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Carbon Monoxide-Induced Activation of Gene Expression in *Rhodospirillum rubrum* Requires the Product of *cooA*, a Member of the Cyclic AMP Receptor Protein Family of Transcriptional Regulators

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Induction of the CO-oxidizing system of the photosynthetic bacterium *Rhodospirillum rubrum* is regulated at the level of gene expression by the presence of CO. In this paper, we describe the identification of a gene that is required for CO-induced gene expression. An 11-kb deletion of the region adjacent to the previously characterized *cooFSCTJ* region resulted in a mutant unable to synthesize CO dehydrogenase in response to CO and unable to grow utilizing CO as an energy source. A 2.5-kb region that corresponded to a portion of the deleted region complemented this mutant for its CO regulation defect, restoring its ability to grow utilizing CO as an energy source. When the 2.5-kb region was sequenced, one open reading frame, designated *cooA*, predicted a product showing similarity to members of the cyclic AMP receptor protein (CRP) family of transcriptional regulators. The product, CooA, is 28% identical (51% similar) to CRP and 18% identical (45% similar) to FNR from *Escherichia coli*. The insertion of a drug resistance cassette into *cooA* resulted in a mutant that could not grow utilizing CO as an energy source. CooA contains a number of cysteine residues substituted at, or adjacent to, positions that correspond to residues that contact cyclic AMP in the crystal structure of CRP. A model based on this observation is proposed for the recognition of CO by CooA. Adjacent to *cooA* are two genes, *nadB* and *nadC*, with predicted products similar to proteins in other bacteria that catalyze reactions in the de novo synthesis of NAD. A mutant with the *cooA-nadBC* region deleted displayed an auxotrophy for nicotinic acid, while the *cooA* insertion mutant did not.

The purple, nonsulfur, phototrophic bacterium *Rhodospirillum rubrum* synthesizes an enzyme system for carbon monoxide (CO) oxidation. Anaerobically in the dark, *R. rubrum* can utilize the CO-oxidizing system to generate energy (28). The system consists of at least three proteins that together couple CO oxidation to H_2 evolution: CooS (CO dehydrogenase), which oxidizes CO; CooF, a CooS-associated Fe-S protein; and CooH, a CO-tolerant hydrogenase (4, 5, 15). CooS and CooF have been purified to homogeneity and biochemically characterized (5, 6, 13–15). The *cooH*, *cooF*, and *cooS* genes are adjacent to one another on the chromosome and have been cloned, sequenced, and mutationally characterized. Mutations in any of these genes eliminate the ability of *R. rubrum* to evolve H_2 from CO and to utilize CO as an energy source (27, 28).

Unlike the case for many other bacteria capable of oxidizing CO anaerobically (11), the presence of the CO-oxidizing system in *R. rubrum* is dependent upon exogenous CO. Induction of this system by CO occurs in cells grown either photoheterotrophically (4, 5) or anaerobically in the dark (28). For cells growing photoheterotrophically in malate-ammonium medium, CooS activity is induced at least 1,000-fold by CO; under these conditions, CooS can constitute 2 to 5% of cellular protein (5). CooS accumulation following exposure to CO requires protein synthesis, indicating that regulation by CO occurs at the level of gene expression. CO-induced gene expression occurs rapidly, with CooS synthesis reaching its maximum rate after only 10 min of exposure to CO (4).

On the basis of the response of *R. rubrum* to CO, we expected that a CO-sensing regulatory factor must exist in this organism, though such a factor had not been reported for either *R. rubrum* or other bacteria. In this study, we have identified a gene linked to the *coo* region whose product is required for CO-induced gene expression of the *coo* region in *R. rubrum* and find that it encodes a member of the cyclic AMP (cAMP) receptor protein (CRP) family of transcriptional regulators.

MATERIALS AND METHODS

Culture of bacterial strains and bacteriophage. *Escherichia coli* was grown in 2× LC (27), Terrific Broth (42), or NZCYM medium (42) at 30 or 37°C with vigorous agitation. Bacteriophage lambda was propagated and manipulated as described by Silhavy et al. (47).

R. rubrum was routinely grown on SMN medium (supplemented malate-ammonium medium; rich medium for *R. rubrum*) (17) plates photoheterotrophically in GasPak jars (Becton Dickinson, Cockeysville, Md.) with tungsten illumination or aerobically. Liquid cultures were grown in SMN medium photoheterotrophically in screw-top tubes or aerobically with shaking.

For the identification of nicotinic acid auxotrophy, *R. rubrum* was plated onto MN medium (33) (malate-ammonium medium; minimal medium for *R. rubrum*) and MN medium supplemented with 100 μM nicotinic acid; the plates were placed in GasPak jars, and the cultures were grown photoheterotrophically for 5 days. The wild-type *R. rubrum* strain, UR2, is capable of growth on MN medium in the absence of nicotinic acid; *R. rubrum* strains that were dependent on added nicotinic acid for growth under these conditions were judged to be auxotrophic for nicotinic acid.

The growth of *R. rubrum* anaerobically in the dark in the absence of a fermentable carbon source is CO dependent (28). To test *R. rubrum* strains for the ability to utilize CO as an energy source, strains were streaked onto SMN medium plates supplemented with 75 μM $NiCl_2$ which were then placed in GasPak jars. After the jars were incubated in the dark overnight, CO was added (via a stopcock in the GasPak jar lid) to a final concentration of 30%, and the jars were incubated in the dark at 30°C for 8 days.

For the maintenance of plasmids in *E. coli*, the following concentrations of antibiotics (in micrograms per milliliter) were used: ampicillin, 50; gentamicin, 7.5; kanamycin, 50; and spectinomycin, 50. For *R. rubrum*, the antibiotics and

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levels used (in micrograms per milliliter) were as follows: gentamicin, 10; kanamycin, 15 for the pUK21-derived (51) resistance gene and 50 for that from Tn*5*; and spectinomycin, 50. Antibiotics were omitted from plates used in *R. rubrum* growth experiments.

Plate overlay assay for a functional CooS protein. The activity of CooS can be measured as the reduction of methyl viologen coupled to CO oxidation (7). To qualitatively determine if the *R. rubrum* mutants constructed in this study could accumulate an active CooS, the following assay was conducted. *R. rubrum* was grown as patches photoheterotrophically on SMN medium plates containing 20 μM $NiCl_2$, and the CO-oxidizing system was induced by adding CO to 30% 12 to 16 h prior to the assay. To start the assay, the plates were removed from the jar and exposed to air for 45 min; this step eliminated subsequent CO-independent background methyl viologen reduction. Ten milliliters of a molten (50°C) agar overlay (50 mM phosphate buffer [pH 7.5], 2 mM EDTA, 5 mM methyl viologen, and 1% agar) was then poured onto each plate and allowed to solidify. After condensate was removed from the lids, the plates were returned to GasPak jars and flushed and evacuated five times with Ar. CO was then added to 30%, and the jars were incubated in the dark at 30°C. CO-dependent methyl viologen reduction was apparent as blue zones surrounding the patches and was typically visible within 2 h; the activity was readily detected in strains that accumulate 2% of the CooS activity of wild type.

Two-dimensional (2-D) PAGE. *R. rubrum* was grown photoheterotrophically in SMN medium supplemented with 10 μM $NiCl_2$ in stoppered serum vials with Ar headspaces. When at an optical density at 680 nm of 6 (late log phase in SMN medium), cultures to be induced for the CO oxidation system received an addition of CO to a final concentration of 30%. Uninduced cultures received no additions. The cultures were agitated under illumination for 30 min, and synthesized proteins were then labeled by addition of 10 μCi of TRAN^{35}S-LABEL (Cys plus Met; ICN Biomedicals, Irvine, Calif.) for 10 min and extracts were prepared by sonication (4). Sample preparation and the first dimension were performed as described elsewhere (4) except that only pH 3 to 10 ampholytes were used and the samples were run for 900 V-h. The second dimension was a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) gel with an acrylamide gradient from 10 to 18% to enhance resolution of smaller proteins.

DNA isolation and manipulation. Unless otherwise noted, the DNA manipulation techniques were those of Sambrook et al. (42). Plasmid DNA used for restriction digestion and cloning was isolated from *E. coli* by the boiling method (25), while plasmid DNA used for sequencing was isolated by a modification (27) of the ammonium acetate method (32). Total cellular DNA was isolated from *R. rubrum* strains as described previously (17). Bacteriophage lambda DNA was isolated by the procedure of Chisholm (9), with minor modifications. DNA was purified from agarose gels with the Geneclean II kit (Bio 101, Vista, Calif.).

Plasmids constructed in vitro were electrotransformed into *E. coli* DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) by using a Gene Pulser apparatus (Bio-Rad, Richmond, Calif.); electrocompetent cells were prepared and electrotransformed as recommended by the manufacturer. For the selection of gentamicin resistance (Gm^r) following transformation of *E. coli*, the length of the incubation following the pulse was extended to 2 h to increase transformation efficiency. For Southern and plaque hybridizations, the Genius kit, which utilizes nonradioactive probe labeling and detection, was used according to the manufacturer's recommendations (Boehringer Mannheim, Indianapolis, Ind.).

DNA sequencing. The 2.5-kb *Bgl*II-*Eco*RI fragment that complemented the regulatory phenotype of UR387 (see below) was sequenced in the following manner. The 2.5-kb fragment was isolated from pLJC24 (10), Klenow DNA polymerase blunted, and ligated to *Eco*RV-cut pBSKS$^-$ (Stratagene, La Jolla, Calif.) to generate pCO16. Nested deletions of the pCO16 insert were prepared in both directions by a modified (27) exonuclease III-dependent procedure (24). Plasmid DNA was sequenced by the chain termination method (43) using the Sequenase 2.0 kit (United States Biochemical Corp., Cleveland, Ohio) with modifications previously noted (27) to eliminate pausing artifacts; deaza-dGTP reactions were used when compression artifacts were not eliminated by the highly denaturing gel system. Persistent pausing artifacts were eliminated by a terminal deoxynucleotidyl transferase treatment (16). Sequencing gels contained 5 or 6% acrylamide (Bio-Rad) and 8.3 M urea plus 40% ultrapure formamide (35). Sequence analyses were performed with the Genetics Computer Group software, Wisconsin package, version 8.FT2-VMS (21), and protein database searches were performed at the National Center for Biotechnology Information by using the BLAST network service (2).

Introduction of mutations into the *R. rubrum* chromosome. Mutations created in cloned regions of *R. rubrum* DNA were introduced into *R. rubrum* from *E. coli* by using suicide vectors derived from pUX19 (34), which is pUK21 (51) to which the *oriT* of pSUP202 (48) has been added. The plasmid to be mobilized was first transformed into *E. coli* S17-1 (48) or SM10 λpir (37) and subsequently mobilized into *R. rubrum* by mating (34). When integration of the entire plasmid by homologous recombination was desired, a vector-encoded resistance was selected. When a gene replacement by reciprocal homologous recombination was sought, the resistance encoded by the desired mutation was selected, and vector-free segregants were identified by screening for the absence of the vector-encoded resistance (27).

Construction of a suicide vector for *R. rubrum* that encodes Sp^r. To generate a vector that could be used to introduce mutations marked with kanamycin resistance (Km^r), a suicide vector, pDWS108, encoding spectinomycin resistance (Sp^r) was constructed. The Sp^r gene of pHP45Ω (40) was excised as a *Hin*dIII fragment, and the ends were filled by Klenow treatment. This fragment was ligated with *Ssp*I-treated pUX19, which disrupted the Km^r gene of that plasmid, generating pDWS108.

Screening a chromosomal library of *R. rubrum* DNA to identify clones that carried DNA 3′ of *cooFSCTJ*. An *R. rubrum* γEMBL4 chromosomal library (17) was plated, and duplicate plaque lifts to nylon membranes were performed as described by Sambrook et al. (42). The two membranes were hybridized with separate probes to the end and to the center of the cloned *coo* region (Fig. 1A, e and c, respectively). Plaques that hybridized to the former, but not to the latter, corresponded to phage that carried inserts extending 3′ of the *cooFSCTJ* region. DNA was isolated from two such phage clones, λCOOR1 and λCOOR2, and the inserts were characterized by restriction digestion and Southern hybridization, with the resulting map shown in Fig. 1A.

Deletion of the region 3′ of *cooFSCTJ*. To create the desired deletion, DNA fragments flanking the region to be deleted were cloned on either side of a selectable gene in the polylinker of a suicide vector. Reciprocal recombination of this region with the chromosome would result in the replacement of the wild-type region with the selectable marker and the deletion.

To construct the deletion, which is termed Δ*coo-8*, the region most distal to the *cooFSCTJ* genes was excised from λCOOR2 as a 4.0-kb *Kpn*I-*Eco*RI fragment and ligated to *Eco*RI-*Kpn*I-treated pDWS108, generating pDWS114. A 1.2-kb *Eco*RV-*Bgl*II fragment, which adjoins the *coo*-proximal end of the desired deletion, was ligated with pDWS108 digested with *Stu*I and *Bgl*II, resulting in plasmid pDWS115. The source of the 1.2-kb *Eco*RV-*Bgl*II fragment was pCO5, a pBSKS$^-$ (Stratagene)-based subclone of pLJC24 (10). The 4.0-kb *Kpn*I-*Eco*RI fragment from pDWS114, which adjoins the *coo*-distal end of the desired deletion, was ligated to similarly cut pDWS115, creating pDWS116. Finally, the Km^r gene of Tn*5* was excised from pSUP2021 (48) with *Hin*cII and ligated to *Bgl*II-digested (Klenow-filled) pDWS116, to generate pDWS118. This plasmid was mobilized into *R. rubrum* UR2 as described above, and four isolates were identified as reciprocal recombinants (and therefore vector free) on the basis of their spectinomycin sensitivity (Sp^s) phenotype. These isolates all lacked the ability to express functional CooS in response to CO as judged by the plate overlay assay (data not shown), and one representative, UR387, was confirmed by Southern analysis (data not shown) and used for further analysis. Figure 1B shows the physical map of the *coo* region of UR387.

Single-copy complementation of UR387. To identify the portion of the deleted region that was involved in *coo* regulation, the *coo*-proximal portion of the deleted region was tested for its ability to complement this phenotype.

Plasmid pCO6R carries an 8.3-kb *Eco*RI fragment of the *coo* region from pLJC24 (10, 27) inserted in the *Eco*RI site of the pUX19 polylinker. This plasmid was altered to add a Gm^r marker (*aacC1*) and to prevent readthrough of plasmid promoters into the *coo* region of the insert: the ΩGm^r element, which incorporates transcriptional and translational terminators, was excised from pGMΩ1 (45) as a *Bam*HI fragment and ligated to *Bam*HI-digested pCO6R, placing the element in the polylinker upstream of the *coo* region (Fig. 1C). The resulting suicide plasmid, pDWS125, was integrated into the chromosome of *R. rubrum* UR387 by selecting Gm^r. A physical map of the resulting merodiploid strain, UR425, is shown in Fig. 1C; the structure of the merodiploid region was confirmed by Southern analysis (data not shown).

Construction of a *cooA* insertion mutant. To construct an insertion mutation in *cooA*, plasmid pDWS119, which carries a 3.4-kb *Pst*I-*Eco*RI fragment of the *cooCTJA-nadBC* region inserted into the polylinker of pUX19, was partially digested with *Fsp*I and ligated to the ΩGm^r element, excised as a *Sma*I fragment from pGMΩ1. A plasmid with the ΩGm^r cassette inserted in *cooA*, pDWS121, was identified and used to replace the wild-type *cooA* gene of *R. rubrum* UR2 as described above, generating strain UR407. The presence of the desired insertion on the chromosome of UR407 was confirmed by Southern blotting (data not shown).

Nucleotide sequence accession number. The nucleotide sequence data reported in this article will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession no. U20508.

RESULTS

The deletion of an 11-kb region 3′ of *cooFSCTJ* results in a mutant (*R. rubrum* UR387) that is incapable of activating *cooS* expression in response to CO. We expected that the CO-responsive regulator of the *coo* regulon would be genetically linked to the *coo* genes. We have preliminary evidence that the region immediately downstream of *cooS*, which appears to include three genes termed *cooCTJ*, does not encode products that are necessary for CO induction (29). We therefore decided to create a deletion of *coo*-distal DNA starting at the *Bgl*II site, which falls just beyond *cooJ* (Fig. 1A), and to test this deletion for any effects on regulation. As the previously cloned region extended only 2.5 kb past the end of *cooJ* to the *Eco*RI

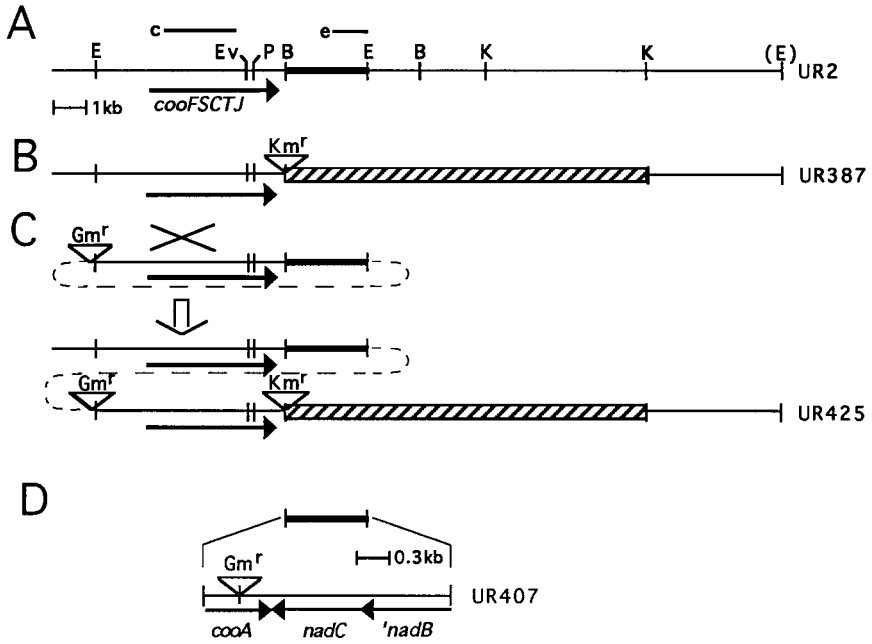

FIG. 1. *coo* region of *R. rubrum*. (A) The wild-type *coo* region with the position of the *cooFSCTJ* transcript indicated (arrow). Relevant restriction sites are indicated as follows: E, *Eco*RI; Ev, *Eco*RV; P, *Pst*I; B, *Bgl*II; and K, *Kpn*I. The 2.5-kb *Bgl*II-*Eco*RI fragment containing *cooA* and *nadBC* (heavy line) is indicated. The chromosomal fragment in λCOOR2 extends from approximately the *Eco*RV site to the right end of the diagram, and the sites shown reflect the restriction map of that fragment. The approximate positions of the two probes (c and e) used in screening the phage library and the *Eco*RI site in the polylinker of the phage vector [(E)] are indicated. In panels B through D, identical regions are maintained in the same vertical register. (B) Location of the Δ*coo-8* deletion in *R. rubrum* UR387. The 11-kb deletion (hatched box) extends from the *Bgl*II site to the most distal *Kpn*I site. The position of the Km^r cassette at the site of the deletion is indicated. (C) Construction of *R. rubrum* UR425. The upper line shows the structure of pDWS125, which carries the 8.3-kb *Eco*RI fragment shown. Also noted is the position of the ΩGm^r cassette that was placed upstream of the *coo* insert to prevent possible transcription from vector promoters. The vector portion of pDWS125 (broken line) is indicated. Recombination in the region of homology indicated (×) with the chromosome of UR387 resulted in the integrant shown in the bottom line of panel C. (D) ORFs in the 2.5-kb *Bgl*II-*Eco*RI fragment (drawn to a larger scale). The apparent transcriptional organization of the region (arrows) and the position of the *cooA9*::*aacC1* insertion (Gm^r) in *R. rubrum* UR407 are indicated.

site (10, 29), we identified a λEMBL4 phage clone that extended the cloned region by approximately 13 kb and used this phage to construct a plasmid that carried an 11-kb deletion of the region located 3′ to *cooFSCTJ* as described in Materials and Methods. The reciprocal recombination of this plasmid into the *R. rubrum* UR2 chromosome led to the replacement of the wild-type region with the deletion Δ*coo-8*, generating strain UR387 (Fig. 1B).

CooS activity in UR387 (Δ*coo-8*) was measured by the plate overlay assay in both the presence and the absence of CO, and activity was negligible in both cases (data not shown), suggesting a defect in the function or regulation of CooS. To distinguish between these two possibilities, protein synthesis in response to CO was monitored by 2-D PAGE of extracts of pulse-labeled cells (Fig. 2), as the mobility of CooS on 2-D PAGE is known (4). After 30 min of CO induction, CooS was one of the most abundantly synthesized proteins in *R. rubrum* UR2 (*coo*$^+$) but was not detectable in UR387 (Δ*coo-8*). Approximately six other proteins, some of which have been tentatively identified as the products of other *coo* genes (23), were CO induced in UR2 but absent in UR387. Similarly, extracts of UR387 contained no CooS antigen as determined by Western blotting (immunoblotting), in contrast to UR2 (data not shown). UR387 also failed to grow with CO as the sole energy source (Fig. 3C). In contrast, UR387 grew similarly to the wild-type strain (UR2) under photoheterotrophic conditions in the presence of CO on plates identical to those used in the CO growth experiment (data not shown); this is consistent with the finding that Coo$^-$ strains tolerate CO when growing photoheterotrophically in rich media (27). Taken together, these results suggest that Δ*coo-8* deletes a gene involved in CO induction of the *coo* regulon.

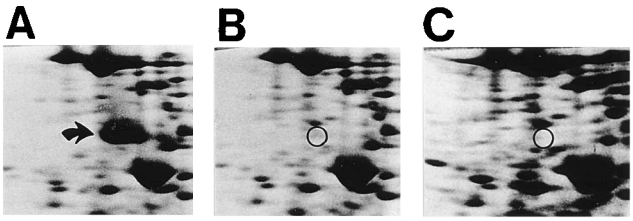

FIG. 2. Detection of CooS synthesis by 2-D PAGE. The presence and absence of CooS in extracts of pulse-labeled cells are noted. (A) *R. rubrum* UR2 (*coo*$^+$) labeled in the presence of CO; CooS (arrow) is indicated. (B) UR2 labeled in the absence of CO; the expected position of CooS (circle) is indicated. (C) *R. rubrum* UR387 (Δ*coo-8*) labeled in the presence of CO.

Introduction of a 2.5-kb fragment of the deleted region in single copy restores the ability of UR387 (Δ*coo-8*) to synthesize active CooS in response to CO. To delineate the specific region necessary for CO-dependent expression, we replaced a portion of the deleted region by mobilizing into *R. rubrum* UR387 a cloned fragment that encompassed part of the deletion as well as a portion of the chromosome that was not deleted (to provide a region of homology for efficient recombination). As described in Materials and Methods, this resulted in a partially diploid strain, UR425, that is diagrammed in Fig. 1C. To avoid possible expression of the *coo* region from promoters in the integrated vector, the ΩGm^r element (45) was placed between the *coo* region and the vector sequences.

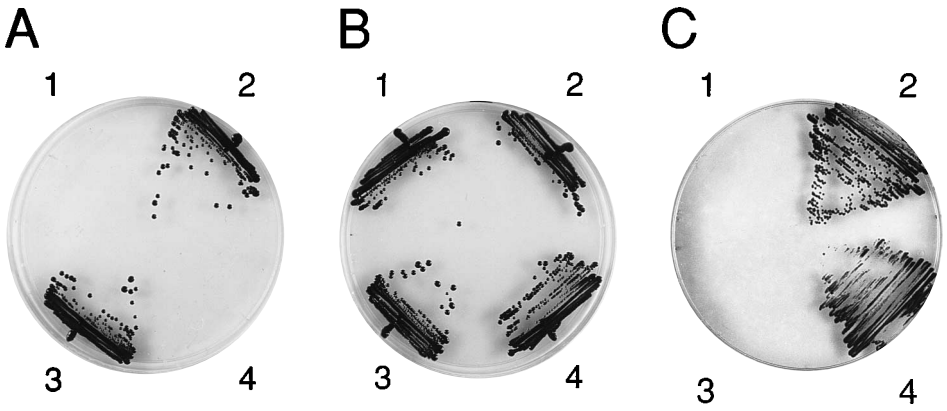

FIG. 3. Growth of *R. rubrum* strains UR387 (1), UR2 (2), UR407 (3), and UR425 (4) streaked on different media. (A) Photoheterotrophic growth on an MN medium plate. (B) Photoheterotrophic growth on an MN medium plate supplemented with 100 μM nicotinic acid. (C) Anaerobic growth on an SMN medium plate in the dark, utilizing CO as an energy source.

Three lines of evidence show that the 2.5-kb fragment complemented the regulatory phenotype of the Δ*coo-8* mutation. (i) UR425 was able to grow with CO as the energy source (Fig. 3C). (ii) UR425 showed CO regulation of CooS activity, as measured by the plate overlay assay; CooS activity was detected following CO induction, while none was detected in the absence of CO induction (data not shown). (iii) CO-regulated synthesis of CooS was apparent, as judged by 2-D PAGE of ^{35}S-pulse-labeled cell extracts (data not shown). These results strongly argue that at least a portion of the regulatory gene is encoded within this 2.5-kb region.

The sequence of the 2.5-kb region predicts three protein products, including a member of the CRP family of transcriptional regulators. The 2.5-kb *Bgl*II-*Eco*RI fragment was sequenced, and the organization of the open reading frames (ORFs) in this region is shown in Fig. 1D. The *coo*-proximal ORF, which is transcribed in the same direction as *cooFSCTJ*, is particularly interesting, as it predicts a transcription factor. Immediately adjacent and transcribed in the opposite orientation are two ORFs that appear to encode products involved in NAD biosynthesis.

The protein predicted from the *coo*-proximal ORF shows similarity to a family of transcription factors, of which the best-characterized members are CRP and FNR (a regulator of anaerobic gene expression) of *E. coli* (30). The mutational analysis of this ORF has identified the loss of this gene as causative for the *coo* regulatory phenotype (see below); on the basis of this finding and the sequence similarity to CRP and FNR, we have designated the gene *cooA*, for CO oxidation activator. A poor Shine-Dalgarno sequence precedes this gene, which appears to initiate with an ATG. The comparison of CooA with CRP and FNR from *E. coli* is shown in Fig. 4. CooA is 28% identical (51% similar) to CRP and 18% identical (45% similar) to FNR. The similarity of CooA to CRP and FNR extends along the lengths of the proteins and includes similarity in the helix-turn-helix DNA-binding portion of CRP (44). CooA is also similar to other members of the CRP family: it is 22% identical (42% similar) to NtcA, which is involved in nitrogen regulation in *Anabaena* sp. strain PCC 7120 (53, 54); 21% identical (50% similar) to AadR, which regulates aromatic acid degradation in the purple nonsulfur bacterium *Rhodopseudomonas palustris* (12); and 21% identical (52% similar) to FixK, which regulates adaptation to nitrogen-fixing conditions in *Rhizobium meliloti* (3).

The other two ORFs appear to encode homologs of proteins involved in NAD biosynthesis. On the basis of both sequence similarities and the auxotrophic requirements caused by mutations in these ORFs (see below), they have been designated *nadB* and *nadC*. The upstream gene, *nadB* (Fig. 1D), is 43% identical (58% similar) to *nadB* of *E. coli* (18), while the downstream gene, *nadC*, is 31% identical (51% similar) to *nadC* from *E. coli* (20). In *E. coli* and other bacteria, NadB (L-aspartate oxidase) and NadC (quinolinic acid phosphoribosyltransferase) catalyze reactions in the de novo biosynthesis of NAD (19, 26, 39). The sequence predicts that the 2.5-kb fragment does not contain the entire *nadB* gene. No start codon was detected for the gene, and the predicted protein is 230 to 240 residues shorter at the N terminus than are its *E. coli* (18) and *Bacillus subtilis* (49) homologs, suggesting that approximately half the gene is present on the sequenced fragment. This also suggests that the promoter for these *nad* genes is not present on this fragment.

Insertion of a drug resistance cassette in *cooA* results in a mutant (*R. rubrum* UR407) unable to grow utilizing CO as an energy source. The similarity of CooA to other transcriptional regulators, as well as the complementation of Δ*coo-8* with the 2.5-kb *Bgl*II-*Eco*RI fragment, suggested that the absence of *cooA* caused the regulatory defect in UR387 (Δ*coo-8*). To verify this, the *cooA9*::*aacC1* mutation was created by the insertion of the ΩGm^r cassette into the *Fsp*I site of *cooA* (Fig. 1D). This mutation was introduced into the *R. rubrum* UR2 chromosome, replacing the *coo*$^+$ allele and generating strain UR407. The lack of genes transcriptionally downstream from *cooA* obviated concerns about polarity. UR407 was unable to express CooS activity in response to CO as measured by the plate overlay assay (data not shown) and failed to grow on CO as the sole energy source (Fig. 3C). This strain was able to grow photoheterotrophically in the presence of CO. This phenotype is consistent with a role of CooA in CO-regulated expression of the *coo* region.

The deletion of *nadB* and *nadC* results in mutants that are dependent on nicotinic acid for growth on minimal medium. If the *nadBC* genes adjacent to *cooA* have a role in de novo NAD biosynthesis in *R. rubrum*, their loss should cause a requirement for nicotinic acid (Nic$^-$ phenotype) (19). The growth requirements of the *R. rubrum nadBC* region mutants (Fig. 3A and B) are consistent with this hypothesis. UR387 (Δ*coo-8*) was dependent on the addition of nicotinic acid for growth on minimal medium, while UR2 (*coo*$^+$ *nad*$^+$) had no such requirement. In UR425 (Fig. 1C), the *cooA-nadBC* region has

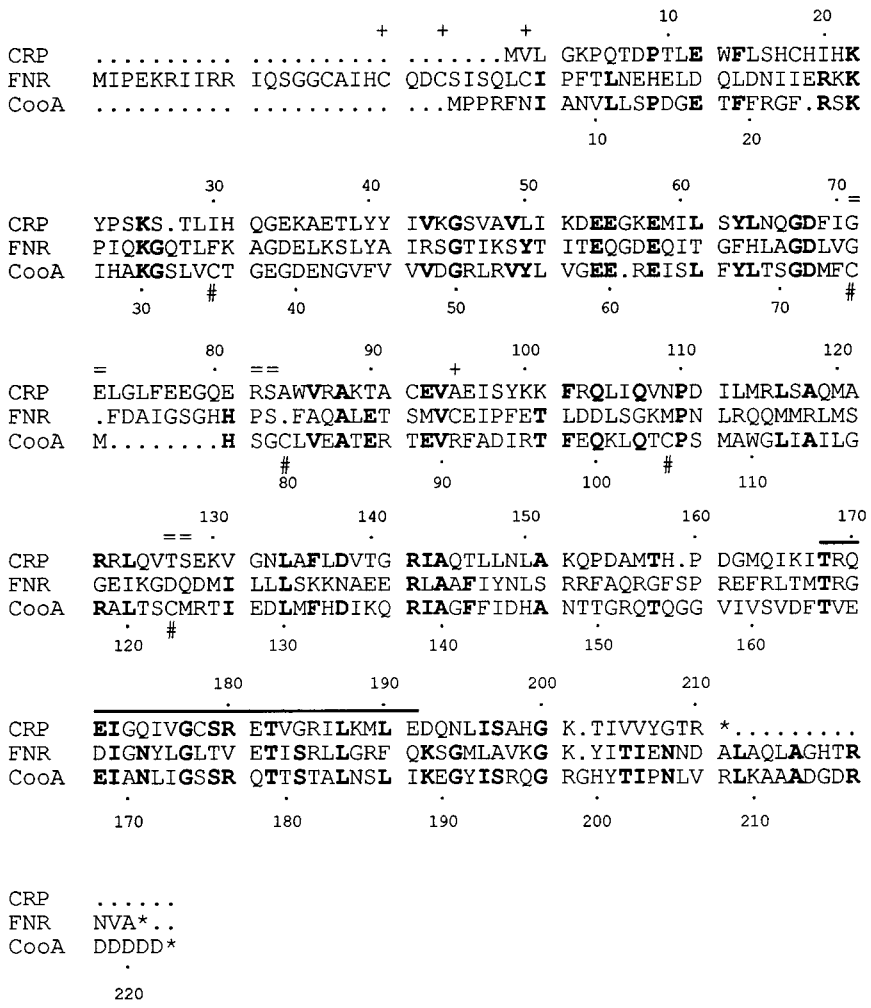

FIG. 4. Comparison of the predicted CooA sequence with those of FNR and CRP of *E. coli*. The positions of CRP and CooA residues are indicated above and below the sequences, respectively. Residues of CooA that are identical to residues of CRP or FNR (boldface), Cys residues in CooA (#), CRP residues that make contacts with cAMP (=), Cys residues essential for the activity of FNR (+), the helix-turn-helix region of CRP (overline), and the stop codons (asterisks) are indicated.

been reintroduced but apparently lacks a complete *nadB* gene, as described above. Not surprisingly, this mutant also displays a Nic$^-$ phenotype. In contrast, UR407 (*cooA9*::*aacC1*) grows normally on minimal medium.

DISCUSSION

In this study, we have shown that CooA regulates CO-induced expression of the *coo* region. Previous studies using inhibitors (4), 2-D PAGE of pulse-labeled extracts (4), and Northern (RNA) blots (29) showed that CO induces transcription of the *coo* region; however, no factors that mediate this induction had been identified. The following data show that CooA is required for CO-dependent expression of the *coo* region. (i) The 11-kb deletion that encompasses *cooA* (UR387) resulted in a regulatory defect, in that CO-induced CooS synthesis and growth with CO as an energy source were abolished. (ii) When introduced into UR387, a 2.5-kb fragment containing *cooA* complemented the Coo$^-$ phenotype of this strain, restoring CO-induced CooS activity and CO-dependent growth. (iii) A cassette insertion in *cooA* resulted in a phenotype identical to that of UR387; this mutant could not utilize CO as an energy source. (iv) The sequence of *cooA* predicts that it is a transcriptional regulator similar to CRP and FNR.

CooA appears to be a new member of the CRP family of transcriptional regulators. CooA lacks the residues that correspond to important effector recognition residues in CRP (22, 38, 52), as well as residues hypothesized to be important in signal sensing in FNR (36). In the crystal structure of CRP complexed with its effector, cAMP, there are six residues in CRP that appear to make specific contacts with cAMP (52), and none of these residues are conserved in CooA (Fig. 4). CooA similarly lacks the critical N-terminal Cys residues of FNR that are thought to be involved in redox sensing (36). In addition, CooA has an unusual run of acidic residues that are not present in CRP or FNR; seven of the nine C-terminal amino acids are aspartic acid. These differences suggest that CooA is not the *R. rubrum* equivalent of FNR or CRP.

The presence of Cys residues in the putative effector recognition region of CooA suggests that a metal or metal cluster may be involved in signal recognition by CooA. When the predicted sequence of CooA is aligned with CRP, there is a Cys residue at, or immediately adjacent to, the positions that correspond to the known cAMP contact sites in CRP. These include Cys residues at position 75 of CooA (CooA75) (replacing the homolog of position 71 of CRP [CRP71], a cAMP contact, and immediately adjacent to cAMP contact CRP72), CooA80 (adjacent to cAMP contacts CRP82 and CRP83), and

CooA123 (replacing cAMP contact CRP127 and adjacent to cAMP contact CRP128). Finally, while the Cys at CooA35 is not homologous to a known cAMP contact, the corresponding position in CRP is in the immediate vicinity of cAMP in the crystal structure (52). In a variety of proteins, Cys residues are known to be ligands for metals and metal centers (8). Since they lie at what corresponds to the effector-binding region of CRP, the Cys residues of CooA may function as ligands for a metal which interacts with an effector. While this molecular modeling of CooA based on CRP is speculative, given the structural differences that could arise from the obvious dissimilarities in the sequences of the two proteins, the positions of these Cys residues in the CooA sequence suggest a testable hypothesis for their role in effector recognition.

If CooA utilizes a metal for effector recognition, then an obvious candidate for the CooA effector is CO. It is a reasonable hypothesis that a metal serves as a CO-binding site in CooA, since CO interacts with proteins by binding to metals or metal centers in virtually all described cases. This is true for proteins that utilize CO as a substrate, such as the CO dehydrogenases from *R. rubrum* (6, 13, 14) and *Clostridium thermoaceticum* (31, 41, 46). CO also interacts with metals in proteins that are inhibited by CO, including hydrogenases from *Clostridium pasteurianum* (1) and *Chromatium vinosum* (50). One could thus envision a simple model in which the binding of CO to a metal center of CooA induces a conformational change in the protein that allows it to activate expression of the *coo* region. However attractive the above model is, we recognize that there will be no direct support for it until CooA can be biochemically characterized. It is possible, for example, that CooA responds to a different effector or to a CO-sensing protein.

In the course of this study, we also identified the *R. rubrum nadBC* genes. This conclusion is based on their very strong sequence similarity to *nadBC* in *E. coli* (18, 20) and on the Nic^- phenotype caused by their deletion from the *R. rubrum* chromosome. In contrast to the homologous genes of *E. coli*, which map to different regions of the chromosome (19), the *nadB* and *nadC* genes of *R. rubrum* appear to form an operon. In *R. rubrum*, the juxtaposition of the *coo* and *nad* genes appears to be coincidental, as no physiological connection between the two has been established: the *nad* deletion confers a Nic^- phenotype in the absence of *coo* expression, and the insertion in *cooA* causes no apparent Nic^- phenotype in a nad^+ background.

This study shows that *cooA* encodes a necessary activator of expression of the *coo* region, and the sequence of CooA suggests that it may sense CO directly through a metal center and subsequently activate transcription. We are currently attempting to purify and biochemically characterize CooA in order to test this model.

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