Burkholderia xenovorans RcoM_{Bx}-1, a Transcriptional Regulator System for Sensing Low and Persistent Levels of Carbon Monoxide

Robert L. Kerby and Gary P. Roberts

Department of Bacteriology, University of Wisconsin—Madison, Madison, Wisconsin, USA

The single-component RcoM transcription factor couples an N-terminally bound heme cofactor with a C-terminal “LytTR” DNA-binding domain. Here the RcoM_{Bx}-1 protein from *Burkholderia xenovorans* LB400 was heterologously expressed and then purified in a form with minimal bound CO (~10%) and was found to stably bind this effector with a nanomolar affinity. DNase I protection assays demonstrated that the CO-associated form binds with a micromolar affinity to two ~60-bp DNA regions, each comprised of a novel set of three direct-repeat binding sites spaced 21 bp apart on center. Binding to each region was independent, while binding to the triplet binding sites within a region was cooperative, depended upon spacing and sequence, and was marked by phased DNase I hyperactivity and protection patterns consistent with considerable changes in the DNA conformation of the nucleoprotein complex. Each protected binding site spanned a conserved motif (5'-TTnnnG-3') that was present, in triplicate, in putative RcoM-binding regions of more than a dozen organisms. *In vivo* screens confirmed the functional importance of the conserved “TTnnnG” motif residues and their triplet arrangement and were also used to determine an improved binding motif [5'-CnnC(C/A)(G/A)TTCAnG-3'] that more closely corresponds to canonical LytTR domain/DNA-binding sites. A low-affinity but CO-dependent binding of RcoM_{Bx}-1 to a variety of DNA probes was demonstrated *in vitro*. We posit that for the RcoM_{Bx}-1 protein, the high CO affinity combined with multiple low-affinity DNA-binding events constitutes a transcriptional “accumulating switch” that senses low but persistent CO levels.

Carbon monoxide (CO) is a prevalent toxin and yet is also a bacterial nutrient, a crucial intermediate in anaerobic central metabolism, and an enzyme cofactor (3, 10, 23, 38). Bacteria sense and respond to CO by using two biochemically characterized non-homologous transcription factors, CooA (CO oxidation activator) and RcoM (regulator of CO metabolism), that have been shown to regulate one of three metabolic processes in different organisms: (i) the expression of *cox*-encoded aerobic CO oxidation, (ii) the expression of *coo*-encoded anaerobic CO oxidation, or (iii) the expression of *cowN*, whose gene product protects nitrogenase function from CO inhibition (21, 22, 43). These single-component transcription factors link an N-terminal domain that enfolds a CO-binding heme cofactor with a C-terminal DNA-binding region, either the common helix-turn-helix domain (CooA) or the rarer “LytTR” domain (RcoM) (14).

The LytTR DNA-binding domain, named for the prototypical LytT and LytR transcription factors (34), typically occurs as a DNA-binding response regulator component of two-component signal transduction systems. These systems often regulate virulence functions, including the synthesis of exopolysaccharides, bacteriocins, toxins, excreted enzymes, and fimbriae (13, 34), and given their absence from eukaryotes, the interference of LytTR domain protein function is considered a useful therapeutic target. The first structural analysis of a LytTR domain, that of the *Staphylococcus aureus* AgrA C terminus (“AgrA_C”) (47), showed that the domain bound to a 15-bp double-stranded-DNA (dsDNA) target (+1-base 5' overhangs) via a novel protein-DNA interaction: two protein loops insert into successive major grooves, and residues at the loop tips make two specific DNA contacts (H169/G₁₃ and R233/G₆'). These DNA contacts flank a conserved core (positions T₉T₁₀) that acts as a hinge and permits the DNA target to conform to the adhered protein (47).

Although there is ample evidence of more complex LytTR domain-DNA interactions, typical activated promoters possess dual

direct-repeat-binding sites spaced 21 bases (two helical turns) apart on center that are located immediately adjacent to a σ^{70} RNA polymerase (RNAP) –35/–10 binding sequence (4, 6, 7, 13, 19, 24–26, 30, 34, 35, 39, 41, 50, 57, 58). Precise spacing between the dual sites is crucial for functions in systems where the binding of the transcription factor stimulates transcription (4, 6, 24, 41), indicating that the protein binds one face of the helix, and protein binding to the dual sites is usually cooperative. However, the overall stoichiometry and structure of the biologically relevant nucleoprotein complex and the nature of RNAP interactions remain undetermined.

We previously demonstrated the CO-binding spectral characteristics of the RcoM paralogues cloned from *Burkholderia xenovorans* LB400, designated RcoM_{Bx}-1 and RcoM_{Bx}-2, and their CO-dependent function *in vivo* (22, 29). Here we report the purification of RcoM_{Bx}-1 largely free of bound CO, demonstrate the CO-dependent DNA binding of purified protein *in vitro*, define the RcoM_{Bx}-1-binding motif, and show the functional cooperativity of RcoM_{Bx}-1 binding to regions comprised of triplet, evenly spaced, direct-repeat sites. We consider the biological significance of a regulatory system with high effector sensitivity linked to multiple low-affinity DNA-binding events and the implications of this binding arrangement on the broader class of two-component LytTR domain regulators.

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Address correspondence to Gary P. Roberts, groberts@bact.wisc.edu.

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TABLE 1 Primer and probe sequences^a

Primer(s) or probe	Sequence(s) (5'→3')
Primers for construction of strain UQ5853 ^b	
ypdA-cat-F	AGAGGTTTAATCCTTATTTCAGAGTCACCCGTGCACGAAATATTCAACATG TGTGACGGAAGATCACTTCG
ypdB-cat-R	AAGCCTGGTGCTTTTCATGAAAGTTCCCGATTAAAGATGCATTAAGTGGCG ACCAGCAATAGACATAAGCG
Primer pairs used to amplify FAM-labeled DNAs employed in footprinting assays	
Bx1-FAM-F + Bx1bind-R	FAM-GGCTCAGACGATTTTCATGCG + GTCGACGGATCACACTGGC
Bx1-FAM-R + Bx1bind-F	FAM-GTCGACGGATCACACTGGC + GGCTCAGACGATTTTCATGCG
Primers used for randomization of the “e” binding site ^c	
Bx1ex1-F	CCGGGGATCCTACGTTTTCNNNNNTTGGCGCGCGTGAACCCGCCGTTTC
Bx1ex2-F	GATCCTACGTTTTCGCCGTAGTTNNNGNNNGTGAACCCGCCGTTTCGGG
Probe DNAs used for fluorescent polarization assays of RcoM _{Bx-1} -DNA binding ^d	
“b + c”	TR-CCGGGTCGTTTCACGCAGGTAAACCCGCCGTTTCGGGAAACCGGCATG
“e + f”	TR-TTTCGCCGTAGTTGGCGCGCGTGAACCCGCCGTTTCGGGAAATTCG
“e ↑”	TR-TTTCCTACAGTTTCACGCACGTG
“e ↑ + f”	TR-TTTCCTACAGTTTCACGCACGTGAACCCGCCGTTTCGGGAAATTCG
“e ↑ + e ↑”	TR-TTTCCTACAGTTTCACGCACGTGAACCTACAGTTTCACGCACCTTGC
Binding-region sequencing primer lacZ-R1	GACGTTGTAAAACGACG

^a Sequences of primers utilized for reporter constructions are presented in Table SA2 in the supplemental material.
^b Underlined bases hybridize to the pACT3 *cat* gene, and the remaining residues hybridize to *ypdA* or *ypdB*.
^c The complement strand is not shown. Shaded residues highlight the conserved TTnnnG motif on the forward DNA strand (Fig. 1), and double-underlined “N” residues indicate the positions randomized during mutagenesis.
^d The unlabeled complement strand is not shown. “TR” indicates the 5′ Texas Red label, shaded residues highlight the conserved TTnnnG motif on the forward DNA strand (Fig. 1), and double-underlined positions indicate differences from the wild-type sequence.

MATERIALS AND METHODS

Reagents, strain cultivation, DNA isolation, and sequence verification. Carbon monoxide gas (99.5% minimum) was supplied by Airgas (Madison, WI). The high-performance liquid chromatography (HPLC)-purified and Texas Red (TR)-labeled oligonucleotides (Integrated DNA Technologies, Coralville, IA) used to prepare dsDNA probes were annealed in a buffer (10 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1 mM EDTA) according to the manufacturer’s instructions. Footprint assay templates were PCR amplified by using 5′–6-carboxyfluorescein (FAM)-labeled oligonucleotides purchased from the University of Wisconsin Biotechnology Center (UWBC, Madison, WI). Desalted oligonucleotides obtained from either of these sources were used with the QuikChange mutagenesis protocol (Stratagene, La Jolla, CA), and all PCRs employed *Pfu* Turbo polymerase (Stratagene). Following isolation by using the QIAprep spin column protocol (Qiagen Inc., Valencia, CA), plasmid sequences were verified using BigDye v. 3.1 reaction chemistry (Applied Biosystems, Carlsbad, CA), with analysis performed by the UWBC.

The routine cultivation of *Escherichia coli* strains was done by utilizing 2× LC medium (20). Cells prepared for β-galactosidase activity screens or assays were grown in 3-(*N*-morpholino)propanesulfonic acid (MOPS)-buffered medium (52) containing 0.1% tryptone and either 10 mM glucose (for plates) or 40 mM sodium acetate (for liquid cultures). The antibiotics used (with their resistance designations in parentheses) included 20 μg/ml kanamycin sulfate (Km^r), 75 μg/ml ampicillin (Na salt) (Ap^r), 20 μg/ml spectinomycin dihydrochloride (Sp^r), and 10 μg/ml chloramphenicol (Cm^r).

Strain and plasmid constructs. Extensive information concerning the construction of plasmids, strain and plasmid designations and characteristics, and the sequences of primers used for plasmid constructions are presented in supplemental methods and Tables SA1 and SA2 in the sup-

plemental material. Strains of the greatest utility are available at the Add-gene repository (www.addgene.org/). As described previously (22), *B. xenovorans* LB400 bears two RcoM paralogues, encoded by *rcoM*₁ and *rcoM*₂, and these genes are transcribed divergently from the adjacent *coxM*₁ and *coxM*₂ genes, as illustrated for *rcoM*₁ and *coxM*₁ (see Fig. 1). Plasmid pUX2410 (Ap^r), routinely used for the controlled expression of the RcoM_{Bx-1} protein (C-terminally 6×His tagged), was described previously(22); however, new compatible and low-copy-number-plasmid *coxM*₁::*lacZ* transcriptional fusion reporter systems (Sp^r Km^r) were developed. These differed from previous usage in (i) the introduction of unique BamHI and SalI sites to permit the directional replacement of the RcoM_{Bx-1}-binding region and (ii) the deletion of unnecessary *tac* promoter (*P*_{tac}) and *lacI*^q regions (they remain on the pUX2410 protein expression system). A schematic representation of the dual-plasmid *in vivo* reporter system is presented in Fig. SA1 in the supplemental material. In addition, all *in vivo* activity assays were performed in the *E. coli* strain UQ5853 background, which lacks both endogenous LytTR domain protein regulatory systems (encoded by *yehTU* and *ypdAB*). This strain was generated from BW29858 (*ΔyehTU*) (61) by using pSIM6-dependent recombineering (9), wherein the *ypdAB* chromosomal sequence was replaced by a cassette created by the PCR amplification of the pACT3 (11) *cat* gene using primers (Table 1) that also annealed to the 5′ terminus of *ypdA* (*ypdA*-cat-F) and the 3′ terminus of *ypdB* (*ypdB*-cat-R). The final UQ5853 construct was verified by the amplification of the chromosomal *ypdA*::*cat*::*ypdB* region and sequencing. Although there is no evidence that the endogenous LytTR systems affected any RcoM_{Bx-1}-dependent reporter, this host strain was prepared to avoid the possibility that altered reporter systems might be adventitiously regulated. **Identification of an improved “e” binding-site sequence.** Template DNA (pUX3176) (see Table SA1 in the supplemental material) derived

FIG 1 Organization of the *B. xenovorans* *rcoM*₁-*coxM*₁ (chromosome I) intergenic DNA. Sequence analysis (see Fig. SA4 in the supplemental material) indicates two parallel RCoM_{Bx}-1-binding regions, each comprised of three successive direct-repeat motifs with the invariant sequence TTnnnG (shaded), designated “a + b + c” and “d + e + f” (showing the “forward” strand). The two binding regions are separated by a 104-bp interval, and they align with 71% identity (underlined residues). The “f” motif is adjacent to a putative RNAP σ^{70} -35/extended -10 sequence (boxed residues) located upstream of *coxM*₁.

from a very low activity reporter system with an altered “e” motif (T₉T₁₀nnnC₁₄ [numbering conventions are defined in Results; double underlining indicates the non-wild-type {WT} residue]) was mutagenized by using primers that randomized groups of 6 residues adjacent to the T₉T₁₀ residues, both upstream (Bx1ex1-F plus complement) and downstream (Bx1ex2-F plus complement) (Table 1). Both sets of primers also restored WT residue G₁₄; this residue was both conserved and necessary for RcoM_{Bx}-1-binding activity, and the use of this template, with the restoration of G₁₄ during mutagenesis, ensured that clones with even marginal activity had resulted from the mutagenesis procedure (see Results). Pools of randomized binding regions were excised by BamHI/Sall digestion, directionally cloned into reporter plasmid pUX3007, and ultimately transformed as plasmid pools into UQ5854 (UQ5853/pUX2410) by means similar to those used for the construction of the individual reporter systems (see supplemental methods in the supplemental material). More than 14,000 clones from each randomization were screened for maximal activity (+CO) on plates prepared with MOPS-buffered medium containing minimal (5 μM) isopropyl-β-D-thiogalactopyranoside (IPTG), 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, glucose, Ap, and Km. Plates were made anaerobic under a reducing H₂-CO₂ atmosphere in GasPak jars (Becton, Dickinson, and Co. [BD], Franklin Lakes, NJ) held at room temperature overnight, CO gas was then injected to ~1% as required, and the plates were incubated at 27°C for an additional day. Plates were exposed to air to allow color formation, and plasmids of the most active clones, showing CO-dependent activity when rescreened, were isolated and sequenced by using a primer that hybridized only to *lacZ* in the reporter construct (*lacZ*-R1) (Table 1). Low-activity colonies were selected from plates held at 4°C for at least 1 month. About 30% of these clones returned the template sequence (i.e., retained residue C₁₄) and were not included in the evaluation of sequences that promoted low activity.

Protein accumulation, purification, and CO binding. RcoM_{Bx-1} protein expression was performed by use of *E. coli* host strain UQ2892 (VJS6737 [49]) and growth conditions that maximized the formation of heme-containing protein and minimized the accumulation of the CO-bound form; these conditions differed from a protocol used previously that resulted in the isolation of predominantly CO-bound protein (22). Specifically, the growth medium contained (per liter of tap-distilled water) 20 g tryptone (Fisher Scientific, Fair Lawn, NJ), 2 g yeast extract (Fisher), 3 g Difco nutrient broth (BD), and 5 g NaCl. The medium pH was adjusted to 6.8, 5 ml of a 2-g/liter iron(III)-citrate (Sigma-Aldrich, St. Louis, MO) stock solution was slowly mixed in, and 400 ml was dispensed per 2.8-liter flask (Pyrex, catalog number 4420; Corning Life Sciences, Lowell, MA). These flasks were covered with gauze and autoclaved for 30 min at 121°C. Flasks were amended with a low level of IPTG (to 6 μM) and Ap and then inoculated at an optical density at 550 nm (OD₅₅₀) of 0.060 with a log-phase culture growing in the same medium (without IPTG). Cultivation occurred at 28°C with vigorous agitation (220 rpm/2.5-cm

throw) for 19 to 20 h, and cultures were then placed on ice and harvested by centrifugation, yielding ~10 g cells (wet weight) per liter of medium.

Cell pellets were resuspended at a ratio of ~ 1 g (wet weight)/3 ml buffer "A" (50 mM MOPS, 500 mM KCl, 0.5 mM dithiothreitol [DTT] [pH 7.4]) and then lysed by two passages through a French press at 18,000 lb/in². Supernatants were prepared by centrifugation (4°C for 60 min at an $R_{\max}$ of 20,800 $\times$ g), immediately frozen on crushed dry ice, and stored at -80°C . For RcoM_{Bx}-1 purification, the supernatant was amended with imidazole (pH 8.0) to 10 mM, and settled Ni-nitrilotriacetic acid (NTA) agarose resin (Qiagen) was gently mixed in at a ratio of 4 ml/10 ml extract. This suspension was held on ice, periodically remixed for 30 min, and centrifuged (5 min at $\sim 150 \times$ g), and the retained resin was then resuspended in 5 volumes of buffer A containing 10 mM imidazole. This suspension was similarly processed, and a second wash with buffer A containing 10 mM imidazole was performed, except that the slurry was poured into a column and the buffer was eluted. The matrix was next rinsed with 5 to 7 volumes of room-temperature buffer "B" (50 mM imidazole, 50 mM MOPS, 500 mM KCl [pH 7.7]), and the RcoM_{Bx}-1 protein was eluted in this buffer containing 220 mM imidazole. The protein solution was then adjusted to 40% saturation in (NH₄)₂SO₄, and the protein was pelleted. The isolated protein was dissolved in a minimal amount of sample buffer "C" (40 mM MOPS, 500 mM KCl, 5% glycerol [pH 7.4]), applied onto a buffer C-equilibrated Sephadex G-25 column (GE Healthcare, Piscataway, NJ), eluted, distributed into aliquots, frozen, and stored at -80°C . The sample protein concentration was determined with a bicinchoninic acid assay (Pierce, Rockford, IL), using bovine serum albumin as the standard, and the heme content was measured by the formation of a reduced pyridine hemochrome (55). Protein purities were determined to be $>90\%$ by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis.

The preparation and UV-visible (UV-Vis) spectral analysis of Fe(III), Fe(II), and Fe(II)-CO RcoM_{Bx}-1 forms were performed by using buffer C as previously specified (22), with the crucial addition of DTT to 2 mM to stabilize the Fe(II) and Fe(II)-CO forms. The analysis of CO binding to low concentrations of protein was done by microliter-scale injections of 0.5 or 1% CO-equilibrated anaerobic water into the completely sample-filled, dithionite-reduced, and sealed cuvette (2, 16). For these procedures, the spectrophotometer slit was increased to 2 nm, and 3 scans were averaged to reduce noise in the resulting spectra.

DNAse I protection (footprint) reactions. We used fluorescently (FAM)-labeled DNA targets and automated fragment analysis (62) to analyze the binding of RcoM_{Bx-1} to the *rcoM*₁-*coxM*₁ intergenic sequence (Fig. 1). Singly labeled (forward- or reverse-strand) dsDNA targets were prepared by the PCR amplification of pUX2424 (see Table SA1 in the supplemental material) using one fluorescently labeled primer plus the unlabeled cognate primer (Table 1). These reactions produced 397-bp DNAs that corresponded to the 367-bp WT sequence plus 17 and 13 bases of the *rcoM*₁ and *coxM*₁ coding regions, respectively. Non-WT FAM-

labeled DNA targets were similarly prepared, using the combination of Bx1-FAM-F plus the unlabeled cognate primer with template DNAs that introduced the desired alteration (pUX3236, pUX3295, pUX3296, pUX3300, or pUX3301) (see Table SA1 in the supplemental material). The FAM-labeled DNA fragments were purified by using QIAquick columns (Qiagen) and spectrophotometrically quantified.

Typically, aerobic reaction mixes (35 μ l) containing 250 ng (875 fmol) target DNA were combined with 10 μ l of RcoM_{Bx-1} protein (0, 25, 50, and 125 μ M in sample buffer C plus 2 mM DTT, dithionite reduced and CO bound as appropriate) and incubated at 22°C for 20 min. (As explained in Results, the Fe(II)-CO protein form was completely stable under these reaction conditions.) Next, 5 μ l of diluted RQ1 DNase I (0.2 units; Promega, Madison, WI) was mixed in. With these additions, the pH 7.1 reaction mix (50 μ l) consisted of 250 ng DNA, RcoM_{Bx-1} protein (0 to 25 μ M), and DNase I in a solution containing 40 mM MOPS buffer, 125 mM KCl, 5% glycerol, 2 mM DTT, 10 mM MgCl₂, and 2 mM CaCl₂. DNase activity was terminated at 1 or 2 min by transferring 20- μ l reaction samples into 80 μ l of ice-cold 150 mM EDTA. Stopped reaction mixtures were mixed with 500 μ l of "PB" buffer (Qiagen), applied onto QIAquick columns, rinsed with two 700- μ l "PE" buffer washes (Qiagen), and then eluted with 50 μ l sterile purified water. The purified samples were amended with carboxy-X-rhodamine (ROX)-labeled internal size standards and analyzed by using an Applied Biosystems 3730 analyzer (UWBC). FAM-labeled primers and plasmid pUX2424, used to prepare the WT footprint targets, were also used for Thermo Sequenase dye primer manual cycle sequencing reactions (USB Corp., Cleveland, OH), which were similarly analyzed. These data, aligned to the footprint results using internal size standards, served to precisely correlate footprint data with the DNA sequence (see Fig. SA2 in the supplemental material).

Fluorescence polarization assays for DNA binding. Reactions were performed by using 6- by 50-mm glass tubes containing 500 μ l (final volume) of a pH 7.2 solution containing 60 mM MOPS, 125 mM KCl, 5% glycerol, 48 μ g sheared salmon sperm DNA, 2 mM DTT, and 8 nM TR-labeled probe (Table 1). Fe(III) RcoM_{Bx-1} samples (25 μ l in sample buffer C) were added, and the tubes were septum sealed, mixed by gentle inversion, and then equilibrated to 25°C. Fe(II) protein was prepared by flushing the tube headspace with Ar and then injecting a freshly prepared anaerobic solution of Na dithionite to 0.5 mM, and the Fe(II)-CO form was prepared by flushing the Fe(II) samples with CO and mixing. The fluorescence polarization of each form was measured with a Beacon 2000 system (Invitrogen, Carlsbad, CA), with 594-nm excitation and 620-nm emission filters, using a 2-min equilibration delay and a 6-cycle read average.

Assay of RcoM_{Bx-1} and CO-dependent *lacZ* expression *in vivo*. Aerobic cultures of host strain UQ5853 bearing an RcoM_{Bx-1} expression plasmid (typically pUX2410, except as noted) plus a low-copy-number compatible reporter construct were assayed for activity by using MOPS-buffered medium containing 40 mM sodium acetate, as described previously (22), except that the 120-ml serum vials contained 4 ml of culture. Validation experiments demonstrated (i) that cell growth was not oxygen limited in these cultures, (ii) that the activity in Miller units (MU) remained constant in cultures harvested at OD₅₅₀ values of between 1.2 and 2.2 (and all samples were obtained within this range), and (iii) that cell growth and measured activities were unaffected by added CO levels of 0.9 to 3.4% (1.7% was routinely used) with both 10 and 100 μ M IPTG (i.e., at limiting and excess levels of RcoM_{Bx-1} accumulation) (data not shown). Activities were markedly affected by the level of RcoM_{Bx-1} accumulation, and therefore, assays were performed with different RcoM_{Bx-1} expression levels to establish that the reporter systems were not saturated or potentially affected by the accumulation of heme-free protein (see Results). β -Galactosidase activities were determined according to a standard protocol (40) and represent averages of data from at least 3 independent cultures per condition.

RESULTS

The preparation of heme-containing RcoM_{Bx-1} with minimal bound CO. Expression conditions that generated stable, high-quality, heme-containing, functional RcoM_{Bx-1} proteins are detailed in Materials and Methods, and we summarize two key points here. First, cultivation with minimal IPTG (6 μ M) was found to maximize the percentage of heme-containing (*holo*-) RcoM_{Bx-1}. IPTG levels of up to 50 μ M afforded more total *holo*-RcoM_{Bx-1} but with an increasing degree of the hemeless (*apo*-) form (data not shown). Second, the level of CO-bound RcoM_{Bx-1} that arose during culturing, which previously constituted the majority of the isolated protein (22), was minimized, and the preparation consistently generated pure RcoM_{Bx-1} (C-terminally 6 $\times$ His-tagged) protein with $\sim$ 10% in the CO-bound form. We previously used photolysis to slowly remove bound CO prior to spectral analysis (22), but this process was not practical for the more concentrated solutions of RcoM_{Bx-1} utilized for the *in vitro* DNA-binding assays described here. While the low level of the CO-bound form likely increased the basal activity, this background was insufficient to confound the data or alter the conclusion that RcoM_{Bx-1} binding to the DNA target was CO dependent (see below).

RcoM_{Bx-1} binds CO with high affinity and stability. Given the previous difficulty in isolating non-CO-bound RcoM_{Bx-1} (22), it is not surprising that equilibrium binding assays confirmed a high CO affinity. Assays performed using approximately 100 nM purified RcoM_{Bx-1} proteins (both N- and C-terminally His-tagged forms) resulted in a nearly linear, stoichiometric binding of CO at nanomolar concentrations (see Fig. SA3 in the supplemental material). This level of protein was the minimum feasibly assayed by spectrophotometric titration, and the results indicate a high CO affinity but do not permit its precise determination (2, 16). The finding that RcoM_{Bx-1} forms His-tagged at either the N or C terminus exhibited a high CO affinity suggests that this affinity is indeed a property of the RcoM_{Bx-1} PAS domain heme rather than a consequence of the appended His tag. Moreover, in an appropriate buffer that included the sulfhydryl protectant DTT, the purified Fe(II)-CO RcoM_{Bx-1} form was very stable: the spectrum of a dilute aerobic solution of this form remained unchanged for several hours when incubated at room temperature in the absence of CO or a heme-reducing agent (e.g., sodium dithionite) (data not shown). Under the same conditions, the Fe(II) form was less stable, as approximately 40% was oxidized to the Fe(III) state in 1 h, without the formation of a stable Fe(II)-O₂ intermediate (22). As a practical matter, this permitted the DNase I protection (footprinting) experiments to be performed reliably under conditions where Fe(II)-CO RcoM_{Bx-1} samples, prepared under strictly anaerobic conditions, were then introduced into aerobic footprinting reaction samples (total reaction time of <30 min).

Organization of the RcoM-binding region. The inspection and alignment of putative RcoM-regulated promoters upstream of the *cowN* and *coxM* genes of over a dozen organisms indicated three successive direct-repeat motifs with the invariant residues 5'-TTnnnG-3' (see Fig. SA4 in the supplemental material). This motif is repeated with a 21-base periodicity and bears some similarity to the consensus LytR domain DNA-binding motif (34) and the duplex oligonucleotide sequence utilized for determinations of the AgrA_C-DNA crystal structure (47), namely, 5'-T₁T₂T₃A₄A₅C₆A₇G₈T₉T₁₀A₁₁A₁₂G₁₃T₁₄A₁₅T₁₆-3' (one strand is indi-

cated; in this paper, binding-site residues are numbered according to this sequence). The conserved RcoM-binding-region T₉T₁₀nnnG₁₄ motifs invariably preserve the AgrA_C target T₉T₁₀ residues, centered at a 2-helical-turn periodicity, but the specific flanking AgrA_C-DNA contacts G₆' and G₁₃ are generally absent. However, in the AgrA_C structural analysis, it was noted that the two residues making these DNA contacts occurred within protein loops that are not highly conserved in the LytTR family, and therefore, variations in protein-DNA interactions were likely (47).

The set of three RcoM TTnnnG motifs appears to be duplicated in systems where adjacent *rcoM* and *coxM* genes are divergently transcribed. In *B. xenovorans* LB400, for example, duplicate sets of triplet motifs (designated motifs “a + b + c” and “d + e + f”; here a set of motifs is designated a “region”) appear between *rcoM*₁ and *coxM*₁, separated by a 104-bp interval (Fig. 1). Within each region, the motifs are consistently spaced exactly 21 bp apart and unidirectionally oriented, as indicated in the alignment, and the regions are 71% identical to each other (40 out of 56 bp) (underlined bases in Fig. 1). The designated “f” motif occurs adjacent to the putative σ⁷⁰ RNAP −35 binding sequence (Fig. 1), and as demonstrated below, the “d + e + f” region plus the RNAP-binding site constitute a functional promoter where RcoM_{Bx}-1 binding activates the expression of *coxM*₁. This activity was monitored *in vivo* by creating *coxM*₁::*lacZ* transcriptional fusions (see supplemental methods and Fig. SA1 in the supplemental material). The “a + b + c” region was bound by RcoM_{Bx}-1 but unnecessary for *coxM*₁ transcription (see below); we hypothesize that the binding of RcoM_{Bx}-1 to the “a + b + c” region negatively autoregulates *rcoM*₁ expression.

How does purified RcoM_{Bx}-1 interact with DNA? We used DNase I protection “footprint” reactions to assess the binding of purified RcoM_{Bx}-1 with FAM-labeled (forward- or reverse-strand) dsDNAs corresponding to the 367-bp *rcoM*₁-*coxM*₁ intergenic sequence plus 17 and 13 bp of the *rcoM*₁ and *coxM*₁ genes, respectively (Fig. 1). The pools of FAM-labeled fragments generated in these reactions were analyzed and precisely aligned with the DNA sequence (see Fig. SA2 in the supplemental material).

Footprint reactions performed in the presence of 10 μM Fe(II) RcoM_{Bx}-1 were very similar to control reactions (no RcoM_{Bx}-1) and showed evidence of only slight hyperactive cutting (Fig. 2A), which presumably reflects the ~10% level of the contaminating Fe(II)-CO form present in the protein as purified. (Robust evidence of the inactivity of the Fe(III) and Fe(II) forms and DNA binding of the Fe(II)-CO protein under more controlled conditions were established by fluorescence polarization assays, as described below.) Footprint reactions performed with different levels of the Fe(II)-CO RcoM_{Bx}-1 protein defined protected segments (Fig. 2A and B) that overlapped the designated “a” through “f” motifs (Fig. 1). Adjacent to and interspersed among the protected sequences are sites where DNase I activity is enhanced by RcoM_{Bx}-1 binding: these sites were categorized as being of modest (3- to 10-fold increase in fragment intensity) or substantial (>10-fold increase) hyperactivity. In contrast, the DNase I-generated fragment pattern of the flanking DNA and the interval between the “c” and “d” binding sites was unaffected by the presence of the RcoM_{Bx}-1 protein. The patterns of protection and hyperactive cutting are designated below each set of overlapped footprint electropherograms by sets of bar-shaped and triangular symbols in Fig. 2A and B and were aligned with DNA sequence data obtained by using the same FAM-labeled primers (see Fig.

SA2 in the supplemental material), as summarized in Fig. 2C. Within the recognized limitations of DNase I footprinting, which cuts DNA unevenly and modestly overstates protection in the 3' direction (18, 53), this analysis shows protection that spans each TTnnnG motif. Where this protection appears to be less extensive—for example, for the “a” binding site of the reverse strand and the “f” binding site of both strands—one notes the general absence of DNase I-generated fragments regardless of the level of RcoM_{Bx}-1 rather than evidence indicating the absence of RcoM_{Bx}-1 binding. In addition, in both the “b” and “e” sites, the hyperactive DNase I cuts show a regular staggered pattern averaging 5 bp (±1 standard deviation) apart along both DNA strands. This pattern is consistent with an arrangement along one face of the DNA helix opposite the positions protected by bound RcoM_{Bx}-1.

Several lines of evidence indicated independent “a + b + c” and “d + e + f” interregion binding but synergistic intraregion binding. First, for both forward and reverse WT template DNAs, 5 and 10 μM Fe(II)-CO RcoM_{Bx}-1 additions that fully protected the “a + b + c” region only partially protected the “d + e + f” region, suggesting that these regions have different binding affinities (Fig. 2A and B). In addition, several non-WT templates bearing individual “d,” “e,” or “f” conserved-residue alterations (TTnnnG changed to GGnnnG) or a spacing change (+10 bases added between the “d” and “e” binding sites, at the position indicated by the arrow in Fig. 2C) significantly reduced binding to the “d + e + f” region, while binding to the “a + b + c” region served as an unchanged control (Fig. 3). Notably, although the RcoM_{Bx}-1 protection of the native “f” site is difficult to assess because of the minimal DNase I-generated fragment profile, the altered “f” sequence both modifies cleavage at this site and diminishes the protection of itself and of the unaltered “d” and “e” sites (Fig. 3), confirming the significance of the “f” site for RcoM_{Bx}-1 binding and demonstrating intraregion cooperativity. Similarly, the TTnnnG-to-GGnnnG alteration of the “b” motif diminished protection within the “a + b + c” region, while protection within the “d + e + f” region was unaffected (data not shown).

Finally, a template bearing an enhanced “e” binding site (designated “e ↑” [the derivation is described below]) shows an improved protection of the “d” and “e ↑” sites plus typical hyperactive DNase I activity flanking the “d” and “f” sites when assayed at a lower level of Fe(II)-CO RcoM_{Bx}-1 protein (Fig. 3). This result is consistent with the *in vivo* activity and *in vitro*-binding properties of the “e ↑” site (see below) and is noted here as further support of intraregion binding cooperativity. In summary, the footprint results substantiate specific but independent RcoM_{Bx}-1 binding to the “a + b + c” and “d + e + f” regions, indicate higher-affinity binding to the former region, and reveal synergistic RcoM_{Bx}-1-binding interactions within each region.

Importance of *holo*-RcoM_{Bx}-1 and the triple-motif-binding region for *in vivo* function. We created a dual-plasmid *in vivo* system for the analysis of RcoM_{Bx}-1-dependent activity: the protein was expressed under the control of IPTG from one plasmid, while a second low-copy-number construct bore an RcoM_{Bx}-1-binding target *coxM*₁::*lacZ* transcriptional fusion. The binding target was flanked by unique BamHI and SalI sites to permit substitutions (see also supplemental methods and Fig. SA1 in the supplemental material). Regardless of the reporter system, the accumulation of β-galactosidase was similarly affected by the concentration of IPTG ([IPTG]) in the medium: for example, with the

FIG 2 Identification of the *RcoM_{Bx-1}*-binding sites on the forward and reverse strands of the *rcoM1-coxM1* intergenic DNA. (A and B) The 397-base FAM-labeled forward (A) and reverse (B) dsDNAs were incubated with various levels of purified *RcoM_{Bx-1}* protein [Fe(II)-CO form, except where an Fe(II) sample is noted (*)], digested with DNase I, and then analyzed as described in Materials and Methods. The electropherograms indicate sized fragment pools between 60 and 340 bases with peak intensities in absorbance units (vertical scale bar = 1,000 absorbance units). These assays identified two ~60-base protected regions and several hyperactive DNase I sites, indicated beneath each figure by horizontal bars (protected positions) and triangles (▲ for the forward strand and ▼ for the reverse strand). The smaller triangles indicate sites with 3- to 10-fold increases in DNase I activity, while larger triangles indicate sites with >10-fold increases in DNase I activity compared to the zero-*RcoM_{Bx-1}* control. The precise positions of DNase I protection and hyperactivity were determined by an alignment with sequencing data prepared by using the same FAM-labeled primers (see Fig. SA2 in the supplemental material). (C) Summary of the protection assays for both DNA strands. The six TTnnG invariant motifs are indicated by their designation indicated above the top (forward) strand (e.g., "a-----"), protected bases on both strands are boxed, and *RcoM_{Bx-1}*-binding-dependent hyperactive DNase I sites are denoted by triangles, as described above for panels A and B. The ↓ symbol indicates the position of the 10-bp insertion (see the text). nt, nucleotides.

standard pUX2410 *RcoM_{Bx-1}* expression plasmid plus a compatible pUX3010 reporter system plasmid that incorporated the native "a to f" (i.e., "a + b + c" and "d + e + f") binding regions (strain UQ5877), activities (with CO) rapidly increased with an IPTG concentration of up to 50 μ M, a trend which correlates with the accumulation of heme-containing *RcoM_{Bx-1}* measured in the

same medium (Fig. 4A). Similar activity trends were observed for other reporter configurations (Fig. 4B and C), and in each instance, reporter constructs showed limited β -galactosidase activity at 10 μ M IPTG and response saturation at around 50 μ M IPTG. Given their CO dependence, these activities presumably represent accumulations of *holo-RcoM_{Bx-1}* and the formation of

FIG 3 Effect of template alterations on the binding of purified RcoM_{Bx-1}. FAM-labeled forward-strand templates were prepared with (i) wild-type DNA ("WT" Template); (ii) an alteration of the conserved TTnnnG motif to GGnnnG in the "d," "e," or "f" binding sites ("TT→GG" Templates); (iii) a 10-base insertion between the "d" and "e" binding sites ["d-(+10)-e" Template]; or (iv) an "e" binding-site sequence that improved activity *in vivo* ("e↑" Template). The electropherograms represent fragment pools of between 60 and 340 bases obtained after the binding of the indicated level of the Fe(II)-CO RcoM_{Bx-1} form and DNase I digestion.

the functional RcoM_{Bx-1} oligomer, which remains undefined. At minimum, we used IPTG levels of 10 and 50 μM to ensure that assay mixtures were not saturated and assays reflected a dynamic response of the particular reporter system and expressed RcoM_{Bx-1} protein.

The *in vitro* footprint evidence for independent RcoM_{Bx-1} binding to the "a + b + c" and "d + e + f" regions and synergistic binding within the "d + e + f" region was substantiated *in vivo* by using strains that differed only in the extent of the RcoM_{Bx-1}-binding region fused to the *lacZ* reporter. The "a + f" and "d +

FIG 4 *In vivo* reporter system activity depends upon RcoM_{Bx-1} accumulation, the CO effector, and reporter configuration. (A) The IPTG-regulated accumulation of heme-containing RcoM_{Bx-1} (C-terminally 6×His tagged, in nmol heme/mg soluble protein) from expression plasmid pUX2410 is superimposed on the β-galactosidase activity (in Miller units [MU]) expressed from the pUX3010 reporter system that bears the native "a + b + c" and "d + e + f" binding regions. (B and C) Accumulation of β-galactosidase activity in strains containing pUX2410 plus different RcoM_{Bx-1}-binding-region reporter systems cultivated in the absence (open symbols) or presence (filled symbols) of CO at different levels of IPTG. (B) Low activity of strains UQ5874 (squares) (the reporter bears the "f" binding site) and UQ5875 (diamonds) (the reporter bears the "e + f" binding region). (C) High activity of strains UQ5876 (triangles) (the reporter bears the "d + e + f" binding region) and UQ5877 (circles) (same as described above for panel A).

TABLE 2 Reporter system comparisons

Reporter system and strain (expressed RcoM) ^a	Avg β -Galactosidase activity (MU) (95% confidence interval) ^b			
	10 μ M IPTG		50 μ M IPTG	
	–CO	+CO	–CO	+CO
Three-binding-site (“d + e + f” region) reporter systems				
UQ6172 (expresses RcoM _{Bx} -1 [untagged])	12	282 (29)	78 (9)	317 (20)
UQ6175 (expresses RcoM _{Bx} -1 [N-terminally 6 $\times$ His tagged])	18 (3)	279 (4)	112 (6)	243 (10)
UQ5876 (expresses RcoM _{Bx} -1 [C-terminally 6 $\times$ His tagged])	4 (1)	167 (17)	13 (2)	236 (12)
UQ6173 (expresses RcoM _{Bx} -2 [untagged])	4 (1)	83 (3)	10 (1)	169 (1)
Equivalence of binding regions and effect of their fusion ^c				
UQ5880 “a + b + c” binding region substituted for the “d + e + f” binding region	6 (2)	289 (36)	54 (12)	331 (33)
UQ6148 fused “a + b + c + d + e + f” binding region	4 (1)	34 (2)	11 (1)	42 (3)
Evaluation of the conserved TTnnnG motif in the “d,” “e,” or “f” binding site ^{c,d}				
UQ6018 “dC + e + f” reporter (“d” motif, TTnnn <u>C</u>)	3 (1)	39 (6)	4 (1)	83 (3)
UQ6020 “d + eC + f” reporter (“e” motif, TTnnn <u>C</u>)	3 (1)	57 (7)	5 (1)	118 (3)
UQ5964 “d + e + fC” reporter (“f” motif, TTnnn <u>C</u>)	4 (1)	9 (1)	4 (1)	15 (1)
UQ6046 “dx + e + f” reporter (“d” motif, <u>G</u> GnnnG)	3 (1)	18 (5)	4 (1)	30 (2)
UQ6211 “d + ex + f” reporter (“e” motif, <u>G</u> GnnnG)	3 (1)	5 (1)	3 (1)	9 (1)
UQ6218 “d + e + fx” reporter (“f” motif, <u>G</u> GnnnG)	3 (1)	7 (2)	4 (1)	11 (2)

^a Except as noted (strains UQ6172, UQ6173, and UQ6175), all systems pair the indicated reporter construct with pUX2410, a compatible plasmid that provides IPTG-dependent expression of RcoM_{Bx}-1 (C-terminally 6 $\times$ His tagged).

^b Data indicate averages of data from at least three independent cultures per condition.

^c Strain UQ5876 is the appropriate control for the data presented.

^d Double-underlined positions indicate differences from the wild-type sequence.

e + f” reporter systems showed similar CO-dependent responses over a range of [IPTG]-dependent RcoM_{Bx}-1 accumulations, suggesting that the “a + b + c” region is dispensable for *coxM*₁ regulation (Fig. 4C). In contrast, reporter strength was diminished 15-fold in a two-binding-site (“e + f”) reporter system, and the CO- and [IPTG]-dependent response was eliminated in the single-site (“f”) reporter system (Fig. 4B). Given the independence of RcoM_{Bx}-1 binding to the “a + b + c” and “d + e + f” regions demonstrated by footprinting and the equivalent *in vivo* activities of the “a to f” and “d + e + f” reporter systems, assays were routinely performed using the “d + e + f” reporter system or the “e + f” reporter system, when a lower level of activity was desired.

Both *in vitro* and *in vivo* assays routinely employed RcoM_{Bx}-1 (C-terminally 6 $\times$ His tagged) expressed from pUX2410. Other protein forms were tested for activity *in vivo*: the untagged (UQ6172) and N-terminally 6 $\times$ His-tagged (UQ6175) RcoM_{Bx}-1 versions proved to be highly active, while the untagged RcoM_{Bx}-2 protein (UQ6173) appeared to be less active (Table 2). The finding that these different RcoM proteins did not provide equivalent CO-dependent activities, especially at the 10 μ M IPTG level, likely reflects differences in the accumulation of the *holo*-RcoM proteins and their capacity to form a functional oligomer as well as possible differences in their inherent activities. Indeed, under the conditions used for protein expression, we observed that the *holo*-RcoM_{Bx}-2 protein accumulates approximately half as well as the *holo*-RcoM_{Bx}-1 protein (data not shown), consistent with the lower level of activity seen for the former protein (Table 2). Among these proteins, the differences in apparent CO-independent activity at higher levels of protein expression (50 μ M IPTG) might reflect several undetermined factors, including differences

in the affinity for ambient CO or differences in accumulation levels: given sufficient expression, even a low percentage of CO-bound protein would effect a significant response. Nevertheless, with limiting accumulation, all RcoM proteins clearly demonstrate CO-dependent activity.

Thus far, the data had indicated a high level of sequence identity (Fig. 1) and similar patterns of RcoM_{Bx}-1 binding to the “a + b + c” and “d + e + f” regions (Fig. 2C) but no evidence that the two regions would be functionally similar if placed in the same context. To test this, we prepared a reporter system where the “d + e + f” region of pUX3009 (UQ5876) was precisely replaced by the “a + b + c” region, creating pUX3080 (UQ5880). This reporter system was highly active, indicating that a properly positioned “a + b + c” region in its native orientation was transcriptionally competent (Table 2). Finally, given that the reporter activity markedly increased with the number of adjacent binding sites (“d + e + f” > “e + f” > “f”) (Fig. 4B and C), we prepared a six-site reporter where the “a + b + c” region was positioned upstream of the “d + e + f” region such that all six sites were contiguously spaced 21 bp apart on center (in the WT, the two regions are separated by 104 bp) (Fig. 1). Despite the similarities in RcoM_{Bx}-1 binding to each region (Fig. 2C) and despite their functional equivalence (compare UQ5876 and UQ5880) (Table 2), the concatenated “a + b + c + d + e + f” reporter system had poor activity (UQ6148) (Table 2). This result is inconsistent with a model where RcoM_{Bx}-1 proteins bind simply as a linear complex along the DNA and, along with the footprinting evidence of RcoM_{Bx}-1 binding along one face of the DNA helix (Fig. 2C) and evidence of LytTR domain-induced DNA bending, would be more easily explained by formation of a curved nucleoprotein complex (see Discussion).

TABLE 3 Alterations of the putative $-35/-10$ sequence to create RcoM-independent variants

Strain and alteration(s) ^a	Avg β -Galactosidase activity (MU) (95% confidence interval) ^b			
	0 μ M IPTG		50 μ M IPTG	
	–CO	+CO	–CO	+CO
UQ5876 “d + e + f” reporter (control)	3 (1)	5 (1)	13 (2)	236 (12)
UQ6177 “d + e + f” reporter with improved $-35/-10$ sequence [5'-TTGACA-(18n)-TATCCT-3']	722 (18)	715 (36)	616 (29)	490 (23)
UQ6178 (same as UQ6177 but with the “f” binding motif altered to TTnnnC)	777 (73)	768 (70)	743 (107)	610 (40)

^a All systems pair the indicated reporter construct with pUX2410, a compatible plasmid that provides IPTG-dependent expression of RcoM_{Bx-1} (C-terminally 6×His tagged). Double-underlined positions indicate differences from the wild-type sequence.

^b Data indicate averages of data from at least three independent cultures per condition.

Functional evidence for the $-35/-10$ sequence designation and limits of maximal β -galactosidase activity. Reporter variants were also constructed in the putative $-35/-10$ RNAP-binding sequence to substantiate its functional significance, distinct from the adjacent RcoM_{Bx-1}-binding sites (Fig. 1), and alterations that made this sequence more like the σ^{70} consensus (46) indeed resulted in high RcoM_{Bx-1}- and CO-independent activities (UQ6177) (Table 3). The finding that the constitutive activities were 2- to 3-fold higher than the maximum seen with the RcoM_{Bx-1}-dependent systems (e.g., UQ5876 and UQ5877) (Fig. 4C) is consistent with the supposition that the RcoM_{Bx-1}-dependent maxima reflect the ability of functional RcoM_{Bx-1} to promote stable RNAP binding rather than an inherent limitation of the reporter constructs. Moreover, in contrast to data indicating that the “f” motif G₁₄ residue is crucial for RcoM_{Bx-1} function (see below), the activities of the constitutive reporter (UQ6177) were not reduced by the alteration of this position (UQ6178) (Table 3). This supports a model of transcriptional organization wherein RcoM_{Bx-1}- and RNAP-binding events occur at adjacent but distinct sites (Fig. 1).

Functional significance of the conserved T₉T₁₀nnnG₁₄ RcoM_{Bx-1}-binding-motif residues. Akin to the results of the footprinting experiments performed with templates in which individual “d,” “e,” or “f” conserved motifs were altered (Fig. 3), we assessed the activities of reporter systems with similar changes. Both single-base alterations (UQ6018, UQ6020, and UQ5964) and dual-position changes (UQ6046, UQ6211, and UQ6218) diminished activities (Table 2). The T₉T₁₀→G₉G₁₀ alterations were generally more deleterious than the G₁₄→C₁₄ changes, although the latter mutation was particularly detrimental when introduced at the “f” motif, reducing activity by >15-fold. This contrasted with the result for the UQ6178 constitutive variant described above (Table 3) and confirmed the importance of the “f” motif for RcoM_{Bx-1}-dependent transcriptional activity. These data are consistent with the footprinting results and affirm the necessity of each motif in the “d + e + f” region for RcoM_{Bx-1} binding and function.

The “e” binding site is nonoptimal and readily improved. To gain insight into the RcoM_{Bx-1}-DNA interaction, we sought DNA target sequences that provided improved reporter activity because of increased RcoM_{Bx-1} affinity. The initial *in vivo* screen utilized an “e + f” binding-region template (pUX3025) (see Table SA1 in the supplemental material) and primers that randomized six “e” site bases (for the forward strand, 5'-T₁C₂C₃G₄N₅N₆N₇G₈T₉T₁₀G₁₁N₁₂N₁₃N₁₄C₁₅G₁₆-3', where the randomized residues are indicated by “N”). After transferring the mutagenized binding-re-

gion pool into the reporter system and ultimately into the UQ5854 host, followed by screening for CO-dependent activity using a low level of IPTG to limit RcoM_{Bx-1} accumulation (5 μ M) (see Materials and Methods), we found that active clones invariably presented a “G” residue at position N₁₄ (21 out of 21 reporter constructs), while none of the six randomized positions was conserved in the inactive clones (17 reporter constructs sequenced). The absolute conservation of residue G₁₄ in active clones is consistent with its invariance in the TTnnnG motif (Fig. 1) and functional importance in the “d,” “e,” and “f” motifs (Table 2). There was also modest evidence in the active reporter constructs for the conservation of residue C₆ (11 out of 21 reporter constructs), but the requirement for G₁₄ dominated.

To expand on these results and avoid the dominating requirement for residue G₁₄, we subsequently started with an “e + f” binding-region template DNA bearing an “e” motif C₁₄ residue and then utilized primers that mutagenized “e” site residues but restored residue G₁₄. The low background activity afforded by this template and the restoration of residue G₁₄ ensured that even clones with low activity were indeed the product of mutagenesis and permitted the evaluation of “e” site residues of secondary importance to the overriding requirement for G₁₄ (see Materials and Methods). Two sets of 6-base randomizations were performed by using primer Bx1ex1-F (for N₃-to-N₈ randomization) or Bx1ex2-F (for N₁₁-to-N₁₃ plus N₁₅-to-N₁₇ randomization) and their complements (Table 1), and pools of randomized binding regions were screened as described above. Sequences of 37 different CO-dependent high-activity clones resulting from the N₃-to-N₈ randomization returned reporters with a near-universal conservation of residues C₃ (35/37 clones) and C₆ (36/37) and a high level of conservation of residues C/A₇ (36/37) and G/A₈ (33/37). Sequences of 20 different CO-dependent high-activity clones resulting from the N₁₁-to-N₁₇ randomization returned reporters with a high level of conservation of residues C₁₁ (17/20 clones) and A₁₂ (19/20). As expected, sequences of >16 randomly picked clones with low reporter activity (nontemplate carryover) from each randomization showed no binding sequence base preferences (data not shown).

Based upon these data, we constructed a reporter that merged higher-activity sequences from the randomizations to create an enhanced “e” site (here designated “e[↑]”), 5'-T₁C₂C₃T₄A₅C₆A₇G₈T₉T₁₀C₁₁A₁₂C₁₃G₁₄C₁₅A₁₆-3' (double-underlined residues denote differences from the WT “e” sequence). When assayed under conditions of limiting RcoM_{Bx-1} expression (10 μ M IPTG) the “e[↑] + f” reporter proved to be 6-fold more active than the “e + f” control (compare UQ6139 and UQ5875) (Table 4), and the

TABLE 4 Enhanced-activity “e ↑” binding site

Reporter system and strain ^a	Avg β-Galactosidase activity (MU) (95% confidence interval) ^b					
	2 μM IPTG		10 μM IPTG		50 μM IPTG	
	–CO	+CO	–CO	+CO	–CO	+CO
Dual-binding-site reporters						
UQ5875 “e + f” (control)	ND	ND	2 (1)	6 (1)	3 (1)	13 (2)
UQ6139 “e ↑ + f” (“e ↑” = <u>CTACAGTT</u> <u>CACGCAC</u>)	ND	ND	2 (1)	37 (8)	6 (1)	75 (7)
UQ6140 “e + e ↑” (“e ↑” = <u>CTACAGTT</u> <u>CACGCAC</u>)	ND	ND	2 (1)	2 (1)	2 (1)	4 (1)
UQ6116 “e ↑ + e ↑”	ND	ND	2 (1)	14 (1)	3 (1)	32 (3)
Triple-binding-site reporters						
UQ5876 “d + e + f” (control)	3 (1)	22 (6)	4 (1)	167 (17)	13 (2)	236 (12)
UQ6141 “e ↑ + e + f” (“e ↑” = <u>CTACAGTT</u> <u>CACGCAC</u>)	ND	ND	10 (1)	122 (8)	48 (5)	149 (5)
UQ6142 “d + e ↑ + f” (“e ↑” = <u>CTACAGTT</u> <u>CACGCAC</u>)	2 (1)	104 (25)	8 (3)	398 (19)	76 (12)	461 (21)
UQ6143 “d + e + e ↑” (“e ↑” = <u>CTACAGTT</u> <u>CACGCAC</u>)	ND	ND	3 (1)	38 (2)	5 (1)	55 (1)

^a Note that in all instances, the enhanced-activity “e ↑” binding sequence (5′-CTACAGTTACGCAC-3′) is identical; the double-underlined positions denote differences from the wild-type sequence. All systems pair the indicated reporter construct with pUX2410, a compatible plasmid that provides IPTG-dependent expression of RcoM_{Bx}-1 (C-terminally 6×His tagged). ND, not done.

^b Data indicate averages of data from at least three independent cultures per condition.

“d + e ↑ + f” reporter was 2-fold more active than the “d + e + f” control (compare UQ6142 and UQ5876) (Table 4). The enhanced activity of the “d + e ↑ + f” reporter was more significant (5-fold increase) under conditions of very low levels of RcoM_{Bx}-1 accumulation (2 μM IPTG), where the enhanced binding (as demonstrated by the footprinting assay [Fig. 3]) presumably afforded a much fuller occupancy of the “d + e ↑ + f” region than of the “d + e + f” control. We do not claim that the “e ↑” sequence represents an optimal-affinity binding site given its derivation from the independent randomizations of two portions of this binding site, yet it is clear that this “e ↑” sequence both improves RcoM_{Bx}-1 binding assayed *in vitro* (Fig. 3) and enhances activity *in vivo* (Table 4). Conversely, the native “e” site does not provide maximal RcoM_{Bx}-1 binding and function, although it may be optimal for proper regulation in its native organism (see Discussion). In addition, while the conserved motif evident in an alignment of RcoM-binding regions is limited to 3 residues (TT nnnG) (see Fig. SA4 in the supplemental material), the N₃-to-N₈ randomization results clearly demonstrate that RcoM_{Bx}-1 interactions extend well upstream of this motif.

Are enhanced binding sites equivalent and additive? The “d,” “e,” and “f” motif alterations detailed in Table 2 indicated that changes to the “f” motif were particularly deleterious but that all TTnnnG motif changes diminished reporter function. We therefore asked the converse, i.e., whether the improved “e ↑” sequence could be substituted at other positions with similar results. This proved not to be the case, as the “e ↑” substitution at the “f” site in either the “e + f” or “d + e + f” reporter system nearly eliminated activity in the former system (UQ6140) (Table 4) or reduced it more than 4-fold in the latter (UQ6143) (Table 4), even under conditions of maximal RcoM_{Bx}-1 expression (50 μM IPTG). The “e ↑ + e ↑” reporter construct in strain UQ6116 provided increased activity relative to that of the WT control but half the activity of the “e ↑ + f” system. The substitution of the “e ↑” sequence for the “d” site in the “d + e + f” reporter (UQ6141) (Table 4) reduced activity relative to that of the control by less than 2-fold. Thus, in a functional assay, the context of the enhanced binding sequence matters: the “e” and “f” sites in particular are not equivalent. Presumably, this reflects nonequivalent

interactions within the nucleoprotein complex where RcoM_{Bx}-1 bound at the “e” site interacts with flanking RcoM_{Bx}-1 bound at the “d” and “f” positions, while RcoM_{Bx}-1 bound at the “f” site likely interacts with the “e” site-bound RcoM_{Bx}-1 and RNAP.

CO dependence of DNA binding. Fluorescence polarization assays of RcoM_{Bx}-1 binding to Texas Red (TR)-labeled DNA targets designated according to the “a” to “f” conventions (Table 1) were employed to assess binding under conditions where the protein form [heme Fe(III), or Fe(II) or Fe(II)-CO] was readily controlled, in order to unambiguously demonstrate CO-dependent binding. Unfortunately, while preliminary tests under various assay conditions with 0.1 to 8 μM purified RcoM_{Bx}-1 protein demonstrated CO-dependent binding, they failed to attain a limiting upper asymptote, which precluded the determination of a dissociation constant (1, 28). This may reflect the propensity of LytTR domain proteins to aggregate (13, 25, 31, 47) and/or the fact that a triple-binding-site probe was not tested. As explained in Discussion, we became concerned that the RcoM_{Bx}-1-induced bending of longer DNA probes would confound an assay that assesses the rotation of the nucleoprotein complex, and we compared only the binding to one- or two-site probes using a single protein concentration (6 μM) that maximized the response differential (with or without added CO). As indicated above, approximately 10% of each RcoM_{Bx}-1 protein preparation was purified in the very stable Fe(II)-CO state and resulted in marginal binding in the “Fe(III)” and “Fe(II)” samples, where binding occurred.

RcoM_{Bx}-1 binding to a “b + c” probe was promoted by the addition of CO to the Fe(II) protein (sample A) (Fig. 5), and when it occurred, binding to the other probes was likewise CO dependent (samples D, E, F, and G). Consistent with footprinting data that showed a better protection of the “a + b + c” binding region than of the “d + e + f” binding region, binding to the “e + f” probe was notably poorer (sample B) than binding to the “b + c” target (sample A). Binding to a shorter single-site “c” (not shown) or “e ↑” (sample C) target was not evident. In accordance with the enhanced protection of the “e ↑”-containing template (Fig. 3) and the increased activity of the “e ↑ + f” reporter (Table 4), the “e ↑ + f” probe showed CO-dependent binding (sample D) which was similar to that of the “b + c” target (sample A) and

FIG 5 RcoM_{Bx-1} binding to DNA targets *in vitro*. DNA binding was measured by fluorescence polarization of samples containing 6 μ M purified protein incubated under three conditions: aerobic (open boxes), reduced (gray bars), and reduced plus CO (black bars). The tested RcoM_{Bx-1} proteins bore either C-terminal or N-terminal 6 $\times$ His tags (designated “C-His” and “N-His,” respectively) and were either wild type (“WT”) or altered in a residue predicted to directly contact DNA (“H185A”). Texas Red-labeled DNA probes corresponded to the “b + c,” “e + f,” “e $\uparrow$,” “e $\uparrow$ + f,” or “e $\uparrow$ + e $\uparrow$ ” sequences, as specified in Table 1. The figure summarizes data from multiple assays, with error bars indicating 95% confidence intervals.

clearly stronger than that with the native “e + f” sequence (sample B). In contrast to the *in vivo* data, however, where the introduction of the “e $\uparrow$ ” sequence at the “f” position reduced reporter activity (Table 4), *in vitro* bindings to the “e $\uparrow$ + f” and “e $\uparrow$ + e $\uparrow$ ” targets (samples D and E) were similar, even with a lower RcoM_{Bx-1} level (1 μ M) (data not shown). This difference between the *in vitro* binding assay and the *in vivo* functional assay again suggests that the “f” site, which lies immediately adjacent to the promoter (Fig. 1), serves dual roles: (i) binding RcoM_{Bx-1} and (ii) positioning RcoM_{Bx-1} appropriately for interactions with RNAP. In this scenario, it is plausible that a higher-affinity binding of RcoM_{Bx-1} to the “e $\uparrow$ + e $\uparrow$ ” target (determined *in vitro*) might interfere with its optimal interaction with RNAP (when measured *in vivo*) and result in different *in vivo/in vitro* assay results.

Finally, samples F, G, and H (Fig. 5) indicate additional tests and controls, all employing the “e $\uparrow$ + e $\uparrow$ ” probe DNA. Unlike the CO-dependent transcription factor CooA, where DNA binding is divalent cation dependent (60), the addition of Mg²⁺ and Ca²⁺ at levels used in the DNase I footprint assay reduced the affinity of RcoM_{Bx-1} for the target DNA (compare samples E and F). The placement of the 6 $\times$ His tag at the N terminus of the protein instead of the C terminus slightly reduced DNA binding (compare samples E and G), indicating again that the appended purification tag does not play a significant role in RcoM_{Bx-1} function. In contrast, the change of a single conserved RcoM_{Bx-1} LytTR domain residue (H185A) that corresponds to residue H169 of AgrA_C (one of only two amino acids that make specific DNA contacts [47]) indeed eliminated DNA binding (sample H). These results demonstrate that the DNA-binding activity of the RcoM_{Bx-1} single-component transcriptional activator depends upon the formation of the Fe(II)-CO state and involves a predicted LytTR domain-DNA target interaction. These results also substantiate differences in binding-region affinities and evidence of cooperative binding within each region, and they confirm the higher-affinity binding engendered by the “e $\uparrow$ ” sequence.

DISCUSSION

A single-component LytTR domain transcription factor. Most LytTR domain regulators are two-component systems wherein

effector binding to the receiver component typically regulates the phosphorylation of a separate DNA-binding component (5, 15, 34). In contrast, the RcoM proteins, and almost certainly the similar but biochemically uncharacterized CoxC/H proteins (12, 22, 45), are single-component LytTR domain transcription factors that merge effector sensing with transcriptional activation. Both functions are readily assessed: spectroscopic assays demonstrated the binding of CO to the RcoM_{Bx-1} or RcoM_{Bx-2} heme cofactor (22, 29), and, as shown here, footprinting and fluorescence polarization measurements defined the iterative binding of the active protein to sets of direct-repeat DNA sites.

These DNA sites were demonstrated by DNase I protection assays to overlap six conserved TTnnnG motifs, designated motifs “a” through “f,” that were evident in the 367-bp *rcoM₁-coxM₁* intergenic sequence (Fig. 1 and 2). Sets of three motifs were grouped into two regions, and RcoM_{Bx-1} bound independently to each region. In contrast, binding within a region was cooperative (Fig. 3), and maximal *in vivo* activity depended upon triplet binding sites (“a + b + c” or “d + e + f”) positioned adjacent to the –35/–10 sequence (Table 2 and Fig. 4). Transcriptional function (Tables 2 and 3 and Fig. 4) and DNA binding (Fig. 5) were CO dependent, although active RcoM_{Bx-1} displayed a low affinity for the DNA target. To some degree, the lower affinity in the footprinting assay reflects the inclusion of divalent cations in the reaction mixture, which is necessary for DNase I activity but inhibitory for RcoM_{Bx-1} binding (Fig. 5), but the micromolar-level affinity of the protein in the fluorescence polarization assays likely also reflects the choice of target DNA probes. Given the binding synergy within regions (Fig. 3) and the significantly increasing activity *in vivo* proportional to the extent of the binding sequences (“d + e + f” > “e + f” > “f”) (Fig. 4), one would predict a higher *in vitro* binding affinity for a “d + e + f” (or “d + e $\uparrow$ + f”) target probe. This has not been assessed out of concern that the fluorescence polarization assay would be confounded by conformational changes in the target DNA (see below).

Variation and novelty of RcoM_{Bx-1}-DNA and LytTR domain-DNA interactions. At first glance, the RcoM_{Bx-1}-DNA TTnnnG conserved motif only modestly resembles the pattern of

FIG 6 Comparison of AgrA_C-DNA and RcoM_{Bx-1}-DNA interactions. (A) The structure of the C-terminal DNA-binding domain of *S. aureus* AgrA (AgrA_C) bound to a consensus sequence indicates two base-specific DNA contacts with residues H169 and R233 (47). (B) The analogous base-specific interaction (dotted line) between the native “e” motif G₁₄ position (circled) with conserved RcoM_{Bx-1} residue H185. (C) The higher-binding-affinity “e↑” DNA sequence incorporates 6 alterations (double underlined) that increase similarity to the AgrA consensus sequence and introduce a potential interaction (dotted line) to conserved RcoM_{Bx-1} residue R254.

the LytTR-DNA-binding motif (34), most notably in maintaining the “TT” core residues precisely spaced at two-helical-turn intervals (Fig. 1). However, other LytTR family regulators interact with the same motif (4), and a structural analysis of the AgrA_C-DNA interaction indicates that only residues H169 and R233 make base-specific DNA contacts, namely, H169/G₁₃ and R233/G₆'. These residues are present within two polymorphic loops that are not highly conserved in the LytTR family (47), and variations in their spatial location could be reflected in the differences in the DNA-binding site. Thus, while RcoM_{Bx-1} residue H185 is conserved in RcoM proteins from aerobic and facultative organisms (22) and is necessary for DNA binding (Fig. 5), it does not exactly align with AgrA_C residue H169, and this modest structural shift is likely reflected in the 1-base deviation of their DNA contacts, namely, G₁₃ (AgrA_C H169) and G₁₄ (RcoM_{Bx-1} H185) (Fig. 6). A second RcoM_{Bx-1}-associated motif characteristic might be indicated by the nonconservation of C₆, whose cognate residue G₆' forms a specific contact with AgrA_C residue R233 (Fig. 6). However, this nucleotide was introduced in 36 out of 37 reporters screened for higher-affinity RcoM_{Bx-1} “e” binding sites and is consistent with a structural and functional correspondence between AgrA_C residue R233 and the conserved RcoM_{Bx-1} residue R254 (Fig. 6). Overall, the conservation and functional requirement of the TTnnnG motif bases (Table 2), their consistent 21-bp spacing (Fig. 1), and the similarities of the AgrA_C DNA target and the higher-affinity RcoM_{Bx-1} “e↑” site (Fig. 6) conform to characterized LytTR-DNA interactions. At the same time, the native RcoM_{Bx-1}-DNA interactions, as least as indicated by the “e” site randomization results and binding assays (Fig. 3 and 5 and Table 4), are not functionally maximized.

In part, these “detuned” RcoM_{Bx-1}-DNA interactions may be compensated for by cooperative binding to a triplet set of direct-

repeat-binding sites located adjacent to the RNAP core promoter sequence. Dual binding sites are common in LytTR protein-activated promoters (4, 6, 7, 19, 24–26, 30, 35, 39, 41, 50, 57, 58), with evidence of cooperative binding (19, 41, 50), although promoter DNAs with unevenly spaced or inversely oriented binding sites, or that bind additional regulators, have also been described (8, 17, 27, 32, 39). There is precedence for evenly spaced triplet binding sites in non-LytTR domain transcriptional systems: the porin expression regulator OmpR binds two such regions (59), although structural details are unknown, and the nitric oxide-sensing NorR regulator binds as a dimer to each site in a triplet series and appears to form a hexameric ring assembly (54). By analogy, RcoM_{Bx-1} might therefore bind to each triplet-site region as a hexamer composed of three homodimers. The ability to purify a functional RcoM_{Bx-1} protein and the elaboration of the DNA target and binding conditions should facilitate further analyses. Finally, it is noteworthy that NorR belongs to a class of homodimeric transcription factors that generally bind to dual DNA sites yet form a hexameric species by recruiting a third partner from solution; perhaps, a similar oligomerization mechanism occurs among some dual-binding-site LytTR proteins.

A predicted result of RcoM_{Bx-1} oligomerization at a triplet-binding-site region is the significant conformational change of the bound DNA. The structural analysis of AgrA_C bound to a single-binding-site target showed an ~38° global bend in the bound oligonucleotide (47), and the binding of full-length AgrA to the dual high-affinity sites of the *agr* P2 promoter caused an ~81° bend (39). These deformations correlate with the appearance of DNase I hyperactivity in footprint assays (25). Conspicuous hyperactive DNase I sites are evident with RcoM_{Bx-1}-bound DNA (Fig. 2) and are particularly frequent within the central “b” and “e” targets, where the hyperactive sites are spaced ~10 bp apart along a given strand and phased by ~5 positions along the complement (Fig. 2C), compatible with their occurrence along one helix face opposite the bound protein. Phased hyperactive DNase I sites are evident in some LytTR protein-DNA interactions (25, 41) as well as other classes of well-characterized protein-DNA interactions where protein binding to one DNA face induces significant bending, including DNA bound by cyclic AMP receptor protein (33), factor of inversion (44, 48), and MerR family regulators (36). Significant RcoM_{Bx-1}-induced DNA bending is also consistent with the surprisingly poor activity of the UQ6148 reporter (Table 2), where the separately functional “a + b + c” and “d + e + f” binding regions were merged with a two-turn “c”-to-“d” interval. We posit that in this construct, the formation of a bent or folded RcoM_{Bx-1}-DNA complex at the higher-affinity “a + b + c” region interferes with the transcriptionally productive occupancy of the “d + e + f” region.

CooA and RcoM have dissimilar roles in prokaryotic CO sensing. As detailed previously (22), both CooA and RcoM are heme-containing, single-component, ~230-residue transcriptional activators bearing nonhomologous N-terminal effector-binding domains and unrelated C-terminal DNA-binding domains. The prototypical CooA transcriptional activator (originating from *Rhodospirillum rubrum*) has a micromolar affinity for CO (37) and a nanomolar affinity for its palindromic DNA target (51), and we hypothesize that it is appropriately tuned to regulate the expression of the anaerobic *coo* system (42). In contrast, *cox*-encoded aerobic CO oxidation systems typically operate at much lower CO levels (23, 56) that necessitate sensitive detection and deterrence

of spurious induction in the event of a transient CO exposure. RcoM_{Bx}-1 appears to be suitable for this task, exhibiting a high CO affinity combined with the transcriptional requirement for multiple binding events that ensure effector persistence. Of further interest is that the role of the “a + b + c” binding region, which is oriented in parallel with the “d + e + f” region (Fig. 1), can functionally substitute for it (Table 2) and more avidly binds RcoM_{Bx}-1 (Fig. 2) yet is unnecessary for *coxM*₁ expression (Fig. 4C). One plausible role for the “a + b + c” region is the negative autoregulation of the divergently transcribed *rcoM*₁ gene (Fig. 1). In this capacity, nascent RcoM_{Bx}-1 would respond to CO, but the binding of Fe(II)-CO RcoM_{Bx}-1 would then interfere with *rcoM*₁ transcription and prevent the accumulation of a large pool of stable and active CO-bound protein.

In conclusion, this report demonstrates the CO-dependent DNA-binding response of the RcoM_{Bx}-1 transcription factor and establishes the nature of the binding target and the functional importance of multiple binding-site interactions. Evidence of a novel triplet-site-binding region may be significant for an understanding of the nucleoprotein complex formed by the large class of medically significant LytTR domain regulators.

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