Pseudomonas syringae Two-Component Response Regulator RhpR Regulates Promoters Carrying an Inverted Repeat Element

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The two-component system RhpRS was identified in Pseudomonas syringae as a regulator of the genes encoding the type III secretion system and type III effector proteins (together called the T3 genes). In the absence of the sensor kinase RhpS, the response regulator RhpR represses the induction of the T3 gene regulatory cascade consisting of hrpRS, hrpL, and the T3 genes in a phosphorylation-dependent manner. The repressor activity of RhpR is inhibited by RhpS, which presumably acts as a phosphatase under the T3 gene inducing conditions. Here, we show that RhpR binds and induces its own promoter in a phosphorylation-dependent manner. Deletion and mutagenesis analyses revealed an inverted repeat (IR) element, GTATC-N6-GTAC, in the rhpR promoter that confers the RhpR-dependent induction. Computational search of the P. syringae genomes for the putative IR elements and Northern blot analysis of the genes with a putative IR element in the promoter region uncovered five genes that were upregulated and two genes that were downregulated in an RhpR-dependent manner. Two genes that were strongly induced by RhpR were assayed for the IR element activity in gene regulation and, in both cases, the IR element mediated the RhpR-dependent gene induction. Chromatin immunoprecipitation assays indicated that RhpR binds the promoters containing a putative IR element but not the hrpR and hrpL promoters that do not have an IR element, suggesting that RhpR indirectly regulates the transcriptional cascade of hrpRS, hrpL, and the T3 genes.

DNA-binding response regulators usually bind directly to promoter elements to mediate gene regulation. Most response regulators bind to DNA elements consisting of direct or inverted repeats (IR) that are separated by a spacer of 2 to 11 base nucleotides (de Been et al. 2008). For example, response regulators in the OmpR family typically bind to direct repeat elements separated by a spacer of four or five nucleotides, whereas response regulators of the NarL family usually bind to IR elements separated by two to six nucleotides (de Been et al. 2008). A direct repeat element, (T/G)GTTTA-N5-(T/G)GTTTA, is defined as the PhoP box (Grosisman 2001), whereas an imperfect IR (CGGCG-N5-GTCGC) is critical for DNA binding of the response regulator RegR of Bradyrhizobium japonicum (Emmerich et al. 2000). Response regulators are believed to form homodimers on the repeat elements (Blanco et al. 2002; Maris et al. 2002). Some promoters have several copies of the repeat elements, and response regulators often form an oligomer on such promoters (Maris et al. 2005).

Some response regulators are capable of positive as well as negative regulation of their target genes. For example, the phosphorylated response regulator OmpR (P-OmpR) of Escherichia coli binds the promoters of ompF and ompC genes and regulates their expression in response to medium osmolarity (Head et al. 1998; Lan and Igo 1998). There are four and three P-OmpR binding sites in the promoters of ompF and ompC, respectively (Yoshida et al. 2006). At low osmolarity, P-OmpR binds to two or three high-affinity sites in the ompF promoter and activates ompF. Under this condition, only one site in the ompC promoter is occupied by P-OmpR, which is insufficient to activate ompC. At high osmolarity, P-OmpR occupies all three sites in the ompC promoter to activate ompC and all four sites in the ompF promoter to inhibit ompF (Yoshida et al. 2006). The response regulator CoVR of Streptococcus pyogenes can also directly activate and repress its target genes (Churchward 2007). CoVR represses its target genes via promoter occlusion, because the CoVR binding site overlaps with the sigma 70 promoter or the transcriptional start site (Gao et al. 2005; Guas and Scott 2005; Guas et al. 2006). CoVR activates the expression of the dipeptide permease gene dppA by interfering with the binding of a repressor protein to the dppA gene promoter (Gusa et al. 2007).

Bacteria primarily rely on two-component systems (TCS) to sense and respond to environmental changes (Hoch 2000). A TCS usually consists of a sensor histidine kinase and a response regulator (Stock et al. 2000). In general, the sensor kinase, upon sensing a specific signal, autophosphorylates at a highly conserved histidine residue in the transmitter domain and, subsequently, transfers the phosphoryl group to an aspartate residue in the receiver domain of its cognate response regulator. Phosphorylation activates the response regulator which, in turn, stimulates or represses the transcription of its target genes (Stock et al. 2000). Many TCS sensor kinases also possess a phosphatase activity that can dephosphorylate the cognate response regulators and retain the later in an inactive state (Bijlsma and Grosisman 2003). The relative kinase and phosphatase activities in bacterial cells are modulated by environmental stimuli and determine the outcome of signal transduction. Response regulators can be phosphorylated by unrelated sensor kinases or by small phosphate donor molecules such as acetyl phosphate in the absence of cognate sensor kinases (McCleary et al. 1993; Laub and Goulian 2007). As a result, many response regulators display a regulatory activity even in the absence of their cognate sensor kinases (Laub and Goulian 2007).

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RESULTS

The rhpR promoter is induced by RhpR.

Our previous studies showed that RhpR represses the T3 genes in the rhpS\textsuperscript{–} mutant, and the presence of RhpS derepresses the T3 genes in MM (Xiao et al. 2007). In an attempt to depict the regulatory pathway, we searched for the RhpR-regulated promoters. Bacterial TCS loci are often subject to direct autoregulation by the response regulators (Bijlsma and Groisman 2003). To determine whether RhpR regulates rhpRS expression, Northern hybridization was performed to compare the expression of rhpR RNA in wild-type (WT) DC3000 and the transposon insertion mutant of the rhpS gene. rhpR RNA was expressed at a much higher level in the rhpS\textsuperscript{–} mutant than in the WT strain in MM as well as in rich King’s B (KB) medium (Fig. 1A).

To test whether RhpR regulates the activity of the rhpR promoter, 540 bp of rhpR promoter DNA (including the start codon ATG of the rhpR gene) was fused to the promoterless luciferase (\textit{lux}) reporter gene, and the resulting plasmid, pHM2::rhpR\textsubscript{sig}–lux (Table 1), was introduced into WT DC3000, the rhpS\textsuperscript{–} mutant, and the \textit{Δ}rhpRS mutant. Reporter activity was 10-fold higher in the rhpS\textsuperscript{–} mutant than in the WT and \textit{Δ}rhpRS mutant strains (Fig. 1B), indicating an autoactivation of the rhpR promoter by RhpR.

RhpR requires the phosphorylation site to activate the rhpR promoter.

RhpR requires the phosphorylation site for repression of the T3 genes in \textit{P. syringae} strains (Xiao et al. 2007). To determine whether the inducing activity of RhpR is also regulated by phosphorylation, the RhpR(D70A) mutant with the predicted phosphorylation site Asp70 substituted by alanine was tested for the activity to induce the \textit{rhpR} promoter. Hemagglutinin (HA)-tagged WT RhpR and RhpR(D70A) mutant proteins were expressed in the \textit{Δ}rhpRS mutant using a constitutive promoter harbored by the pML122 plasmid (Table 1). Western blot analysis indicated that RhpR-HA and RhpR(D70A)-HA proteins were expressed at similar levels (Xiao et al. 2007). The expression of RhpR-HA but not RhpR(D70A)-HA in the \textit{Δ}rhpRS mutant strongly induced the \textit{rhpR\textsubscript{sig}500} promoter in both MM and KB (Fig. 2A), suggesting that phosphorylation of RhpR enhances its regulatory activity. These results also supported our hypothesis that RhpR is phosphorylated by unknown factors in the rhpS\textsuperscript{–} mutant (Xiao et al. 2007).

In addition to the RhpR-dependent induction, the \textit{rhpR\textsubscript{sig}500} reporter gene in WT DC3000, the rhpS\textsuperscript{–} mutant, and the \textit{Δ}rhpRS mutant displayed higher activities in MM than in KB medium (Figs. 1B and 2A), indicating an RhpR-independent induction of the \textit{rhpR} promoter by MM.

The RhpR-dependent induction of the \textit{rhpR} promoter led us to test whether RhpR directly regulates the \textit{rhpR} promoter. Our attempts to purify the recombinant RhpR protein from \textit{E. coli} were unsuccessful, which deterred the in vivo assays of RhpR interaction with the \textit{rhpR} promoter. Thus, we tested whether the expression of the recombinant RhpR protein can induce the \textit{rhpR} promoter in \textit{E. coli} cells. Plasmids expressing GST-RhpR and GST-RhpR(D70A) fusion proteins were transformed into \textit{E. coli} BL21 carrying the pHM2::rhpR\textsubscript{sig}–lux plasmid.
tent with the result in ΔrhpRS mutant (Fig. 2A), the rhpRΔrhpR-luc reporter gene displayed a higher activity in the BL21 strain expressing GST-RhpR protein than in the BL21 strain expressing GST-RhpR(D70A) (Fig. 2B), suggesting direct regulation of the rhpR promoter by RhpR.

**RhpR requires the phosphorylation site for maximal association with the rhpR promoter.**

A chromatin immunoprecipitation (ChiP) assay was performed to test whether RhpR directly binds the rhpR promoter in *P. syringae*, and whether the binding activity of RhpR is affected by phosphorylation. ΔrhpRS mutant strains expressing RhpR-HA and RhpR(D70A)-HA proteins were used for ChiP assay. RhpR-HA and RhpR(D70A)-HA were expressed at similar levels in the ΔrhpRS mutant (Xiao et al. 2007). The ΔrhpRS mutant carrying the empty pML122 plasmid was used as a negative control. A ChiP assay was performed with the anti-HA antibodies, and the enrichment of the rhpR promoter DNA in the immunocomplexes was detected using quantitative real-time polymerase chain reaction (qRT-PCR). The primer used for amplification of the rhpR promoter DNA is listed in Table 2. A ChiP assay was also performed without the use of the anti-HA antibodies to determine nonspecific precipitation of the promoter DNA. The amount of the promoter DNA precipitated by the anti-HA antibodies to determine nonspecific precipitation of the promoter DNA. The amount of the promoter DNA precipitated by the anti-HA antibodies was not detected in PSPTO1489 (Fig. 3), a housekeeping gene that is not regulated by RhpR (L. Lan and X. Tang, unpublished data).

**Identification of the RhpR-regulated element in the rhpR promoter.**

The 540-bp rhpR promoter in the pHM2::rhpRΔrhpR-luc plasmid was deleted to 170, 120, 80, and 40 bp upstream of the rhpR start codon ATG, and the resulting deletions were assayed for the promoter activities in the rhpSΔrhpR mutant (Fig. 4A). The 170-bp promoter (rhpRpro170) had approximately 60% of the activity relative to rhpRpro222. However, the 120-bp promoter (rhpRpro120) had only 7% of the activity. Further deletion to 40 bp completely eliminated the promoter activity. rhpRpro222 and rhpRpro120 promoters displayed low activities at the same level in the ΔrhpRS mutant (data not shown). These indicated the presence of an RhpR-dependent promoter element in the region between –170 and –120.

The region between –170 and –120 has a perfect 5-bp IR sequence (between –147 and –132) with a 6-bp spacer, GTATC-N6-GATAC (Fig. 4C). To test whether this IR element has a role in the RhpR-dependent regulation, two additional deletions were generated in the region between –170 and –120. The 147-bp promoter carrying the IR element displayed strong ac-

Table 1. Plasmids

<table>
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<tr>
<th>Plasmid name</th>
<th>Description</th>
<th>Reference</th>
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</thead>
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<tr>
<td>pML122::rhpR-HA</td>
<td>rhpR-HA in pML122 plasmid, under pNm promoter</td>
<td>Xiao et al. 2007</td>
</tr>
<tr>
<td>pML122::rhpR(D70A)-HA</td>
<td>Derived from pML122::rhpR-HA, with Asp70 replaced by Ala</td>
<td>Xiao et al. 2007</td>
</tr>
<tr>
<td>pML122</td>
<td>Broad-host plasmid</td>
<td>Lubes et al. 1990</td>
</tr>
<tr>
<td>pHM2</td>
<td>Broad-host plasmid</td>
<td>Xiao et al. 2007</td>
</tr>
<tr>
<td>pGEX-KG</td>
<td>plasmid to produce GST-fusion protein</td>
<td>Guan et al. 1991</td>
</tr>
<tr>
<td>pHM2-luc</td>
<td>Broad host plasmid for reporter genes construction</td>
<td>Xiao et al. 2007</td>
</tr>
<tr>
<td>pHM2::rhpRΔrhpR-luc</td>
<td>rhpR-luc (~540 from ATG) reporter in pHM2</td>
<td>This study</td>
</tr>
<tr>
<td>pHM2::rhpRpro170-luc</td>
<td>rhpR-luc (~170 from ATG) reporter in pHM2</td>
<td>This study</td>
</tr>
<tr>
<td>pHM2::rhpRpro120-luc</td>
<td>rhpR-luc (~147 from ATG) reporter in pHM2</td>
<td>This study</td>
</tr>
<tr>
<td>pHM2::rhpRpro132CA-luc</td>
<td>rhpR-luc (~132 from ATG) reporter in pHM2</td>
<td>This study</td>
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<tr>
<td>pHM2::rhpRpro144TG-luc</td>
<td>rhpR-luc (~120 from ATG) reporter in pHM2</td>
<td>This study</td>
</tr>
<tr>
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<td>pHM2::rhpRpro141D-luc</td>
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<td>pHM2::PSPTO2767-pro-222-luc</td>
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<td>This study</td>
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<td>pHM2::PSPTO2767-pro-109-luc</td>
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<td>pHM2::PSPTO2036-pro-93-luc</td>
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<td>This study</td>
</tr>
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<td>pGST::rhpR</td>
<td>rhpR in pGEX-KG plasmid</td>
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</tr>
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<td>pGST::rhpR-D70A</td>
<td>Derived from pGST-rhpR, with Asp70 replaced by Ala</td>
<td>This study</td>
</tr>
</tbody>
</table>
tivity, whereas the 132-bp promoter without the IR element exhibited low activity in the rhpS− mutant (Fig. 4A). These results indicated that the IR element mediates the RhpR-dependent induction of the rhpR promoter.

The IR sequences of the IR element are perfectly conserved in the promoters of rhpR orthologs in P. syringae pv. tomato, P. syringae pv. syringae, and P. syringae pv. phaseolicola (Fig. 4B). However, the 6-bp spacers are variable in sequence (Fig. 4B). All the IR elements are 132 bp upstream of their corresponding rhpR open reading frames (ORF). Additional elements similar to this IR element were not found in the rhpR promoters.

**Determination of the rhpR transcriptional start site.**

To define the position of the IR element in rhpR promoter, a 5′ rapid amplification of cDNA ends (RACE) reaction was performed with RNA samples prepared from WT DC3000 and the rhpS− mutant to determine the transcriptional start site of rhpR. Four clones containing the PCR products derived from each 5′ RACE reaction were sequenced. All the clones derived from WT DC3000 showed that the 5′ end of rhpR RNA starts at T165, which is 165 bp upstream of the rhpR ORF (Fig. 4C). All the clones derived from the rhpS− mutant showed that the rhpR RNA starts at G87, which is 87 bp upstream of the rhpR ORF (Fig. 4C). G87 is conserved in P. syringae pv. tomato and P. syringae pv. syringae but not in P. syringae pv. phaseolicola, whereas T165 is not conserved in the three sequenced P. syringae genomes (Buell et al. 2003; Feil et al. 2005; Joardar et al. 2005). A putative sigma 70 promoter element was predicted at the −10 and −35 regions upstream of T165, and a sigma 54 promoter element of poor homology to the consensus sequence was predicted between the IR element and G87 (Fig. 4C). This sigma 54 element is moderately conserved in the sequenced P. syringae genomes (Buell et al. 2003; Feil et al. 2005; Joardar et al. 2005). Although the promoter reporter assay suggested a transcriptional start site in the region between −40 and −80 bp, a transcript starting at this region was not identified by the RACE analysis. It is common that TCS have more than one transcriptional start site controlled by different promoters (Bijlsma and Groisman 2003). For example, three transcriptional start sites have been reported for the E. coli TCS locus qseBC (Clarke and Sperandio 2005).

**Mutagenesis analysis of the IR element.**

To determine whether the sequence of the IR element is important to the rhpR promoter activity, mutations (G to T, T to G, A to C, and C to A) were generated to each nucleotide of the IR modules in the pHM2::rhpR147-luc reporter plasmid, and the resulting mutant plasmids were introduced into the rhpS− mutant. Each mutation reduced the promoter activity but not in a uniform fashion (Fig. 4D). Generally, mutants of the upstream 5-bp module (−147 to −143) showed better promoter activity than mutants of the downstream 5-bp IR module (−136 to −132), indicating that the downstream IR module is more important in regulating the promoter activity.

To determine whether the spacer length between the two IR modules is critical for promoter activity, four mutants were generated to the spacer in the pHM2::rhpR147-luc plasmid: one mutant carries a deletion of 1 bp (−C142), one mutant carries a deletion of 2 bp (−C142 and −G141), one mutant carries insertion of one adenine between −C142 and −G141, and one mutant carries insertion of four adenosines between −C142 and −G141 (Fig. 4D). A promoter activity assay showed that any change of the spacer length inactivated the promoter (Fig. 4D), indicating that the 6-bp length of the spacer is required for the activity of the IR element.

**Genome-wide search of the genes regulated by the putative IR elements.**

The identification of an RhpR-regulated IR element enabled us to search for putative RhpR-regulated promoters in P. syringae. The DC3000 genome was searched for the perfect IR sequence, GTATC-N6-GATAC, which uncovered only the rhpR promoter. Because mutant IR elements with one nucleotide substitution exhibited partial activities, we also searched the DC3000 genome for putative IR sequences carrying one variable nucleotide in the repeat modules (NTATC-N6-GATAC, GNATC-N6-GATAC, GTNTC-N6-GATAC, GTANC-N6-GATAC, GTATN-N6-GATAC, GTATC-N6-NATAC, GTATC-N6-GNTAC, GTATC-N6-GANAC, GTATC-N6-GATNC, and GTATC-N6-GATAN). The search produced 44 hits (Table 3).

Eighteen genes downstream of these putative IR elements were analyzed using RNA blotting for their expression in WT DC3000, the rhpS− mutant, and the ΔrhpRS mutant (Fig. 5).
These genes were selected because, according to a microarray analysis, they displayed a differential expression in WT DC3000 and the \( \Delta rhpS \) mutant cultured in MM (L. Lan and X. Tang, unpublished data). The PSPT02036 gene promoter contains a putative IR element (GTATC-N\(_6\)-CTTAC) with two variable nucleotides (underlined) in the downstream IR module. This gene was also selected for RNA blot analysis, because it was expressed at a much higher level in the \( \Delta rhpS \) mutant than in WT DC3000 according to the microarray analysis (unpublished data). RNA blot analysis indicated that five genes, including PSPT02767, PSPT02036, PSPT03477, PSPT03574, and PSPT03600, displayed the same expression pattern as that of the \( rhpR \) promoter (e.g., more transcripts in the \( \Delta rhpS \) mutant than in WT DC3000 and the \( \Delta rhpRS \) mutant) (Fig. 5). These genes are probably induced by RhpR. An extra band of larger size was visible in the Northern blot probed by the full-length PSPT02036 cDNA. This band likely resulted from nonspecific hybridization because it was visible when the 3’ fragment of the PSPT02036 cDNA was used as probes but not visible when the 5’ fragment was used as probes (data not shown). The 3’ fragment of the PSPT02036 cDNA is highly rich in GC (approximately 80%). Two genes, PSPT00536 and PSPT00897, were expressed at lower levels in the \( \Delta rhpS \) mutant than in WT DC3000 and the \( \Delta rhpRS \) mutant (Fig. 5). These genes may be suppressed by RhpR. The putative functions of these genes are summarized in Table 3. The remaining genes displayed an expression pattern independent of RhpR.

### Table 2. Primers

<table>
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<th>Primer</th>
<th>Sequence</th>
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<td>PSPT03574-R</td>
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**Function of the putative IR elements in PSPT02767 and PSPT02036 promoters.**

The PSPT02767 and PSPT02036 transcripts were strongly induced in the \( \Delta rhpS \) mutant. The putative IR elements were analyzed for putative IR elements (GTATC-N\(_6\)-CTTAC) with two variable nucleotides (underlined). The PSPT02036 gene promoter contains a putative IR element (GTATC-N\(_6\)-CTTAC) with two variable nucleotides (underlined) in the downstream IR module. This gene was also selected for RNA blot analysis, because it was expressed at a much higher level in the \( \Delta rhpS \) mutant than in WT DC3000 according to the microarray analysis (unpublished data). RNA blot analysis indicated that five genes, including PSPT02767, PSPT02036, PSPT03477, PSPT03574, and PSPT03600, displayed the same expression pattern as that of the \( rhpR \) promoter (e.g., more transcripts in the \( \Delta rhpS \) mutant than in WT DC3000 and the \( \Delta rhpRS \) mutant) (Fig. 5). These genes are probably induced by RhpR. An extra band of larger size was visible in the Northern blot probed by the full-length PSPT02036 cDNA. This band likely resulted from nonspecific hybridization because it was visible when the 3’ fragment of the PSPT02036 cDNA was used as probes but not visible when the 5’ fragment was used as probes (data not shown). The 3’ fragment of the PSPT02036 cDNA is highly rich in GC (approximately 80%). Two genes, PSPT00536 and PSPT00897, were expressed at lower levels in the \( \Delta rhpS \) mutant than in WT DC3000 and the \( \Delta rhpRS \) mutant (Fig. 5). These genes may be suppressed by RhpR. The putative functions of these genes are summarized in Table 3. The remaining genes displayed an expression pattern independent of RhpR.

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**Table 2. Primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>DC-rhpR-F</td>
<td>AACATAGTGATGCAGCAAGACTTCCGAC</td>
<td>PSPT03574-R</td>
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<td>PSPT00897-R</td>
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<td>DC-rhpR-pro-147F</td>
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<tr>
<td>DC-rhpR-pro-147F</td>
<td>TTTAGATCGTGCTGCTCTGCGATAC</td>
<td>PSPT00897-R</td>
<td>ATGCTGCTGCAATGATATG</td>
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</tbody>
</table>

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**Figure 5.** These genes are probably induced by RhpR. An extra band of larger size was visible in the Northern blot probed by the full-length PSPT02036 cDNA. This band likely resulted from nonspecific hybridization because it was visible when the 3’ fragment of the PSPT02036 cDNA was used as probes but not visible when the 5’ fragment was used as probes (data not shown). The 3’ fragment of the PSPT02036 cDNA is highly rich in GC (approximately 80%). Two genes, PSPT00536 and PSPT00897, were expressed at lower levels in the \( \Delta rhpS \) mutant than in WT DC3000 and the \( \Delta rhpRS \) mutant (Fig. 5). These genes may be suppressed by RhpR. The putative functions of these genes are summarized in Table 3. The remaining genes displayed an expression pattern independent of RhpR.
lyzed for their roles in regulating PSPTO2767 and PSPTO2036 promoters. PSPTO2767 and PSPTO2036 promoters with the IR element (238 bp upstream of PSPTO2767 ORF and 109 bp upstream of PSPTO2036 ORF) and without the IR element (222 bp upstream of the PSPTO2767 ORF and 93 bp upstream of the PSPTO2036 ORF) were fused with the luc reporter gene and assayed in WT DC3000, the \( \text{rhp}^S \) mutant, and the \( \Delta \text{hrpRS} \) mutant (Fig. 6A and B). Both promoters with the IR element showed higher LUC activity in the \( \text{rhp}^S \) mutant than in WT DC3000 and the \( \Delta \text{hrpRS} \) mutant. However, both promoters without the IR element showed low activities in all three strains. These results indicated that the putative IR elements in promoters of PSPTO2767 and PSPTO2036 conferred the RhpR-dependent induction of these genes in the \( \text{rhp}^S \) mutant.

PSPTO2767 encodes a lipopolysaccharide core biosynthesis domain protein. The ortholog of PSPTO2767 in \( \text{P. syringae pv. syringae} \), Psy_2496, has two putative IR elements: one is identical to the PSPTO2767 IR element in the repeat modules and the other is identical to the IR element in the promoter of PSPPH_2653, the ortholog of PSPTO2767 in \( \text{P. syringae pv. phaseolicola} \) (Fig. 6C). There is no additional IR element in the DC3000 genome identical to the IR element of PSPTO2767 in the repeat modules.

PSPTO2036 encodes a putative small lipoprotein, and its orthologs in the \( \text{P. syringae pv. phaseolicola} \) and \( \text{P. syringae pv. syringae} \) genomes were not annotated (Feil et al. 2005; Joardar et al. 2005). Tblastn search identified a small ORF (named PSPPH1805^6) between PSPPH1805 and PSPPH1806 in the \( \text{P. syringae pv. phaseolicola} \) genome and a small ORF (named Psy_1846^7) between Psy_1846 and Psy_1847 in the \( \text{P. syringae pv. syringae} \) genome (Buell et al. 2003; Feil et al. 2005; Joardar et al. 2005). Proteins encoded by these small genes are identical in the N-terminal signal peptide but variable in the C-terminal portion following the lipid modification site (data not shown). The IR elements of these genes are identical in the repeat modules (Fig. 6C). No additional IR element was found in the DC3000 genome identical to the IR element of PSPTO2036 in the repeat modules. Northern blot analysis indicated that PSPPH1805^6 was expressed at a higher level in the \( \text{rhp}^S \) mutant than in the WT \( \text{P. syringae pv. phaseolicola} \) strain (data not shown). The promoters of PSPTO2767 and PSPTO2036 and their orthologous genes in \( \text{P. syringae pv. syringae} \) and \( \text{P. syringae pv. phaseolicola} \) all carry a putative sigma 54 element downstream of the IR element (data not shown).

**RhpR binds the promoters containing a putative IR element.**

ChiP and qRT-PCR assays were performed to test whether RhpR binds the promoters carrying a putative IR element. The promoters of 11 genes were tested. Three of these genes (PSPTO2767, PSPTO2036, and PSPTO3477) displayed an RhpR-dependent upregulation, two genes (PSPTO0536 and PSPTO0897) displayed an RhpR-dependent downregulation, and six genes (PSPTO0898, PSPTO0406, PSPTO1066, PSPTO5198, PSPTO5200, and PSPTO3659) displayed an RhpR-independent expression (Fig. 5). Except for the promoter DNA of PSPTO3659, the remaining 10 promoter DNAs all exhibited a clear RhpR-dependent enrichment in ChiP assay (Fig. 3), even though some of the corresponding genes did not show an RhpR-dependent regulation in Northern blot analysis (Fig. 5).

Because RhpR represses the induction of genes in the T3 gene regulatory cascade in MM, we also tested whether RhpR binds the promoters of the known T3 regulatory genes, including \( \text{hrpR} \), \( \text{hrpL} \), and \( \text{rpoN} \) (Xiao and Hutcheson 1994; Xiao et al. 1994; Hendrickson et al. 2000). The promoters of these genes do not contain a putative IR element (Buell et al. 2003). The promoter DNA of these genes did not exhibit an RhpR-dependent enrichment in ChiP assay (Fig. 3), indicating that RhpR does not bind these promoters.

**DISCUSSION**

Like many bacterial TCS genes, the \( \text{rhpRS} \) locus is subject to positive autoregulation by RhpR. ChiP and qRT-PCR assays

---

**Fig. 3.** Chromatin immunoprecipitation (ChiP) quantitative real-time polymerase chain reaction (qRT-PCR) assay of in vivo RhpR binding with promoters. \( \Delta \text{hrpRS} \) strains containing the pML122 empty vector, pML122::rhpR-HA, and pML122::rhpR(D70A)-HA were used in ChiP assay with and without the anti-hemagglutinin (HA) antibodies. The strains were grown in King’s B medium. Promoter regions of the selected genes in the immunocomplexes were examined by qRT-PCR. Enrichments of promoter DNAs in the immunocomplexes by the anti-HA antibodies (expressed as fold changes) were calculated as \( 2^{\Delta \text{Ct}} = \frac{\text{Ct}_{\text{AB}} - \text{Ct}_{\text{No AB}}}{\text{Ct}_{\text{No AB}}} \), where \( \text{Ct} \) = threshold cycle and AB = antibodies. Results are from three independent experiments. Error bars indicate standard deviations.
indicated that RhpR directly binds to the rhpR promoter. RhpR regulates the rhpR promoter in a phosphorylation-dependent manner. Mutation of the putative phosphorylation site in RhpR protein (D70A) almost abolished its regulatory activity and association with the rhpR promoter. Based on these results, we propose that phosphorylation of RhpR facilitates its interaction with the rhpR promoter. The rhpR<sub>540</sub>-<sub>luc</sub> reporter gene displayed low activity in the ΔrhpRS mutant in both MM and KB, indicating an RhpR-independent basal expression of the rhpRS locus. Results from our previous study as well as this study both suggested that, in KB medium, MM, and the plants, RhpS serves as a phosphatase to retain RhpR in an unphosphorylated state (Xiao et al. 2007). Similar interactions between a sensor kinase and the cognate response regulator have been reported.

![Fig. 4. Identification and mutagenesis analysis of the inverted repeat (IR) element in the rhpR promoter. A, rhpR<sub>540</sub> promoters of 540, 170, 147, 132, 120, 80, and 40 bp were cloned into the pHM2-luc plasmid. Resulting constructs were introduced into the rhpS<sup>−</sup> mutant. Bacteria were cultured in King’s B medium and then incubated in minimal medium for 6 h before the measurement of luciferase activities. Gray bars on the right indicate the length of promoter deletions. Luciferase activities were normalized to bacterial cell numbers. B, Sequence features of the rhpR promoter. Translational start codon ATG of rhpR is bold. The transcriptional start sites G87 and T165 are underlined. The IR element is bold and underlined. The putative sigma 70 and sigma 54 sites are bold italic. C, Alignment of the IR elements in the rhpR promoters of Pseudomonas syringae pv. tomato DC3000, P. syringae pv. syringae B728a, and P. syringae pv. phaseolicola 1448A strains. The IR modules are in boxes. D, Mutagenesis analysis of the IR element. The length of the promoters and the mutations of the IR modules are indicated on the left of the figure. Mutant promoters were cloned into the pHM2-luc reporter plasmid. Activities of the mutant promoters were assayed as described in A. Luciferase activities were normalized to bacterial cell numbers. Error bars indicate standard deviations.
for several TCS, including QseBC of *E. coli* (Kostakioti et al. 2009), CovRS of *S. pyogenes* (Dalton and Scott 2004), and VanRS of *Streptomyces coelicolor* (Hutchings et al. 2006). Based on this study and results from other TCS, we speculate that environmental signals stimulate the RhsS kinase activity which, in turn, phosphorylates RhsR, and the phosphorylated RhsR (P-RhsR) binds to the *rhpR* promoter and activates the expression of *rhpRS*, leading to rapid accumulation of RhsS and RhsR proteins and quick response to the signal.

Deletion analysis revealed a perfect IR element in the *rhpR* promoter that confers the RhsR-dependent gene regulation. Although we were unable to demonstrate the direct interaction of RhsR protein with the IR element due to the failure to obtain purified RhsR protein, based on the requirement of the IR element for the RhsR-dependent induction of *rhpR* promoter, we propose that P-RhsR protein forms a homodimer on the IR element. Point mutations of the repeat modules reduced but did not abolish the *rhpR* promoter activity, suggesting that P-RhsR can dimerize on an imperfect IR element. However, alteration of the spacer length between the repeats completely abolished the promoter activity, suggesting that the space between the two repeat modules is crucial either for dimerization of P-RhsR on the IR element or the engagement of P-RhsR protein with the RNA polymerase complex. This result also suggested that a single repeat module sequence in the promoter is unlikely to have an RhsR-dependent regulatory activity.

Identification of the IR element enabled computational search of the RhsR-regulated genes in the DC3000 genome. The IR element in the *rhpR* promoter is the only perfect IR element in DC3000. Because mutagenesis analysis indicated that point mutations of the repeat modules only reduced the IR activity, our initial search focused on putative IR elements with one nucleotide mismatch. In all, 44 putative IR elements of this type were identified. Many of these 44 putative IR elements are in the promoters of conserved genes in the three sequenced *P. syringae*

### Table 3. Genes containing a putative inverted repeat (IR) element in their promoters

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<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Position</th>
<th>IR sequence</th>
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<tbody>
<tr>
<td>Group I</td>
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<td><em>PSPTO2223</em></td>
<td>RhsR</td>
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<td><em>PSPTO2036</em></td>
<td>Lipoprotein, putative</td>
<td>−109 to −94</td>
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<tr>
<td><em>PSPTO2767</em></td>
<td>LPS core biosynthesis domain protein</td>
<td>−238 to −222</td>
<td></td>
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<tr>
<td><em>PSPTO3477</em></td>
<td>Hypothetical</td>
<td>−237 to −225</td>
<td></td>
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<tr>
<td><em>PSPTO3574</em></td>
<td>TonB-dependent siderophore receptor, putative</td>
<td>−103 to −88</td>
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<tr>
<td><em>PSPTO3660</em></td>
<td>Xanthine dehydrogenase</td>
<td>−231 to −216</td>
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<td>−99 to −84</td>
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<td>DNA-binding response regulator, LuxR family</td>
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<td>Group III</td>
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<td>DnaJ domain protein</td>
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<td><em>PSPTO1066</em></td>
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<td>Outer membrane protein Omph</td>
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<td>Response regulator</td>
<td>−252 to −237</td>
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</table>

* *Group I*, upregulated genes in the *rhpS* mutant; *Group II*, downregulated genes in the *rhpS* mutant; *Group III*, unchanged genes in the *rhpS* mutant; and *Group IV*, untested genes. Genes in groups I to III were tested by RNA blotting. Genes indicated by * were tested by chromatin immunoprecipitation analysis.
genomes (Buell et al. 2003; Feil et al. 2005; Joardar et al. 2005). Some of these IR elements may be functional, because the RhpR proteins in _P. syringae_ strains are >98% identical (Xiao et al. 2007), and they are likely to regulate conserved functions.

Further characterization of these putative IR elements was guided by the data from a microarray analysis that was designed to compare gene expression in WT DC3000 and the _rhpS_– mutant cultured in MM. Nineteen genes (including one

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<td>KB-MM</td>
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**Fig. 5.** Northern blot analysis of genes carrying a putative inverted repeat (IR) element in the promoter. Wild-type DC3000 (WT), the _rhpS_ mutant, and the _ΔrhpRS_ mutant were grown in King’s B medium and then incubated in minimal medium for 6 h before RNA extraction. Total RNA (10 µg/sample) was analyzed by RNA blotting with radio-labeled probes derived from the coding regions of the corresponding genes. The ethidium bromide-stained RNA gel indicates the loading of RNA samples. Sequences of the putative IR elements in the gene promoters are shown in the right column.
gene with two nucleotide mismatches) that displayed a differential expression in the microarray analysis were assayed for their expression in WT DC3000, the \( \Delta \text{rhpS} \) mutant, and the \( \Delta \text{rhpRS} \) mutant using RNA blot analysis. This assay identified five genes that showed an RhpR-dependent induction and two genes that showed an RhpR-dependent suppression in the \( \Delta \text{rhpS} \) mutant. The IR elements of PSPTO2036 and PSPTO2767 were further assayed for their activities to regulate the corresponding promoters. Both IR elements were found to be required for the RhpR-dependent induction of the respective promoters. The remaining IR elements have not been characterized for their activities to regulate their corresponding promoters. Further characterization of these IR elements will show whether they, indeed, mediate the RhpR-dependent induction or suppression of their corresponding genes. It should be noted that the IR element in the PSPTO2036 promoter has two nucleotide mismatches in one of the repeat modules but this IR element still confers a strong RhpR-dependent induction, suggesting that a functional IR element can tolerate more than one variable nucleotide in the repeat modules. Thus, future studies of the IR element should test how mutation of two or more nucleotides in the repeat modules affects the activity of the IR element.

Such information is crucial for computational identification of the RhpR-regulated genes. All three confirmed IR elements are upstream of a putative sigma-54 binding site, suggesting that P-RhpR may interact with the sigma 54 protein to activate the transcription of the corresponding genes. The position of a response regulator binding site relative to the sigma-factor binding site is crucial to its regulatory activities. In general, response regulators that bind upstream of the sigma-factor binding site positively regulate gene transcription (Bijlsma and Groisman 2003). For PSPTO2767 and PSPTO2036, the upstream position of the IR elements relative to the putative sigma-54 binding site is consistent with the positive role of RhpR. A sigma-factor-regulated promoter element of high homology was not identified in the promoters of PSPTO0536 and PSPTO0897. However, the putative IR motifs and the translation start codons are closely located in both genes. This implies the possibility that the IR motif is downstream of a core promoter and confers the RhpR-dependent negative regulation of the corresponding gene.

PSPTO2767 encodes a putative lipopolysaccharide core biosynthesis domain protein, whereas PSPTO2036 encodes a putative small lipoprotein (Buell et al. 2003). These genes encode conserved functions in the sequenced \( \text{P. syringae} \) genomes (Buell et al. 2003; Feil et al. 2005; Joardar et al. 2005). It is interesting that both genes seem to have a bacterial cell-wall-related function. The opposite expression patterns of the two cell-wall-related genes and the T3 genes suggested coordination of the T3 gene expression with a cell-wall-related function.

A ChIP assay was used to determine whether RhpR interacts with the promoters carrying a putative IR element. This assay confirmed that RhpR interacts with the three promoters carrying an RhpR-induced IR element (\( \text{rhpS} \), PSPTO2767, and PSPTO2036). RhpR also interacts with the promoters of the two genes repressed by RhpR (PSPTO0536 and PSPTO0897). However, it remains to be determined whether RhpR binds the putative IR element in these promoters and whether the binding of RhpR with these promoters mediates the negative regulation of the corresponding genes. Surprisingly, RhpR also interacts with the putative IR promoters that have a putative IR element but did not show an RhpR-dependent regulation. The result is unlikely an artifact of the ChIP assay, because the interaction is specific to the promoters with the IR element, and promoters without the IR element did not show any interaction with the RhpR protein. It is possible that RhpR does, indeed, interact with these promoters, and the regulation of these promoters requires the function of RhpR. However, activation or suppression of these promoters requires additional proteins that were not present in the test growth conditions (i.e., culture in MM and KB medium). Similar results have been reported for the TCS response regulator CovR that interacts with specific sites not found to be regulated by CovR (Churchward et al. 2009).

Although RhpR has been identified as a suppressor of the \( \text{P. syringae} \) T3 genes, ChIP assays indicated that RhpR does...
not bind directly to the promoters of the T3 regulatory genes, including hrpR, hrpL, and rpoN. These regulatory genes do not have a putative IR element in their promoters. These results suggested that RhpR indirectly regulates the T3 regulatory cascade consisting of hrrs, hrpL, and the T3 genes. One or more of the genes directly regulated by RhpR may serve as the link to connect RhpR and the hrrs-hrpL-T3 gene transcriptional cascade.

MATERIALS AND METHODS

Bacterial strains and media.

Bacterial strains used in this study were P. syringae pv. tomato DC3000 (Buell et al. 2003) and the hrrs- and ΔhrpRS mutant strains derived from DC3000 (Xiao et al. 2007). E. coli DH5α was used for constructing all plasmids. E. coli BL21 was used for testing of RhpR-mediated induction of rhpR promoter. DC3000 and its derivatives were grown at room temperature in KB medium (King et al. 1954) containing appropriate antibiotics. E. coli strains were cultured in Luria-Bertani (LB) medium at 37°C. Antibiotics for selection of P. syringae strains were rifampicin, 25 mg/liter; kanamycin, 10 mg/liter; spectinomycin, 50 mg/liter; tetracycline, 10 mg/liter; and gentamicin, 10 mg/liter. Antibiotics for selection of E. coli were ampicillin, 100 mg/liter; kanamycin, 50 mg/liter; spectinomycin, 100 mg/liter; and gentamicin, 20 mg/liter.

Construction of plasmids for promoter analysis.

All promoter DNA fragments were PCR amplified using the DC3000 genomic DNA as a template. Primers used for PCR amplifications are listed in Table 2. To facilitate cloning, all forward primers were added with an EcoRI site and all reverse primers were added with a BamHI site.

For rhpR promoter deletion analysis, reverse primer rhpR-proR was used with one of the forward primers rhpR-pro540F, rhpR-pro170F, rhpR-pro147F, rhpR-pro132F, rhpR-pro120F, and rhpR-pro40F to PCR amplify the rhpR promoter fragments of 540, 300, 170, 147, 132, 120, and 40 bps upstream of the rhpR ORF.

To create point mutations in the IR element of rhpR promoter, forward PCR primers rhpR-pro147G-TF (−147G to T), rhpR-pro146T-GF (−146T to G), rhpR-pro145A-CF (−145A to C), rhpR-pro144T-GF (−144T to G), rhpR-pro143C-AF (−143C to A), rhpR-pro136G-TF (−136G to T), rhpR-pro135A-CF (−135A to C), rhpR-pro134T-GF (−134T to G), rhpR-pro133A-CF (−133A to C), and rhpR-pro132C-AF (−132C to A) were used with the reverse primer rhpR-proR in PCR amplifications.

To insert or delete nucleotides in the spacer of the IR element of rhpR promoter, the forward primers rhpR-pro141I1F and rhpR-pro141I1R were used with two pairs of primers: DC-rhpR-PE1 and DC-rhpR-S1 as well as two pairs of primers DC-rhpR-PE2 and DC-rhpR-S2 (Table 2). The PCR products were cloned into the pGEM-T vector (Promega Corp., Madison, WI, U.S.A.) and sequence verified.

Analysis of RhpR-mediated regulation of rhpR promoter in E. coli.

pGST::rhpR and pGST::rhpR(D70A) were constructed to express GST-RhpR and GST-RhpR(D70A) proteins in E. coli BL21. The pML122::rhpR-HA and pML122::rhpR-D70A-HA plasmids (Xiao et al. 2007) were used as template DNA for PCR-amplification of rhpR and rhpR(D70A), respectively, with rhpR-GST-F (containing an XbaI site) and rhpR-GST-R (containing an HindIII site) as primers (Table 2). The PCR products were digested with XbaI and HindIII, cloned into pGEX-KG (Guan and Dixon 1991), and sequence verified. pGST::rhpR and pGST::rhpR-D70A were transformed into E. coli BL21 containing the reporter plasmid pHM2::rhpR4ar-luc. To determine the LUC activities, bacterial strains were grown at 37°C in LB medium containing spectinomycin and ampicillin to OD600 = 1. Isopropyl-thio-galactopyranoside (IPTG) was added into the cultures to a final concentration of 1 mM to induce the GST fusion protein production. At 1 h after IPTG induction, 100 µl of culture was mixed with 1 µl of 1 mM luciferin in a 96-well plate, and the luciferase reporter gene activities. Bacterial culture (100 µl) was mixed with 1 µl of 1 mM luciferin in a 96-well plate, and the luciferase activity was determined using a cooled CCD camera (Roper Scientific).

Determination of the rhpR transcriptional start site.

5′ RACE was performed using the 5′-Full RACE core set (Takara, Shiga, Japan) and total RNAs prepared from WT DC3000 and the hrrs- mutant following the manufacturer’s instructions. First-strand cDNAs were prepared from 1 µg of total RNA with the 5′-phosphorylated reverse-transcription primer, DC-rhpR-RTF (Table 2), and Avian myeloblastosis virus reverse transcriptase. The template RNAs were then digested by RNase H, and the cDNAs were circulated by ligations. The circulated cDNAs were then amplified by nested PCR with two pairs of primers: DC-rhpR-PE1 and DC-rhpR-S1 as the first pair and DC-rhpR-PE2 and DC-rhpR-S2 as the second pair (Table 2). The PCR products were cloned into the pGEM-T vector (Promega Corp., Madison, WI, U.S.A.) and sequenced. Homology of the trapped sequences was searched with the BLASTn program.

RNA isolation and Northern blotting.

Procedures described by Lan and associates (2006) were used for RNA extraction and Northern blotting. The bacterial strains were grown in KB broth (King et al. 1954) to approximately OD600 = 2 before being harvested for RNA extraction. For gene expression analysis in MM, the bacteria first were grown in KB to OD600 = 2, then centrifuged, washed twice with MM (Huynh et al. 1989), resuspended in MM to OD600 = 0.3 CFU/ml, and cultured for different periods before RNA extraction. Primers that were used to amplify probe sequences are listed in Table 2. The PCR products were radio-labeled.
with $^{32}$P-dCTP using the Random Primed DNA Labeling kit (Ambion, Austin, TX, U.S.A.) as probes.

**ChiP and qRT-PCR.**

The ChiP experiments were performed using the ChiP-IT Express kit (Active Motif, Carlsbad, CA, U.S.A.) according to the manufacturer’s protocol, and the procedures were modified according to Bruscella and associates (2008). *P. syringae* bacteria were grown overnight in KB medium containing gentamicin. Cross-linking was performed by adding formaldehyde (final concentration, 1%) to the medium for 10 min. The reaction was terminated by adding glycine Stop-Fix solution (Active Motif) and incubating for 10 min at room temperature with gentle agitation. Bacteria were centrifuged for 1 min at 12,000 × g, washed twice with ice-cold phosphate-buffered saline, resuspended in 1 ml of lysis solution supplemented with 5 µl of phenylmethylsulfonyl fluoride (PMSF) and 5 µl of protease inhibitor cocktail, and incubated on ice for 30 min. Digestion buffer (1 ml) containing 5 µl of PMSF and 5 µl of protease inhibitor cocktail was added to the lysate and then heated for 5 min at 37°C. An enzymatic shearing mixture (30 µl at 200 U/ml) was added to the digestion mixture. After incubation for 25 min at 37°C with agitation, the reaction was stopped by the addition of 20 µl of 0.5 M EDTA and incubation for 10 min on ice. After centrifugation at 15,000 × g and 4°C for 10 min, the supernatant was recovered, and the shearing efficiency was examined as described by the manufacturer. Preclearing of chromatin samples, input recovery, immunoprecipitation with or without the anti-HA antibody, addition of Protein G beads, washing, elution of DNA-protein complexes, reverse cross-linking, RNA removal, and proteinase K treatment were performed by following the manufacturer’s instructions. The eluted DNA samples from the ChiP assay were used for qRT-PCR experiments using Bio-Rad iCycler IQ (Bio-Rad, Hercules, CA, U.S.A.). PCR primers (Table 2) for amplification of promoter regions were designed by using the Primer3 software. The PSPT01489 gene (encoding a putative xenobiotic reductase) that is equally expressed in WT DC3000 and the rhpS mutant (L. Lan and X. Tang, unpublished data) was used for normalization. The SYBR green PCR mixture (Bio-Rad) was mixed with appropriate amounts of ChiP samples for qRT-PCR. The amount of PCR product was estimated for different promoter regions using the input DNA (the total sheared DNA prior to ChiP) and the immunoprecipitated DNA with and without the anti-HA antibodies as the matrix. Threshold cycle (Ct) values were obtained for all samples. The ChiP enrichments were determined by the fold change of amplification between the immunoprecipitated DNA with the antibodies (AB) and the immunoprecipitated DNA without the antibodies, and these were calculated by the following formula: 2 $^{-\Delta \Delta Ct} = C_{\Delta Ct} = C_{AB} - C_{\Delta Ct AB}$. A standard curve and a melt curve were drawn for each primer pair. The slope of the standard curve was used to calculate the primer efficiency for each primer pair. Results were collected only from the reactions showing primer efficiencies between 95 and 105%. A melt curve was drawn for each primer pair to ensure that only one specific PCR product was obtained. The results for all reactions were obtained from at least two independent experiments.

**ACKNOWLEDGMENTS**

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