSPTO5620	...	...	...	...	...
PSPTOB0078	...	...	...	...	...

MM. Other mechanisms regulating either gene transcription or RNA stability may differ between WT-MM and *lon*⁻-KB and contribute to the differential induction. For instance, either a positive regulator present in WT-MM but absent in *lon*⁻-KB or a negative regulator present in *lon*⁻-KB but absent in WT-MM would lead to the high induction of these hrp box genes in WT-MM.

The second group contained nine genes that accumulated the same or more RNA in *lon*⁻-KB than in WT-MM. In addition to HrpL, the expression of these genes likely involves a second regulator that functions either positively in *lon*⁻-KB or negatively in WT-MM. Only four HrpL-regulated loci (PSPTO0873-0874-0875, PSPTO0834-0835-0836-0837, PSPTO0044, and PSPTO4703) displayed this type of expression pattern. The first two loci encode metabolic enzymes with predicted roles in secondary metabolism. It was reported that *lon* mutation elevates antibiotic production in *P. fluorescens* (Whistler et al. 2000). Whether these two loci are involved in antibiotic production in DC3000 remains to be determined.

The group 3 genes were induced in *lon*⁻-KB versus WT-KB, but the RNA abundance in WT-MM was less than fourfold

higher than in *lon*⁻-KB. The promoters of these genes may have a very high affinity for HrpL; thus, a small amount of HrpL is sufficient for maximal induction of these genes in *lon*⁻-KB. As such, these genes might be the early genes during the TTSS induction when the *hrpL* level is still low. Alternatively, the induction of genes in group 3, similar to genes of group 2, also may involve a second regulator that is differentially present in *lon*⁻-KB and WT-MM.

All 68 HrpL-regulated genes that display poor or no induction in *lon*⁻-KB versus WT-KB belong to group 4. In general, genes in this group that display a minor induction in *lon*⁻-KB versus WT-KB also display relatively higher HrpL-dependent induction in MM, whereas genes that display no induction in *lon*⁻-KB versus WT-KB also display relatively lower HrpL-dependent induction in MM. Perhaps the promoters of these genes are weak, and the low level of HrpL in *lon*⁻-KB is insufficient to activate these genes to a level significantly above the control. However we cannot rule out the involvement of other regulators that are differentially present in WT-MM and *lon*⁻-KB. Genes representing the first three groups (PSPTO4599 for group 1, PSPTO0873 for group 2, and

Fig. 2. Northern blot analysis of *hrpL* and three HrpL-regulated genes in the wild-type (WT) DC3000 and the derived *lon*⁻, *hrpRS*⁻, *hrpS*⁻, and *hrpL*⁻ mutants in rich medium King's B (KB) and minimal medium (MM). The bacteria were cultured in KB to an optical density at 600 nm (OD_{600}) of 0.7 to 0.8 before harvesting for RNA extraction. For the test in MM, the bacteria first were cultured in KB to OD_{600} = 0.7 to 0.8 and then washed twice with MM, resuspended in MM to OD_{600} = 0.2, and cultured for another 6 h before being harvested for RNA extraction. The coding region of each gene was polymerase chain reaction (PCR) amplified and radiolabeled with ³²P-dCTP for probes. **A**, All the genes were tested with the same RNA samples. The composite bands for each probe were from the same hybridization and autoradiography. **B**, The test was conducted with a different set of RNA samples. The ethidium bromide-stained gel pictures in each panel indicate the loading of the RNA samples.

avrPto for group 3) were analyzed by Northern blotting, and they showed the same expression patterns as indicated by the microarray (Fig. 2A and B).

The *lon*⁻ mutation differently affects the expression of the TTSS regulatory genes in KB and MM.

It is known that the *lon*⁻ mutation allows stabilization of HrpR, which in turn activates *hrpL* in rich medium. To determine whether the *lon*⁻ mutation similarly impacts *hrpL* expression in MM, we conducted Northern blot analysis to compare the *hrpL* RNA in the WT DC3000 strain and the *lon*⁻ mutant cultured in KB and MM (Fig. 2A). In KB, the *lon*⁻ mutant showed more *hrpL* RNA than did the WT strain. Unexpectedly,

the *lon*⁻ mutant displayed lower *hrpL* RNA than did the WT strain after 6 h of growth in MM. To determine whether this is a general phenomenon, three *P. syringae* pv. *phaseolicola* *lon*⁻ mutants were similarly tested, and all exhibited similar results (Fig. 3A and B). The reduced *hrpL* RNA in the *lon*⁻ mutants was unexpected, because *lon*⁻ mutation stabilizes HrpR which, in turn, would induce more *hrpL* expression. This also was different from an earlier report that the DC3000 *lon*⁻ mutant displayed a higher level of *hrpL* expression by 30 min after induction in MM (Bretz et al. 2002). To further investigate *hrpL* expression in MM, we determined the dynamics of *hrpL* expression in *lon*⁻ MM (Fig. 3C). The *hrpL* RNA in the *lon*⁻ mutant was induced to a high level 1 h after culture in MM and then decreased. In con-

Fig. 3. Mutation of *lon* affects the expression of *hrpL* differently in rich medium King's B (KB) and minimal medium (MM). **A**, Three *lon*⁻ mutants of *Pseudomonas syringae* pv. *phaseolicola* NPS3121 strain. The long arrow indicates the open reading frame of *lon*. Three triangles underneath indicate the transposon insertion sites. The numbers represent insertion locations relative to the start codon. **B**, The *hrpL* RNA expression in the *lon*⁻ mutants. The bacteria were cultured in KB to an optical density at 600 nm (OD₆₀₀) of 0.7 to 0.8 before harvesting for RNA extraction. For the test in MM, the bacteria first were cultured in KB to OD₆₀₀ = 0.7 to 0.8 and then washed twice with MM, resuspended in MM to OD₆₀₀ = 0.2, and cultured for another 6 h before being harvested for RNA extraction. The *hrpL* coding region was polymerase chain reaction (PCR) amplified and radiolabeled with ³²P-dCTP as probes. The ethidium bromide gel picture shows the loading of RNA samples. **C**, Comparison of the *hrpL* and *hrpR* expression dynamics in MM. The wild-type (WT) NPS3121 strain and the derived *lon*⁻2 mutant were cultured in KB to an optical density at 600 nm of 0.7 to 0.8 before induction in MM as described in B. Bacteria were harvested after 1, 3, 6, and 8 h in MM; "0" indicated bacteria before treatment with MM. The coding regions of *hrpL* and *hrpR* were PCR amplified and radiolabeled with ³²P-dCTP as probes. The loading of RNA samples is indicated by the ethidium bromide-stained gel. **D**, Analysis of *hrpL* promoter activity in the NPS3121 WT strain and the *lon*⁻ mutants. The *hrpL-luc* reporter gene constructed by Xiao and associates (2004) was introduced in the NPS3121 WT strain and the *lon*⁻2 mutant. Bacteria were cultured in KB and MM as described in B. The luciferase (LUC) activity was measured by mixing 1 µl of 1 mM luciferin with 100 µl of cultured cells (2 × 10⁷ CFU) in MM, and the LUC activity was measured at the indicated times.

trast, in the WT strain, the induction of *hrpL* RNA was delayed. A strong signal was detected approximately 3 h after induction in MM, and it remained high until 8 h. A further test with the *hrpL*-LUC reporter showed that LUC activity was higher in the *lon*⁻ mutant than in the WT strain within 1 h. The LUC activity in the WT strain continued to increase while the LUC activity in the *lon*⁻ mutant decreased after 6 h (Fig. 3D). Compared with the *hrpL* RNA abundance, the peak of LUC activity in both strains was much delayed; this probably is due to stability of the LUC protein.

To determine whether reduced *hrpL* expression resulted from reduced expression of upstream regulators, we examined the RNAs of *hrpR*, *hrpS*, *rpoN*, *gacA*, and *rhpS* in the *lon*⁻

mutant and WT DC3000 strains. GacA is a two-component response regulator that is coupled with the sensor kinase GacS to regulate a variety of traits in bacteria (Heeb and Haas 2001). This system controls expression of TTSS genes through the *hrpRS*-*hrpL* regulatory cascade in *P. syringae* pv. *tomato* strain DC3000. GacA deficiency attenuates the transcription levels of *hrpRS*, *rpoN*, and *hrpL* significantly (Chatterjee et al. 2003). RhpS is a sensor kinase that recently was found to control induction of the TTSS genes in MM and in planta. Mutation of *rhpS* severely diminishes induction of *hrpL* and *hrpRS* (Xiao et al. 2007). In KB, disruption of *lon* marginally increases the RNAs of *hrpR*, *hrpS*, *rpoN*, *gacA*, and *rhpS*. After 6 h of growth in MM, the *lon*⁻ mutation did not affect the *rpoN*, *gacA*, and *rhpS* RNAs, but it significantly reduced the *hrpR* and *hrpS* RNAs (Fig. 4). The time course study in Figure 3C indicated that the level of *hrpR* RNA is well correlated with the *hrpL* RNA in the *lon*⁻ mutant. This result suggested that Lon acts as a positive regulator for the *P. syringae* TTSS genes in MM. Positive regulation of TTSS genes by Lon was reported in *Yersinia* spp. Lon mediates the degradation of YmoA, a histone-like protein that represses expression of LcrF, the positive regulator of the *Yersinia* TTSS genes. The YmoA protein accumulates in the absence of Lon which, in turn, represses the TTSS genes (Jackson et al. 2004). Similarly, *P. syringae* may have a negative regulator repressing the *hrpRS* operon in MM, and this negative regulator might be degraded by Lon. If this is true, the negative regulator would accumulate and repress the *hrpRS* operon in the *lon*⁻ mutant. Because HrpR, but not HrpS, can be degraded by Lon (Bretz et al. 2002), the *lon*⁻ mutation would allow the buildup of HrpR but not HrpS in the mutant. In this scenario, reduced HrpS may account for the reduced *hrpL* expression in the *lon*⁻-MM.

Mutation of *lon* reduces the pathogenicity of *P. syringae* on host plants.

The *lon*⁻ mutants of the *P. syringae* pv. *syringae* 61 strain and the *P. syringae* pv. *tomato* DC3000 strain both were reported to elicit a more rapid hypersensitive response than did the corresponding WT strains on the nonhost tobacco plants; however, the DC3000 *lon*⁻ mutant did not affect the pathogenicity on *Arabidopsis* plants (Bretz et al. 2002). The stronger HR was proposed to be attributed to the stabilization and heightened secretion of effectors in the *lon*⁻ mutants (Losada and Hutcheson 2005). Our Northern blot analysis indicated a reduced expression of the TTSS genes in the *lon*⁻ mutant in MM. Reduction of the TTSS genes under TTSS-inducing conditions may lead to reduced pathogenicity of the *lon*⁻ mutant. To test this possibility, *lon*⁻ mutants of *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *tomato* strains were inoculated on bean and tomato plants, respectively. In several repeat experiments, both mutants consistently produced fewer symptoms and exhibited less growth than did their corresponding WT strains (Fig. 5A through D and Supplementary Fig. 1). Although the growth of the *P. syringae* pv. *phaseolicola* *lon*⁻ mutants was reduced by only 2.5- to 3.5-fold, the reduction was observed in all repeat experiments, and statistical analysis indicated that this reduction was significant. Expression of a plasmid-borne *lon* gene largely complements the defect in pathogenicity. These data indicated that *lon* is required for full pathogenicity of *P. syringae*. The reduced pathogenicity is consistent with reduced TTSS gene expression in *lon*⁻ mutants. However, because the *lon*⁻ mutation is pleiotropic, the effect on the TTSS-independent genes also may contribute to the reduced pathogenicity. The discrepancy between our observation and that of Bretz and associates (2002) may result from the use of different host plants or dif-

Fig. 4. Expression of type III secretion system regulatory genes in wild-type (WT) DC3000 and the *lon*⁻ mutant in rich medium King's B (KB) and minimal medium (MM). Bacteria were grown in KB and induced in MM for 6 h. The coding region of each gene was polymerase chain reaction (PCR) amplified and radiolabeled with ³²P-dCTP as probes. **A**, Expression of *hrpR*, *hrpS*, and *hrpL* genes. **B**, Expression of *gacA* and *rpoN* genes. **C**, Expression of the *rhpS* gene. The composite bands for each probe were from the same hybridization and autoradiography. Loading of the RNA samples is indicated by the ethidium bromide gels.

ferent concentrations of bacteria for inoculation. The requirement of *lon* for full pathogenicity also was reported in many other bacterial strains such as *Salmonella enterica* (Takaya et al. 2005), *A. tumefaciens* (Su et al. 2006), and *Brucella abortus* (Robertson et al. 2000).

MATERIALS AND METHODS

Plant materials and bacterial strains.

Bean (*Phaseolus vulgaris* cv. Red Kidney) and tomato (*Lycopersicon esculentum* cv. Rio-Grande PtoS) plants were

Fig. 5. Pathogenicity assay of the *lon*⁻ mutants derived from strains DC3000 and NPS3121. The wild-type (WT), *lon*⁻ mutant, and complemented *lon*⁻ mutant strains were grown in rich medium King's B to an optical density at 600 nm of 2.0 to 3.0, washed twice with sterile water, and resuspended in water (plus silwet L-77 at 10 μ l/liter for DC3000 and its derived strains) to the final concentration of 2×10^4 CFU/ml for inoculation. The bacteria were vacuum infiltrated into tomato and syringe infiltrated into bean primary leaves. The disease symptoms on **A**, tomato and **B**, bean leaves were photographed at 5 days after inoculation. Representative leaf areas from the inoculated bean leaf were $\times 3$ magnified to show the disease lesions (**B**). Bacterial populations in **C**, tomato and **D**, bean leaves were measured at the indicated times. The error bars indicate standard error.

grown in a greenhouse and used for pathogenicity assays. Bacterial strains used in this study were *Pseudomonas syringae* pv. *tomato* DC3000 and *P. syringae* pv. *phaseolicola* NPS3121 and the *lon*⁻ mutant strains derived from them. The DC3000 *lon*⁻ mutant was isolated by screening a DC3000 Tn5<TET-1> transposon insertion mutant library by nested polymerase chain reaction (PCR) following the procedures described by Xiao and associates (2007). Two *lon* gene-based primers, *lon*⁻-G1 and *lon*⁻-G2, and two transposon-based primers, TN-Tet-L1 and TN-Tet-L2 (Table 5), were used for nested PCR. The mutant carried the Tn5<TET-1> transposon inserted at 1,691 bp downstream of the translation initiation site. The three NPS3121 *lon*⁻ mutants were identified from an EZ-Tn^{KM}<Kan-2> transposon insertion mutant library by screening for mutants with high levels of *avrPto-luc* expression in the KB medium. The transposon insertion sites were determined using a two-stage semidegenerated PCR according to Xiao and associates (2007). Transposons were inserted at 507, 1,044, and 2,018 bp downstream from the translation initiation site. Southern blot analysis indicated that all mutants carried a single-copy transposon insertion (data not shown). The KB medium (per liter) contained 29 g of Bacto proteose peptone, 1.5 g of K₂HPO₄, 8 ml of glycerol, and 0.74 g of MgSO₄. The MM (per liter) contained 6.8 g of KH₂PO₄, 1 g of (NH₄)₂SO₄, 345 mg of MgCl₂ · 6H₂O, 100 mg of NaCl, and 1.8 g of fructose. The pH was adjusted to 5.7 with KOH.

Microarray design and analysis.

A high-density oligonucleotide array was designed to cover 5,608 predicted ORF of DC3000. Each probe pair had a perfect-match oligonucleotide of 24 nucleotides and a mismatch oligonucleotide of the same sequence except substitutions at positions 6 and 12. The substitutions were A to T, C to G, G to C, and T to A. Seventeen distinct high-quality probe pairs were designed for each ORF. The synthesis of cDNA probes and microarray hybridization, washing, and staining were performed by NimbleGen Systems Inc. (Madison, WI, U.S.A.) according to the standard protocol. Arrays were scanned with an Axon Genepix 4000B scanner at 532 nm and a resolution of 5 µm.

Microarray data analysis.

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 robust multichip average algorithm (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 pairwise sample comparison, *P* values were calculated for each ORF using the two-sided *t* test.

RNA isolation and Northern blotting.

The bacterial strains were grown in KB broth (King et al. 1954) to approximately 7×10^8 CFU/ml before being harvested for RNA extraction. For gene expression analysis in MM, the bacteria first were grown in KB to 1×10^9 CFU/ml, then centrifuged, washed twice with MM (Huynh et al. 1989), resuspended in MM to 3×10^8 CFU/ml, and cultured for different periods before RNA extraction. Procedures described by Lan and associates (2006) were used for RNA extraction and Northern blotting.

Construction of the pHM2::lon for complementation.

The NPS3121 *lon* gene under the native promoter (210 bases upstream of the ORF) was PCR amplified using the primers 5'-TTAAGCTTCACACGCAGGTGTATGGG-3' (containing *Hind*III) and 5'-CTAGCTAGCATGCGTGCTAATTCTGTC-3' (containing *Nhe*I). The PCR product was digested with *Hind*III and *Nhe*I and cloned into the pBS-HA vector (X. Deng, unpublished). After sequence verification, the insert was released with *Hind*III and *Bam*HI and cloned into pHM2 (Xiao et al. 2007).

Bacterial growth and disease symptoms assays.

Preparation of bacterial inoculum and the bacterial growth assay were performed as described by Shan and associates (2000). NSP3121 at 2×10^4 CFU/ml was hand injected into the fully expended primary leaves of bean plants 10 days after emerging from soil. DC3000 at 2×10^4 CFU/ml was vacuum infiltrated into tomato plants 3 weeks after emergence from

Table 5. Polymerase chain reaction (PCR) primers used in this study^a

Primers	Forward	Reverse
PSPTO4224	AACCCAGGAAGAGGATCAGG	ACCAACAGGACACTGCATGA
PSPTO40039	GTGGGGGCAAAAATAAAGG	TCGTACTGCGGGTATCTGTT
PSPTO3566	TGATACTCACTCGCAAAGTAGGC	TATTGTGGGTCCTGGTTTGG
PSPTO2298	AAGAATTGATGCGCCGTTAC	GATTCCCGCAAATTTCTCAC
PSPTO3123	tgggcactgtactaccagca	aggcttgaagcatcttaccg
hrpL (DC3000)	ttgtgatcctcgactcaacc	gggtcgatttgcctgtg
avrPto	gagggtatcacgaatgggaatatatgtgc	TTGCCAGTTACGGTACGGGCTAGGAGAAAG
PSPTO4599	cgggacaaagacgtttgc	ctgtccctgtccatagcat
PSPTO0873	ggaggttatcgctcggaatca	agtgttgaaaggcgcaaga
PSPTO4589	ctaaagcggccagggcatc	acactctggggccagatgc
hrpL (NPS3121)	gactcttgcctgcccgtat	gggtcaactctgctgctcaa
hrpR (NPS3121)	taatgaacagcgcgtttctg	agcaactcccaactcctca
hrpR (DC3000)	gtcggagtatcgagcgtctg	agcgataccctgggtgaact
hrpS (DC3000)	caagctggatatctgcgtga	agcagtgggcaaacatcg
rpoN (DC3000)	gaactcgacgaatcgaagc	tcaatcgcttactgctactg
gacA (DC3000)	gtcgtatgacctgaccttg	gtgacgtacagcgagcaag
rhpS (DC3000)	TTGAATTCCAGGGAGCTGGGTTGA	TTTCTAGAGTCAAAGGCGCGGCAG
PSPTO1453	ATGTCCAATAGCAGCTCT	TCAGCTCAGCGTCACGTC
PSPTO0507	ATGGACATCATTCGTATC	TTACTTACGCGAAACGAT
lon-1-G2	AGTCTTAATGCGTACTAATTCTATCCTTA	...
lon-1-G1	AGCAGATGCAGCGATAGCATCAATCAGTT	...
TN-Tet-L1	GTACCGGCATAACCAAGCCTATGCCTACAG	...
TN-Tet-L2	GAGGATGACGATGAGCGCATTTGTTAGATTTC	...

^a The PCR primers were designed based on DC3000 genomic sequence.

soil. Disease symptoms were photographed 5 days after inoculation. Student's *t* test was used for statistical analysis of the bacterial growth difference.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

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