Altered-Function Mutations in the *Agrobacterium tumefaciens* OccR Protein and in an OccR-Regulated Promoter

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OccR is a LysR-type transcriptional activator that controls the occQ and *traR* promoters of octopine-type Ti plasmids. The opine octopine converts OccR from a repressor to an activator of occQ, shortens the protein’s DNase I footprint, and decreases the angle of an OccR-caused DNA bend at the occQ promoter. In this study we first localized the cis-acting DNA sequences required for regulated expression of occQ. To understand better the mechanism of activation of OccR, we isolated mutations both in the *occQ* promoter and in the *occR* gene which function differently from the wild type. An *occQ* promoter mutation that changes the putative −35 region of *occQ* from TTGACC to TTGACA increases the basal expression of *occQ* about 15-fold. Three mutations in *occR* were also identified, one of which activates *occQ* at fully constitutive levels in both the absence and presence of octopine. This mutation (E23G) is located in the first helix of a putative helix-turn-helix DNA-binding motif. The other two *occR* mutations cause the protein to detect much lower concentrations of octopine than wild-type OccR protein does. These mutations (F113L and G148D) are located in a region of the protein that is predicted to contain the ligand-binding site.

*Agrobacterium tumefaciens* is a widespread plant pathogen that causes crown gall tumors by conjuring transferring a DNA element (T-DNA) from the 200-kb Ti plasmid to the nuclei of infected plant cells (11, 15, 25). Certain T-DNA genes direct the overproduction of phytohormones, resulting in neoplastic cell division, while other T-DNA genes direct the synthesis and secretion of derivatized amino acids and sugars called opines that are used as nutrients by the bacteria. The Ti plasmid also contains several operons of nontransfered genes that are responsible for the uptake and catabolism of opines. Octopine-type Ti plasmids direct the synthesis in plant cells of four opines: octopine, octopinic acid, lysopine, and histopine (20, 21). Catabolism of these opines as carbon and nitrogen sources is mediated by the occ operon, which encodes seven proteins, including an ATP-dependent peroxidase and three catabolic enzymes (17, 18, 22). This operon is transcriptionally induced by octopine, octopinic acid, and lysopine, and induction is mediated by the product of the *occR* gene (7).

OccR is a member of the LysR family of transcriptional regulators and has properties similar to those of other members of this family (16). Biochemical and genetic analyses of various LysR-type proteins have suggested that these proteins have similar domain organizations (9, 16). The N-terminal region contains a putative helix-turn-helix (HTH) DNA-binding motif that has been suggested to be a DNA-binding domain (1). Most of the amino acid substitutions reported to affect DNA binding of LysR family members are clustered in or near the putative HTH, while a few others cluster in the C terminus (2, 16). The C-terminal region is also suggested to be involved in DNA binding and transcription activation. The central portion of LysR-type proteins is proposed to be involved in the recognition of the inducer and/or in the response to the inducer that results in transcription activation.

In vitro band shift, footprint, and transcription experiments with purified OccR showed that the protein binds with high affinity to a single site overlapping the *occR* and *occQ* promoters, repressing the *occR* gene and either repressing or activating the divergent *occQ* operon, depending on octopine availability (24). Octopine also shortens the DNase I footprint of OccR and increases the gel mobility of OccR-DNA complexes by relaxing an OccR-caused DNA bend. Complexes lacking octopine have a 62 bp bend, while complexes containing octopine have a 46 bp bend. It was proposed that octopine-induced alterations in DNase I protection patterns and DNA bending might be linked to regulation of transcription activation. It was hypothesized that the longer DNA bend protection pattern and high-angle DNA bend were consequences of an OccR conformation that repressed *occQ* transcription, while the short footprint and low-angle bend resulted from an OccR conformation that promoted transcription. We are currently trying to understand how these ligand-induced alterations in OccR-DNA interactions are linked to regulation of the target promoter.

The minimal segment of DNA required for *occQ* function. Although an OccR operator site was previously determined, the possibility of a second regulatory site somewhat removed from the *occQ* promoter was not excluded. By comparing β-galactosidase expression from three different *occQ-lacZ* fusions, we have now defined more precisely the minimal DNA segment required for the induction of *occQ*. One of our goals in these experiments was to identify any OccR-binding sites that are well removed from the *occQ* promoter, as are found with the *lac* and *gal* operons (12, 14). pLH505 (7) carries Ti plasmid DNA extending from approximately 1,200 bp downstream of the *occQ* transcription start site (24) to 930 bp upstream (Fig. 1). pKY119 is a similar plasmid containing 930 bp upstream of the transcription start site and 100 bp downstream (Fig. 1). This plasmid was constructed by digesting a 1,134-bp polymerase chain reaction (PCR) product containing *occR* (described below) with BamHI and EcoRI to liberate a 1,030-bp fragment that was cloned into BamHI-EcoRI-digested pMC1403 (5). pKY118 is similar to pKY119 and pLH505 except that it contains only 111 bp upstream and 100 bp downstream of the *occQ* start site (Fig. 1). This plasmid contains a 211-bp PCR fragment synthesized with the oligonucleotides 5'-GCAGAGATCCGGGCGAAGCCCATGAGT TGA-3' and 5'-GCAGAAATCCGGGGAACGGTCGCACCTG

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CCT-3′ and digested with BamHI and EcoRI. Plasmid pLH506 or pKY125 (described below) was provided to supply functional OctR protein.

The basal and induced levels of β-galactosidase expression of the strain carrying pLH505 were about half those of the strain carrying pKY119 (Table 1). We believe that the differences in β-galactosidase expression can be partly or fully explained by the fact that in pLH505, the lacZ reporter is fused to the second gene of the operon (occM), while in pKY118 and pKY119 lacZ is fused to the first gene (occQ). β-Galactosidase expression in the strain carrying pKY118 was almost identical to that in the strain carrying pKY119 under all conditions tested, and the induction ratios of all three plasmids were nearly identical. This indicates that all critically important regulatory elements are present in pKY118. We conclude therefore that the DNA sequences spanning nucleotides +100 to −111 on either side of occQ are sufficient for fully regulated expression and that there are no crucial cis-acting operator sites lying outside of this region. Virtually all of this 211-bp interval was previously assayed for OccR-binding sites by DNase I protection experiments (24). In the absence of octopine, a single binding site was identified lying from −21 to −80 bp (Fig. 2A), while in the presence of octopine, the sequences from −28 to −80 were protected (24). The removal of even 10 bp from the upstream end of the occ operator prevents OctR binding and would almost certainly abolish regulated occQ expression (24a).

![Diagram of plasmids](https://example.com/plasmid_diagram.png)

FIG. 1. Physical maps of plasmids described in this article. Plasmids pLH505 and pKY108 are derivatives of pMC1403 containing occM-lacZ fusions and a truncated (nonfunctional) occR gene (5). pKY118, pKY119, and pKY120 are similar but contain occQ-lacZ fusions. pKY108 and pKY120 contain a point mutation in the −35 region of the occQ promoter. The remaining five plasmids are derivatives of pSW208 carrying functional copies of occR. pSW208 is a derivative of PACYC184 which contains that plasmid's cat and rep genes, as well as the lacZa and multiple cloning site of pTZ18R (7). pLH506 contains wild-type occR (7), while pKY109 contains occRG148D, pKY110 contains occRF113L, and pKY129 contains occRE23G.

### Table 1. Induction of different occ-lacZ fusions by octopine

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>β-Galactosidase activity†</th>
<th>Induction ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without octopine</td>
<td>With octopine</td>
</tr>
<tr>
<td>pLH505, pLH506</td>
<td>6.7</td>
<td>56.6</td>
</tr>
<tr>
<td>pLH505, pSW208</td>
<td>9.8</td>
<td>9.3</td>
</tr>
<tr>
<td>pKY119, pKY125</td>
<td>11.2</td>
<td>110.2</td>
</tr>
<tr>
<td>pKY119, pSW208</td>
<td>17.5</td>
<td>17.4</td>
</tr>
<tr>
<td>pKY118, pKY125</td>
<td>12.7</td>
<td>108.4</td>
</tr>
<tr>
<td>pKY118, pSW208</td>
<td>18.1</td>
<td>17.6</td>
</tr>
</tbody>
</table>

†β-Galactosidase activities were measured for derivatives of E. coli MC4100 containing the indicated plasmid pairs. Strains were cultured for 2 h in the absence or presence (400 μM) of octopine.

‡In Miller units (13).

An occQ promoter mutation showing elevated expression.

We devised a genetic selection for altered-function mutations either in occR or in the occQ promoter on the basis of the observation that the Δlac Escherichia coli strain MC4100 (pLH505) (pLH506) requires inducing levels of octopine to form colonies on defined medium containing lactose as a sole carbon source (data not shown). This is presumably because the occQ-lacZ fusion of pLH506 can synthesize sufficient levels of β-galactosidase only if the occQ promoter is induced. To obtain constitutive mutants, we mutagenized this strain with germicidal UV radiation (UVGL-25; UVP, Inc., San Gabriel, Calif.) (4) for an interval of 1 to 30 min and allowed it to grow to stationary phase in minimal lactose broth without octopine. Turbid cultures were subcultured four times in the same medium and plated on AB agar plates (3) containing glucose, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), chloramphenicol, and carbenicillin to screen for mutants that form blue colonies.

Three mutant strains (possibly siblings) that formed dark blue colonies on this medium were obtained. To determine whether the mutations were plasmid borne and, if so, on which plasmid they occurred, plasmid DNA was isolated from the mutants, reintroduced into unmutagenized E. coli MC4100 (pLH505) or MC4100 (pLH506), and plated on minimal plates containing X-Gal. All three mutations were localized on pLH505, which contains the occQ-lacZ fusion, suggesting that the mutations might be in the occQ promoter-operator region. The DNA sequences of the occQ promoter-operator regions of these mutant plasmids were determined. All three had the same point mutation in the −35 region of the occQ promoter which changed a cytosine residue to adenosine. This alteration changes the −35 sequence from TTGACC to TTGACΔ, which is the consensus −35 motif of E. coli vegetative promoters (Fig. 2A) (8), thus providing direct evidence that this sequence is indeed a functional part of the occQ promoter. A representative plasmid containing this mutation was designated pKY108 (Fig. 1).

To confirm that constitutive expression of β-galactosidase was caused by pKY108, this promoter was cloned by PCR amplification with the primers used to construct pKY118 (see above). The resulting 229-bp PCR product was cloned into
pMC1403 to construct pKY120. pKY120 was confirmed by DNA sequencing to be identical to pKY118, except for the single point mutation. Strain MC4100(pKY120) showed 16 times greater  β-galactosidase activity than MC4100(pKY118) and was not octopine responsive (Fig. 3). When OccR was supplied in trans by introducing pKY125 into these strains, β-galactosidase expression became slightly repressed (Fig. 3), in agreement with earlier observations that OccR can repress as well as activate transcription (24). In the presence of octopine, OccR slightly induced the mutant promoter (Fig. 3).

This mutation simultaneously disrupts a dyad symmetry (from CGGTCA/N2TGACCG to C/TATCA/N2TGACCG) at the left end of the OccR footprint (6, 23). It seemed possible that this mutation might in some way interfere with the ability of OccR to repress or to activate occQ transcription. However, the observations that the mutant occQ promoter is active in the absence of OccR, still repressed by OccR in the absence of octopine, and induced by OccR in the presence of octopine indicate that the elevated basal expression level was caused not by disruption of the left dyad symmetry but rather by the improved −35 consensus sequence. These data also suggest that at least this base in the dyad symmetry is not required for normal contacts between OccR and the operator. We conclude that this mutation increases the ability of RNA polymerase to bind to and to initiate transcription at the occQ promoter and has little effect on the ability of OccR protein to repress or activate transcription.

Constitutive and sensitized OccR mutations. Since the genetic selection described above did not yield mutants in occR, we devised a method to screen for mutants on X-Gal plates. We first subcloned this gene by PCR amplification (10), with the oligonucleotides 5′-GCAGGATCGGCGGCGAAGC CCAATGAGTGA-3′ and 5′-GCACCCGTTGCTAATC CGCACAC-3′ as primers and pH506 as the DNA template. This 1,134-bp DNA fragment was digested with BamHI and Smal and ligated with BamHI-Smal-digested pSW208. The resulting plasmid, pKY125 (Fig. 1), was sequenced to confirm that it had the wild-type nucleotide sequence.

Mutagenesis of the occR structural gene was carried out by introducing plasmid pKY125 or pH506 into the E. coli mutator strain ES1578 (mutD5) (26) and cultivating the resulting strains for 40 generations. Plasmids were isolated, transformed into E. coli MC4100(pKY118), which contains an occQ-lacZ fusion, and plated on AB agar plates without octopine and containing glucose (0.5%), X-Gal (20 µg/ml), chloramphenicol (100 µg/ml), and carbenicillin (100 µg/ml). Mutants exhibiting elevated occQ expression were expected to form blue colonies on these plates, while wild-type colonies would remain white or light blue. Ten strains that formed dark blue colonies were isolated after approximately 105 colonies were screened. To identify the replicon containing the mutation, plasmid DNA was isolated from each strain and used to transform E. coli MC4100(pKY118) to Cam' on AB agar plates containing X-Gal. All of the resulting transformants formed dark blue colonies, indicating that the occR-containing plasmid was responsible for the elevated  β-galactosidase expression.

The DNA sequences of these occR alleles were determined by subcloning the mutant alleles as BamHI-KpnI fragments into the phagemid pTZ19R (U.S. Biochemicals, Cleveland, Ohio), purifying these plasmids in single-stranded form, and sequencing them with Sequenase version 2.0 (U.S. Biochemicals). Two plasmids had a mutation that alters codon 23 from GAA to GGA, resulting in a change from glutamate to glycine (E23G). Five plasmids had an occR point mutation that alters codon 113 from TTC to CTC, resulting in a change from phenylalanine to leucine (F113L) (Fig. 2B). Finally, three
plasmids had a mutation that alters codon 148 from GCC to GAC, resulting in a change from glycine to aspartate (G148D).

The β-galactosidase activities of representatives of each class were measured in AB media containing different concentrations of octopine. The occRE23G allele conferred a fully constitutive OccR phenotype, activating occQ-lacZ in the absence of octopine as strongly as in its presence (Fig. 4). This activation is also approximately as strong as that of the wild-type protein in the presence of high levels of octopine. In contrast, occRF113L and occRG148D strains showed only slightly elevated β-galactosidase activity in the absence of octopine. However, both of these mutants required far less octopine than did the wild type for full induction (Fig. 4). For example, in broth containing 3 μM octopine, the strain with the wild-type occR gene showed little if any induction of β-galactosidase, while a strain containing occRF113L or occRG148D showed almost full induction (Fig. 4). We designate this a “sensitized” phenotype.

The mutation occRE23G is located in the first α-helix of the HTH DNA-binding motif. The DNA sequence specificities of different HTH motifs are often conferred primarily by the second helix, known as the recognition helix, while the first α-helix of the HTH motif lies across the major groove to position the second helix and makes nonspecific contacts with the DNA (6). Mutations in the HTH motif of other LysR homologs have invariably resulted in a decreased protein function (16). It was somewhat surprising that this mutation would result in a “locked” active phenotype, given that glycine residues generally disrupt helical secondary structure (19).

The fact that OccRF113L and OccRG148D are sensitive to low concentrations of octopine while they are still largely octopine dependent implies that these mutations might alter some aspect of inducer recognition and response. Several studies have supported the hypothesis that residues 100 to 150 of other LysR-type regulatory proteins are also involved either in recognition of inducing ligands or in the responses to inducing ligands that lead to transcriptional activation (16). The two sensitized OccR mutants are located in this central region of OccR. These alterations might result in an increased binding affinity for octopine or might cause the protein to be more easily converted from a repressive to an active conformation.

It has been proposed that the ligand-induced alternations in the OccR DNase I footprint and in the DNA bend angle described above might be intimately linked to the conversion of OccR from a repressor to an activator. The fact that OccRE23G is functionally locked in an active state in vivo suggests that this protein may be locked into an active conformation in vitro, that is, a conformation that causes a short footprint and a low-angle DNA bend. Therefore, our model predicts that the mutant protein may have properties in the absence of octopine that are similar to those assumed by the wild-type protein in the presence of octopine. Alternatively, if the octopine-influenced bending of DNA is not obligately linked to regulation of occQ, then the mutant protein may still undergo ligand-responsive conformational changes similar to those of the wild-type protein. Testing these predictions will be the focus of future studies.

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