Two Global Regulatory Systems (Crp and Arc) Control the Cobalamin/Propanediol Regulon of *Salmonella typhimurium*

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The genes for cobalamin (vitamin B₁₂) biosynthesis (*cob*) are coregulated with genes for degradation of propanediol (*pdu*). Both the *cob* and *pdu* operons are induced by propanediol by means of a positive regulatory protein, PocR. This coregulation of a synthetic and a degradative pathway reflects the fact that vitamin B₁₂ is a required cofactor for the first enzyme in propanediol breakdown. The *cob/pdu* regulon is induced by propanediol under two sets of growth conditions, i.e., during aerobic respiration of a poor carbon source and during anaerobic growth. We provide evidence that, under aerobic conditions, the Crp/cyclic AMP system is needed for all induction of the *pocR*, *cob*, and *pdu* genes. Anaerobically, the Crp/cyclic AMP and ArcA/ArcB systems act additively to support induction of the same three transcription units. The fact that these global control systems affect expression of the gene for the positive regulatory protein (*pocR*) as well as the *pdu* and *cob* operons is consistent with our previous suggestion that these two global controls may act directly only on the *pocR* gene; their control over the *cob* and *pdu* operons may be an indirect consequence of their effect on the level of PocR activator protein. The reported experiments were made possible by the observation that pyruvate supports aerobic growth of all of the mutants tested (*cya*, *crp*, *arcA*, and *arcB*); pyruvate also supports anaerobic growth of these mutants if the alternative electron acceptor, fumarate, is provided. By using pyruvate as a carbon source, it was possible to grow all of these mutant strains under identical conditions and compare their expression of the *cob/pdu* regulon. The role of Crp in control of vitamin B₁₂ synthesis suggests that the major role of vitamin B₁₂ in *Salmonella* spp. is in catabolism of carbon sources; the coregulation of the *cob* and *pdu* operons suggests that propanediol is the major vitamin B₁₂-dependent carbon source.

The cobalamin (*cob*) operon of *Salmonella typhimurium* encodes most of the biosynthetic enzymes for the enzyme cofactor cobalamin (vitamin B₁₂) (19, 20, 25). This operon maps at min 41, adjacent to the divergently transcribed *pdu* operon (18) which encodes enzymes for utilization of propanediol as a carbon and energy source. Both operons are induced by propanediol; this control is mediated by a positive regulatory protein (PocR) encoded by a gene that maps between the *cob* and *pdu* operons (6, 23). The proximity of these operons and their coordinated control probably reflect the fact that vitamin B₁₂ is an essential cofactor for propanediol dehydratase, the first enzyme of propanediol degradation (18, 28). The coregulation also supports the idea that the major function of vitamin B₁₂ in *Salmonella* spp. is in propanediol degradation.

Insight into the physiological significance of the *cob/pdu* regulon might come from knowing what global regulatory systems control transcription. Fragmentary information has suggested that two such systems are involved. Before propanediol was identified as the inducer, it was observed that mutations in the Crp/cyclic AMP (Crp/cAMP) system cause a slight reduction in *cob* operon expression during anaerobic growth on glucose; such mutations do not eliminate the increase in *cob* operon expression seen following a shift from aerobic to anaerobic conditions (13). These results suggested that the *cob* operon is subject to global control by Crp and by some additional system sensing anaerobic conditions; the effects of Crp on the *pdu* operon were not tested.

Before identification of propanediol as an inducer, the *cob* operon was found to be maximally expressed during anaerobic respiration when an alternative electron acceptor was used;

optimal expression was observed during anaerobic growth on glycerol and fumarate. Evidence was presented for a redox control system which responds to a reduced cell interior rather than oxygen per se (3). This redox control system appears to be the ArcA/ArcB system, a two-component system that acts under anaerobic conditions to repress synthesis of proteins involved in aerobic respiration (16, 17). Although the redox effect seen earlier (3) is confirmed, the high expression seen with glycerol (compared with that of other carbon sources) is probably not due to the fact that more rapid metabolism of glycerol generates more reducing equivalents but rather to the fact that glycerol can substitute for propanediol as an inducer (6). Mutations in the *arcA* and *arcB* genes cause a slight reduction in anaerobic expression of the *cob* operon (1). The effects of *crp* and *arc* mutations on induction of the *cob* operon by propanediol have not been tested nor have the effects of these mutations on the *pdu* operon.

We have determined the effects of *crp*, *cya*, *arcA*, and *arcB* mutations on induction of *cob* and *pdu* operons by propanediol. We were able to compare the effects of these mutations both aerobically and anaerobically by finding a carbon source, pyruvate, that supports growth of *Salmonella* *crp* and *arc* mutants during both aerobic respiration and anaerobic respiration (with fumarate as the electron acceptor). Even strains with defects in both the *arc* and *crp* genes can grow on pyruvate under these conditions. This has permitted us to determine the effect of these global control mutations on the *cob/pdu* regulon by comparing regulon expression in isogenic strains grown under the same conditions.

We present evidence here that the Crp and Arc systems affect both the *pdu* and *cob* operons. Under aerobic conditions, induction of these operons requires the Crp/cAMP system but not the ArcA/ArcB system. Under anaerobic conditions, in-

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TABLE 1. Strain list

Strain	Genotype
TT10852	<i>metE205 ara-9 cob-24::MudJ</i>
TT17131	<i>metE205 ara-9 pdu-12::MudJ</i>
TT17167	<i>metE205 ara-9 DUP1736[(pocR12)*MudA*(hisD9952)]</i>
TT17439	<i>metE205 ara-9 DEL833 cob-24::MudJ</i>
TT17445	<i>metE205 ara-9 zhc-3729::Tn10dCm</i>
TT17447	<i>metE205 ara-9 crp-773::Tn10 cob-24::MudJ</i>
TT17449	<i>metE205 ara-9 crp-773::Tn10 pdu-12::MudJ</i>
TT17452	<i>metE205 ara-9 cya-961::Tn10 zhc-3729::Tn10dCm</i>
TT17456	<i>metE205 ara-9 cya-961::Tn10 zhc-3729::Tn10dCmcrp*-661</i>
TT17458	<i>metE205 ara-9 crp-773::Tn10 zhc-3729::Tn10dCm</i>
TT17459	<i>metE205 ara-9 zhc-3729::Tn10dCm cob-24::MudJ</i>
TT17460	<i>metE205 ara-9 zhc-3729::Tn10dCm cya-961::Tn10 cob-24::MudJ</i>
TT17461	<i>metE205 ara-9 zhc-3729::Tn10dCm crp-773::Tn10 cob-24::MudJ</i>
TT17462	<i>metE205 ara-9 zhc-3729::Tn10dCm cya-961::Tn10 crp*-661 cob-24::MudJ</i>
TT17463	<i>metE205 ara-9 zhc-3729::Tn10dCm pdu-12::MudJ</i>
TT17464	<i>metE205 ara-9 zhc-3729::Tn10dCm cya-961::Tn10 pdu-12::MudJ</i>
TT17465	<i>metE205 ara-9 zhc-3729::Tn10dCm crp-773::Tn10 pdu-12::MudJ</i>
TT17466	<i>metE205 ara-9 zhc-3729::Tn10dCm cya-961::Tn10 crp*-661 pdu-12::MudJ</i>
TT17471	<i>metE205 ara-9 zhc-3729::Tn10dCm DUP1736[(pocR12)*MudA*(hisD9952)]</i>
TT17472	<i>metE205 ara-9 zhc-3729::Tn10dCm cya-961::Tn10 DUP1736[(pocR12)*MudA*(hisD9952)]</i>
TT17473	<i>metE205 ara-9 zhc-3729::Tn10dCm crp-773::Tn10 DUP1736[(pocR12)*MudA*(hisD9952)]</i>
TT17474	<i>metE205 ara-9 zhc-3729::Tn10dCm cya-961::Tn10 crp*-661 DUP1736[(pocR12)*MudA*(hisD9952)]</i>
TT17480	<i>metE205 ara-9 cob-24::MudJ arcA201::Tn10dTc</i>
TT17481	<i>metE205 ara-9 cob-24::MudJ zgj-7303::Tn10dCm</i>
TT17482	<i>metE205 ara-9 cob-24::MudJ zgj-7303::Tn10dCm arcB1</i>
TT17483	<i>metE205 ara-9 pdu-12::MudJ arcA201::Tn10dTc</i>
TT17484	<i>metE205 ara-9 pdu-12::MudJ zgj-7303::Tn10dCm</i>
TT17485	<i>metE205 ara-9 pdu-12::MudJ zgj-7303::Tn10dCm arcB1</i>
TT17486	<i>metE205 ara-9 cob-24::MudJ crp-773::Tn10 arcA201::Tn10dTc</i>
TT17487	<i>metE205 ara-9 pdu-12::MudJ crp-773::Tn10 arcA201::Tn10dTc</i>
TT17492	<i>metE205 ara-9 DUP1736[(pocR12)*MudA*(hisD9952) pocR1::Tn10dTc]</i>
TT17493	<i>metE205 ara-9 DUP1736[(pocR12)*MudA*(hisD9952)] arcA201::Tn10dTc</i>
TT17494	<i>metE205 ara-9 DUP1736[(pocR12)*MudA*(hisD9952)] crp-773::Tn10</i>
TT17495	<i>metE205 ara-9 DUP1736[(pocR12)*MudA*(hisD9952)] crp-773::Tn10 arcA201::Tn10dTc</i>

duction of the regulon is stimulated by additive effects of both the Crp and the Arc systems.

The positive regulatory protein PocR is controlled in a manner that parallels the control of the *pdu* and *cob* operons. This is consistent with an earlier proposal that global control systems may act by directly regulating the level of the positive regulatory protein PocR (6). By controlling the level of PocR protein, these global systems may indirectly exert their control over the *cob/pdu* regulon.

MATERIALS AND METHODS

Bacterial strains and transposons. All strains are derivatives of *S. typhimurium* LT2 (Table 1). The *arcB1* point mutation was provided by D. Andersson (1). The *cya-961::Tn10* and *crp-773::Tn10* mutations were provided by P. Postma. The MudA and MudJ elements are transposition-defective derivatives of phage Mu d1 used to create *lacZ* operon fusions (8, 9, 15). *Tn10dTc* and *Tn10dCm* are transposition-defective derivatives of transposon *Tn10* (12, 30).

Media. Nutrient broth (0.8%) with 85 mM NaCl was used as the rich medium. The minimal medium was NCE (4) supplemented with 1 mM MgSO₄ and one or more of the following compounds: D-glucose, 0.2% for aerobic growth and 0.4% for anaerobic growth; Na pyruvate, 0.44%; Na₂ fumarate, 0.32%; Na₂ succinate, 1.0%; glycerol, maltose, D-sorbitol, and DL-1,2-propanediol, 0.2%. Antibiotics were used at concentrations described previously (6). cAMP was used at a concentration of 5 mM. The chromogenic β -galactosidase substrate X-Gal

(5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid) was used at a concentration of 25 mg/ml.

Genetic techniques. Transductional crosses were mediated by the high-frequency generalized transducing phage mutant P22 HT105/1 *int-201* (27) as described previously (6). Transductants were purified and made phage free by streaking for single colonies on nonselective green indicator plates (10).

β -Galactosidase assays. Cells were grown under either aerobic or anaerobic conditions as described previously (6). Activity was determined by the method of Miller (22). Numbers in our tables are in Miller units; although these strains were assayed many times, the presented number is the result of a single experiment. For particular strains and conditions, the enzyme levels were variable. These values are marked with asterisks in the tables and are discussed later.

Manipulation of *cya* and *crp* mutants. Both *cya* and *crp* mutants are unstable in that strains with these mutations accumulate suppressor and modifier mutations or lose the original mutation. On NB medium, our *cya::Tn10* and *crp::Tn10* insertion mutants frequently reverted to Cya⁺ or Crp⁺ by loss of the *Tn10*. The addition of tetracycline did not eliminate the appearance of revertants. Revertants (Cya⁺ or Crp⁺) that remained Tc^r appeared to arise by duplication of the original mutant gene, which was followed by loss of the *Tn10* insertion from one copy of the gene. Such revertants were invariably unstable and gave rise to frequent Tc^s segregant clones. To minimize selection for Crp⁺ and Cya⁺ revertants, strains were grown on E-glucose medium (29) for all routine procedures.

TABLE 2. Growth phenotypes of *cya* and *crp* mutants

Strain	Relevant genotype	Growth in liquid NCE minimal medium with the indicated carbon source ^{a,b}								
		Aerobic					Anaerobic			
		Succinate	Glycerol	Maltose	Pyruvate	Glucose	Maltose	Glycerol-fumarate	Pyruvate-fumarate	Glucose
TT17445	Wild type	+	+	+	+	+	+	+	+	+
TT17452	<i>cya::Tn10</i>	—	—	—	+	+	—	—	+	+
TT17458	<i>crp::Tn10</i>	—	—	—	+	+	—	—	+	+
TT17456	<i>cya::Tn10 crp*</i>	+	+	+	+	+	+	+	+	+

^a Cells were grown at 37°C in liquid medium with methionine; on solid medium, *cya* and *crp* mutants failed to grow anaerobically, even on glucose.

^b Symbols: +, turbidity approximately equivalent to that of a fully grown glucose culture scored visually after 24 h of incubation; —, no growth.

In the course of constructing strains, we noticed that *cya* and *crp* null mutants have a characteristic phenotype on green indicator plates (medium described above); both mutant types form colonies that are lighter in color than those of wild-type colonies. Colonies of a *crp** mutant (with or without a *cya* mutation) are indistinguishable from those of the wild type. These colony phenotypes are not understood but proved useful in strain construction.

Isolation of *crp mutants.** The Crp protein is a positive regulator required for maximal expression of a variety of genes involved in carbon source breakdown. Normally, activation of these genes occurs only in the presence of cAMP. Mutant forms of the Crp protein (termed Crp*) are able to activate their target genes to some extent even in the absence of cAMP. The Crp/cAMP system has been reviewed recently (7).

The *crp** mutations used here were identified by chemical mutagenesis of strain TT17452, which contains a *cya::Tn10* insertion and a *Tn10dCm* insertion close to the *crp** gene (>95% cotransduction). After diethylsulfate (DES) mutagenesis (24), new *crp** mutants were selected as Mal⁺ revertants of the parent *cya::Tn10* strain (Mal[−]). Mal⁺ revertants (arising at a frequency of 10^{−5}) were screened for retention of Tc^r and ability to grow on glycerol, sorbitol, and succinate. Putative *crp** mutants grew on all such carbon sources. To ensure that the mutation permitting growth was in the *crp* gene, linkage to the *Tn10dCm* near the *crp* locus was confirmed. In this article, all reported data are for strains with mutation *crp*661*.

Isolation of *arcA::Tn10* mutants. By using a transposase with reduced target site specificity (21), 400,000 random *Tn10dTc* insertion mutants were isolated and pooled. A P22 transducing lysate was prepared on this pool. A recipient strain (TT17439) with a *cob-lacZ* fusion and a large deletion of the *arcA* region (*serB* to *thr*) was transduced to tetracycline resistance and prototrophy (Ser⁺ Thr⁺) on NCE glycerol X-Gal plates. Transductants showing reduced expression of *cob* (light blue colonies) were tested for dye sensitivity, a phenotype characteristic of strains with mutations in the *arcA* gene. Dye sensitivity was checked on solid L-broth medium containing 200 mg of toluidine blue per ml (17). The mutant phenotypes, dye sensitivity and reduced expression of *cob*, were always cotransduced with the Tc^r phenotype of the *arcA* mutation. This insertion was 95% linked to the *thr* locus.

Growth on pyruvate for doubling time measurements. Cells pregrown aerobically in E-glucose medium were used to inoculate 2 ml of NCE pyruvate (with or without fumarate). This culture was grown aerobically to full density. For aerobic growth rate determination, 0.2 ml of this culture was used to inoculate 5 ml of NCE pyruvate in a 125-ml flask, and culture turbidity was measured regularly with a Klett-Summerson photoelectric colorimeter. For anaerobic growth, 0.2 ml of the aerobic pyruvate-fumarate culture was used to inoculate 5 ml

of the same medium in an anaerobic chamber (Forma Scientific model 1024) as described previously (6). The tubes were sealed and culture turbidity was measured regularly in a Bausch & Lomb Spectronic 20 spectrophotometer. This series of inoculations was also used to grow cells for β -galactosidase assays.

RESULTS

Growth phenotypes of the *cya*, *crp*, and *crp** mutations used.

Since strains with *cya* and *crp* null mutations accumulate suppressors and modifiers (see Materials and Methods), we transduced these *Tn10* insertion mutations into a fresh genetic background and retested growth phenotypes. The phenotypes of our *cya* and *crp* mutants appear to be consistent with previously described phenotypes for such mutations in *Escherichia coli* and *S. typhimurium* (Table 2).

As shown in Table 2, *cya* and *crp* null mutants were able to grow on pyruvate aerobically and on pyruvate-fumarate anaerobically as long as liquid cultures were tested. For unknown reasons, strains with *cya* and *crp* mutations did not grow anaerobically on any solid medium tested, including nutrient broth and minimal medium with pyruvate or even glucose as a carbon source. The ability of these strains to grow on pyruvate in liquid cultures was critical to later experiments.

Isolation and characterization of the *arcA* and *arcB* mutants. Andersson previously reported that several *arcA* and *arcB* point mutations cause reduced expression of the *cob* operon (1). We isolated an *arcA::Tn10dTc* insertion mutation and used it to test the effect of such a mutation on the *pocR* and *pdu* transcripts. This allele is likely to be null and is easy to move into new genetic backgrounds (see Materials and Methods). The *arcB1* mutation used here was isolated by Andersson on the basis of its deficiency in anaerobic expression of the *cob* operon; he demonstrated that this mutation affects the *arcB* gene (1).

Growth of *crp*, *cya*, and *arc* mutants on pyruvate. To compare the effects of various defects in global control systems on expression of the *cob/pdu* regulon, we needed to find a carbon source that would support both aerobic and anaerobic growth of strains with mutations in the *crp*, *cya*, and *arc* genes. We found that pyruvate was such a carbon source.

To characterize the growth phenotypes of *cya*, *crp*, *crp**, and *arc* mutations, we measured growth rates on pyruvate aerobically and on pyruvate-fumarate anaerobically, conditions we later used for performing β -galactosidase assays. As shown in Table 3, pyruvate supported growth of all strains tested, but both *arc* and *crp* mutations caused a reduction in growth rate that was more marked under anaerobic conditions. During anaerobic growth on pyruvate-fumarate, the combined effect of the two mutations on doubling time is approximately equal

TABLE 3. Doubling times of *crp* and *arc* mutants on pyruvate

Strain	Relevant genotype ^a	Doubling time (h) under indicated conditions ^b	
		Aerobic, pyruvate	Anaerobic, pyruvate-fumarate
TT17445	Wild type	1.7	2
TT17452	<i>cya::Tn10</i>	2.8	4.5
TT17458	<i>crp::Tn10</i>	2.8	4.5
TT17456	<i>cya::Tn10 crp*</i>	1.7	2
TT10852	Wild type	1.5	2
TT17480	<i>arcA::Tn10</i>	1.6	3.5
TT17486	<i>crp::Tn10 arcA::Tn10</i>	3	7

^a The four strains listed first in the table are an isogenic set; the last three strains are an isogenic set in a slightly different genetic background.

^b Cells were grown at 37°C in NCE medium with methionine as described in Materials and Methods.

to the sum of the individual effects, suggesting that the two systems affect growth rate independently.

Effects of *cya* and *crp* mutations on aerobic expression of the *cob/pdu* regulon. Isogenic strains containing a *cob-lac* or *pdu-lac* operon fusion and a mutation in the *cya* or *crp* gene were assayed for β -galactosidase activity after growth on glucose or pyruvate as carbon sources and with cAMP or propanediol as possible additions to the growth medium. As shown in Table 4, the Crp/cAMP system is essential for induction of the *cob/pdu* regulon in cells grown on poor carbon sources aerobically.

Both the basal and induced expression levels of the *cob* operon are stimulated by the addition of exogenous cAMP to cultures growing on either glucose or pyruvate. Exogenous cAMP restored wild-type expression to a *cya* mutant (unable to make cAMP) but had no effect on a *crp* mutant. In a *cya* mutant, induced expression of the *cob* operon was increased by a *crp** mutation. Expression was higher in a *crp** mutant than in a Crp⁺ strain and was further stimulated by adding exogenous cAMP, indicating that the particular *crp** allele used encodes a protein that is still responsive to cAMP.

Propanediol did not stimulate aerobic *cob* operon expression during growth on glucose unless a *crp** mutation was present; the effect of this mutation is equivalent to increasing

cAMP levels in a Crp⁺ strain. During growth on pyruvate, propanediol stimulated *cob* expression in wild-type or *crp** backgrounds (a higher level was achieved in the latter), but no induction was seen in the *cya* or *crp* mutants. Therefore, the Crp system seems essential for aerobic induction by propanediol.

The *pdu* operon generally showed the same responses to *cya* and *crp* mutations as the *cob* operon; however, uninduced levels of transcription were lower and induced levels were higher. The addition of cAMP alone did not stimulate the basal expression of the *pdu* operon during growth on either glucose or pyruvate. In cells grown on glucose, propanediol (presented alone) induced the *pdu* operon only in the strain carrying a *crp** mutation. The *pdu* operon was induced in Crp⁺ cells growing on glucose with the addition of both propanediol and cAMP, but there was no induction in a *crp* null mutant. During growth on pyruvate, induction by propanediol was eliminated by *crp* or *cya* null mutations. Thus, propanediol induction of both the *cob* and *pdu* operons is absolutely dependent on the Crp system during aerobic growth.

Several values in Table 4 (and subsequent tables) are followed by asterisks. These β -galactosidase activities varied within the ranges indicated in the footnotes, and the numbers given in the table are median values. Possible causes of this variation will be discussed later (see Discussion).

Effects of *cya* and *crp* mutations on anaerobic expression of the *cob/pdu* regulon. The same strains used above were assayed under anaerobic conditions (Table 5). During anaerobic growth on glucose (leading to a low cAMP level), the *crp* and *cya* mutations had little effect on induction of the *cob/pdu* regulon by propanediol; in this experiment, expression levels were as high in *cya* or *crp* mutants as they were in the wild-type control. In a separate experiment (see Table 8), a *crp* mutation reduced expression but did not eliminate induction by propanediol. During anaerobic growth on pyruvate-fumarate medium (higher cAMP levels), the Crp/cAMP system is partially responsible for induction by propanediol; strains with *cya* or *crp* mutations were induced by propanediol but to a lower maximal level.

The basal level of anaerobic *cob* operon expression in cells grown on glucose or pyruvate-fumarate is increased by the addition of exogenous cAMP or by the presence of a *crp**

TABLE 4. Effects of *crp* and *cya* mutations on aerobic expression of the *cob/pdu* regulon

Strain	Relevant genotype ^a	β -Galactosidase activity (Miller units) under indicated conditions ^b							
		Glucose				Pyruvate			
		None	cAMP	PD	PD-cAMP	None	cAMP	PD	PD-cAMP
TT17459	<i>cob::lac</i>	2	15	3	95	5	54	75* ^c	400
TT17460	<i>cob::lac cya</i>	<1	14	<1	96	<1	50	<1	500
TT17461	<i>cob::lac crp</i>	<1	<1	<1	<1	<1	<1	<1	<1
TT17462	<i>cob::lac cya crp*</i>	10	22	30* ^d	240	18	42	300	640
TT17463	<i>pdu::lac</i>	<1	<1	<1	95	<1	<1	280* ^e	1,300
TT17464	<i>pdu::lac cya</i>	<1	<1	<1	71	<1	<1	<1	1,500
TT17465	<i>pdu::lac crp</i>	<1	<1	<1	<1	<1	<1	<1	<1
TT17466	<i>pdu::lac cya crp*</i>	<1	<1	34	370	<1	<1	530	1,400

^a All strains carry a MudJ insertion either in the *cob* (*cob::lac*) or *pdu* (*pdu::lac*) operon; these insertions fuse the *lac* operon of the inserted material to the relevant chromosomal operon.

^b Cells were grown aerobically at 37°C in NCE medium with methionine and the indicated sources of carbon and energy. None, no addition to the growth medium; cAMP, PD, and PD-cAMP, additions of cAMP, propanediol, and propanediol-cAMP; respectively. Values marked with an asterisk were found to be variable in replicate experiments.

^c Enzyme levels varied from 15 to 130 Miller units.

^d Enzyme levels varied from 10 to 50 Miller units.

^e Enzyme levels varied from 70 to 700 Miller units.

TABLE 5. Effects of *crp* and *cya* mutations on anaerobic expression of the *cob/pdu* regulon

Strain	Relevant genotype ^a	β -Galactosidase activity (Miller units) under indicated conditions ^b							
		Glucose				Pyruvate-fumarate			
		None	cAMP	PD	PD-cAMP	None	cAMP	PD	PD-cAMP
TT17459	<i>cob::lac</i>	4	24	37	530	73	310	760	1,200
TT17460	<i>cob::lac cya</i>	3	42	59 ^{*c}	340	9	290	92	1,100
TT17461	<i>cob::lac crp</i>	3	3	81 ^{*c}	61 ^{*c}	10	9	64	120
TT17462	<i>cob::lac cya crp*</i>	19	52	220	300	140	320	1,000	1,100
TT17463	<i>pdu::lac</i>	1	14	74	1,300	1	6	3,500	3,100
TT17464	<i>pdu::lac cya</i>	1	5	160 ^{*d}	1,700	1	4	260	4,700
TT17465	<i>pdu::lac crp</i>	1	1	150 ^{*d}	160 ^{*d}	1	1	200	140
TT17466	<i>pdu::lac cya crp*</i>	1	33	1,100	1,100	1	21	4,300	4,300

^a All strains carry a MudJ insertion either in the *cob* (*cob::lac*) or *pdu* (*pdu::lac*) operon; these insertions fuse the *lac* operon of the inserted material to the relevant chromosomal operon.

^b Cells were anaerobically grown at 37°C in NCE medium with methionine and the indicated sources of carbon and energy. None, no addition; cAMP, PD, and PD-cAMP, additions of cAMP, propanediol, and propanediol-cAMP, respectively.

^c Enzyme levels varied from 10 to 80 Miller units.

^d Enzyme levels varied from 50 to 250 Miller units.

mutation. On pyruvate-fumarate, the basal level of expression is reduced by *cya* or *crp* mutations, indicating that the higher basal level of expression in cells grown on a poor carbon source is largely due to stimulation by Crp/cAMP. Propanediol stimulates *cob* operon expression about 10-fold on either glucose or pyruvate-fumarate. While propanediol does not induce expression aerobically on glucose (Table 4), anaerobic growth on glucose allows induction. This could reflect higher cAMP levels anaerobically (7) or the involvement of an additional global system. We suggest below that the ArcA/ArcB system permits the observed anaerobic induction in the presence of glucose.

For an unknown reason, the *crp** strain shows a higher induced level on pyruvate-fumarate than on glucose even though the strain has a *cya* mutation and should not produce cAMP during growth on either of the carbon sources. It is possible that this is due to increased expression of the *arcA* gene during the slow growth on pyruvate-fumarate.

The *pdu* and *cob* operons respond similarly to all of the changes in genotype and growth conditions tested. While Crp/cAMP was required for all induction aerobically, this

system is only partially responsible for the anaerobic induction of the regulon.

Effect of the ArcA/ArcB system on aerobic expression of the *cob/pdu* regulon. The ArcA/ArcB system plays little or no role in controlling the *cob/pdu* regulon under aerobic conditions (Table 6). During aerobic growth on succinate in the presence of the inducer (propanediol), an *arcA* insertion mutation caused no reduction in expression of the *cob* and *pdu* operons compared to strains with a wild-type *arcA* allele. Mutations in the *arcB* gene similarly had no effect on the expression of *cob-lac* and *pdu-lac* fusions under these conditions. Thus, neither the regulatory protein (*arcA*) nor the membrane-associated sensor protein (*arcB*) of the Arc system seems to be required for normal aerobic expression of the regulon.

Effect of the ArcA/ArcB system on anaerobic expression of the *cob/pdu* regulon. The ArcA/ArcB system is essential for induction of the *cob* and *pdu* operons by propanediol during anaerobic growth on glucose (Table 7). Under these conditions, mutations in either the *arcA* or *arcB* gene completely eliminate propanediol induction of the *cob* and *pdu* operons.

During anaerobic growth on pyruvate-fumarate, the ArcA/

TABLE 6. Lack of a role for the ArcA/ArcB system in aerobic expression of the *cob/pdu* regulon

Strain	Relevant genotype ^a	β -Galactosidase activity (Miller units) under indicated conditions ^b					
		Glucose		Pyruvate		Succinate	
		None	PD	None	PD	None	PD
TT10852	<i>cob::lac</i>	8	5	13	65	34	450
TT17480	<i>cob::lac arcA</i>	3	5	10	39	25	410
TT17481	<i>cob::lac</i>	7	7	15	66	29	470
TT17482	<i>cob::lac arcB</i>	6	8	12	77	33	460
TT17131	<i>pdu::lac</i>	1	1	2	57	8	1,500
TT17483	<i>pdu::lac arcA</i>	1	1	2	43	12	1,400
TT17484	<i>pdu::lac</i>	2	1	1	64	2	1,700
TT17485	<i>pdu::lac arcB</i>	<1	1	11	73	5	1,800

^a The strains used are isogenic pairs. Each such pair is separated by a space. All strains carry a MudJ insertion either in the *cob* (*cob::lac*) or *pdu* (*pdu::lac*) operon; these insertions fuse the *lac* operon of the inserted material to the relevant chromosomal operon.

^b Cells were grown aerobically at 37°C in NCE medium with methionine and the indicated sources of carbon and energy. None, no addition to the medium; PD, propanediol was added.

TABLE 7. Effects of the ArcA/ArcB system on the anaerobic expression of the *cob/pdu* regulon

Strain	Relevant genotype ^a	β-Galactosidase activity (Miller units) under indicated conditions ^b			
		Glucose		Pyruvate-fumarate	
		None	PD	None	PD
TT10852	<i>cob::lac</i>	9	34	87	710
TT17480	<i>cob::lac arcA</i>	8	5	60	480
TT17481	<i>cob::lac</i>	2	28	67	590
TT17482	<i>cob::lac arcB</i>	1	1	54	500
TT17131	<i>pdu::lac</i>	<1	61	15	2,400
TT17483	<i>pdu::lac arcA</i>	2	<1	24	1,600
TT17484	<i>pdu::lac</i>	<1	54	1	2,100
TT17485	<i>pdu::lac arcB</i>	2	9	<1	1,700

^a The strains used are isogenic pairs. Each such pair is separated by a space. All strains carry a MudJ insertion either in the *cob* (*cob::lac*) or *pdu* (*pdu::lac*) operon; these insertions fuse the *lac* operon of the inserted material to the relevant chromosomal operon.

^b Cells were grown anaerobically at 37°C in NCE medium with methionine and the indicated sources of carbon and energy. None, no addition to the medium; PD, propanediol was added.

ArcB system has a partial role in induction of the *cob* and *pdu* operons (Table 7). Under these conditions, mutations in the *arcA* gene cause a 33% reduction in the induced level of expression of the *cob* and *pdu* operons; mutations in *arcB* cause a 17% reduction in expression of these operons. The finding that *arcA* mutations reduce but do not prevent expression of the *cob* and *pdu* operons indicates that a global regulator other than the ArcA protein may assist in induction of these operons during anaerobic growth on poorer carbon sources. The data presented above in Table 5 suggest that this regulator is the Crp/cAMP complex.

Combined effects of *arcA/arcB* and *crp* mutations on induction of the *cob* and *pdu* operons. During anaerobic growth on poor carbon sources, induction of the *cob* and *pdu* operons requires either the Arc or Crp global system; maximal induction requires both the Arc and Crp systems. During anaerobic growth on pyruvate-fumarate, the *arcA* mutation reduced the maximum induced level of the *cob* and *pdu* operons by 33%

(Table 8). The *crp* mutation reduced expression of the two operons by 76 and 87%, respectively. Double mutants with both a *crp* and an *arcA* mutation are unable to induce either the *cob* or *pdu* operon during anaerobic growth on pyruvate-fumarate. These results show that either the Crp/cAMP complex or the ArcA/ArcB system can mediate induction of the *cob* and *pdu* operons during anaerobic growth on pyruvate-fumarate and that the additive effects of these regulators are needed for maximal expression.

Effects of *cya* and *crp* mutations on expression of the *pocR* gene. The PocR regulatory protein, which is required for induction of the *cob* and *pdu* operons, is itself subject to global regulation (6). Expression of *pocR* is induced by propanediol in strains that are phenotypically PocR⁺ but not in strains that are PocR⁻ (6). To provide a *pocR*⁺ gene to strains carrying a *pocR::lac* insertion mutation, a duplication was constructed as described previously (6); the duplication strain has a wild-type *pocR* allele in one copy of the duplicated region and a *pocR::lac* insertion in the other copy of the *pocR* gene. Mutations (*cya* and *crp*) were added to this duplication strain to test global control of *pocR* transcription.

As shown in Table 9, the *pocR* gene is induced under conditions analogous to those that result in induction of the *cob* and *pdu* operons. Both aerobically and anaerobically, there is a two- to threefold increase in *pocR* expression during growth on pyruvate compared with growth on glucose. This increase is eliminated in strains carrying either a *cya* or *crp* mutation. Thus, Crp/cAMP seems to be responsible for the carbon source effect on *pocR* expression. Moreover, *pocR* expression is stimulated by cAMP or by the presence of a *crp*^{*} mutation. In strains lacking the PocR protein (data not shown), no induction by propanediol was observed, but the global control mutations showed the same effects noted above for the merodiploid strains in the absence of propanediol. Thus, the Crp/cAMP system regulates the *pocR* gene independently of autoinduction.

Aerobic autoinduction of the *pocR* gene by propanediol occurs only in PocR⁺ strains and is greater during growth on poor carbon sources unless cAMP is also added. During aerobic growth on pyruvate, expression of *pocR* is induced about 2.5-fold by propanediol. Maximal expression is seen when both propanediol and cAMP are added. No autoinduction is seen in strains carrying either a *cya* or a *crp* mutation,

TABLE 8. Additive effects of the ArcA/ArcB system and Crp/cAMP complex on the anaerobic expression of the *cob/pdu* regulon

Strain	Relevant genotype ^a	β-Galactosidase activity (Miller units) under indicated conditions ^b			
		Glucose		Pyruvate-fumarate	
		None	PD	None	PD
TT10852	<i>cob::lac</i>	9	34	87	710
TT17480	<i>cob::lac arcA</i>	8	5	60	480
TT17447	<i>cob::lac crp</i>	2	9	17	170
TT17486	<i>cob::lac arcA crp</i>	2	4	7	7
TT17131	<i>pdu::lac</i>	<1	61	15	2,400
TT17483	<i>pdu::lac arcA</i>	2	<1	24	1,600
TT17449	<i>pdu::lac crp</i>	1	16	6	320
TT17487	<i>pdu::lac arcA crp</i>	1	<1	7	7

^a All strains carry a MudJ insertion either in the *cob* (*cob::lac*) or *pdu* (*pdu::lac*) operon; these insertions fuse the *lac* operon of the inserted material to the relevant chromosomal operon.

^b Cells were grown anaerobically at 37°C in NCE medium with methionine and the indicated sources of carbon and energy. None, no addition to the medium; PD, propanediol was added.

TABLE 9. Effects of *crp* and *cya* mutations on expression of *pocR*

Strain	Relevant genotype ^a	β -Galactosidase activity under indicated conditions ^b															
		Aerobic								Anaerobic							
		Glucose				Pyruvate				Glucose				Pyruvate-fumarate			
		None	cAMP	PD	cAMP-PD	None	cAMP	PD	cAMP-PD	None	cAMP	PD	cAMP-PD	None	cAMP	PD	cAMP-PD
TT17471	Wild type	39	150	60	250	76	150	180	350	110	210	250	400	420	510	800	1,100
TT17472	<i>cya</i>	28	190	36	360	18	260	18	500	110	180	120	570	130	850	210	1,200
TT17473	<i>crp</i>	23	30	31	32	20	19	18	15	87	98	86	110	430	300	310	240
TT17474	<i>cya crp</i> *	110	170	130	330	130	230	190	520	200	380	590	640	510	950	1,100	1,200

^a All strains are *pocR::lac/pocR*⁺.^b Cells were grown at 37°C in NCE medium with methionine and the indicated sources of carbon and energy. None, no addition to the medium; cAMP, PD, and cAMP-PD, additions of cAMP, propanediol, and cAMP-propanediol, respectively.

indicating that the Crp/cAMP system is the primary global regulator controlling aerobic autoinduction of *pocR*. This pattern of regulation is the same as that seen for the *cob* and *pdu* operons. Similar to the control of *cob* and *pdu*, the Crp/cAMP system appears to be only partially responsible for anaerobic autoinduction of *pocR* by propanediol since *cya* and *crp* mutations do not completely eliminate autoinduction during anaerobic growth on pyruvate-fumarate.

Combined effects of the ArcA/ArcB and Crp/cAMP systems on expression of the *pocR* gene. The Crp/cAMP system is essential for all aerobic expression of the *pocR* gene, while both the ArcA/ArcB and Crp/cAMP systems control anaerobic *pocR* expression (Table 10). A *crp* mutation completely eliminates aerobic autoinduction of *pocR*, while an *arcA* mutation has little effect. Neither *arcA* nor *arcB* mutations affected expression of *pocR* during aerobic growth on glucose or pyruvate, in strains with or without functional PocR protein (data not shown). We conclude that the Crp/cAMP complex is the primary global regulator controlling aerobic autoinduction of the *pocR* gene.

During anaerobic growth on pyruvate-fumarate, both the Crp/cAMP complex and the ArcA/ArcB system play a role in autoinduction of the *pocR* gene. Under these conditions, strains with mutations in either the *arcA* or *crp* gene show partial autoinduction of the *pocR* gene. Strains with mutations in both the *arcA* and *crp* genes are unable to autoinduce the *pocR* gene during anaerobic growth on pyruvate-fumarate. The above results indicate that the general pattern of *pocR* gene expression is the same as that of the *cob* and *pdu* operons. Crp

is the primary global regulator under aerobic conditions, while both the Crp and Arc systems act additively to stimulate anaerobic expression.

DISCUSSION

Previous studies have shown that the adjacent, divergently transcribed *cob* and *pdu* operons are coregulated by the positive regulatory protein PocR, by using propanediol as an effector (6, 23). Induction by propanediol occurs during aerobic growth on poor carbon sources and during anaerobic growth. In the experiments presented here, we provide evidence that two global regulatory systems, Crp/cAMP and ArcA/ArcB, control induction of both the *cob* and *pdu* operons.

During aerobic growth, the Crp/cAMP complex is essential for all induction of these operons. During aerobic growth on pyruvate, null mutations in either the *cya* or *crp* gene eliminated induction of the *cob* and *pdu* operons by propanediol, while *arcA* and *arcB* mutations had virtually no effect. During aerobic growth on glucose, the addition of cAMP to the growth medium allowed induction of both operons.

During anaerobic growth on glucose (leading to a low level of endogenous cAMP), the ArcA/ArcB system is the primary global activator of the *cob* and *pdu* operons; neither operon was induced under these conditions in strains carrying null mutations in either the *arcA* or *arcB* gene. However, during anaerobic growth on the poor carbon source pyruvate (leading to a high level of cAMP), both the ArcA/ArcB system and the Crp/cAMP complex are needed for maximal induction of the *cob* and *pdu* operons. Both *arcA* and *crp* mutations individually caused reduced induction of the regulon during anaerobic growth on pyruvate-fumarate. Strains with null mutations in both the *arcA* and *crp* genes showed virtually no expression of *cob* or *pdu* under these conditions. These results show that under anaerobic conditions, the Crp/cAMP complex and the ArcA/ArcB system have additive effects on induction of the *cob* and *pdu* operons.

Previous findings suggested that the *pocR* regulatory gene, like the *cob* and *pdu* operons, is subject to both global control and induction by propanediol (6). The global control of *pocR*, but not its induction by propanediol, is seen in strains that lack functional PocR protein. Here we show that both the Crp/cAMP and the ArcA/ArcB systems regulate expression of the *pocR* gene. As was found for the *cob* and *pdu* operons, the Crp/cAMP complex is the primary global regulator of *pocR* under aerobic conditions, while maximal anaerobic induction requires the additive effects of both the Crp/cAMP complex and the ArcA/ArcB system. Thus, the general pattern of *pocR* regulation is the same as that seen for the *cob* and *pdu* operons.

TABLE 10. Effects of the ArcAB system and Crp/cAMP complex on the *pocR* gene

Strain	Relevant genotype ^a	β -Galactosidase activity under indicated conditions ^b			
		Aerobic, on pyruvate		Anaerobic, on pyruvate-fumarate	
		None	PD	None	PD
TT17167	(<i>pocR::lac/pocR</i> ⁺)	130	310	400	900
TT17492	(<i>pocR::lac/pocR</i> ⁻)	160	140	370	370
TT17493	(<i>pocR::lac/pocR</i> ⁺) <i>arcA</i>	140	290	170	590
TT17494	(<i>pocR::lac/pocR</i> ⁺) <i>crp</i>	39	36	160	280
TT17495	(<i>pocR::lac/pocR</i> ⁺) <i>arcA crp</i>	40	38	57	46

^a All strains carry a MudJ insertion in one copy of the *pocR* gene; this insertion fuses the *lac* operon of the inserted material to the *pocR* transcript.^b Cells were grown at 37°C in NCE medium with methionine and the indicated sources of carbon and energy. None, no addition to the growth medium; PD, propanediol was added.

FIG. 1. Model for control of the *cob/pdu* regulon. We propose that the global regulatory systems Crp/cAMP and ArcA/ArcB directly stimulate *pocR* gene expression, which in turn allows induction of the two operons by propanediol. Some direct interactions of these global regulatory proteins with the *cob* and *pdu* promoters are not yet excluded.

We have suggested previously that global control of the *cob/pdu* regulon may be mediated by the *pocR* gene. The proposal is that the global controls cause an increase in the internal level of PocR protein, which in turn permits the *cob* and *pdu* operons to respond to propanediol. This model is diagrammed in Fig. 1. The results presented here are consistent with this model but do not eliminate the possibility that one or both of these global systems also acts directly on the *cob* and *pdu* operons. If one compares Tables 6 and 7, it can be seen that *cob* operon expression is stimulated by a shift from aerobic to anaerobic conditions (on pyruvate) in the absence of an added inducer. This could be interpreted as global controllers acting directly at the *cob* promoter. However, these uninduced levels of operon expression are reduced by a *pocR* mutation (6) and thus still seem to depend on transcription stimulated by this protein. We have shown previously (6) that glycerol can act as an inducer. Thus, endogenous metabolites may act as effectors of PocR that induce low-level transcription of the *cob* operon, even in the absence of added propanediol. If this is the case, variation in *pocR* gene expression may still be responsible for changes in *cob* operon expression seen in the absence of propanediol.

The use of pyruvate as a carbon source was crucial to the experiments described in this article. Induction of *cob* and *pdu* by propanediol occurs only on poor carbon sources. Strains carrying *cya* and *crp* null mutations cannot grow on most poor carbon sources; they can grow on pyruvate both aerobically and anaerobically (with fumarate as the electron acceptor). With pyruvate, it was possible to grow mutant and wild-type strains under the same growth conditions. Induction of the regulon does not occur during aerobic growth on glycerol (6) but can occur during growth on pyruvate. This suggests that cAMP levels are higher on pyruvate than on glycerol but that the Crp protein is not needed to induce genes required for breakdown of pyruvate.

As noted earlier, some of our β -galactosidase assay values

varied from experiment to experiment. To pursue the possible causes of this variability, we assayed strains at various times in the growth of a culture (data not shown). The level of operon expression was fairly constant during the exponential phase of growth but increased as cells entered the stationary phase. We feel that the observed variability is probably due to slight differences in cAMP and active ArcA levels caused by harvesting cells at slightly different times. Variability was seen aerobically in Crp⁺ strains during growth on glucose or pyruvate. Under these conditions, ArcA should be inactive and slight changes in cAMP levels could affect induction; no variability was seen when exogenous cAMP was added. Anaerobically, variability was seen only during growth on glucose, when the cAMP level is low and Crp is largely inactive; slight changes in active ArcA levels due to differences in oxygenation of dense cultures could affect induction. No variability was seen under growth conditions that activate both global regulators. The variability seen under particular growth conditions does not interfere with the main conclusions regarding the roles of the Crp/cAMP and ArcA/ArcB systems.

We initiated this investigation in the hope that the behavior of this system would suggest reasons for the genetic investment of *S. typhimurium* in vitamin B₁₂ synthesis. The question is based on the paradoxical observation that *S. typhimurium* has invested nearly 1% of its genome in the synthesis and import of vitamin B₁₂, yet *cob* mutants, which are unable to synthesize vitamin B₁₂, have no laboratory phenotype in an otherwise wild-type cell. The findings that the *pdu* operon is adjacent to the *cob* operon and that propanediol induces expression of both the *pdu* and *cob* operons suggest that the major significance of vitamin B₁₂ synthesis to *S. typhimurium* involves propanediol metabolism. The global regulatory pattern of the *cob* and *pdu* operons by Crp/cAMP and ArcA/ArcB further suggests that *S. typhimurium* has use for propanediol as a carbon and/or energy source under both aerobic and anaerobic conditions. However, *S. typhimurium* can use propanediol as a

sole carbon and energy source only when oxygen is present as an electron acceptor (18). *S. typhimurium* cannot synthesize vitamin B₁₂ under the aerobic conditions tested, even when the *cob* operon is fully induced (2). Hence, the synthesis of vitamin B₁₂ (anaerobic) and the ability to grow on propanediol (aerobic) seem mutually exclusive.

The paradox described above is based on a comparison of strains growing under strictly anaerobic conditions with strains growing under heavy aeration. It seems likely that the natural environment of *S. typhimurium* might frequently provide intermediate (microaerobic) oxygen levels or periodic exposure to oxygen. We have tested the ability of *S. typhimurium* to grow in an oxygen gradient with propanediol as the sole carbon source and no added vitamin B₁₂. Growth occurred at an intermediate oxygen concentration (5). Apparently, low oxygen levels can permit B₁₂ synthesis and still provide sufficient electron acceptor to support respiration of propanediol. *S. typhimurium*, like all enteric bacteria, might frequently encounter microaerobic environments. In the large intestine, which is primarily anaerobic, gases from the bloodstream pass through the epithelium, and nearby regions are microaerobic (26). The global regulatory system that controls induction of the *cob* and *pdu* operons would be expected to allow highest induction of these operons during microaerobic growth on poor carbon sources. The ArcA/ArcB system has already been shown to play a role in the microaerobic induction of the *cyd* operon (11, 14). The availability of propanediol is likely to depend on dietary fucose or rhamnose, whose fermentative breakdown produces propanediol as a by-product.

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