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In Vitro Analysis of the Interactions between the PocR Regulatory Protein and the Promoter Region of the Cobalamin Biosynthetic (*cob*) Operon of *Salmonella typhimurium* LT2

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The PocR protein of *Salmonella typhimurium* LT2 was overexpressed and used to demonstrate in vitro that it specifically binds to the cobalamin biosynthetic operon (*cob*) promoter region. Evidence is presented to show that PocR DNA-binding activity in vitro is regulated by the effector molecule 1,2-propanediol. Deletion analysis of the *cob* promoter (P_{cob}) suggested that two regions upstream of the promoter are needed for optimal activation of P_{cob} by PocR in vivo. DNase I footprinting experiments demonstrated that PocR binds to two sites within P_{cob}. The transcription initiation site of *cob* mRNA in response to 1,2-propanediol was identified and shown to be different from the one reported for transcription initiation under anoxic conditions in the absence of 1,2-propanediol.

The adenosyl-cobalamin (Ado-CBL) biosynthetic (*cob*) genes of *Salmonella typhimurium* LT2 encode proteins required for the synthesis of Ado-CBL (14, 19). The majority of the *cob* genes are located at min 41 of the linkage map of this bacterium and are organized into a large operon (referred to as the *cob* operon) of approximately 17 kb in size containing 17 *cbi* genes needed for the synthesis of adenosylcobinamide and 3 *cob* genes needed for the assembly of the nucleotide loop (20, 32). Hereafter, the genes in this operon are collectively referred to as *cob*. Four additional *cob* genes, *cobA*, *cobB*, *cobC*, *cobD*, have been located outside the min 41 operon (14, 16, 27, 42). Except for *cobC* and *cobD*, which are adjacent to one another (27), these genes are unlinked to each other and to the *cob* operon, and there is no indication that they are part of a regulon.

The ability of *S. typhimurium* LT2 to synthesize Ado-CBL represents a large investment of genomic information whose transcription needs to be carefully regulated. From a functional perspective, CBL is required for four enzymatic reactions in *S. typhimurium* (15, 17, 18), one of which is needed for the catabolism of 1,2-propanediol (1,2-PDL) as the sole carbon and energy source (18). The 1,2-PDL utilization (*pdu*) genes map adjacently to, and are transcribed divergently from the *cob* operon (see Fig. 1) (18). The metabolic link between the two pathways is reflected in the transcriptional regulation of the two operons. *cob* and *pdu* expression is induced by the presence of 1,2-PDL in the environment (4, 30), suggesting that the major function of CBL is to support 1,2-PDL utilization in *S. typhimurium*.

Regulation of *cob* and *pdu* expression by 1,2-PDL is thought to be mediated by the PocR protein, a transcription factor in the AraC family of regulators. The *pocR* gene was located by genetic means between the *cob* and *pdu* operons, and its nucleotide sequence has been established (4, 9, 30, 32).

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To better understand the importance of *cob* and *pdu* to the physiology of *S. typhimurium*, we have undertaken the molecular analysis of PocR and its mechanism of action. We have analyzed PocR activity in vitro and have begun to characterize the elements required for PocR function in vivo. Physical evidence is presented to document that PocR is a DNA-binding protein that interacts specifically with the *cob* promoter region in vitro and that binding to its target is enhanced by 1,2-PDL.

(Part of these results was presented at the General Meeting of the American Society for Microbiology, Las Vegas, Nev., 1994.)

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The genotypes of strains and plasmids used in this work are listed in Table 1. The insertion elements Mu d1-8, Mu dI1734 (6, 7), and Tn*10*DEL*16*DEL*17* (43) are abbreviated MudA, MudJ, and Tn*10d*(Tc), respectively. The media used in this study have been described elsewhere (30, 31). The *cob* operon consists of the *cbi* and *cob* genes (32). For the purpose of clarity, we will refer to it as the *cob* operon and to the promoter as the *cob* promoter (P_{cob}). Fusion *cbi-24*::MudJ is within the *cob* operon. Expression of *cbi-24*::MudJ, measured as β-galactosidase activity, served as a reporter of *cob* transcription.

General recombinant DNA techniques. (i) General methods. DNA was introduced into recipient cells either by electroporation (26) or by $CaCl_2$ transformation (39). Plasmids were prepared by an alkaline lysis method (22) or with the Wizard Prep kit (Promega, Madison, Wis.). Transductions were performed as previously described (10, 30).

(ii) DNA sequencing. DNA sequencing was performed with the Sequenase kit, version 2.0 (U.S. Biochemical Co., Cleveland, Ohio). The primer 5′-ATGTGTA TCCACCTTAAC-3′ (Genosys, The Woodlands, Tex.) was used to sequence the DNA flanking the insertion site of the Tn*10d*(Tc) elements. This primer hybridizes to a sequence near the end of the IS*10* element present at both ends of the transposon. The primer 5′-GCATCAACTGACAAAACGGC-3′ was used to confirm N-terminal PocR sequences in PocR fusion plasmid constructs. This primer hybridizes approximately 140 nucleotides downstream of the PocR initiation codon. Primer ARP-3 (5′-ATAATGTGCTACGTTTTACA-3′ [28]) was used to determine the site of *cob* transcription initiation by using primer extension (see below).

(iii) Plasmid constructions. (a) Cloning of *pocR* and *poc*::Tn*10d*(Tc) elements. DNA was isolated from phage heads of induced lysates of the appropriate Mud-P22 strain (Table 1) as previously described (44). The DNA was digested to completion with *Pst*I [which does not cut within *pocR* or within the Tn*10d*(Tc) element (9, 32, 43)] and cloned into *Pst*I-digested pSU19 (23). Clones containing the *pocR* gene were identified by transforming JE1945 (*pocR pdu*::MudA) and screening the transformants for complementation of *pocR* function by colony color on plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyr-

TABLE 1. Strains and plasmids

Strain	Genotype	Reference or source
Strains		
S. typhimurium		
TR6583	*metE205 ara-9*	K. Sanderson via J. Roth
JE1945	*pdu-8*::MudA *pocR106*::Tn*10d*(Tc)	30
JE3157	*metE205 ara-9* DEL*299*/pDA3 pMR18	This study
JE3315	*metE205 ara-9* DEL*299*/pDA9 pMR18	
JE3321	*metE205 ara-9* DEL*299*/pDA7 pMR18	
JE3323	*metE205 ara-9* DEL*299*/pDA8 pMR18	
JE3751	*metE205 ara-9 recA1 cbi-24*::MudJ *pocR106*::Tn*10d*(Tc) pGEX-2T	
JE3752	*metE205 ara-9 recA1 cbi-24*::MudJ *pocR106*::Tn*10d*(Tc) pMR35	
JE3753	*metE205 ara-9 recA1 cbi-24*::MudJ/pGEX-2T	
JE3754	*metE205 ara-9 recA1 cbi-24*::MudJ/pMR35	
TT14383	*cob-343*::MudQ	J. Roth
JE3749	*cob-343*::MudQ *pduF501*::Tn*10d*(Tc)	
JE3750	*cob-343*::MudQ *pocR106*::Tn*10d*(Tc)	
JR501	*hsdSA29 hsdSB121 hsdL6 trpC2 metA22 metE551 ilv452 leu3121 rpsL 120 galE719 xyl-404 H1-b H2-e,n,x nml* $(Fels2)^-$ *fla-66*	41
DA2515	DEL*299*/pDA3	28
E. coli		
DH5αF′	*F′/endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ*(lacIZYA-argF)U169 deoR* [ϕ*80dlac*Δ(*lacZ*)*M15*]	
BL21	F^- *ompT* r_B^- m_B^-	37
JE3610	BL21/pGEX-2T	This study
JE2611	BL21/pMR35	This study
Plasmids		
pRS551	High-copy-number promoter analysis vector, Amp^r Kan^r	34
pUC19	Cloning vector, Amp^r	25
pSU19	Cloning vector, Cm^r	23
pGEX-2T	GST fusion vector, Amp^r	36
pT7-5	T7 overexpression vector	38
pMR16	$pocR^+$ in pSU19	This study
pMR18	$pocR^+$ in pSU19	
pMR20	$pocR^+$ in pSU19	
pMR31	P_{cob} region from pDA3 in pUC19	
pMR35	$pocR^+$ in pGEX-2T	
pDA3	*cob* promoter −192 to +134 in pRS551	28
pDA7	*cob* promoter −121 to +134 in pRS551	
pDA8	*cob* promoter −93 to +134 in pRS551	
pDA9	*cob* promoter −73 to +134 in pRS551	

anoside) and 1,2-PDL. The resulting plasmid, pMR7, contained a 3.7-kb *Pst*I insert, as predicted by the sequence (9, 32).

Clones containing the Tn*10d*(Tc) elements were identified by transformation of the ligation mixture into strain JR501 (restriction deficient and modification proficient; Table 1), plating to Luria-Bertani (LB)-chloramphenicol plates to select for inheritance of the plasmid, and replica printing to LB-chloramphenicol plates containing tetracycline at a concentration of 2 μg per ml. Coinheritance of both markers was confirmed by retransformation of the parent strain, selecting chloramphenicol-resistant cells and assessing coinheritance of the tetracycline resistance marker.

For the plasmid containing *poc-102*::Tn*10d*(Tc), two *Eco*RI fragments were subcloned to separate the two halves of the transposon. For the *poc-106*::Tn*10d* (Tc)-containing plasmid, an *Eco*RI fragment and an *Eco*RI-*Pst*I fragment were subcloned into pUC19 (25).

Plasmid pMR16. Plasmid pMR7 was digested with *Bst*NI, treated with Klenow enzyme to blunt the ends, and ligated to *Hin*cII-digested pSU19. The *Bst*NI site is located between the putative −35 and −10 regions of the *pocR* promoter (8). Thus, this clone did not express *pocR* from its own promoter.

(b) Plasmid pMR18. Plasmid pMR18 is a derivative of pMR16, constructed by digestion of pMR16 with *Eco*RV and *Sma*I, followed by ligation to *Hin*cII-digested pSU19. In this manner, we were able to isolate clones of *pocR* in which expression of *pocR* was not increased by transcription from P_{lac} on the plasmid, since high-level expression of *pocR* by P_{lac} led to constitutive (1,2-PDL-independent) PocR activity in vivo.

(c) Plasmid pMR20. The *pocR* gene was cloned into plasmid pT7-5 (38) by digesting pMR16 with *Hin*dIII and *Bam*HI and ligating to *Hin*dIII-*Bam*HI-digested pT7-5. Transformants of *E. coli* DH5αF′ were screened by restriction analysis of plasmid clones.

(d) Plasmid pMR31. pDA3 was digested with *Eco*RI and *Bam*HI, and the 330-bp fragment was isolated from a gel slice and ligated to *Eco*RI-*Bam*HI-digested pUC19. Transformants of *E. coli* DH5αF′ were screened by colony color on X-Gal-containing plates and by sequence analysis.

(e) Plasmid pMR35. Plasmid pMR18 was digested with *Bsp*HI and *Eco*RV, filled in with Klenow enzyme, and ligated to *Bam*HI-digested, filled-in pGEX-2T. *Bsp*HI digested pMR18 uniquely at a site containing the putative initiation codon of *pocR*. Thus, this construct did not create any additional amino acids at the N terminus of PocR. Clones containing the desired insertion were identified by restriction analysis. Putative fusion plasmid-containing strains were first analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of IPTG (isopropyl-β-D-thiogalactopyranoside)-induced cultures. Plasmids from strains overproducing a protein of the appropriate size were sequenced across the fusion site to confirm that the correct sequence had been generated.

Enzyme assays. (i) β-Galactosidase. β-Galactosidase assays were performed as previously described (30).

(ii) β-Lactamase. β-Lactamase activity was detected by the method described elsewhere (40), with one exception: sodium deoxycholate was included at a final concentration of 0.3% in the lysis buffer.

Cell extracts. Overexpression of PocR was achieved by using either *E. coli* BL21 (for pGEX-2T derivatives) or BL21/(DE3) (for pT7-5 derivatives). Five hundred milliliters of LB was inoculated with a 4% (vol/vol) inoculum (ca. 8×10^7 CFU) of overnight culture grown at 30°C without shaking (ca. 2×10^9 CFU/ml), grown in LB medium containing ampicillin (50 μg/ml) and 1,2-PDL at 25 mM at 30°C for 4 to 5 h. PocR synthesis was induced by the addition of IPTG (0.1 mM for pT7-5 derivatives and 0.15 mM for pGEX-2T derivatives). Induced cultures were grown at 25°C for 2 to 3 h, and cells were harvested by centrifugation (8,000 × *g* for 10 min), washed once in buffer, and resuspended in 5 ml of buffer (ca. 5×10^{10} cells). Cell lysis was achieved by using a pressure disruption bomb (Parr Instrument Company, Moline, Ill.) or by sonication on ice (twice for 90 s at 50% duty cycle, Branson Sonicator; VWR Scientific, Chicago, Ill.). Crude extracts were clarified by centrifugation at 26,900 × *g* for 1 h with a Sorvall SS-34 rotor in an RC5B refrigerated centrifuge (Dupont Instruments, Wilmington, Del.). The buffer used was 50 mM Tris-Cl, pH 8.0, containing 0.1 mM EDTA, 1 mM dithiothreitol, and 25 mM 1,2-PDL (except where noted). The serine protease inhibitor phenylmethylsulfonyl fluoride (Kodak Chemicals) was added to 1 mM immediately after cell lysis, using a 100 mM solution in isopropanol.

Purification of GST-PocR. Glutathione-*S*-transferase (GST) fusion proteins were purified as previously described (36), with some modifications. Briefly, extracts were incubated with glutathione-agarose beads (*S*-linkage; Sigma Chemical Co., St. Louis, Mo.) at 1.5 ml of 50% beads per 5 ml of extract, for 30 min on ice with gentle mixing. The beads were spun at 750 × *g* for 5 min, washed three times with 15 ml of MTPBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.3), and eluted three times with 1 ml of 50 mM Tris-Cl (pH 8.0)–25 mM, 1,2-PDL–10 mM glutathione. The beads were incubated for 5 min on ice during each wash and 10 min on ice during each elution step, with gentle mixing.

Thrombin cleavage of fusion proteins. Thrombin was added at 100 ng/25 μg of fusion protein in elution buffer containing 150 mM NaCl and 2.5 mM $CaCl_2$ as previously described (36) and incubated for 2 h at 30°C. The products were analyzed by SDS-PAGE (21).

SDS-PAGE. Protocols for analysis of proteins were as described in reference 33. Gels contained 12% (wt/vol) acrylamide. A Mini-PROTEAN II apparatus (Bio-Rad Laboratories, Hercules, Calif.) was used to separate proteins.

Gel retardation assay. Protein was incubated for 15 min at room temperature with labeled DNA in assay buffer (20 mM Tris-Cl [pH 7.6], 5% sucrose, 1 mM dithiothreitol 10 mM EDTA, 30 μg of sheared salmon sperm DNA ml^{-1} 40 μg of bovine serum albumin ml^{-1}, 10 mM magnesium acetate, 100 mM potassium glutamate) and then loaded onto a 5% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer with the power on. 1,2-PDL was added to the running buffer at 25 mM, except for the experiment whose results are shown in Fig. 2. DNA was prepared by restriction enzyme cleavage of the appropriate plasmid and labeled with [α-^{32}P]dATP (800 Ci/mmol; NEN Radiochemicals, Wilmington, Del.) and Klenow enzyme. Labeled DNA was separated from unincorporated nucleotides by means of a spin column (22). DNA was electrophoresed for 1.5 h at 100 V at room temperature, and the gels were dried under vacuum.

DNase I footprinting. We used a protocol adapted from the one reported by Newlands et al. (24). DNA samples were prepared as described in reference 5 by PCR. pMR31 (5 ng) was used as the template, and the mp18/19 −40 (5′-

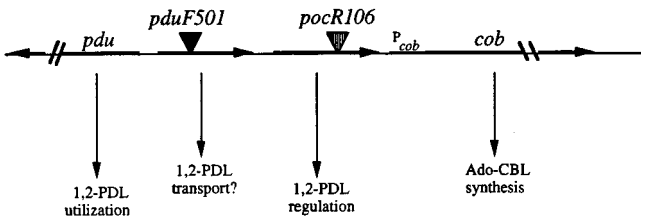

FIG. 1. The *cob-pdu* regulon and location of *pocR* and *pduF* insertions. Transcribed regions are indicated by large arrows in the appropriate direction. The putative function of each gene or region is indicated. The Tn*10d*(Tc) insertions in *pocR* and *pduF* are indicated by inverted triangles. *pocR* and *pduF* are drawn as independent transcriptional units. The figure is not to scale.

GTTTTCCCAGTCACGAC-3′) and reverse (5′-AACAGCTATGACCATG-3′) sequencing primers (obtained from Genosys) were used to amplify the *cob* promoter region. The DNA was purified with a spin column. Protein was incubated as for the gel retardation assay in 50-μl reaction volumes, and then DNase I was added to 1 μg/ml. The reaction mixture was incubated for 30 s, and then the reaction was stopped by the addition of 4 μl of 0.125 M EDTA–2 M sodium acetate followed by the addition of 60 μl of phenol and vortexing. Samples were then ethanol precipitated and resuspended in 10 μl of loading dye (7 M urea, 1× Tris-borate-EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). A sample of 5 μl was resolved on an 8% acrylamide–7 M urea gel for 2 h at 55 W. Gels were dried and analyzed by using a PhosphorImager 4451 (Molecular Dynamics, Sunnyvale, Calif.) or exposed to X-ray film at −80°C.

Primer extension. RNA was prepared with the RNeasy kit (Qiagen Inc., Chatsworth, Calif.). Primer extension reactions were done by following a published protocol (35), with some modifications. RNA was combined with 2 pmol of primer and 5.5 μl of labeling mix (7.5 μM [each] dTTP, dCTP, and dGTP). This mixture was boiled for 2 min and then cooled to room temperature. One microliter of 50 mM dithiothreitol (50 nmol), 1 μl of 10× reaction buffer (300 nmol of Tris-HCl [pH 8.3], 300 nmol of NaCl, 50 nmol of $MgCl_2$), and 0.63 μg of actinomycin D (dissolved in methanol) were added. The mixture was incubated for 10 min at 42°C; this was followed by the addition of 8 U of avian myeloblastosis virus reverse transcriptase (Molecular Genetics Resources, Tampa Fla.) and 1 μl of [α-^{32}P]dATP (800 Ci/mmol). After 30 min of incubation at 42°C, 3 μl of a mix of nucleotides, each at 2.5 mM, was added, and the mixture was incubated for an additional 30 min. The reaction was stopped by the addition of 13 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% xylene cyanol, and 0.05% bromophenol blue). After heating to 80°C for 3 min, a 5-μl sample was electrophoresed on a 6% polyacrylamide–7 M urea gel for 2 h at 60-W constant power.

RESULTS

Identification of the location of *poc* insertions. We previously reported the isolation of two classes of insertion mutations which affected 1,2-PDL-dependent transcription of *cob* and *pdu* but which were phenotypically distinct from one another (30). To determine what function these insertions affected, the Tn*10d*(Tc) insertions were cloned from the chromosome and the sequence of the junction of the insertions with the surrounding chromosomal DNA was determined.

As shown in Fig. 1, using the reported sequence of the *pduF* and *pocR* genes (9), we determined that *poc-102*::Tn*10d*(Tc) was located in the *pduF* gene and that *poc-106*::Tn*10d*(Tc) was located in the *pocR* gene. Strains carrying insertion *poc-102*::Tn*10d*(Tc) [hereafter renamed *pduF501*::Tn*10d*(Tc)] displayed reduced but not abolished transcription of *cbi*::MudJ and *pdu*::MudA fusions in response to 1,2-PDL. Considering that *pduF* is postulated to encode a 1,2-PDL transport protein, the phenotype of a *pduF* mutant may be due to a decreased amount of 1,2-PDL entering the cell. Additionally, since it has been suggested that insertions in *pduF* may exert polarity onto the downstream *pocR* gene, *pduF* mutants may also have decreased amounts of PocR protein (8).

In contrast, the mutation determined to be in the *pocR* gene [*pocR106*::Tn*10d*(Tc)] completely eliminated 1,2-PDL-dependent gene expression (30). This was consistent with the proposed role of PocR as a transcriptional activator protein (4, 9, 30, 32). A strain unable to synthesize the PocR protein would be completely unable to sense and respond to the presence of 1,2-PDL in the medium. Since the phenotype of strains containing the *pocR106*::Tn*10d*(Tc) insertion can be complemented in *trans* by a plasmid containing only the *pocR* gene (pMR18; data not shown), it seems likely that the effect of the *pocR106*::Tn*10d*(Tc) insertion on expression of the downstream *cob* genes is not due to polarity.

Overexpression of PocR. To isolate biochemically useful quantities of pure PocR protein, we cloned the *pocR* gene into the T7 RNA polymerase-based overexpression vector, pT7-5 (38). In this system, PocR was overproduced from the strong T7 promoter by using the native ribosome binding site of *pocR*. When cell extracts of PocR-overproducing strains were examined by SDS–12% PAGE, significant amounts of a 34-kDa protein were detected (data not shown). We determined that this protein was PocR on the basis of its size (predicted by the translated DNA sequence to be 34 kDa [9]) and by its absence in control cell extracts obtained from strains carrying plasmid pT7-5 lacking *pocR*. PocR proved to be highly insoluble when overexpressed (data not shown). The amount of soluble PocR in cell extracts increased when overexpression was performed in strain BL21(DE3). We found that expression of PocR at 25°C also improved the yield of soluble PocR (data not shown). By using these conditions, we were able to generate cell extracts which showed the predicted DNA-binding activity of PocR in vitro (see below).

DNA-binding activity of PocR. On the basis of the homology between PocR and AraC, it was likely that PocR is a transcription factor that regulates expression of *cob* and *pdu* in response to 1,2-PDL. The DNA-binding activity of PocR was investigated by means of a gel retardation assay. The substrate for this assay was a 330-bp fragment containing the *cob* promoter region (the *cob* promoter region from pDA3, shown in Fig. 6). This region was found to contain the anaerobically induced promoter (28), and we determined that it included the sequences necessary for 1,2-PDL-dependent transcription (see below). PocR-containing extracts produce specific protein-DNA complexes with P_{cob}-containing DNA (see Fig. 2). This activity was observed in PocR-containing extracts but not in control extracts, showing that the binding activity requires the presence of PocR (not shown). Complex formation required that the DNA substrate contain the *cob* promoter region, since only the P_{cob}-containing band was shifted and not the vector DNA band.

Three distinct protein-DNA complexes were consistently observed when PocR-containing cell extracts were employed (Fig. 2). This suggested either that PocR bound to multiple sites within the target DNA or that additional proteins bound to the target DNA. If the multiple complexes represented the effect of an additional protein, this would imply that such a protein binds in a PocR-dependent manner, since no binding at all was observed in the absence of PocR. Further experiments support the idea that these bands represent interactions of PocR with the DNA (see below).

Regulation of PocR DNA-binding activity by 1,2-PDL. In our initial experiments, 1,2-PDL was included in all the media and buffers used to prepare PocR-containing extracts, as well as in the assay mixture and the gel running buffer. This was done to ensure maximal activity of PocR. To determine whether the DNA-binding activity of PocR was responsive to 1,2-PDL in vitro, cell extracts containing PocR were prepared under conditions where 1,2-PDL was omitted at a given step during the preparation. DNA-binding activity in the extracts was then determined by the gel retardation assay. As shown in Fig. 2, the presence of 1,2-PDL strongly affected the DNA-binding activ-

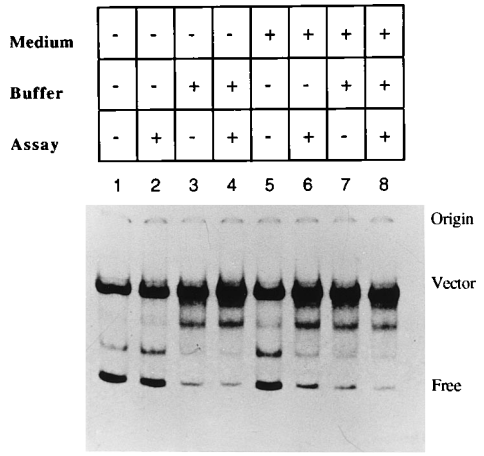

FIG. 2. Regulation of PocR activity by 1,2-PDL. The table indicates whether 1,2-PDL was (+) or was not (−) added to the cell growth medium, extract preparation buffer, or assay buffer, respectively. The DNA template is that contained within pDA3 (−192 to +134). The positions of free P_{cob} DNA, labeled vector, and the origin are indicated. Equivalent amounts of total protein were used in each reaction, and PocR levels were similar as judged by SDS-PAGE.

ity of PocR. Extracts prepared in the presence of 1,2-PDL or in its absence showed distinctly different binding activities (Fig. 2, lanes 1 and 7). Addition of 1,2-PDL to the extract buffer was sufficient to alter the activity of PocR (Fig. 2, compare lanes 1 and 3). Addition of 1,2-PDL only to the medium produced an activity which could be stimulated by the addition of 1,2-PDL during the reaction (Fig. 2, compare lanes 5 and 6). All extracts used contained comparable concentrations of PocR, as determined by SDS-PAGE analysis (not shown). Although we have not yet determined the molecular nature of the different complexes, such an analysis will provide information as to how 1,2-PDL affects PocR activity.

Production of GST-PocR. Although we were able to produce active PocR in crude extracts, scaled-up production of soluble PocR suitable for purification was erratic. The yield of soluble PocR varied, and extensive control experiments failed to identify the source of the observed variability. To circumvent this problem, PocR was produced as a fusion protein whose purification would be facilitated by affinity chromatography. The GST system (Pharmacia, Piscataway, N.J.) was used. GST-PocR contains the GST protein from *Shistosoma japonicum* fused to the N terminus of PocR, resulting in a fusion protein of approximately 60 kDa (36). The fusion site was sequenced to ensure that the correct amino acid sequence was generated and that the *pocR* gene was in frame. Cells expressing GST-PocR directed by plasmid pMR35 produced a new partially soluble polypeptide with the expected molecular weight (data not shown).

In vivo activity of GST-PocR. GST-PocR was active in vivo as judged by complementation of a *pocR* mutant. Plasmid pMR35 was introduced into strain JE1951 [*pocR106*::Tn*10d* (Tc) *cbi-24*::MudJ), and β-galactosidase activity was measured in cells grown with or without 1,2-PDL in the medium. The GST-PocR fusion protein was able to activate *cob* expression in response to 1,2-PDL (140 U of β-galactosidase), relative to the control cells grown in medium lacking 1,2-PDL (20 U of β-galactosidase). This showed that GST-PocR was functional in vivo and that the GST moiety did not interfere with PocR activity. For these assays, cells were grown in the absence of IPTG, the inducer of GST-PocR expression, suggesting that the level of protein made under uninduced conditions was sufficient to activate *cob* transcription.

Activity of GST-PocR in vitro. Binding of the GST-PocR fusion protein to the *cob* promoter region was detectable in crude cell extracts with the gel retardation assay. GST-PocR was purified, and its DNA-binding activity was tested in vitro. The purified fusion protein was able to bind to and shift target DNA containing the *cob* promoter region, whereas a similarly purified control GST preparation showed no DNA-binding activity. As shown in Fig. 3, use of GST-PocR resulted in the same number of protein-DNA complexes as previously observed with the native PocR protein (Fig. 2). The fact that purified GST-PocR protein produced multiple complexes suggested that PocR bound to multiple sites in the target DNA and not that the different complexes were due to binding of other proteins in addition to PocR. Although the GST-PocR preparation was not homogeneous, it seemed unlikely that another protein capable of binding to the target DNA was purified along with GST-PocR.

The GST-PocR protein contained a cleavage site for the protease thrombin at the junction between the two protein sequences (36), providing a convenient method for removing the GST moiety. Cleavage of GST-PocR with thrombin resulted in loss of DNA-binding activity (data not shown). Since there was no thrombin site within PocR, it was concluded that after cleavage, PocR activity was unstable.

Determination of the transcription start site at P_{cob}. The transcription initiation site of the anaerobically induced *cob* message was previously identified (28). To determine whether the PocR-dependent transcript initiated from the same promoter, primer extension analysis was performed with the same plasmids and primer system (28). As shown in Fig. 4, the PocR-dependent message appears to initiate at a point which is different from that of the anaerobically induced message. We repeatedly saw a new signal produced when *cob* expression was induced by 1,2-PDL and PocR (Fig. 4, lanes 3 and 4) that was distinct from that seen when *cob* expression was induced by anaerobiosis (lanes 5 and 6). Under anaerobic conditions, we identified the same initiation site as had previously been determined (28). For the purpose of discussion, we have named the putative PocR-dependent promoter P1 and the anaerobically induced promoter P2. This result suggested that there may be two promoters controlling *cob* expression. Since the cells in this experiment were grown under different conditions, it is possible that the different transcript size reflected differential processing or degradation in vivo. We noted that the addition of 1,2-PDL not only produced the P1 transcript but

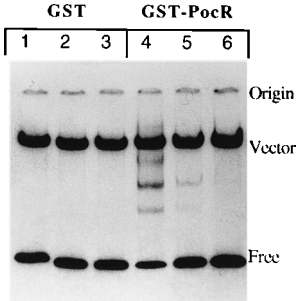

FIG. 3. GST-PocR DNA-binding activity in vitro. The first three lanes contain GST control extracts, and the second three lanes contain GST-PocR. The concentrations of GST-PocR added are 180 nM (lane 4), 18 nM (lane 5), and 3.6 nM (lane 6). Control (GST only) extracts were used at the same volume of extract as in the corresponding GST-PocR lane. The positions of the origin, vector, and free P_{cob} DNA are indicated.

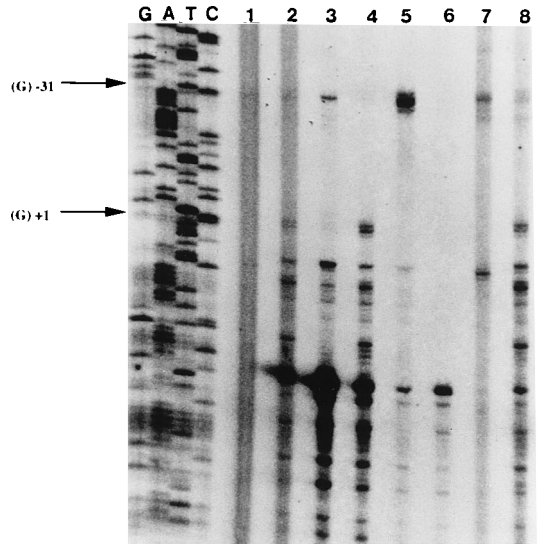

FIG. 4. Primer extension analysis of P_{cob}. Reaction mixtures in lanes 1 and 2 contained 10 μg of RNA prepared from strain TR6583, grown either without (lane 1) or with (lane 2) 1,2-PDL. Those in lanes 7 and 8 were similar, except that 20 μg of RNA was used. Reaction mixtures in lanes 3 and 4 contained 5 μg of RNA prepared from strain JE3157 (pDA3 pMR18), grown either without (lane 3) or with (lane 4) 1,2-PDL. All of the preceeding RNA samples were obtained from strains grown aerobically. Reaction mixtures in lanes 5 and 6 contained 10 μg of RNA prepared from strain DA2515 (pDA3) grown either in the presence (lane 6) or in the absence (lane 5) of oxygen. The guanine residue where transcription starts is shown for P1 (+1) and P2 (−31) (see the text). A sequencing ladder generated with the same primer was loaded next to lane 1.

also inhibited transcription from P2. This may suggest that there is a competition between the two promoters for initiation. We will return to this result in Discussion.

Elements in the *cob* promoter region required for PocR activity. To understand how PocR activates transcription, the region of DNA upstream of the *cob* promoter required for PocR activity was identified. We tested P_{cob} activity when the *cob* promoter was present on pDA3, a multicopy plasmid containing the *cob* promoter region upstream of a promoterless *lacZ* gene, such that β-galactosidase activity reflected *cob* promoter activity. Regulation of *cob* promoter activity in these plasmids by 1,2-PDL was only detected if PocR was overexpressed with pMR18. The level of induction of pDA3 (Table 2) in response to 1,2-PDL was comparable to that seen with the chromosomal fusions and to that of pDA6 (which contains an additional 700 bp upstream and 600 bp downstream of the DNA included on pDA3), indicating that pDA3 likely contained all the information needed for PocR-dependent expression of *cob*.

Deletion analysis of the *cob* promoter region. We determined the *cob* promoter activity in response to 1,2-PDL from derivatives of pDA3 containing 5′ deletions of the *cob* promoter region to further define regions important for regulation by PocR. In the discussion of these results, the numbering of nucleotide positions is in reference to the start of the putative PocR-dependent transcript P1. As shown in Table 2, the results strongly suggested that multiple regions are required for PocR activation. Expression from pDA3 (the full-length fragment, −192 to +134) resulted in a 31-fold increase in β-galactosidase activity in response to 1,2-PDL. Deletion of −192 to −121 resulted in a drastic reduction in *cob* expression, since only a fourfold induction was seen when expression from plasmid pDA7 (−121 to +134) was measured. This suggested that the region between −192 and −121 was important for maximal activation by PocR. The residual level of induction seen with pDA7 suggested that an additional site was present to allow PocR activation of *cob* expression in the absence of the upstream site, albeit at a much lower efficiency. Removal of DNA to −93 abolished induction. The increase in activity seen in the absence of 1,2-PDL is not PocR dependent, as the same result was obtained when control strains containing pSU19 instead of pMR18 (*pocR*$^+$) were used.

These results suggested that PocR might bind at multiple sites within the *cob* promoter region, a suggestion that was supported by the in vitro data showing that multiple protein-DNA complexes were detected by the gel retardation assay. Additionally, we found that GST-PocR was able to bind specifically to the shorter fragments tested, pDA7 (deletion to −121) and pDA9 (deletion to −73) (data not shown), suggesting that these sequences contain PocR binding sites.

PocR binds to two sites at the *cob* promoter. To determine specifically where PocR binds at the *cob* promoter, DNase I protection assays with purified GST-PocR were performed under the same conditions as were used for the gel retardation assay. As shown in Fig. 5, GST-PocR protects the DNA at two sites. One site is located close to the promoter (site I), with protections or enhancements from positions −50 to −97. The other site for PocR binding is further upstream (site II), located at nucleotides −149 to −169. These findings agreed with our earlier results which suggested the presence of multiple binding sites for PocR in this region (Fig. 2 and Table 2). At the concentrations tested, we did not detect preferential binding to site I versus site II. Analysis of the sequence of this region reveals numerous homologous sequences, of both direct and indirect repeats. However, we feel that additional experimental evidence is needed prior to identification of a PocR target sequence.

Effect of CRP on PocR activity. Previous genetic results have demonstrated a role for cyclic AMP (cAMP) receptor protein (CRP)/cAMP in *cob* and *pdu* regulation (1, 13). The level at which CRP acts has not been determined. We tested the role of CRP in PocR-dependent transcription activation by constructing a strain with a deletion in the *crp* gene. We introduced into this strain either the *cbi-24*::MudJ and *pocR106*::Tn*10d*(Tc) insertions or the *pdu-8*::MudJ and *pocR106*::Tn*10d* (Tc) insertions. PocR was provided in a CRP-independent manner by introduction of pMR35 (*gst pocR*$^+$) into these strains. β-Galactosidase activity in cultures with either fusion was dependent on PocR and 1,2-PDL. However, no effect of the *crp* mutation was seen: *crp*$^+$ and *crp* strains produced similar levels of activity under all conditions. In the presence of 1,2-PDL, we

TABLE 2. Promoter activity of P_{cob} 5′ deletion plasmids

Plasmid tested[a]	5′ end	Activity[b]		Fold increase
		Without 1,2-PDL	With 1,2-PDL	
pDA3	−192	25	697	31
pDA7	−121	26	105	4
pDA8	−93	50	85	1.5
pDA9	−73	38	27	<1

[a] The 3′ end of the fragment carried by all plasmids is position +134. Numbering is relative to P1 (see the text).

[b] Activity was determined by measuring β-galactosidase (nanomoles of ONPG hydrolyzed per minute per OD_{650}) and β-lactamase (nanomoles of nitrocefin hydrolyzed per minute per OD_{650}) activities in the same culture and then dividing the former by the latter. Results are the averages from two experiments. Although there was variability between the two experiments, the fold increase in transcription due to 1,2-PDL was constant in both experiments.

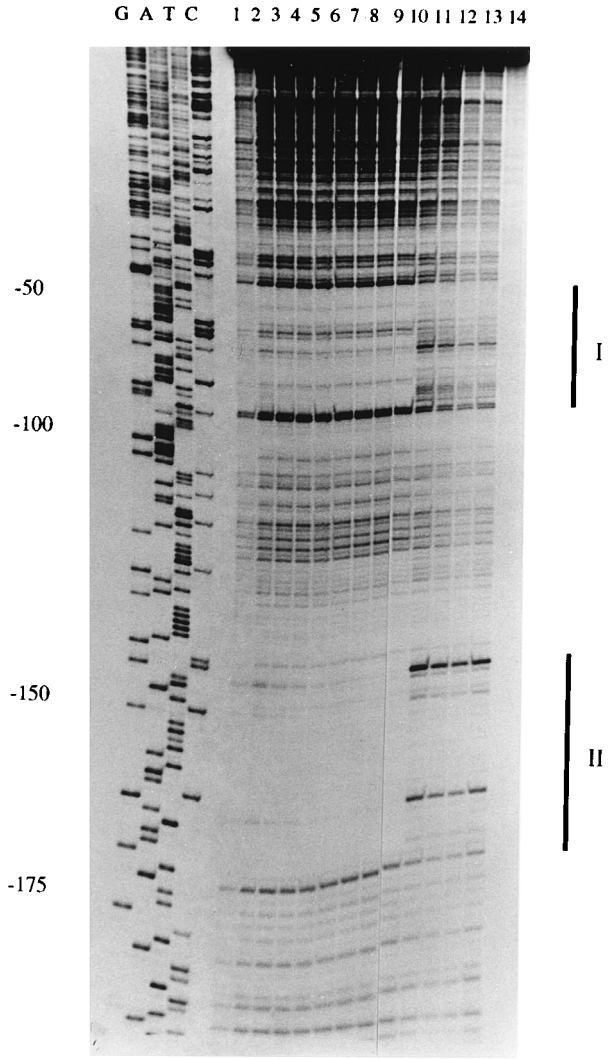

FIG. 5. In vitro footprinting of the *cob* promoter region in the presence of GST-PocR. The top strand is shown. The sequencing ladder was generated with the same end-labeled primer as that used to generate the fragment for footprinting analysis (see Materials and Methods). The concentrations of GST-PocR are 35 nM (lanes 1 and 2), 70 nM (lanes 3 and 4), 135 nM (lanes 5 and 6), 270 nM (lanes 7 and 8), and 410 nM (lane 9). Lanes 10 and 11 contained no extract, and lanes 12 and 13 contained GST control extracts, at the same volume of extract as in lanes 7 and 8. The DNA in lane 14 was not treated with DNase I. Numbers on the left indicate nucleotide positions relative to the P1 initiation site (see the text). Binding sites are indicated by vertical bars.

measured 160 U (crp^+) versus 210 U (*crp*) of β-galactosidase activity, using *cbi-24*::MudJ, and 160 U (crp^+) versus 120 U (*crp*) of β-galactosidase activity, using the *pdu-8*::MudJ fusion. Expression in the absence of 1,2-PDL was likewise unaffected by the *crp* allele tested. This showed that CRP is not required for GST-PocR activity at either the *cob* or *pdu* promoter and suggested that the only role of CRP in this system may be to regulate *pocR* expression.

DISCUSSION

PocR is a DNA-binding protein. We demonstrated in vitro binding of PocR to the *cob* promoter region, consistent with the proposed role of PocR as a transcriptional activator of *cob* and *pdu* expression, and we showed that binding was responsive to 1,2-PDL. This result lends support to our previous findings which suggested that 1,2-PDL was the effector required by PocR (30). We used the GST-PocR fusion protein to determine the probable binding sites for PocR within P_{cob}. We were unable to show GST-PocR-dependent transcription in an in vitro assay with purified RNA polymerase. Additionally, using the DNase I footprinting technique, we were not able to detect binding of RNA polymerase to the *cob* promoter region, either in the presence or in the absence of GST-PocR (data not shown). This suggests either that the purified system is lacking some additional component required for RNA polymerase binding and transcription activation or that something in the GST-PocR preparation prevents RNA polymerase from binding to the *cob* promoter. Clearly, in vivo, GST-PocR activates *cob* transcription. We have recently identified an additional chromosomal locus required for PocR-dependent transcription of *cob* and *pdu* (29). Analysis of this locus may enable us to further understand the requirements for PocR activity.

PocR binds to two sites within Pcob. DNA binding sites of proteins in the AraC family vary, although certain patterns have emerged (12). Binding generally involves two 17-bp half sites either directly or inversely repeated. The site usually overlaps the −35 region of the promoter. Comparison of PocR binding with this pattern reveals that PocR seems to have different requirements: the presence of two well-spaced sites, both of which are required for PocR activation of *cob*, seems to represent a unique mechanism of binding within this family.

We note that the two binding sites for PocR at P_{cob} which we identified by DNase I footprinting with purified GST-PocR do not correlate with those proposed in reference 8, in which three PocR binding sites at P_{cob} were predicted on the basis of computer analysis. Binding of PocR at the upstream site coincides well with the upstream-most site identified by Chen et al. (8), and similarly, the downstream-most site predicted is included within the binding region we determined. A third site for PocR binding was predicted to be located between the two sites identified by our analysis (8). Binding of PocR to this site was not detected in our DNA footprinting experiments. It is possible that footprinting experiments using native PocR, instead of GST-PocR, may detect the predicted third site. This seems unlikely, given that PocR and GST-PocR produce similar binding patterns in the gel retardation assay. We point out that the sizes of the footprints we obtained (20 bp at the upstream site and 40 bp at the downstream site) suggest that PocR binding to the two regions may have different characteristics.

PocR-dependent transcription initiation site. Previous analysis suggested that the anaerobically induced transcript initiated at the guanine residue labeled P2 in Fig. 6 (28). We note the presence of several putative ArcA binding sites (11) (data not shown). This agrees with previous results obtained in vivo (1, 2) and suggests direct activation of *cob* by ArcA. However, no in vitro data to support direct interaction of ArcA with P_{cob} are available.

Using the same plasmid system and primer, we have found a new putative transcription start site at the *cob* promoter, one that is responsive to 1,2-PDL (called P1). Although further analysis is needed to determine whether this truly represents a new promoter, indirect evidence suggests that it may.

First, analysis of the region shows a sequence with homology to the −35 and −10 consensus sequences of a σ70-dependent promoter, although the similarity is low. At this point, we do not have any evidence that the promoter may be transcribed by RNA polymerase containing a different σ factor.

Second, *cob* promoter mutants with increased transcription initiation at P1 have been isolated (3). Sequence analysis of

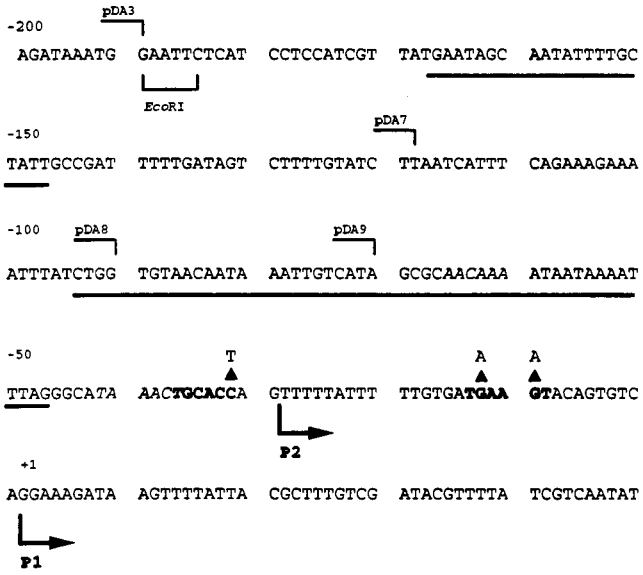

FIG. 6. Nucleotide sequence of the *cob* promoter region. P1 and P2 indicate transcription initiation sites defined by primer extension analysis. The end points of the deletion plasmids are noted above the sequence. PocR binding sites are underlined. The putative −35 and −10 regions for P2 are italicized, and those for P1 are in boldface type. Mutations leading to increased initiation at P1 are indicated as letters above the sequence. The 3′ end of the promoter fragments is located approximately 75 nucleotides downstream of the sequence shown.

these mutants reveals that the mutations alter residues that we suggest might make up the −35 and −10 regions of P1, altering this regions toward consensus, which would explain the increased transcription seen by Andersson (3).

Third, the downstream PocR binding site would be located at −50 to −97 with respect to P1, which seems a more plausible relationship than that involving initiation at P2, which would result in the PocR binding site extending from −17 to −64 relative to the transcription initiation site. This information is summarized in Fig. 6.

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