Naringenin Regulates Expression of Genes Involved in Cell Wall Synthesis in *Herbaspirillum seropedicae*


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Five thousand mutants of *Herbaspirillum seropedicae* SmR1 carrying random insertions of transposon pTnMod-OGmKmlacZ were screened for differential expression of LacZ in the presence of naringenin. Among the 16 mutants whose expression was regulated by naringenin were genes predicted to be involved in the synthesis of exopolysaccharides, lipopolysaccharides, and auxin. These loci are probably involved in establishing interactions with host plants.

The betaproteobacterium *Herbaspirillum seropedicae* is an endophytic diazotroph that forms nitrogen-fixing associations with maize (*Zea mays*), rice (*Oryza sativa*), sorghum (*Sorghum bicolor*), and sugar cane (*Saccharum officinarum*), as well as such diverse plants as bananas (*Musa spp.*) and pineapple (*Ananas comosus*) (1, 5, 27). As such, *H. seropedicae* is a potential nitrogen biofertilizer that also produces phytohormones that may stimulate the growth of plants (4). Studies demonstrated that the inoculation of rice with *H. seropedicae* promoted a yield increase equivalent to treatment with 40 kg N/ha (2). Coinoculation of micropropagated sugar cane with *H. seropedicae* resulted in increased yield equivalent to treatment with 40 kg N/ha (2). Synthesis in *H. seropedicae* was encoded by the promoterless lacZ reporter gene in the presence of naringenin. Among the 16 mutants whose expression was regulated by naringenin were genes predicted to be involved in the synthesis of exopolysaccharides, lipopolysaccharides, and auxin. These loci are probably involved in establishing interactions with host plants.

To identify bacterial genes whose expression is under the control of the flavonoid naringenin, the chromosome of *H. seropedicae* strain SmR1 (23) was randomly mutagenized using a lacZ-Km-Gm cassette carried by the plasmid pTnMod-OGmKmlacZ, and strains resistant to both kanamycin and gentamicin were selected (30). Twelve thousand mutant strains were obtained, and 5,000 screened for differential expression of the promotereless lacZ reporter gene in the presence of naringenin (50 μmol/liter) using NFFβHP-malate agar medium (19) containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (30 μg/ml). One hundred ninety-six mutants bearing mutated genes potentially controlled by naringenin were preselected, and their β-galactosidase activities determined (7, 24). Of these, 16 isolates carried mutated genes that reacted differently to the presence of naringenin. Four of the mutants carried genes that were upregulated, and 12 carried genes that were downregulated by naringenin (Table 1). The mutated genes of these strains were identified by sequencing as described previously (30).

The fraction of the genome covered by the set of insertion mutants was calculated according to the method of Jacobs et al. (17). The total length of the *H. seropedicae* genome is 5,513,887 bp, containing 4,737 open reading frames (ORFs) with an average length of 1,029 bp. Since a total of 5,000 mutants were tested and assuming that half of these (2,500) would have the lacZ gene inserted in the mutated gene transcription orientation, approximately 38% of the genome was covered by the mutant collection screened (17).

Among the mutants in genes downregulated (2- to 3-fold) by naringenin, four were affected in genes probably involved in the synthesis of cell wall components. Strain MHS01 was mutated in epsG, which is involved in exopolysaccharide biosynthesis; MHS02 in a gene for a probable O-antigen acetylase; MHS05 in ampG, which codes for a mureopeptide permease of the major facilitator superfamily; and MHS15 in a gene coding for a probable glucosyltransferase bearing low similarity to GumH, a glucosyl transferase from *Xylella fastidiosa* that is involved in exopolysaccharide biosynthesis. Interestingly, strain MHS03 was mutated in a gene coding for a conserved protein with no function assigned but located in an operon together with two genes coding for putative outer membrane proteins. One of these is a probable outer membrane porin of the OmpA family, suggesting that the mutated gene may also play a role in outer membrane structure. LPSs consist of an O-antigen, a lipid A, and core oligosaccharides...
TABLE 1. Identification of genes from H. seropedicae that are regulated by the flavonoid naringenin

<table>
<thead>
<tr>
<th>Mutant strain</th>
<th>Accession no. of the mutated locus</th>
<th>Deduced gene product</th>
<th>Relative naringenin regulation (avg expression ± SD) shown by:</th>
<th>β-Gal activity</th>
<th>RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHS01</td>
<td>YP_003775401.1</td>
<td>EPS biosynthesis protein (EpsG)</td>
<td>Downregulated (0.31 ± 0.05)</td>
<td>Downregulated (0.07 ± 0.045)</td>
<td></td>
</tr>
<tr>
<td>MHS02</td>
<td>YP_003775614</td>
<td>Probable O-antigen acetylase</td>
<td>Downregulated (0.34 ± 0.09)</td>
<td>Downregulated (0.23 ± 0.11)</td>
<td></td>
</tr>
<tr>
<td>MHS03</td>
<td>YP_003773926.1</td>
<td>Conserved hypothetical protein</td>
<td>Downregulated (0.50 ± 0.01)</td>
<td>Downregulated (0.52 ± 0.082)</td>
<td></td>
</tr>
<tr>
<td>MHS04</td>
<td>YP_003774406</td>
<td>Probable acyltransferase protein</td>
<td>Downregulated (0.44 ± 0.07)</td>
<td>Downregulated (0.43 ± 0.047)</td>
<td></td>
</tr>
<tr>
<td>MHS05</td>
<td>YP_003773531.1</td>
<td>Probable muropeptide permease of the major facilitator superfamily protein (AmpG)</td>
<td>Downregulated (0.33 ± 0.05)</td>
<td>Downregulated (0.12 ± 0.043)</td>
<td></td>
</tr>
<tr>
<td>MHS06</td>
<td>YP_003778011.1</td>
<td>Probable acyl CoA-acetate/3-ketoacid CoA transferase, beta subunit</td>
<td>Upregulated (1.65 ± 0.21)</td>
<td>Upregulated (1.23 ± 0.012)</td>
<td></td>
</tr>
<tr>
<td>MHS07</td>
<td>YP_003774121.1</td>
<td>Probable tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase protein (TrmU)</td>
<td>Downregulated (0.66 ± 0.11)</td>
<td>Downregulated (0.24 ± 0.063)</td>
<td></td>
</tr>
<tr>
<td>MHS08</td>
<td>YP_003776865.1</td>
<td>Hypothetical protein</td>
<td>Upregulated (1.66 ± 0.07)</td>
<td>Upregulated (1.13 ± 0.09)</td>
<td></td>
</tr>
<tr>
<td>MHS09</td>
<td>YP_003775366.1</td>
<td>Probable 5-aminovaleric acid synthase protein</td>
<td>Upregulated (2.04 ± 0.03)</td>
<td>Downregulated (0.93 ± 0.046)</td>
<td></td>
</tr>
<tr>
<td>MHS10</td>
<td>YP_003776866.1</td>
<td>Hypothetical protein</td>
<td>Downregulated (0.18 ± 0.001)</td>
<td>Downregulated (0.71 ± 0.01)</td>
<td></td>
</tr>
<tr>
<td>MHS11</td>
<td>YP_003778130.1</td>
<td>Conserved hypothetical protein</td>
<td>Upregulated (1.22 ± 0.015)</td>
<td>Downregulated (0.81 ± 0.056)</td>
<td></td>
</tr>
<tr>
<td>MHS12</td>
<td>YP_003775661.1</td>
<td>Probable NAD-dependent aldehyde dehydrogenase protein</td>
<td>Downregulated (0.27 ± 0.03)</td>
<td>Downregulated (0.258 ± 0.075)</td>
<td></td>
</tr>
<tr>
<td>MHS13</td>
<td>YP_003777134.1</td>
<td>Hypothetical protein</td>
<td>Downregulated (0.54 ± 0.12)</td>
<td>Downregulated (0.21 ± 0.038)</td>
<td></td>
</tr>
<tr>
<td>MHS14</td>
<td>YP_003777655.1</td>
<td>Probable indole pyruvate ferredoxin oxidoreductase, alpha and beta subunits protein</td>
<td>Downregulated (0.042 ± 0.02)</td>
<td>Downregulated (0.15 ± 0.015)</td>
<td></td>
</tr>
<tr>
<td>MHS15</td>
<td>YP_003777590.1</td>
<td>Glucosyltransferase hypothetical protein</td>
<td>Downregulated (0.54 ± 0.12)</td>
<td>Downregulated (0.23 ± 0.04)</td>
<td></td>
</tr>
<tr>
<td>MHS16</td>
<td>YP_003775234.1</td>
<td>Conserved hypothetical protein</td>
<td>Downregulated (0.12 ± 0.04)</td>
<td>Downregulated (0.17 ± 0.053)</td>
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</tbody>
</table>

* Sixteen insertion mutants of H. seropedicae SmR1 carry genes whose expression is modulated by naringenin.
* BLASTn and SMART comparisons of the genes with publicly available databases (accessed on 28 July 2010) are shown.
* Expression levels were measured as β-galactosidase (β-Gal)-specific activities of the parental strain and lacZ fusion mutants, and relative expression is reported as the ratio of β-galactosidase activity in the presence of 50 μmol/liter naringenin to β-galactosidase activity in the absence of naringenin.
* Relative expression was determined by the 2−ΔΔCT (threshold cycle) method (22); the relative expression of the epsG gene located in the same operon as ampG was 0.082 ± 0.01. The results represent three replicate experiments. The RT-PCR experiments were performed using Power SYBR green master mix (Applied Biosystems, Carlsbad, CA), and reactions were run on a Step One Plus real-time PCR system (Applied Biosystems).

and are present as a monolayer on the outer membrane of Gram-negative bacterial cells. Strain MHS02 carries a mutation that may affect the acetylation of the O-antigen part of the LPS of H. seropedicae. Similar changes to the O-antigen of rhizobia result in altered symbiotic phenotypes (9, 21).

Changes in bacterial outer surface structures, such as LPS and EPS, often result in increased sensitivity to cationic peptide antibiotics, detergents, and various stresses and affect symbiotic development (9, 10, 18, 20, 21, 31). Accordingly, we challenged the collection of mutants with increasing concentrations of sodium dodecyl sulfate (SDS). In contrast to the parental strain, isolates with mutations in genes related to the synthesis of EPS (MHS01) and muropeptide permease (MHS05) were unable to grow in the presence of 0.5% (wt/vol) SDS (data not shown). Since mutants affected in LPS synthesis had an altered sensitivity to SDS, we analyzed the LPS profiles of isolates MHS01 and MHS05. LPSs were extracted using the phenol procedure (13) and separated on denaturing polyacrylamide gels (20). Only mutation of the muropeptide permease gene caused significant changes to the LPS profile (Fig. 1A). Although the band pattern is similar to that of the parental strain, the amount of LPS recovered per cell of strain MHS05 is clearly reduced, suggesting that LPS may not be qualitatively different but is present in a lower concentration in the ampG strain's outer membrane. The AmpG protein is located in the...
membrane of Gram-negative bacteria and is necessary for cell wall peptidoglycan recycling (11). The peptidoglycan is both a highly complex and essential macromolecule of bacterial cell walls (6). The results suggest that the lack of AmpG in strain MSH05 leads to an anomaly in the peptidoglycan layer affecting the synthesis or targeting of LPS in *H. seropedicae*.

To check whether any of the mutations influenced the colonization of maize (*Zea mays*), the protocol of Balsanelli et al. (3) was used to test bacterial cell adhesion to root surface and internal colonization. No difference was observed between the parental strain (SmR1) and mutants in growth and root surface attachment (data not shown). On the other hand, the endophytic population of MHS05 was reduced by 10-fold compared to that of the parental strain both 1 and 3 days after inoculation, suggesting that the modification in LPS in this strain is important for bacterial establishment in plant tissues (Fig. 1B). Previously, Balsanelli et al. (3) showed that strains unable to synthesize LPS were severely impaired in maize colonization. These authors also showed that the *mlb* gene, whose product is involved in LPS biosynthesis, was upregulated by naringenin (4-fold), in agreement with our results showing that naringenin can modulate genes involved in the biosynthesis and modification of LPS. All other strains had the same pattern of colonization as the parental strain (data not shown).

Genes coding for a putative acetyltransferase (MHS07), a probable acyltransferase (MHS04), an aldehyde dehydrogenase (MHS12), and indole pyruvate ferredoxin reductase (MHS14) were also downregulated by naringenin. Since the nase (MHS12), and indole pyruvate ferredoxin oxidoreductase probably acyltransferase (MHS04), an aldehyde dehydrogenase of LPS. All other strains had the same pattern of colonization as the parental strain (data not shown).

Four strains were mutated in genes upregulated by naringenin. These include those coding for a putative acyl coenzyme A (CoA):acetate/3-ketoacid CoA transferase (MHS06), a hypothetical protein (MHS08), an aminotransferase similar to S-aminolevulinic acid synthase (MHS09), and a MarR transcription regulator that may be involved in multidrug resistance (MHS11). The deduced product of the gene mutated in strain MHS08 is similar to a protein of *Vibrio cholera* of the type II secretory pathway. This system is used by many Gram-negative bacteria, mainly to secrete extracellular enzymes that are associated with the degradation of host tissue (28, 29). The gene coding for the aminotransferase (MHS09) is located in an operon together with a gene coding for a permease of the MFS family, which is possibly related to the tetracyclomycin C resistance protein. Cho et al. (12) identified a similar multidrug resistance permease gene of *Escherichia coli* that is upregulated by an infusion containing polyphenols. In strain MHS16, the gene mutated is located in an operon together with another acetyltransferase, an organization that is conserved in *Burkholderia vietnamiensis*. Thus, altogether, at least three different genes coding for acyltransferases seem to have their expression affected by naringenin.

Four other mutations were in hypothetical genes of *H. seropedicae* whose products had no significant matches in all databases (MHS03, MHS10, MHS13, and MHS16). The genes mutated in strains MHS03 and MHS10 probably code for membrane proteins.

Real-time PCR analysis was used to validate the data obtained with lacZ fusions. To do this, total RNA was isolated from cultures grown in the presence and absence of naringenin (50 μmol/liter) using the Trizol procedure (Sigma Corp., St. Louis, MO). Two micrograms of RNA was used to synthesize cDNA with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). The 16S rRNA gene was used as the internal control. Primers for the gene epsB located in the same operon as epsG were also used, to confirm the coregulation of genes related to EPS synthesis.

The RT-PCR analyses confirmed regulation of the mutated genes by naringenin except for strains MHS08, MHS09, and MHS11 (Table 1). In these three strains, the β-galactosidase activity indicated upregulation by naringenin, whereas RT-PCR indicated no regulation (MHS08 and MHS09) or even slight downregulation (MHS11), raising the possibility of posttranscriptional regulation of these genes. Finally, the epsB and epsG genes responded similarly to naringenin, suggesting that the eps operon is regulated by the flavonoid.

Our results show that *H. seropedicae* responds to the plant flavonoid naringenin by modifying the pattern of gene expression. The products of four genes with altered expression seem to be involved in the synthesis of the outer membrane of the cell wall. Changes in the cell surface are documented in other bacteria that interact with plants (e.g., rhizobia) (9, 20, 31) and probably play a role in the interaction between *H. seropedicae* and its host plants. Furthermore, the data indicate that naringenin may also regulate genes involved in phytohormone production in *H. seropedicae*.

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REFERENCES


