Most Mutant OccR Proteins That Are Defective in Positive Control Hold Operator DNA in a Locked High Angle Bend

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OccR is a LysR-type transcriptional regulator of *Agrobacterium tumefaciens* that positively regulates the octopine catabolism operon of the Ti plasmid. Positive control of the *occ* genes occurs in response to octopine, a nutrient released from crown gall tumors. OccR also functions as an autorepressor in the presence or absence of octopine. OccR binds to a site between *occQ* and *occR* in the presence or absence of octopine, although octopine triggers a conformational change that shortens the DNA footprint and relaxes a DNA bend. In order to determine the roles of this conformational change in transcriptional activation, we isolated eleven OccR mutants that are defective in activation of the *occQ* promoter but are still able to autorepress. These mutations spanned most of the length of the protein. Two additional positive control mutants were isolated using site directed mutagenesis. Twelve mutant proteins, in the presence or absence of octopine, displayed a high angle DNA bend. One mutant, L26A, showed the ligand-responsive DNA binding similar to wild type OccR, and therefore must be impaired in a subsequent step in activation.
Introduction

LysR type regulators (LTTRs) comprise the largest family of transcriptional regulators found in most proteobacteria (15, 22). Most LTTRs are involved in the regulation of metabolic functions such as amino acid synthesis or in carbon catabolism. These proteins contain a highly conserved N-terminal DNA binding domain and a less conserved C-terminal ligand recognition domain. The activities of LTTRs are generally regulated by low molecular weight ligands, but these proteins are highly unusual among transcription factors in that most are able to bind to their DNA recognition sites with similar affinity in the presence or absence of these ligands. With a few exceptions, LTTRs regulate transcription by undergoing conformational changes that have little effect on binding affinity, but that nonetheless alter their ability to recruit RNA polymerase (RNAP). Without the inducing ligand, at least some LTTRs bind to a region of DNA that spans five helical turns, with one dimer centered at or near nucleotide -62 and the other centered at or near -32 (11, 16, 27, 29, 36, 38). In this conformation, LTTRs cause a high-angle DNA bend. Upon ligand binding, the dimer centered at -32 shifts to a position centered at nucleotide -42, a position at which many transcriptional regulators bind in order to recruit RNA polymerase. This change of conformation exposes the promoter to solvent, allowing RNA polymerase recruitment, and relaxes a high-angle DNA bend. Genes encoding LTTRs are often transcribed divergently from the target promoter, and are negatively autoregulated (15, 22), and in such cases, both ligand-bound and ligand-free forms of the protein are equally able to carry out autorepression. A small number of LTTRs have been shown to bind as a dimer in the absence of ligands, centered at approximately -62, and as tetramers in their presence,
Agrobacterium tumefaciens is well known for its ability to perform horizontal gene transfer across biological kingdoms. During the colonization of higher plants, this plant pathogen transfers oncogenic DNA fragments called T-DNA into the plant cytoplasm and also injects a number of proteins that ensure the targeting of T-DNA to the plant cell nucleus and its integration into nuclear DNA (13, 23). The transferred DNA directs transformed cells to produce a set of compounds called opines (9), that can serve as bacterial nutrients. Opines cause the induction of genes encoding cognate uptake and catabolic proteins within the bacterium, and this induction requires dedicated opine detection systems. The OccR protein is encoded by some tumor-inducing (Ti) plasmids, including pTiA6, pTiR10, pTiAch5, and pTi15955, and is responsible for detecting one such opine, called octopine, which is synthesized by a reductive condensation of arginine and pyruvate (14). OccR binds DNA in the presence or absence of octopine, but binding of octopine causes the protein to activate a nearby promoter of a 14-gene operon that encodes two ABC-type permeases, four opine catabolic genes, and traR, which encodes a quorum sensing transcriptional regulator (12, 38).

Purified OccR binds upstream of the occQ promoter in the presence or absence of octopine (1, 2, 38, 40). In the absence of octopine, OccR protects a region of DNA extending from -80 to -28 nucleotides upstream of the occQ promoter, and causes a high angle DNA bend. Addition of octopine has only minor effects on the binding...
affinity, but shortens the protected region by 10 nucleotides at the downstream end
(protection extends from nucleotides -80 to -38) and partially relaxes the DNA bend.
OccR binds as a tetramer in both cases (2). Gel shift assays also showed that the
upstream 20 nucleotides is crucial for binding affinity, while the downstream 30
nucleotides have minor effect on binding affinity, but are essential in the absence of
octopine for the longer protected region and for high angle DNA bending (40).

We have previously described the isolation of a large number of OccR constitutive
mutants that are able to activate the \textit{occQ} promoter in the absence of octopine (1) and
showed that these mutant proteins are predisposed to form a low angle DNA either in
the absence of octopine or in the presence of very low octopine concentrations. Here
we asked the converse question. We isolated a large number of OccR mutants that
are unable to activate the \textit{occQ} promoter, and asked whether these mutants are locked
in an inactive conformation even when octopine is present.

\textbf{Materials and Methods}

\textbf{Strains, plasmids and reagents.} All the strains and plasmids used in this study are
listed in Table 1. Mutagenesis of a plasmid-borne \textit{occR} gene was carried out using \textit{E. coli} XL1red (Agilent Technologies). Oligonucleotides were purchased from Integrated
DNA Technologies (Coralville, IA). Antibiotics and octopine were purchased from
Sigma. Restriction enzymes and T4 DNA kinase were purchased from New England
Biolabs. PCR fragment purification kits were purchased from Qiagen.
Radionucleotides were purchased from Perkin Elmer.
Protein overexpression and purification. Crude cell lysates were prepared using *E. coli* strain BB101 containing mutant *occR* derivatives cloned into pCST301. These plasmids contain a PT7-*occR* fusion and a His6 tag at the C terminus. Cells were cultured at 37°C in 300 ml of LB containing 1 mg/ml ampicillin. When the OD$_{600}$ of the cultures reached 0.6, IPTG was added to a final concentration of 1 mM. Cells were then cultured for an additional 6 hours at 28°C. Cells were centrifuged and resuspended in 1 ml of TEDG buffer (50 mM Tris $\cdot$ HCl, pH 7.9, 0.5 mM EDTA, 1 mM DTT, 5% glycerol) plus 0.15 M NaCl, and disrupted using a French pressure cell (20,000 psi). The lysate was clarified by ultracentrifugation (150,000 × g for 10 min at 4°C). OccR proteins were purified using Talon resin (Clontech), which contains immobilized cobalt ions, according to a protocol supplied by the manufacturer.

DNA bending assays. For DNA bending assays with mutant proteins, a fragment containing the OccR binding site was PCR amplified from plasmid pLW132 (using primers GGATCCTCTAGAGCATATCATA and GATCCCTCGATTCCATGGGGTA), and end-labeled using $\gamma$-32P-dATP and T4 polynucleotide kinase (NEB). Purified OccR mutant proteins (50 nM final concentration) and 0.12 pmol of labeled DNA were combined at room temperature (RT) in a buffer containing 10 mM Tris-HCl (pH 7.9), 1 mM EDTA, 1 mM DTT, 60 mM potassium glutamate, 30 μg/ml calf thymus DNA, 20 μg/ml BSA, and 10% glycerol, and incubated at RT for 30 min. Samples were size-fractionated using 6% polyacrylamide gels in 1X TAE buffer (20 mM Tris-acetate, 1 mM EDTA, pH 8.5) at 4°C. In some cases octopine was added to the binding reaction,
β-Glucuronidase and β-Galactosidase assays. To quantify the activities of the mutant occR alleles on the occQ and occR promoters, derivatives of strain KYC1211(pSS101) containing the mutant occR derivatives of pSS102 were cultured overnight in AB minimal medium (3) at 28°C with appropriate antibiotics. Cultures were diluted 100-fold into fresh AB medium without antibiotics and containing octopine at the indicated concentrations. β-glucuronidase and β-galactosidase specific activities (24) were measured when the cell density reached an OD600 of approximately 0.5.

Results

Mutagenesis of OccR. In order to mutagenize occR, we introduced by transformation plasmid pSS102 (which expresses OccR, see Table 1) into the E. coli strain XL1-Red. Thirty transformants were isolated and in cultured independently in LB medium containing appropriate antibiotics for approximately 40 generations. At the end of this interval, plasmid DNA was isolated from each culture and introduced by electroporation into A. tumefaciens strain KYC1211(pSS101). KYC1211 carries a null mutation in occR and a Tn5gus insertion in occQ that creates an occQ-uidA fusion. Plasmid pSS101 carries an occR-lacZ fusion. Electroporated cells were cultured on solid AT medium containing 100 µg/ml spectinomycin, 100 µg/ml gentamycin, 1 µM octopine, and 40 µM X-Glu. Clones that were defective for activation of the occQ gene formed white or pale blue colonies. Approximately 1000 such clones were then streaked on
AB medium containing the same antibiotics as above and X-Gal. Since this strain contains an occR-lacZ fusion, clones containing OccR alleles still able to repress this promoter will form white colonies on this medium. Of the 1000 clones so tested, 16 appeared to be proficient in autorepression.

Derivatives of pSS102 thought to contain positive control mutations were introduced into E. coli strain DH5α and then reintroduced into KYC1211(pSS101) to ensure that the observed phenotypes were attributable to the pSS102 plasmid. Quantitative assays of β-glucuronidase and β-galactosidase expression in response to varying octopine concentrations were performed using these reconstructed strains (Tables 2 and 3).

Eleven clones having a PC phenotype and isolated from independent pools of mutagenized plasmids were retained for further study (Fig. 1). All mutants were strongly defective in expression of the occQ promoter, though the mutant A141V showed a residual induction (Table 2). All mutants also repressed the occR-lacZ fusion, indicating that they must retain the ability to fold correctly, resist proteolysis, and to bind to operator DNA (Table 3).

DNA bending by OccR positive control mutants. Plasmid pCST301 is a derivative of pET22b that contains a PT7-occR-His6 fusion. Fragments of the occR gene of this plasmid were replaced using the corresponding occR fragments containing the mutations described above, and the resulting mutant proteins were overexpressed and purified using IMAC as described above. Purified OccR proteins were used in DNA bending assays as described previously (1). The OccR binding site was previously
placed into plasmid pBend3 (42), which is designed to measure DNA bending, creating pLW132. We used PCR amplification to construct a 334 nucleotide fragment with the OccR binding site at the center of the fragment (virtually identical to a fragment excised using BamHI, see ref (1)). OccR-DNA complexes with a high bend angle migrate more slowly on electrophoretic gels than similar complexes with low bend angles. In previous studies, slow electrophoretic migration of complexes containing this fragment has been invariably associated with a 52 nucleotide DNase I footprint while rapid gel migration was invariably associated with a 42 nucleotide footprint (1, 2, 38-40).

We hypothesized that OccR mutants with defects in octopine detection or octopine-dependent conformational changes would have a high-angle DNA bend even in the presence of octopine, while PC mutants with defects in RNA polymerase contacts or some other step in initiation should behave like wild type OccR in these bending assays, that is, they were predicted to show a high angle bend in the absence of octopine and a low angle bend in its presence. The DNA fragment described above was combined with wild type or mutant OccR proteins, and resulting complexes were size-fractionated using 6% native polyacrylamide gels. In the absence of octopine, complexes containing any of the mutant proteins migrated at the same rate as complexes containing wild type OccR (Fig. 2). In the presence of octopine, complexes containing wild type OccR migrated considerably more rapidly than in its absence (compare wild type complexes to the molecular weight standards). This indicates that octopine caused OccR to undergo a conformational change that resulted in a low bend angle, as previously reported (40). In contrast, the PC mutants showed little or no
change in gel mobility in response to octopine. Mutants A90V, R140G, A141V, and G196S appear to show some residual relaxation of the bend angle, but far less than wild type OccR. Of these four mutants, R140G, A141V, and G196S showed residual induction of the occQ promoter in vivo (Table 2), which may help to explain their residual relaxation of the DNA bend. In contrast, mutant A90V showed no induction of the occQ promoter in vivo. Also of interest is mutant G91E, which showed residual induction (Table 2) but which formed fully locked complexes (Fig. 2). The reasons for this are not clear.

We performed a control experiment to ensure that the mobility shifts that we had detected were due to changes in DNA bending rather than differences in the number of bound protein subunits. Gel retardation assays were repeated using DNA fragments with the binding site near one end of the fragment rather than at the middle of the fragment. Complexes made using these DNA fragments all migrated at the same (more rapid) rate in the presence or absence of octopine (data not shown). We conclude that these PC mutations altered the conformation of bound protein rather than the number of bound OccR monomers.

**Site-Directed PC mutants.** Previous studies of two other LysR-type proteins (CysB and GcvA, both of *E. coli*) have identified PC mutants in a predicted turn between helices α2 and α3 (17, 19). We performed site directed mutagenesis to individually alter six residues between amino acids 23 and 29, which are predicted to lie within or near the corresponding turn of OccR (Fig. 3). Each mutant was tested for defects in
occQ activation and occR autorepression (Table 4). Of these seven mutants, L24A and L26A showed defects in the former but were proficient in the latter, therefore showing defects in positive control.

The mutations that cause the L24A and L26A mutations were introduced into pCST301, creating PT7-occR-His6 fusions. The two mutant proteins were purified and used for mobility shifts in the presence and absence of octopine. In the absence of octopine, both resembled wild type OccR (Fig. 4). In the presence of 10 µM octopine, complexes containing the L24A mutant migrated considerably more slowly than wild type, and resembled the migration rate of complexes lacking octopine (Fig. 4). In contrast, complexes containing the L26A mutation migrated only slightly more slowly than complexes containing wild type OccR (Fig. 4). This indicates that this mutant is still able to undergo octopine-mediated changes in conformation. Its defect in activation must therefore have other causes, and is quite likely due to an inability to interact with RNA polymerase.

Discussion

The present study focuses on the isolation of positive control mutations of OccR, and complements an earlier study that focused on constitutively active (octopine-independent) OccR alleles (1). It is striking that both classes of mutations span virtually the entire length of the protein (Fig. 5). A few PC mutations were even found adjacent to the sites of constitutive mutations. In the former study, many of the strongest constitutive alleles contained two point mutations that were strongly
synergistic (1). In contrast, all the mutants described here contained just one amino acid substitution. We conclude that mutations at many sites can perturb the delicate balance between the two OccR conformations. In neither study did we isolate a particular mutation more than once, suggesting that many more alleles of each phenotype remain unidentified.

Several members of this family have been studied at the structural level. Full length structures are available for CbnR of *Ralstonia eutropha* (26), TsaR of *Comamonas testosterone* (25), PA0218 of *Pseudomonas aeruginosa* (unpublished), BenM and CatM of *Acinetobacter* sp. (8, 10), DntR of *Burkholderia* sp. (35), ArgP of *Mycobacterium tuberculosis* (41), and CrgA of *Neisseria meningitidis* (32). Of these, CbnR, TsaR, and PA0218 have been solved in tetrameric forms, though only in the absence of ligand (25, 26). TsaR has been solved as a dimer in the presence and absence of the inducing ligand (25). Fragments of several other LTTRs have been reported, including the ligand-recognition domains of CysB and OxyR of *E. coli* (7, 37), RovM of *Yersinia enterocolitica* (30) and CynR of *E. coli* (unpublished).

The structures of full-length proteins show an N-terminal DNA binding domain (approximately 60 residues in length) composed of three helices followed by two beta strands. This domain is joined to the less conserved C-terminal ligand recognition domain by a long (30 residue) helix, called the linker helix. The two linker helices of a protein dimer are adjacent to each other in an antiparallel conformation, and form a coiled-coil that is the major dimerization determinant.
The ligand recognition domains have a structural similarity to periplasmic substrate binding proteins of various ABC-type uptake systems (31). They are composed of two subdomains, designated RD I and RD II, which have been proposed to contain a pair of jaw-like structures that are flexible in the absence of ligand and are immobilized by ligand. RD I is composed of two non-contiguous amino acid sequences, residues 91-161 and 265-294 of CbnR, while domain II is composed of one contiguous sequence composed of residues 164-259 (Fig. 5). The cavity formed between domains I and II is the likely ligand binding site (7, 25, 37). OccR might therefore bind octopine at a similar site.

We used the crystal structures described above, especially that of CbnR, to model the mutations isolated in the current study (Fig. 5). Of the 13 positive control mutations, nine are found at predicted turns between alpha helices or beta strands. Four of the 13 mutations are predicted to lie within the DNA binding domain. Mutation R10Q lies in helix α1, and is predicted to contact the linker helix. This mutation might therefore impact the transmission of conformational changes between the domains. Mutations L24A and L26A lie at or near a turn between helices α2 and α3, a region that has been implicated in positive control in CysB and GvcA (17, 19). Mutant R51H lies between strands β1 and β2, and is predicted to be highly exposed to solvent.

The remaining nine mutations lie within the ligand recognition domain. Of these, seven mutations lie in turns rather than helixes or strands. Four mutations, M98T, P107L,
G196S, and D238N, lie near the probable octopine binding site and might therefore affect ligand recognition. Two mutations, A90V and G91E, lie at the extreme N-terminus of the ligand recognition domain and could be impaired in transmission of the signal between domains. The remaining two mutants are R140G and A141V. Because the two subunits of the CbnR dimer show a pronounced asymmetry (26), these residues are predicted to lie in different environments on the two subunits. In one subunit, these residues are predicted to contact the linker helix, and the mutations might therefore affect the transfer of information between the ligand recognition domain and the DNA binding domain. In the other subunit of the dimer, these residues face toward a large channel within the complex.

We had predicted that two classes of positive control mutants would be found, one class unable to bind octopine or unable to transmit octopine-induced conformational changes, and the other unable to make productive contacts with RNA polymerase. The two classes were to be distinguished by their DNA bending phenotype, as the first class should be locked in a conformation that causes a high-angle DNA bend, while the second class should be unimpaired in octopine-induced relaxation of the bend. However, we did not anticipate that virtually all of the mutants we isolated would be of the first class. Of the eleven mutants obtained in the initial screen, all had defects in ligand recognition. Even when we targeted a suspected activation region, only one of the two mutants was octopine responsive in DNA bending assays. These findings underscore the importance of ligand-responsive changes in DNA bending and the large fraction of the protein involved in this process.
Positive control mutants have been isolated in three other LysR-type proteins. The F31L mutation of GcvA was impaired for activation of a target promoter but still proficient in autorepression (17). NahR mutants defective in activation but proficient in DNA binding ability were found to be distributed across the full length of the protein (33). Only one allele (P35S) was found in the DNA binding domain, and it was only partially competent for DNA binding. All the other mutants were in the ligand recognition domain, and may have affected the ability to detect the naphthalene.

Positive control mutants of CysB were described in two studies. In one report, five mutations (Y27G, M160I, T196I, A244V and A247E) were defective for positive control. Of these, only mutant Y27G was competent for ligand recognition (as judged by DNA bending assays). A subsequent study focused on the region near residue Y27. All mutations tested (L26A, Y27A, Y27G, T28A, S29A, and Q30A) were impaired in activating a target promoter. Of these L26A was also defective in DNA binding. The other five mutants were proficient in DNA binding and ligand responsive DNA bending, but were unable to recruit RNA polymerase (20). Several mutants in the “determinant” of the RNA polymerase alpha subunit also showed defects in activation (20).

The overall structures of the CbnR, TsaR, and PA0218 tetramers may provide important clues about the functions of this family of proteins. For convenience, we have labeled the four subunits of the CbnR tetramer A, B, C, and D, and have labeled the DNA
binding domains A’-D’ and the ligand recognition domains A”-D”. The DNA binding
domains A’ and B’ form a dimer, as do domains C’ and D’. Surprisingly, domains A”
dimerizes not with B”, but rather with D”. Likewise, domain B” dimerizes with C” (see
Fig. 6A). Structural studies strongly suggest that the DNA binding domains and ligand
recognition domains can both dimerize only when four subunits form a tetramer.

The structures of CbnR, TsaR, and PA0218 were obtained in the absence of inducing
ligands and are presumed to represent or resemble the inactive forms of these proteins
(26). Fig. 6B shows the N-terminal DNA binding domains of CbnR in green, except for
the probable DNA recognition helices, which are in yellow. The four DNA recognition
helices are all parallel or antiparallel, such that the DNA could bind all four
simultaneously. Binding to all four helices would cause a significant DNA bend, as has
been observed for several family members. The first two DNA recognition helices
could bind consecutive major grooves of the DNA, as could the last two helices.
However, the second and third helices are separated by approximately two helical turns
of DNA. This is exactly the conformation we had predicted for OccR-DNA complexes
in the absence of octopine (1, 2, 39, 40).

We have predicted that octopine would reduce the space between the second and third
helices so that the four DNA recognition helices can bind four consecutive major
grooves. The structure of CbnR provides a possible mechanism for this
conformational change. In the structure of CbnR, DNA binding domains C’ and D’ are
forced apart from A’ and B’ by the four ligand recognition domains (A””, B””, C”” and D””).
which form a C-shape (Fig. 6C and 6D). Interestingly, this C-shape is not conserved in TsaR or in PA0218 (Fig. S1). This complex of ligand recognition domains therefore forces DNA recognition helices C' and A' apart. These helices can move closer to each other only if the steric hindrance caused by the ligand recognition domains is removed (arrows in Fig. 6E). TsaR and PA0218 resemble CbnR in this respect, in that the central two DNA binding helices are 50 Å or 80 Å apart, respectively, and are prevented from drawing closer by steric hindrance from the four ligand recognition domains (Fig. S1). It would be extremely interesting to crystallize an LTTR in the presence of ligand and/or operator DNA.

Acknowledgments

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References


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<th>Relevant genotype</th>
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<td>BL21/DE3 with a slyD deletion</td>
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<td>XL1-Red</td>
<td>endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutT Tn10 (Tetr)a</td>
<td>Agilent Technologies</td>
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<td>CST101 A. tumefaciens KYC1211 (pSS101)(pSS102)</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>KYC1211</td>
<td>A. tumefaciens R10 (occQ::Tn5-gusA7)(occR::pKY135) KmR, SmR</td>
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<td>pBBR1MCS5</td>
<td>Broad host range cloning vector</td>
<td>(18)</td>
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<td>pBEND3</td>
<td>Derivative of pBluescript for assays of DNA bending, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
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* Only vectors, host strains, and plasmids containing wild type occR genes are shown.

Construction of plasmid derivatives containing mutant occR genes is described in detail in the Materials and Methods.
Table 2. Activation of the occQ promoter by OccR positive control mutants. 

<table>
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<th>OccR Allele</th>
<th>occQ-gus Expression at Indicated Octopine Concentrations</th>
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<td></td>
<td>0 μM</td>
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<tr>
<td>no OccR</td>
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<td>w.t. OccR</td>
<td>2.6</td>
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<tr>
<td>R10Q</td>
<td>2.1</td>
</tr>
<tr>
<td>R51H</td>
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<td>A90V</td>
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<tr>
<td>M98T</td>
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<tr>
<td>P107L</td>
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<tr>
<td>E134K</td>
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</tr>
<tr>
<td>A141V</td>
<td>2.8</td>
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<tr>
<td>G196S</td>
<td>2.4</td>
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<td>D238N</td>
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β-glucuronidase activities were measured for A. tumefaciens strain CST101 containing derivatives of pSS102 containing the indicated occR mutations. Strains were cultured until mid-log phase in the absence or presence of the indicated concentrations of octopine and assayed for β-glucuronidase specific activity (6).
Table 3. Repression of the occR promoter by OccR positive control mutants.\textsuperscript{a}

<table>
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<tr>
<th>OccR Allele</th>
<th>no Octopine</th>
<th>Fold Repression</th>
<th>1 μM Octopine</th>
<th>Fold Repression</th>
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<td>(1)</td>
<td>134</td>
<td>(1)</td>
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<tr>
<td>WT OccR</td>
<td>15</td>
<td>9</td>
<td>9</td>
<td>14</td>
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<td>R10Q</td>
<td>16</td>
<td>9</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>R51H</td>
<td>17</td>
<td>8</td>
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<td>8</td>
<td>19</td>
<td>7</td>
</tr>
<tr>
<td>M98T</td>
<td>8</td>
<td>17</td>
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<tr>
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<td>3</td>
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\textsuperscript{a} β-galactosidase activities were measured for \textit{A. tumefaciens} strain CT101 containing derivatives of pSS102 containing the indicated occR mutations. Strains were cultured until mid-log phase in the presence of the indicated concentrations of octopine and assayed for β-galactosidase specific activity (24).
Table 4. Phenotypes of site-directed OccR mutants

<table>
<thead>
<tr>
<th></th>
<th>PoccQ-uidA</th>
<th></th>
<th>PoccR-lacZ</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>No octopine</td>
<td>1 µM octopine</td>
<td>No octopine</td>
<td>1 µM Octopine</td>
</tr>
<tr>
<td>None</td>
<td>1.3</td>
<td>1.9</td>
<td>(1)</td>
<td>160</td>
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<tr>
<td>W.T.</td>
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<td>128</td>
<td>67</td>
<td>18</td>
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<tr>
<td>E23Q</td>
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<td>96</td>
<td>19</td>
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<tr>
<td>L24A</td>
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<td>7.5</td>
<td>4</td>
<td>21</td>
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<tr>
<td>L26A</td>
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<td>29</td>
<td>15</td>
<td>19</td>
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<tr>
<td>V27A</td>
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<td>14</td>
<td>69</td>
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<tr>
<td>T28A</td>
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<tr>
<td>T28Y</td>
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<td>1.8</td>
<td>0.95</td>
<td>81</td>
</tr>
<tr>
<td>Q29R</td>
<td>1.9</td>
<td>2.1</td>
<td>1.1</td>
<td>133</td>
</tr>
</tbody>
</table>
Figure Legends

**Fig. 1.** Positive control mutations of OccR described in this study. The helix-turn-helix DNA binding motif is shown in black. The ligand-recognition domain is composed to two halves. Of these, ligand-recognition domain II (LR II) is composed of one contiguous amino acid sequence, while ligand-recognition domain I (LR I) is composed to two non-contiguous sequences.

**Fig. 2.** DNA bending assays of positive control mutants in the absence or presence of octopine (10 μM). In this assay, OccR-DNA fragments are size-fractionated by electrophoresis. The DNA fragments are all identical and have an OccR-induced bend located near the center of the molecule, such that bending greatly retards the migration rate of the complex. Therefore, slow-migrating complexes are inferred to have a high angle DNA bend, while faster-migrating complexes have a low angle DNA bend.

**Fig. 3.** Site directed mutagenesis OccR, at the predicted turn between helix α2 and α3. Residues of CysB and GcvA required for positive control are underlined and in bold face.

**Fig. 4.** DNA bending assays of positive control mutants obtained by site-directed mutagenesis, in the absence and presence of octopine (10 μM). See Fig. 2 legend for details.

**Fig. 5.** Locations of all positive control mutants described in this study (A) modeled
using the CbnR structure. Constitutive mutants described in a previous study (1) are shown for comparison in part B.

Fig. 6. A. Possible OccR quaternary structure, based upon the known quaternary structures of CbnR (26), TsaR (25), and PA0218 of *P. aeruginosa* (unpublished). Dimerization of DNA binding domains (A'-D', in green) and ligand-recognition domains (A"-D" in light cyan, medium cyan, dark cyan, and grey). Note that no two subunits dimerize along their entire lengths. B-E. X-ray crystal structure of CbnR. The DNA binding regions are shown in green, except for the DNA recognition helices, which are shown in yellow. The ligand recognition domains are shown in three shades of cyan and in dark grey. DNA is modeled in orange. C. CbnR tetramer oriented with DNA binding domains on the right. D. CbnR tetramer showing residues 89-292, revealing the C-shape made by the four ligand recognition domains. E. CbnR tetramer oriented with the DNA binding domains closest to the viewer. The four ligand recognition domains prevent DNA recognition helices D' and C' from approaching A' and B'. Activation of the target promoter requires that these four helices bind four consecutive major grooves of the DNA.
DNA Recognition   Linker                        Ligand Recognition Domain
OccR 1 MN-----LRQVEAFRAVMLTG-QMTAAAELMLVTQPAISRLIKDFEQATKLQLFERRGNHI-IPTQE 60

α1 α2 α3 β1 β2

CyeB 1 MK-----LQQLRYIVEVVNVHSSTABGLYTQPQGSKGVRMLEDIGIQIFSRSQKHLIGMVTPA 62
GcVA 1 MSKRLPPNALRVFAARHL-SFTRAEEELPITQAAVSHQIKSLEDGLKLFLRRNRLS-LLTEE 64
CbnR 1 MB-----FRQLKYFIAVAEAG-NMAAKRLHVQPPTITROMQALEADLGTVSIMHRSOG-IETTA 60
A (positive control) B (constitutive)