The MarR family transcriptional regulator CouR, from the soil bacterium *Rhodopseudomonas palustris* CGA009, has recently been shown to negatively regulate a *p*-coumarate catabolic operon. Unlike most characterized MarR repressors that respond to small metabolites at concentrations in the millimolar range, repression by CouR is alleviated by the 800-Da ligand *p*-coumaroyl–CoA with high affinity and specificity. Here we report the crystal structures of ligand-free CouR as well as the complex with *p*-coumaroyl–CoA, each to 2.1-Å resolution, and the 2.85-Å resolution cocrystal structure of CouR bound to an oligonucleotide bearing the cognate DNA operator sequence. In combination with binding experiments that uncover specific residues important for ligand and DNA recognition, these structures provide glimpses of a MarR family repressor in all possible states, providing an understanding of the molecular basis of DNA binding and the conformational alterations that accompany ligand-induced dissociation for activation of the operon.

Proteins of the multiple antibiotic resistance regulator (MarR) family constitute a large group of transcription factors that are widespread in bacteria and archaea. These transcription factors control gene expression to regulate such diverse processes as antibiotic resistance, stress response, aromatic carbon source catabolism, and virulence. An analysis of 19 major families of bacterial transcription factors recently implicated the MarR family as a significant contributor, comprising roughly 8% of the total proteins analyzed. With nearly 130,000 deposited bacterial genomes to date, many MarR family members have been and will be annotated as such; however, less than 1% of these sequences have been fully characterized with respect to physiological function. The founding member of this family was first identified in multidrug resistant *Escherichia coli*, where the MarR protein regulates an operon encoding the AcrAB-ToLC multidrug efflux system, in response to a wide range of antibiotics and phenolic compounds (1–7).

Most MarR homologs act as gene repressors, and a typical organization of the locus orient the mar gene distally from the genes that are under transcriptional control. The intergenic region, typically at sites that overlap the −10 and −35 promoter elements, contains a 16- to 20-bp palindromic DNA sequence that harbors the binding site for the transcriptional regulator. Binding of a MarR homodimer to this palindromic region represses expression of both the gene that is regulated as well as the repressor itself. Dissociation of the repressor from DNA and activation of gene expression occurs when binding of a small-molecule ligand alters the conformation of the MarR homodimer. As a result of this autoregulated expression, the physiological concentrations of the repressor itself change very slightly, allowing for an exceptionally sensitive response to ligand concentrations.

Structural studies of MarR homologs reveal two sets of N- and C-terminal α-helices that facilitate dimerization and two winged helix–turn–helix motifs (wHTH) that bind to the palindromic DNA duplex via spacing that is established by dimer formation. Each monomer binds to one half-site of the palindromic DNA inverted repeat sequence, and residues along the dimerization interface help to establish the spacing between the two half-sites. Disruption of dimer formation results in a loss in DNA binding affinity and a loss of the corresponding drug resistance phenotype. A clade of MarR members responds to oxidative stress through the oxidation of Cys residues, which results in change to a conformation that is incompatible with DNA binding. Biochemical and biophysical studies of MarR family members are limited by the low binding affinity for cognate ligands, which often results in confounding data. For example, several small-molecule ligands can bind to MarR factors at multiple sites, and the physiological relevance of these interactions is unclear (8, 9).

A recent analysis of the pathway for the catabolism of the aromatic compound *p*-coumarate by the alphaproteobacterium *Rhodopseudomonas palustris* CGA009 revealed that genes encoding the enzymes for this pathway are regulated by a MarR-like transcriptional repressor. This soil bacterium uti-
lizes plant-derived phenylpropanoids, including p-coumarate, as carbon sources by first converting them to acyl/aryl-CoA and subsequently to p-hydroxybenzoates, which are degraded aerobically by an oxidative meta-ring cleavage pathway or anaerobically by a reductive aromatic ring degradation pathway (10, 11). In *R. palustris*, the transcription of an operon that encodes for enzymes involved in the catabolism of p-coumarate to p-hydroxybenzoate is under the control of a MarR family regulator named CouR. Recombinant CouR was shown to bind to an inverted repeated sequence in the −10 region of the promoter, and DNA binding was disrupted by addition of low micromolar concentrations of p-coumaroyl–CoA (pCC) (Fig. 1, B and C) (1, 10, 12).

An orthologous pathway for aromatic acid catabolism has been identified in *Rhodococcus jostii* RHA1, which also utilizes a similar strategy of CoA-thioesterification, followed by β-oxidative deacetylation to generate hydroxybenzoates. However, the constituent enzymes encoded by this operon are different from those of *R. palustris*. For example, the *R. jostii* RHA1 cluster encodes an aryl-CoA dehydrogenase and an aryl-CoA ligase (CouL) that can utilize dihydroferulate as a substrate, suggesting that the pathway functions on dihydro-p-hydroxycinnamic acids rather than their unsaturated p-hydroxycinnamate counterparts. Last, although the MarR family repressor that regulates the *R. jostii* RHA1 operon has been named CouR, the sequence identity of this polypeptide to *R. palustris* CouR is only 36%, and the DNA-binding site is divergent in sequence and size, as it contains a 5-nucleotide (nt) spacer between the two half-sites rather than the 3-nt spacer in the *R. palustris* promoter (13).

The *R. palustris* CouR represents a rare example of a MarR family regulator for which a physiologically relevant ligand has been verified, and the cognate ligand is shown to bind to the receptor with low micromolar affinity. Competition studies indicate that only pCC (and not p-coumarate, benzoyl-CoA, or acetyl-CoA) can induce dissociation of CouR from its promoter DNA. To elucidate the mechanism for recognition of the target promoter and how binding of a large ligand affects protein–DNA interactions to relieve transcriptional repression, we determined the cocrystal structures of CouR in complex with a 23-nt duplex inverted repeat corresponding to the physiological DNA binding site (to 2.85-Å resolution, Fig. 2A) and the corresponding structure of the repressor in the inactive state, bound to pCC (to 2.1-Å resolution; Fig. 3, A and B). The function of specific amino acids in DNA or ligand recognition were probed by analysis of structure-based site-specific variants using electrophoretic mobility shift assays (EMSA, for DNA binding) or differential scanning fluorimetry (DSF) assays (for ligand binding). The combined biochemical and structural biological data provide a framework for understanding transcriptional regulation by CouR, which further extends the existing knowledge of MarR family repressors.

**Results**

**Structure determination and overall structure**

Cocrystallization efforts with CouR and various synthetic oligonucleotides bearing the inverted repeat DNA recognition sequence yielded several candidates, but most of these crystals did not diffract beyond 3.5-Å resolution. Altering the length and identity of the nucleotides flanking the recognition sequence finally produced crystals that diffracted to 2.85-Å resolution at an insertion device synchrotron beamline. Structures of ligand-free CouR and a complex with synthetic pCC each produced crystals that diffracted to 2.1-Å resolution. Because of ease of reproducibility, structure determination focused on using crystals of the CouR–DNA complex, and crystallographic phases were determined by the single-wavelength anomalous diffraction method from data collected on crystals of selenomethionine-labeled CouR. The apo-CouR and CouR-pCC structures were determined by molecular replacement using the coordinates of the polypeptide from the DNA-bound structure, followed by manual rebuilding of the polypeptide.

The overall structure of the CouR homodimer is triangular in shape with a pseudo 2-fold axis of symmetry. Each monomer consists of six α-helices and two β-strands containing a wHTH motif, where helices α3–α4 and strands β1–β2 define the DNA binding elements (Fig. 1, A and D). Dimerization between the two monomers is mediated by interlocking interactions between helices α1, α5, and α6 from each monomer, resulting in a burial of 4,487–4,497 Å² of solvent-accessible surface area (depending on the conformational state). Dimerization between the two monomers is mediated by extensive burial of numerous hydrophobic residues to form the structural core.

Residues at this interface include Leu-51, Leu-55, Val-62, and Phe-66 from helix α1; Val-152, Leu-160, and Leu-164 from helix α5; and Leu-172, Leu-176, and Ile-179 from helix α6. Additional interactions include intramolecular (Asp-65–Arg-159) as well as intermolecular (Glu-46–Lys-149 and Glu-49–Arg-169) salt bridges. Most notably, electron density is sparse for the β-strands that contain the wHTH motif, and the wings show a different conformation for each of the two monomers. There is also weak density in the presumptive binding pocket for one of the monomers, which may reflect a weakly bound molecule of HEPES from the buffer component, but this was too poorly defined and not modeled.

A DALI search of the CouR monomer against the Protein Data Bank reveals a strong conservation of secondary structural elements with other transcriptional regulators. The closest homologs include a putative regulator of unknown function from *Pseudomonas aeruginosa* (PDB code 2NNN, Z score of 17.5, RMSD of 1.4 Å over 133 aligned Cα atoms), the *Streptomyces coelicolor* β-ketoacid regulator PcaV (PDB code 4FHT, Z score of 17.1, RMSD of 2.7 Å over 141 aligned Cα atoms), *E. coli* MarR (PDB code 3VOE, Z score of 17.1, RMSD of 2.7 Å over 136 aligned Cα atoms), and the multidrug efflux regulator MexR from *P. aeruginosa* (PDB code 1LNW, Z score of 17.1, RMSD of 1.7 Å over 140 aligned Cα atoms).

**Cocrystal structure of CouR with operator DNA**

The cocrystal structure of CouR bound to a 23-bp duplex bearing the two half-sites was determined to 2.85-Å resolution from crystals containing two copies of the CouR dimer–DNA duplex complex in the asymmetric unit (ASU). The CouR homodimer is situated on the pseudo-palindromic duplex so that helix α4 from the wHTH motif is positioned into each of
the two half-sites along consecutive major grooves that are roughly 34 Å apart. The DNA duplex is roughly B-form but is under-twisted by 1.3°, resulting in a shortening of the end-to-end distance by roughly 3.8 Å, relative to canonical B-DNA. Notably, these deviations arise from a widening of the major grooves, to accommodate binding of the CouR α4 recognition helix, and a corresponding slight narrowing of the minor groove (Fig. 2).

Each CouR monomer contacts each half of the inverted repeat sequence through interactions with the wHTH motif, with the α4 helix positioned in the major groove and strands β1-β2 of the wHTH making contacts with nucleotides on the outer periphery of the recognition sites. As in other structures of MarR family regulators in complex with their cognate operator sequences, the number of contacts between CouR and DNA bases of the recognition sequence are mediated via the additional residues that make contact with the DNA backbone. Last, Gln-94 base, and equivalent residues in MepR (Thr-63) and OhrR (Thr-70) are also involved in base pair recognition. Last, Gln-94 (equivalent to Gln-50 in MepR) is located in helix α4 between the carboxamide of Asn-107 and N6 and N7 of A15. Van der Waals contacts with nucleotides at the periphery of the recognition sequences. Electron density for both strands as well as for the intervening loop is clear and continuous in the DNA cocrystal structure. Residues within the loop of this region are situated in the minor groove and are involved in extensive nonspecific contacts with the DNA backbone. The phosphate backbone of the DNA duplex is within hydrogen bonding distance of several residues, including the side chains of Arg-123, Arg-125, Arg-130, Arg-131, Ser-132, and His-133, as well as the main chain carbonyl of Ser-128. The side chain of Arg-131 is poised within hydrogen-bonding distance of T3, but this nucleotide is outside of the recognition half-site and is not part of the inverted repeat sequence of the naturally occurring couA operon. Arg-131 interacts with the side chain carboxylate of Asp-129 via a salt bridge, and this Asp-X-Arg pairing, along with a similar interaction with thymine, is observed across various MarR family members (13–17).

Outside of interactions with the wHTH motif, there are a few additional contacts that make contact with the DNA backbone. Specifically, the side chain of Lys-56 (equivalent to Arg-10 in MepR and Tyr-19 in OhrR) from helix α1 is situated directly above the phosphate of T14, which is located at the inner periphery of the inverted repeat. As a result, interactions between equivalent Lys-56 residues from each monomer occur at either side of the minor groove. In the CouR cocrystal structure, Asn-107 makes direct interactions with the A15 nucleobase, and equivalent residues in MepR (Thr-63) and OhrR (Thr-70) are also involved in base pair recognition. Last, Gln-94 (equivalent to Gln-50 in MepR) is located in helix α3 and is part...
of a framework of interactions with the phosphate of T6, which also engages Arg-123 and His-133 from each of the strands of the wing (for a detailed CouR–DNA interaction map, see Fig. S4).
in the ligand-free CouR and CouR–DNA cocrystal structures (Fig. 3, A and B). The ligand-free and pCC-bound structures are very similar, demonstrating that binding of the ligand does not cause any appreciable changes in global or local structure of CouR (Fig. 4A). A major difference between the two structures is reflected in the orientation of the β-strands of the wHTH motif, which are partially disordered in the ligand-free structure but are well-resolved in the pCC-bound structure. Moreover, the strands of each monomer in the pCC-bound structure are nearly superimposable, suggesting that ligand binding may restrict the flexibility of these strands.

Recognition of pCC appears to be driven primarily by hydrophobic interactions between the coumaroyl moiety of pCC and one of two equivalent clefts (one per monomer) in addition to some hydrogen bonding and hydrophobic interactions along the pantetheine group of pCC. The cleft exists adjacent to the adenine ring and is spurious. Overall structure of CouR bound to pCC retains the homodimeric oligomerization state and binds pCC symmetrically in a 1:1 fashion (see “Experimental procedures” and Fig. 52 for individual binding curves).

The location and secondary structural composition of this hydrophobic cleft is analogous to those observed in structures of MarR family members bound to various phenolic acids and aldehyde ligands lacking CoA (3, 8, 18, 19). Sufficient electron density was observed to model four pCCs bound to four CouR monomers in the crystallographic ASU. Additional interactions near the opening of the cleft include hydrophobic contacts between the side chain of Ile-103 and the geminal dimethyl group of pCC as well as hydrogen bonding between the side chain of Thr-76 and the β-phosphate of pCC (Fig. 3A).

The electron density corresponding to the adenosine nucleotide of pCC is ambiguous, indicative of disorder because of the minimal interactions of this moiety with the protein and its residence within a solvent pocket in the crystal lattice. Similar disorder has been observed for the adenosine group in other CoA-bound structures (20, 21). These cross-subunit interactions with the pCC ligand result in an inward constriction of the second subunit upon binding of the ligand to the first subunit (Fig. 3, A and B, and Fig. 4A).

The overall structures of the CouR monomer in either DNA- or pCC-bound forms do not differ appreciably, with an RMSD of 0.85 Å over 891 atoms. However, superimposition of CouR dimers (RMSD of 2.35 Å over 2,061 atoms) reveals notable changes that occur in the quaternary structure, especially with regards to the wHTH motif that mediates DNA binding. Specifically, a superposition of dimers of the DNA-bound CouR with that of the pCC-bound structure reveals that the wHTH motif is shifted outwards by 5 Å in the former structure, which facilitates a disposition compatible with binding of the homodimer across consecutive major grooves of the operator (Fig. 4). Binding of DNA resulted in an outward shift of helix α4, which forms a portion of the pCC binding cavity, so that the wings of the wHTH motif are also suitably positioned for interactions with the duplex. The collapse of helix α4 necessary to form a viable pCC binding pocket would result in a CouR dimer that is not optimally aligned for binding across consecutive major grooves, suggesting a mechanism for ligand-mediated attenuation of operator binding. There are no relative rotational movements of the two CouR monomers between the
Structural basis for regulation of coumarate catabolism

DNA- and pCC-bound structures, such as that observed in comparisons of ligand and DNA-bound structures of the MarR-related Rv2887 from *Mycobacterium tuberculosis* (22).

**DSF**

On the basis of the observed crystallographic contacts between CouR and pCC, the residues involved in hydrophobic contacts (Phe-63 and Ile-103) and hydrogen-bonding interactions (Thr-76 and Asn-107) were mutated to Ala. To probe the contributions of each residue to ligand binding, we carried out DSF analyses for each variant. As the protein–ligand complex demonstrated an increased melting temperature \( T_m \), melting curves were monitored as a function of pCC concentration, enabling the measurement of dissociation constants \( K_{D} \) between pCC and each CouR variant (23).

Analysis of WT CouR with pCC yielded a \( K_{D} \) value of 68 ± 8 \( \mu \text{M} \). The Phe-63→Ala and the Thr-76→Ala variants showed the greatest effect on the \( K_{D} \) values for pCC binding. This result is consistent with crystallographic observations, which shows that Phe-63 serves as a major contributor to the hydrophobic pocket that houses the ligand, and an Ala substitution at this site would likely compromise the integrity of the pCC binding pocket. Likewise, Thr-76 is within hydrogen bonding distance of pCC, with a distance of 2.7 Å between the Oγ of this residue and the β-phosphate of pCC (Fig. 3).

Notably, the thermal denaturation midpoint \( T_m \) of the Thr-76→Ala variant had decreased by 10 °C relative to the WT. Consequently, this variant was tested to ensure that it was properly folded and formed the requisite homodimeric assembly. Analytical size exclusion chromatographic (SEC) analysis performed on CouR WT and the Thr-76→Ala variants showed in retention times expected for a 40-kDa homodimer (see “Experimental procedures” and Fig. S3). The Ile-103→Ala variant displays a slight binding impairment upon replacement of the sec-butyl group with a methyl group in the position found to associate with the geminal dimethyl group of pCC. No appreciable binding impairment was observed for the Asn-107→Ala mutant, suggesting that proximity of Asn-107 to the amide carbonyl of pCC may not be critical for tight binding of the pCC ligand (Fig. 3).

**Discussion**

Detailed biochemical studies of MarR family proteins are often hampered because of a lack of details regarding the physiological effector. Even though surrogate small molecules may function at the protein level, the relevance of binding by such effectors is sometimes not clear. Here we present a detailed characterization of CouR, a repressor of a p-coumarate catabolic pathway for which genetic and microbiological analyses have been carried out in detail (1, 12). We complement the earlier studies using biochemical and structural biological approaches to elucidate the details of a MarR member visualized in three different states: without any bound ligand, bound to the cognate operator element, and bound to the physiological effector molecule. Unlike other studies of MarR family members, pCC, the effector for CouR, is not metabolically ubiquitous, and the extensive contacts made throughout the entirety of this structurally unique ligand afford an unbiased delineation of the structural details with a bound ligand. To further dissect the mechanism of ligand-induced dissociation, additional studies will be necessary to confirm whether pCC binds directly to the CouR–DNA complex or free CouR.

A prior effort also characterized a CouR-like pathway encoded in the soil bacterium *R. josti* RHA1 (CouR-RJ) (13). That system is distinct in that not all of the genes proposed in the catabolic pathway are within the same operon. Based on the sequences of the different promoters, it was proposed that a single repressor may regulate transcription even though the promoters are divergently transcribed. Notably, the promoter sequence identified in *R. josti* RHA1 (cATTGAnnnn-TCAATg) is entirely distinct from the *R. palustris* CGA009 CouR (CouR-RP)-responsive promoter (GGTTAAnnnTATAAC), and the two MarR-type regulators share only 36% sequence identity. The authors provide a molecular basis for DNA binding attenuation that invokes the sequestration of two Arg residues (numbered 36 and 38 in CouR-RJ, Fig. 1D) by pCC; however, these residues are not present in CouR-RP, and similar contacts were not observed in the CouR structures presented here. Moreover, superimposing the CouR-RP DNA-bound structure with the orthologous CouR-RJ pCC-bound structure reveals that the separations of helices α4 are nearly equidistant, suggesting compatibility with major groove binding and a unique mechanism of repression (Fig. S5).

The crystal structure of CouR bound to the operator supports the idea of an indirect sequence readout, first suggested by Dolan et al. (15) in the context of the SlyA-DNA structure. This is a result of primarily nonbase-specific contacts mediating DNA recognition and is corroborated by existing MarR family/DNA cocrystal structures in the Protein Data Bank (14–17). The unexpected finding that many of the protein–DNA contacts are actually nonbase-specific lends credence to the notion of an indirect sequence readout and suggests that newer *in silico* techniques may be necessary to predict three-dimensional topologies for correct assignment of protein–DNA binding partners. Indeed, computational techniques involving comparative and machine learning strategies have been utilized to predict protein–DNA binding interactions (24). Predictive methods will hopefully benefit from the experimental structures in this work to understand similar MarR family regulators for which experimental data are lacking.

**Experimental procedures**

**Cloning, expression, and purification of CouR**

The CouR gene was amplified by the PCR from purified *R. palustris* genomic DNA using the following set of primers: 5′ GCACAGGATCCGTCGACCTCGAACAAGGATC 3′ (forward) and 5′ TAAACCTGAGTCGACACTCGCGGGG-ATGG 3′ (reverse). The PCR product was digested with BamHI and XhoI and ligated into a similarly digested pET28-MBP expression vector using T4 DNA ligase (New England Biolabs). The presence of CouR was tested by restriction digestion analysis and verified by di-deoxy sequencing (ACGT Inc.). The resulting construct encodes a fused maltose-binding protein (MBP) protein followed by a His10 affinity tag preceding the CouR reading frame.
Structural basis for regulation of coumarate catabolism

The recombinant plasmid described above was used to transform *E. coli* (Rosetta) for overexpression of MBP-CouR. *E. coli* cultures were grown with shaking at 250 rpm in LB medium supplemented with kanamycin (50 µg/ml) and chloramphenicol (25 µg/ml) until an A_{600} of 0.5–0.6 was reached. The cultures were induced with 0.5 mM isopropyl-1-thiogalactopyranosidase after cooling on ice for 20 min and grown for an additional 16 h at 18 °C. Cells were harvested by centrifugation at 3,500 rpm and resuspended in buffer A (500 mM NaCl, 25 mM Tris-HCl (pH 8.0), and 10% glycerol) before lysing with a French press at 8,000–10,000 pounds/square inch for 4 cycles. The lysate was cleared by centrifugation at 14,000 rpm for 1 h at 4 °C, and the supernatant was loaded on a 5-ml HisTrap nickel–nitrilotriacetic acid affinity column (GE Healthcare) equilibrated with buffer B (1 M NaCl, 25 mM Tris-HCl (pH 8.0), and 30 mM imidazole). The column was washed with 40 ml of buffer B before eluting with a linear gradient from 0–100% buffer C (1 M NaCl, 25 mM Tris-HCl (pH 8.0), and 250 mM imidazole) over 20 min at 2 ml/min. CouR was then subjected to tobacco etch virus protease proteolysis at 4 °C to remove the MBP tag while being dialyzed into 250 mM NaCl, 20 mM Tris (pH 7.5), and 1 mM DTT. The reaction ran to near-completion after 24 h (as monitored by SDS-PAGE) before dialyzing into 250 mM NaCl and 20 mM Tris-HCl (pH 7.5) to remove DTT before subtractive nickel purification. Reaction contents were loaded onto a nickel–nitrilotriacetic acid column equilibrated with buffer A, followed by a 30-ml wash with buffer A. Proteins were eluted in 4-ml increments using the following stepwise gradient of increasing buffer B: 5%, 10%, 20%, 30%, and 100%. This was followed by additional stepwise elution steps of increasing buffer C: 50% and 100%. Fractions containing CouR were dialyzed into 100 mM NaCl, 20 mM Tris-HCl (pH 7.5) at 4 °C before loading onto a 5-ml HiTrap SP HP column (GE Healthcare) equilibrated with buffer D (100 mM NaCl, 50 mM Na,N,N,N-tetrakis(2-hydroxyethyl)glycine (pH 7.5)). The column was washed with 20 ml of buffer D before eluting with a linear gradient of increasing buffer E (1 M NaCl and 50 mM bicine (pH 7.5)) for 35 min at a flow rate of 1.5 ml/min. CouR-containing fractions indicated >95% purity by SDS-PAGE analysis (Fig. S1).

**DSF binding assay**

CouR WT and mutant proteins were purified as described above followed by gel filtration on a 120-ml Superdex 75 column (GE Healthcare) in 50 mM KCl, 0.5 mM 2-mercaptoethanol, and 20 mM HEPES (pH 7.0). The DSF assay was performed on a StepOnePlus RT-PCR instrument (Applied Biosystems) using a 96-well plate format. WT and mutant proteins were subjected to increasing concentrations of pCC from 0–10 mM while maintaining a constant protein concentration of 68 µM and 6× SYPRO orange dye (prepared from a 5,000× stock in DMSO). Samples were allowed to equilibrate for 30 min prior to initiating the melt curve. The melt curve program initiated with a 2 min 25 °C hold step followed by a ramp up to 99 °C at 1.7 °C/min (step and hold) and a final hold for 2 min at 99 °C. First derivatives of the melting curves were used to determine the T_m for each sample well. Plots were fit to a dose–response curve using OriginPro 2015, and the K_D was taken to be equal to the substrate concentration at which melting temperature achieved 50% of the total change.

**EMSA**

EMSAs were performed as described previously (1). The same DNA probe, spanning the –300 to +17 bp relative to the couA start codon, was PCR-amplified, and 0.3 pmol of DNA probe was mixed with various amounts (2, 4, or 8 pmol) of CouR variants in a 15-µl reaction mixture (binding buffer: 20 mM Tris (pH 7.5), 50 mM KCl, 1 mM DTT, and 8% glycerol) and incubated for 25 min at room temperature. The samples were loaded on a 5% nondenaturing acrylamide Tris/glycine-EDTA gel and electrophoresed in Tris/glycine-EDTA buffer (10 mM Tris (pH 8.0), 380 mM glycine, and 1 mM EDTA) at 4 °C. The gel was soaked in 10,000-fold-diluted SYBR Green I nucleic acid stain (Lonza, Walkersville, MD), and DNA was visualized under UV light.

**Analytical SEC**

Analytical SEC was performed using a 40-ml Superdex 200 10/300 GL column (GE Healthcare) equilibrated with 0.1 mM NaCl and 20 mM Tris (pH 7.0). A similar running buffer was used to elute protein standards prepared from commercially available dry lyophilized powders (Sigma-Aldrich) at 1 ml/min. CouR variants were eluted similarly (Fig. S3).

**Crystallization and structure solution of the CouR–operator complex**

Single-stranded DNA oligomers were ordered from Integrated DNA Technologies and were made up to 10 mM by solvating in 20 mM Tris (pH 7.5) and 50 mM NaCl. The complementary single-stranded DNA solutions were mixed 1:1 before using a thermocycler to heat at 95 °C for 5 min, followed by a decrease to 25 °C at a rate of 1 °C per minute. A series of dsDNA sequences ranging from 16–29 bp and containing either blunt end or single-nucleotide overhangs were screened for cocrystallization with CouR before arriving at the optimally diffracting 23-nt sequence (23-mer) used in the final model. Crystals of CouR bound to the 23-mer were obtained by mixing 15 mg/ml CouR with 1.1 stoichiometric amounts of 23-mer and incubating on ice for 20 min. This solution was mixed with an equal volume of reservoir solution containing 0.1 M Tris (pH 8.5), 0.1 M ammonium phosphate, and PEG 6000 to generate 2-µl hanging drops. Thin plate-shaped crystals grew at 16 °C to maximum size (about 100 × 100 µm) in less than a week.

Crystals were cryoprotected in crystallization buffer supplemented with 25% glycerol and vitrified by direct immersion into liquid nitrogen prior to data collection. Structure determination was carried out using single-wavelength anomalous dispersion data collected from crystals of selenomethionine-derivatized CouR bound to DNA using Phenix AutoSol with a Bayesian estimate of the map quality (25). Data were collected at the Advanced Photon Source, Argonne National Laboratory using the Life-Science Collaborative Access Team 21-ID-F, 21-ID-G, and 21-ID-D beamlines. After determining the phases and initial model from Phenix AutoSol, each structure was rebuilt using Phenix AutoBuild with multiple rounds of manual intervention (26). This new model was further refined using
Structural basis for regulation of coumarate catabolism

CCP4 refmac5 in combination with additional manual rebuilding in COOT (27, 28).

Crystallization and structure solution of the CouR–pCC complex

CouR was concentrated to 24 mg/ml, combined with 3 mM pCC, and crystallized at 9 °C in similar 2-μl hanging drops containing a reservoir solution of 0.17 M Li₂SO₄, 23.5% PEG 4000, 1 mM DTT, 0.085 M Tris-HCl (pH 8.5), and 15% glycerol. Rod-shaped crystals grew to maximum size in less than a week to about 200 × 50 μm at the largest face. Prior to data collection, crystals were directly flash-frozen in liquid nitrogen. Data were collected at the Advanced Photon Source, Argonne National Laboratory using the Life-Science Collaborative Access Team 21-ID-G beamline. The structure was solved by molecular replacement using the protein coordinates from the CouR–couR operator structure. Molecular replacement–derived phases were used to build an initial model in Phenix AutoBuild, followed by similar refinement procedures as described for the CouR–couR operator complex. Prior to fitting pCC into electron density difference maps, restraint parameters and geometry optimizations were produced by Phenix Refine (29). Water molecules were incorporated in the CouR–pCC structure using Phenix Refine (30).

Crystallization and structure solution of apo CouR

CouR was concentrated to 15 mg/ml and crystallized at 9 °C in 1 μl sitting drops by mixing 1:1 (v/v) protein solution and reservoir solution containing 60% (v/v) Tacisimate (pH 7.0). Diamond-shaped crystals grew to maximum size in less than a week. The structure was solved by molecular replacement in a similar manner as described for the CouR–pCC cocystal structure. Additional model building and refinement were carried out as described above.

References
1. Hirakawa, H., Schaefer, A. L., Greenberg, E. P., and Harwood, C. S. (2012) Anaerobic p-coumarate degradation by Rhodopseudomonas palustris and identification of CouR, a MarR repressor protein that binds p-coumaroyl coenzyme A. J. Bacteriol. 194, 1960–1967 CrossRef Medline
2. Haque, M. M., Kabir, M. S., Aini, L. Q., Hirata, H., and Tsuyumu, S. (2009) SlyA, a MarR family transcriptional regulator, is essential for virulence in Dickeya dadantii 3937. J. Bacteriol. 191, 5409–5418 CrossRef Medline
3. Davis, J. R., Brown, B. L., Page, R., and Sello, J. K. (2013) Study of PcaV from Streptomyces coelicolor yields new insights into ligand-responsive MarR family transcription factors. Nucleic Acids Res. 41, 3888–3900 CrossRef Medline
4. Kaatz, G. W., DeMarco, C. E., and Sea, S. M. (2006) MepR, a repressor of the Staphylococcus aureus MATE family multidrug efflux pump MepA, is a substrate-responsive regulatory protein. Antimicrob. Agents Chemother. 50, 1276–1281 CrossRef Medline
5. Hao, Z., Lou, H., Zhu, R., Zhu, J., Zhang, D., Zhao, B. S., Zeng, S., Chen, X., Chan, I., He, C., and Chen, P. R. (2014) The multiple antibiotic resistance regulator MarR is a copper sensor in Escherichia coli. Nat. Chem. Biol. 10, 21–28 CrossRef Medline
6. Perez-Rueda, E., Hernandez-Guerrero, R., Martinez-Nunez, M. A., Armenta-Medina, D., Sanchez, I., and Ibarra, J. A. (2018) Abundance, diversity and domain architecture variability in prokaryotic DNA-binding transcription factors. PLoS ONE 13, e0195332 CrossRef Medline
7. Land, M., Hauser, L., Jun, S.-R., Nookaew, I., Leuze, M. R., Ahn, T.-H., Karpinetis, T., Lund, O., Kora, G., Wassenaar, T., Poudel, S., and Ussery, D. W. (2015) Insights from 20 years of bacterial genome sequencing. Funct. Integr. Genomics 15, 141–161 CrossRef Medline
8. Chang, Y.-M., Jeng, W.-Y., Ko, T.-P., Yeh, Y.-J., Chen, C. K., and Wang, A. H. (2010) Structural study of TcaR and its complexes with multiple antibiotics from Staphylococcus epidermidis. Proc. Natl. Acad. Sci. U.S.A. 107, 8617–8622 CrossRef Medline
9. Alekshun, M. N., Levy, S. B., Mealy, T. R., Seaton, B. A., and Head, J. F. (2001) The crystal structure of MarR, a regulator of multiple antibiotic resistance, at 2.3 Å resolution. Nat. Struct. Biol. 8, 710–714 CrossRef Medline
10. Larimer, F. W., Chain, P., Hauser, L., Lamerdin, J., Malfatti, S., Do, L., Land, M. L., Pelletier, D. A., Beatty, J. T., Lang, A. S., Tabita, F. R., Gibson, J. L., Hanson, T. E., Bobst, C., Torres, J. L., et al. (2004) Complete genome sequence of the metabolically versatile photosynthetic bacterium Rhodopseudomonas palustris. Nat. Biotechnol. 22, 55–61 CrossRef Medline
11. Egland, P. G., Pelletier, D. A., Dispensa, M., Gibson, J., and Harwood, C. S. (1997) A cluster of bacterial genes for anaerobic benzene ring biodegradation. Proc. Natl. Acad. Sci. U.S.A. 94, 6484–6489 CrossRef Medline
12. Phattarasukul, S., Radey, M. C., Lappala, C. R., Oda, Y., Hirakawa, H., Brittnacher, M. J., and Harwood, C. S. (2012) Identification of a p-coumarate degradation regulon in Rhodopseudomonas palustris by xpression, an integrated tool for prokaryotic RNA-seq data processing. Appl. Environ. Microbiol. 78, 6812–6818 CrossRef Medline
13. Otani, H., Stogios, P. J., Xu, X., Noces, B., Li, S. N., Savchenko, A., and Eltis, L. D. (2016) The activity of CouR, a MarR family transcriptional regulator, is modulated through a novel molecular mechanism. Nucleic Acids Res. 44, 595–607 CrossRef Medline
14. Hong, M., Fangthong, M., Helmann, J. D., and Brennan, R. G. (2005) Structure of an OhrR-ohrA operator complex reveals the DNA binding mechanism of the MarR family. Mol. Cell 20, 131–141 CrossRef Medline
15. Dolan, K. T., Duguid, E. M., and He, C. (2011) Crystal structures of SlyA protein, a master virulence regulator of Salmonella, in free and DNA-bound states. J. Biol. Chem. 286, 22178–22185 CrossRef Medline
16. Liu, G., Liu, X., Xu, H., Liu, Z., Zhou, H., Huang, Z., Gan, J., Chen, H., Lan, L., and Yang, C. G. (2017) Structural insights into the redox-sensing mechanism of MarR-type regulator AbfR. J. Am. Chem. Soc. 139, 1598–1608 CrossRef Medline
17. Birukov, I., Sea, S. M., Schindler, B. D., Kaatz, G. W., and Brennan, R. G. (2014) Structural mechanism of transcription regulation of the Staphylococcus aureus multidrug efflux operon mepRA by the MarR family repressor MepR. Nucleic Acids Res. 42, 2774–2788 CrossRef Medline
18. Duval, V., McMurry, L. M., Foster, K., Head, J. F., and Levy, S. B. (2013) Mutational analysis of the multiple-antibiotic resistance regulator marR reveals a ligand binding pocket at the interface between the dimerization and DNA binding domains. J. Bacteriol. 195, 3341–3351 CrossRef Medline
19. Kim, Y., Joachimiak, G., Bigelow, L., Babnigg, G., and Joachimiak, A. (2016) How aromatic compounds block DNA binding of HCAR catabolite regulator. J. Am. Chem. Soc. 138, 13243–13256 CrossRef Medline
20. Davies, C., Heath, R. J., White, S. W., and Rock, C. O. (2000) The 1.8 Å crystal structure and active-site architecture of β-ketoacyl-acyl carrier protein synthase III (FabH) from Escherichia coli. Structure 8, 185–195 CrossRef Medline
21. Edwards, T. E., Leibly, D. J., Bhandari, J., Statnekov, J. B., Phan, I., Diet-erich, S. H., Abendroth, J., Staker, B. L., Van Voorhis, W. C., Myler, P. J., and Stewart, L. J. (2011) Structures of phosphopantetheine adenylyltransferase from Burkholderia pseudomallei. Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 67, 1032–1037 CrossRef Medline

22. Gao, Y. R., Li, D. F., Fleming, J., Zhou, Y. F., Liu, Y., Deng, J. Y., Zhou, L., Zhou, J., Zhu, G. F., Zhang, X. E., Wang, D. C., and Bi, L. J. (2017) Structural analysis of the regulatory mechanism of MarR protein Rv2887 in M. tuberculosis. Sci. Rep. 7, 6471 CrossRef Medline

23. Vivoli, M., Novak, H. R., Littlechild, J. a., and Harmer, N. J. (2014) Determination of protein-ligand interactions using differential scanning fluorimetry. J. Vis. Exp. 91, 51809 CrossRef Medline

24. Xiong, Y., Zhu, X., Dai, H., and Wei, D.-Q. (2018) in Computational Systems Biology: Methods and Protocols (Huang, T., ed), pp. 223–234, Springer, New York

25. Terwilliger, T. C., Adams, P. D., Read, R. J., McCoy, A. J., Moriarty, N. W., Grosse-Kunstleve, R. W., Afonine, P. V., Zwart, P. H., and Hung, L. W. (2009) Decision-making in structure solution using Bayesian estimates of map quality: the PHENIX AutoSol wizard. Acta Crystallogr. D Biol. Crystallogr. 65, 582–601 CrossRef Medline

26. Terwilliger, T. C., Grosse-Kunstleve, R. W., Afonine, P. V., Moriarty, N. W., Zwart, P. H., Hung, L. W., Read, R. J., and Adams, P. D. (2008) Iterative model building, structure refinement and density modification with the PHENIX AutoBuild wizard. Acta Crystallogr. D Biol. Crystallogr. 64, 61–69 Medline

27. Vagin, A. A., Steiner, R. A., Lebedev, A. A., Potterton, L., McNicholas, S., Long, F., and Murshudov, G. N. (2004) REFMAC5 dictionary: organization of prior chemical knowledge and guidelines for its use. Acta Crystallogr. D Biol. Crystallogr. 60, 2184–2195 CrossRef Medline

28. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. Acta Crystallogr. D Biol. Crystallogr. 66, 486–501 CrossRef Medline

29. Moriarty, N. W., Grosse-Kunstleve, R. W., and Adams, P. D. (2009) Electronic ligand builder and optimization workbench (eLBOW): a tool for ligand coordinate and restraint generation. Acta Crystallogr. D Biol. Crystallogr. 65, 1074–1080 CrossRef Medline

30. Afonine, P. V., Grosse-Kunstleve, R. W., Echols, N., Headd, J. J., Moriarty, N. W., Mustyakimov, M., Terwilliger, T. C., Urzhumtsev, A., Zwart, P. H., and Adams, P. D. (2012) Towards automated crystallographic structure refinement with phenix.refine. Acta Crystallogr. D Biol. Crystallogr. 68, 352–367 CrossRef Medline

31. Lim, D., Poole, K., and Strynadka, N. C. (2002) Crystal structure of the MexR repressor of the mexRAB-oprM multidrug efflux operon of Pseudomonas aeruginosa. J. Biol. Chem. 277, 29253–29259 CrossRef Medline