Regulation of Cobalamin Biosynthetic Operons in *Salmonella typhimurium*

JORGE C. ESCALANTE-SEMERENA* AND JOHN R. ROTH

University of Utah, Department of Biology, Salt Lake City, Utah 84112

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Transcription of cobalamin (*cob*) biosynthetic genes in *Salmonella typhimurium* is repressed by cobalamin and by molecular oxygen. These genes seem to be subject to catabolite repression, and they are maximally expressed under conditions of anaerobic respiration of glycerol-fumarate. A 215-fold increase in the expression of *cob* genes occurs when *S. typhimurium* shifts from aerobic growth on glucose to anaerobic respiration of glycerol-fumarate under strictly anoxic growth conditions. Exogenous cyclic AMP substantially stimulates the transcription of *cob-lac* fusions during aerobic growth. However, cyclic AMP is not absolutely required for the expression of the pathway, nor does it mediate the aerobic control. Cobalamin biosynthesis is not seen under aerobic growth conditions, even when transcription is stimulated by the addition of cyclic AMP. Hence, additional control mechanisms triggered by the presence of molecular oxygen must operate independently from transcription effects on the *cob* operons.

The metabolic role of cobalamin (vitamin B₁₂) in bacteria has been studied most extensively in bacteria of the genera *Clostridium*, *Rhodopseudomonas*, *Rhizobium*, *Lactobacillus*, and *Propionibacterium* (1, 3-5, 11, 12, 15, 16, 21, 24, 26, 29, 30, 33, 39, 40, 42). Relatively little is known about the general metabolic significance of cobalamin, how its synthesis is regulated, or why some bacteria produce such large quantities of this cofactor. The finding that cobalamin is synthesized in *Salmonella typhimurium* (19) makes it possible to apply genetic methods of assessing the metabolic significance of cobalamin and the regulation of its synthesis. What importance of cobalamin accounts for the maintenance of a pathway estimated to require 30 biosynthetic enzymes? Why does *S. typhimurium* synthesize cobalamin only under anaerobic conditions?

Of all the chemical reactions known to be catalyzed by cobalamin-dependent enzymes (14), only two have been shown to occur in the enterobacteria *Escherichia coli* and *S. typhimurium*. One of these reactions is the last step in the biosynthesis of methionine (38). In this reaction the methyl group of N⁵-methyltetrahydrofolate is transferred to L-homocysteine to yield methionine. Methylcobalamin is the intermediate in this reaction. The methyltransferase enzyme catalyzing this reaction is the product of the *metH* locus, which is thought to play an important role in the transcriptional regulation of the alternative methyltransferase (the *metE* gene product), which is known to catalyze the same reaction in a cobalamin-independent way (18, 25, 35-37). The second cobalamin-dependent reaction known to occur in enterobacteria involves the catabolism of ethanolamine as a carbon or nitrogen source (6, 30). This reaction is catalyzed by ethanolamine-ammonia lyase to yield acetate and ammonia. This enzyme requires adenosyl-cobalamin as a coenzyme (7). To date, no other cobalamin-dependent reactions have been reported to occur in *S. typhimurium*. Neither of the known reactions is essential, in that *S. typhimurium* mutants lacking the ability to synthesize cobalamin grow normally on glucose both aerobically and anaerobically. These facts suggest that cobalamin may play a role in metabolism that is yet to be discovered.

To approach the metabolic significance of cobalamin we have investigated the biosynthetic genes, thinking that knowledge of their regulation might suggest clues to the general metabolic importance of this cofactor. Mutants defective in cobalamin biosynthesis have been nutritionally classified into three groups (CobI, CobII, and CobIII) (19). CobI mutants are defective in the synthesis of the intermediate cobinamide and can synthesize cobalamin only if cobinamide is provided. CobII mutants are defective in the synthesis of 5,6-dimethylbenzimidazole (DMB) but can make cobalamin if DMB is supplied. CobIII mutants fail to make cobalamin even if both DMB and cobinamide are supplied; they are presumed to be defective in late steps of the pathway involved in joining cobinamide and DMB to form cobalamin. The *cob* biosynthetic genes are located near the *his* operon at min 41 of the chromosome, and they are transcribed counterclockwise (19; R. Jeter and J. Roth, submitted for publication). The *cobI* biosynthetic genes appear to comprise a single operon, since synthesis of cobinamide (the *cobI* reactions) occurs only under anaerobic conditions. The *cobII* and *cobIII* operons are very close to each other but separated (~10 kilobases) from the *cobI* operon as judged by genetic linkage. The number of operons involved in the *cobII* and *cobIII* clusters is uncertain.

Here we report the effect of growth conditions on the transcription of the *cob* operons. The results are interpreted to suggest that the most important role of cobalamin may be to promote anaerobic catabolism of nonfermentable carbon sources.

MATERIALS AND METHODS

Bacteria, media, and growth conditions. All bacterial strains used are derivatives of *S. typhimurium* LT2. The genotypes of all bacterial strains used in this study are listed in Table 1. Difco nutrient broth (0.8%) containing NaCl at a final concentration of 85 mM was used as complex medium. The E medium of Vogel and Bonner (41) supplemented with 11 mM glucose or 22 mM glycerol was used as minimal medium. No-carbon E medium (10) supplemented with MgSO₄ (10 mM) was used for studying the expression of *lac* operon fusions during fermentative growth on citrate. Citrate (trisodium salt) was added to a final concentration of 40

* Corresponding author.

TABLE 1. *S. typhimurium* and *E. coli* strains used

Strain	Genotype	Source ^a
<i>S. typhimurium</i>		
LT2	Prototrophic	
TR6583	<i>metE205 ara-9</i>	
TT172	<i>cysG1510::Tn10</i>	
TT10270	<i>trp-3477::Mu dII173</i>	
TT10326	<i>metE205 ara-9 cob-23::Mu dI-8 (branch I)</i>	
TT10327	<i>metE205 ara-9 cob-24::Mu dI-8 (branch I)</i>	
TT10328	<i>metE205 ara-9 cob-25::Mu dI-8 (branch I)</i>	
TT10365	<i>metE205 ara-9 cob-62::Mu dI-8 (branch II)</i>	
TT10369	<i>metE205 ara-9 cob-66::Mu dI-8 (branch III)</i>	
TT10852	<i>metE205 ara-9 cob-24::Mu dII1734 (branch I)</i>	This work
TT10853	<i>metE205 ara-9 cob-24::Mu dII1734 cya::Tn10</i>	This work
TT10854	<i>metE205 ara-9 cob-24::Mu dII1734 crp-773::Tn10</i>	This work
TT10855	<i>metE205 ara-9 cya::Tn10</i>	This work
TT10856	<i>metE205 ara-9 crp-773::Tn10</i>	This work
TT10857	<i>metE205 ara-9 cob-62::Mu dII1734</i>	This work
TT10858	<i>metE205 ara-9 cob-66::Mu dII1734</i>	This work
TT10859	<i>metE205 ara-9 cob-62::Mu dII1734</i>	This work
TT10860	<i>metE205 ara-9 cob-66::Mu dII1734</i>	This work
TT10861	<i>metE205 ara-9 cob-62::Mu dII1734 crp-773::Tn10</i>	This work
TT10862	<i>metE205 ara-9 cob-66::Mu dII1734 crp-773::Tn10</i>	This work
TT10863	<i>metE205 ara-9 cob-62::Mu dII1734 cysG1510::Tn10</i>	This work
TT10864	<i>metE205 ara-9 cob-66::Mu dII1734 cysG1510::Tn10</i>	This work
TT10865	<i>metE205 ara-9 cob-24::Mu dII1734 cysG1510::Tn10</i>	This work
TT10874	<i>metE205 ara-9 cob-24::Mu dII1734 cob-10::Tn10</i>	This work
TT10875	<i>metE205 ara-9 cob-23::Mu dII1734 (branch I)</i>	This work
TT10876	<i>metE205 ara-9 cob-25::Mu dII1734 (branch I)</i>	This work
TT11295	<i>metE205 ara-9 cob-62::Mu dII1734 crp*-771 cya::Tn10</i>	This work
TT11296	<i>metE205 ara-9 cob-66::Mu dII1734 crp*-771 cya::Tn10</i>	This work
TT11297	<i>metE205 ara-9 cob-24::Mu dII1734 crp*-771 cya::Tn10</i>	This work
TT11775	<i>metE205 ara-9 crp*-771 cya::Tn10</i>	This work
PP914	<i>crp*-771</i>	P. W. Postma
PP1002	<i>trpB223 cya::Tn10</i>	P. W. Postma
PP1037	<i>trpB223 crp-773::Tn10</i>	P. W. Postma
<i>E. coli</i> K-12		
	Prototrophic	

^a Unless otherwise stated, all strains used in this work were obtained from our laboratory's culture collection.

mM. Auxotrophic supplements were added at concentrations recommended elsewhere (10). The final concentrations of antibiotics in complex medium were 20 µg of tetracycline, 50 µg of kanamycin, and 30 µg of ampicillin per ml. The final concentrations of antibiotics in minimal medium were 10 µg of tetracycline, 125 µg of kanamycin, and 15 µg of ampicillin per ml. Solid media contained 1.5 g of Bacto-Agar (Difco Laboratories) per liter. When added to the culture medium, the final concentrations were 1.5×10^{-8} M cyanocobalamin, 1.8×10^{-8} M cobinamide dicyanide, and 3×10^{-4} M DBM. High aeration of the cultures was achieved by growing the cells in 5 ml of medium in a 125-ml culture flask (Bellco Glass, Inc., Vineland, N.J.) at a speed setting of 8 in a Gyrotory shaker (New Brunswick Scientific Co.). Cell density was monitored with a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., Inc.).

(i) **Growth under low-oxygen conditions.** All manipulations of anaerobic cultures were performed inside an anaerobic chamber (Forma Scientific; model 1024) whose atmosphere contained N₂-H₂-CO₂ (90:5:5). Culture medium was rendered low in (but not completely devoid of) dissolved molecular oxygen by overnight degassing inside the anaerobic chamber. Inocula were prepared by single-colony inoculation of 2 ml of low-oxygen medium in 13- by 100-mm culture tubes. Culture tubes were stoppered with sterile rubber stoppers. The rubber stoppers were individually wrapped in aluminum foil, autoclaved, and degassed over-

night inside the anaerobic chamber before use. Larger volumes of anaerobic cultures (e.g., 5 ml) were obtained in 125-ml culture flasks as described above, but fitted with rubber stoppers to maintain low-oxygen conditions. All 5-ml cultures were started at a cell density of approximately 10 Klett units.

(ii) **Growth under anoxic conditions.** To completely remove O₂ gas dissolved in the culture medium, we prepared it as described elsewhere (2) for growing strict anaerobes. Briefly, the culture medium was brought to a boil under a blanket of O₂-free N₂ gas; the boiled medium was brought into the anaerobic chamber and dispensed into tubes (5 ml each). Then, the tubes were fitted with previously degassed rubber stoppers, removed from the chamber, and crimp-sealed with an aluminum seal. The atmosphere in the headspace of the tubes (22 ml) was exchanged for O₂-free N₂ gas to remove residual CO₂ and H₂ gas. The pressure of gas inside the tubes was maintained at 101 kPa (101 kPa is equal to 1 atm). For the experiment showing expression of *cob-lac* fusions as a function of the partial pressure of oxygen, premeasured amounts of air were injected into the tubes after removal of an equivalent volume of N₂ gas from the headspace of the tubes. Partial pressure of oxygen was calculated by assuming 21% O₂ in air. The tubes were then autoclaved and inoculated by means of syringes. Cell turbidity was monitored with a Bausch & Lomb Spectronic 20 spectrophotometer at 650 nm.

TABLE 2. Regulation by molecular oxygen of *cob-lac* fusions

Strain	Relevant genotype	Phenotype of fusion	Beta-galactosidase activity ^a during growth on:			
			Glucose		Glycerol	
			+O ₂	-O ₂	+O ₂	+ Fumarate
TT10852	<i>cob-24::lac</i>	CobI ⁻	4	20	10	430
TT10857	<i>cob-62::lac</i>	CobII ⁻	30	40	40	280
TT10858	<i>cob-66::lac</i>	CobIII ⁻	20	30	30	240

^a Values represent beta-galactosidase activity measured in early log phase cultures. Culture conditions, and substrate concentrations are described under Materials and Methods. Culture medium containing low amounts of oxygen was obtained by overnight degassing of the medium inside an anaerobic chamber whose atmosphere contained 95% N₂, 5% H₂, and 5% CO₂. No further efforts were made to remove residual oxygen from the medium. Values shown represent the means of duplicate determinations. A unit of activity is defined as nanomoles of *o*-nitrophenyl- β -D-galactoside per minute per A₆₅₀ unit. All strains carry a *metE205* mutation.

Genetic techniques. All transductional crosses were performed with a derivative of bacteriophage P22 which contains the mutation HT 105/1 (HT = high transducing), which increases the frequency of generalized transduction (31, 32), and the mutation *int-201*, which prevents the formation of stable lysogens (34). All transductional crosses were performed at a multiplicity of infection of about 1. A rapid procedure for obtaining P22 phage lysates has been described (10). In most cases, phage and bacteria were mixed directly on solid media. Crosses to be plated on kanamycin-containing media were first plated on drug-free medium to allow expression of the drug resistance phenotype and then replica printed onto selective medium. Tetracycline and ampicillin resistance selections did not require any preincubation on drug-free medium. Transductants were freed of phage by streaking on green indicator plates (10). Sensitivity to phage infection was tested by cross-streaking against P22 clear-plaque-forming mutant H5.

Construction of *cob::Mu dII1734* fusions. The isolation of a transposition-defective derivative of the original Casadaban and Cohen *Mu lac* bacteriophage (8) has been reported (17). Operon fusions of the transposition-defective bacteriophage *Mu d* 18 (hereafter referred to as *Mu dA*) were obtained in each of the branches of the cobalamin biosynthetic pathway (Jeter and Roth, submitted). To further reduce the frequency of transposition of these insertion mutations, a deletion of the transposition genes was added to each insert by recombination with Casadaban's *Mu dII1734* (9). This conversion (originally described by Castilho et al. [9]) occurs by homologous recombination between sequences present in *Mu dA* and in *Mu dII1734* to yield *Mu dII1734* (hereafter referred to as *Mu dJ*). The resulting *Mu dJ* derivative carries kanamycin resistance instead of ampicillin resistance and is 11.3 kilobases in size instead of 37.2 kilobases. More importantly, *Mu dJ* lacks the A and B *Mu* functions necessary for transposition, which prevents further transposition of the inserted material.

Beta-galactosidase assay. Beta-galactosidase activity was assayed as described by Miller (23) using CHCl₃-sodium dodecyl sulfate to permeabilize whole cells. The final assay volume was 1.7 ml. Enzyme activity was expressed as nanomoles of *o*-nitrophenyl- β -D-galactoside per minute per unit of optical density at 650 nm (A₆₅₀). All assays were performed in duplicate in early-log-phase cultures (70 to 80 Klett units). *E. coli* K-12 was used as a positive and negative control for the expression of the *lacZ* gene. A culture grown in the presence of the gratuitous inducer isopropyl- β -D-thiogalactopyranoside (1 mg/ml) was used as the positive control. A culture grown in the absence of isopropyl- β -D-thiogalactopyranoside was used as the negative control. Typical values for the amount of beta-galactosidase synthe-

sized by *E. coli* K-12 in the presence of IPTG ranged between 1,100 and 1,300 U of activity per A₆₅₀ unit. In the absence of the inducer, 3 to 5 U of activity per A₆₅₀ unit were recorded.

Chemicals. Isopropyl- β -D-thiogalactopyranoside, *o*-nitrophenyl- β -D-galactoside, cyclic AMP sodium salt (cAMP), antibiotics, trimethylamine-*N*-oxide, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.). Dimethyl sulfoxide was purchased from EM Science (Cherry Hill, N.J.).

RESULTS

Regulation of cobalamin synthetic genes by growth conditions. The effect of molecular oxygen on the transcriptional regulation of *cob-lac* fusions was investigated. Separate strains carrying operon fusions of the *lacZ* gene to a gene in each one of the branches of the cobalamin biosynthetic pathway (*cobI*, *cobII*, and *cobIII*) were tested. Table 2 illustrates the regulatory effect of molecular oxygen on the expression of *cob* genes as measured by the synthesis of beta-galactosidase. These assays were performed under the low-oxygen conditions described in Materials and Methods. The expression of the fusions was assayed for cells grown under conditions of anaerobic fermentation of glucose and anaerobic respiration of glycerol-fumarate. These results were compared to those obtained for cells grown aerobically on glucose or glycerol as the energy source. All the assays presented in Table 2 are on cells unable to synthesize cobalamin, and cultures were grown without cobalamin in the growth medium. L-Methionine was added to the culture medium to satisfy the auxotrophic requirement of all the strains.

Several points should be noted. Maximum expression of the *cob* genes was seen under conditions of anaerobic respiration with fumarate as the electron acceptor. Compared with this level, expression is strongly reduced in the presence of glucose (glucose minus oxygen) or oxygen (glycerol plus oxygen). Also a small but repeatable glucose effect is seen in aerobic cells grown with glucose versus cells grown with glycerol as the carbon source. The effect was twofold on *cobI* fusions. The *cobII* and *cobIII* fusions remained unaffected. This effect suggested the possibility of cAMP control, which was tested and is discussed below.

Fusions to the *cobI* operon routinely show a wider range of expression than *cobII* and *cobIII* fusions; that is, under repressed conditions, *cobI* fusions are shut off more tightly, and under induced conditions they are expressed more highly. This observation was made with three independently isolated fusions in the *cobI* operon (strains TT10852, TT10875, and TT10876; Table 1); these fusions are known to

TABLE 3. Expression of *cob-lac* fusions during fermentation and anaerobic respiration

Strain	Relevant genotype	Phenotype of fusion	Beta-galactosidase activity ^a during growth on:					
			Glucose	Citrate ^b	Glycerol			
					Fumarate (R/F) ^c	TMAO ^d (R/F)	DMSO ^e (R/F)	Nitrate (R/F)
TT10852	<i>cob-24::lac</i>	CobI ⁻	40	110	860 (21.5)	750 (18.8)	570 (14.3)	480 (12.0)
TT10857	<i>cob-62::lac</i>	CobII ⁻	50	100	680 (13.6)	520 (10.4)	440 (8.8)	300 (6.0)
TT10858	<i>cob-66::lac</i>	CobIII ⁻	50	80	620 (12.4)	490 (9.8)	380 (7.6)	170 (3.4)

^a Values represent the average of duplicate determinations of enzymatic activity.

^b All cultures were grown in anoxic NCE medium supplemented with MgSO₄ (10 mM) and trisodium citrate (40 mM).

^c R/F is the ratio of units of enzyme activity per A₆₅₀ unit measured during growth under anaerobic respiration divided by the number of enzyme units per A₆₅₀ unit measured during fermentative growth on glucose. All strains carry an *metE205* mutation. All culture were grown in E medium under strictly anoxic conditions. The concentration of electron acceptors in the culture medium was 10 mM. Concentrations of carbon sources in the culture medium: glucose (11 mM) and glycerol (22 mM).

^d TMAO, Trimethylamine-*N*-oxide.

^e DMSO, Dimethyl sulfoxide.

map at different locations in the operon (Jeter and Roth, submitted). Only one fusion to *cobI*, *cobII*, or *cobIII* is presented, since no difference in their regulatory response was found in parallel experiments. Under conditions of anaerobic respiration (glycerol-fumarate), the level of transcription of a *cobI* fusion (strain TT10852) was 22-fold higher than the level measured under fermentative growth on glucose. The same pattern of regulation was seen for fusions to *cobII* and *cobIII* operons. However, the levels for these operon fusions were only seven- to eightfold higher on glycerol-fumarate versus glucose (Table 2). The most dramatic increase in the transcription of a *cobI-lac* fusion was seen for anaerobic respiration of glycerol-fumarate, where over 400 U of beta-galactosidase activity was produced. This represents an increase of more than 2 orders of magnitude. Compared with the level seen for aerobic growth on glucose, the stimulatory effect of anaerobic expression on *cobII* and *cobIII* fusions was 9- and 12-fold, respectively.

Under strictly anoxic growth conditions, the expression of *cob-lac* fusions varied according to the electron acceptor available to the cell (Table 3). Maximum expression was observed with fumarate, followed by trimethylamine-*N*-oxide, dimethyl sulfoxide, and nitrate. A 215-fold increase in the expression of the *cobI-lac* fusion was observed when we compared transcription level under strictly anoxic conditions on glycerol-fumarate to that measured under aerobic growth on glucose. This value is slightly higher than that seen in

Table 2 because of traces of oxygen present in the early experiments (see below). The growth rate of *S. typhimurium* under each set of conditions was glucose > glycerol-nitrate > glycerol-fumarate > glycerol-trimethylamine-*N*-oxide > glycerol-dimethyl sulfoxide (data not shown). We found no correlation between growth rate and expression of the fusions.

Unlike growth of *E. coli* (22), fermentative growth of *S. typhimurium* on citrate does not require the presence of a cosubstrate, and under these growth conditions the expression of all the *cob-lac* fusions was higher than the expression of the same fusions during fermentative growth on glucose. This, like the stimulation of transcription by aerobic growth on glycerol (Table 2), suggested that *cob* genes might be subject to catabolite repression.

cAMP effect. The results reported in Table 2 and 3 suggested that *cob* genes might be subject to catabolite repression and raised the further possibility that the respiration-fermentation differences might be mediated by changes in the cAMP levels. To test the involvement of cAMP in cobalamin biosynthesis, we constructed strains carrying both *cob-lac* fusions and mutations affecting adenylate cyclase (*cya*⁻), the cAMP receptor protein (*crp*⁻), or both (Table 4). The addition of cAMP to glucose-supplemented medium resulted in increased levels of transcription of all the fusions tested under both aerobic and anaerobic growth conditions. Aerobically and anaerobically, a *cobI-lac cya*⁺

TABLE 4. Role of cAMP in the expression of *cob-lac* fusions

Strain	Relevant genotype	Phenotype of fusion	Beta-galactosidase activity ^a during growth on:					
			Glucose		Glucose + cAMP		Glycerol	
			+O ₂	-O ₂	+O ₂	-O ₂	+O ₂	+ Fumarate
TT10852	<i>cob-24::lac</i>	CobI ⁻	4	20	10	70	10	430
TT10853	<i>cob-24::lac cya::Tn10</i>	CobI ⁻	3	6	20	40	NG ^b	NG
TT10854	<i>cob-24::lac crp-773::Tn10</i>	CobI ⁻	3	6	4	10	NG	NG
TT11297	<i>cob-24::lac crp*-771 cya::Tn10</i>	CobI ⁻	4	20	20	50	10	350
TT10857	<i>cob-62::lac</i>	CobII ⁻	30	40	50	90	40	280
TT10859	<i>cob-62::lac cya::Tn10</i>	CobII ⁻	30	50	50	80	NG	NG
TT10861	<i>cob-62::lac crp-773::Tn10</i>	CobII ⁻	40	50	40	80	NG	NG
TT11295	<i>cob-62::lac crp*-771 cya::Tn10</i>	CobII ⁻	30	40	40	70	30	320
TT10858	<i>cob-66::lac</i>	CobIII ⁻	20	30	30	70	30	240
TT10860	<i>cob-66::lac cya::Tn10</i>	CobIII ⁻	30	40	40	60	NG	NG
TT10862	<i>cob-66::lac crp-773::Tn10</i>	CobIII ⁻	30	40	20	40	NG	NG
TT11296	<i>cob-66::lac crp*-771 cya::Tn10</i>	CobIII ⁻	20	30	30	60	30	290

^a One β-galactosidase unit = 1 nmol of ONPG per min per A₆₅₀ unit.

^b NG, No growth.

TABLE 5. Role of cAMP in the expression of *cob-lac* fusions under anoxic growth conditions

Strain	Relevant genotype	Phenotype of fusion	Beta-galactosidase activity ^a during growth on:				
			Glucose	Glucose + cAMP	Citrate ^b	Citrate + cAMP	Glycerol-fumarate
TT11297	<i>cya::Tn10 crp*-771 cob-24::lac</i>	CobI ⁻	40	110	90	480	810
TT11295	<i>cya::Tn10 crp*-771 cob-62::lac</i>	CobII ⁻	50	80	110	200	660
TT11296	<i>cya::Tn10 crp*-771 cob-66::lac</i>	CobIII ⁻	50	90	80	230	540

^a Units of enzyme activity have been defined in Materials and Methods. Cells were grown in anoxic E culture medium prepared as described in Materials and Methods.

^b All culture were grown in anoxic NCE medium supplemented with MgSO₄ (10 mM) and trisodium citrate (40 mM). Nitrogen gas was the only gas present in the headspace of the culture tubes. All strains carry a *metE205* mutation.

crp⁺ strain showed a three- to fourfold increased transcription of the fusion in the presence of exogenous cAMP. The *cobI-lac cya::Tn10* double mutant expressed basal levels of transcription of the fusion in the absence of cAMP; these levels were stimulated six- to sevenfold by the addition of cAMP (Table 4). A *cobI-lac crp::Tn10* double mutant showed the same behavior as the *cobI-lac cya::Tn10*, except that the addition of cAMP failed to stimulate transcription of the fusion (Table 4). These results suggested a strong cAMP effect on *cob* transcription.

To test whether cAMP mediates the stimulation of transcription seen during anaerobic respiration, a *crp*^{*} mutation was tested. This mutation alters the Crp protein so it can activate transcription even in the absence of cAMP (13, 27, 28). A strain was constructed that had a *crp*^{*} mutation, a *cob* fusion, and a *Tn10* insertion in *cya* (Table 4). In this strain, which cannot make cAMP, the expression of the fusion was similar to that measured for a *cobI-lac cya*⁺ *crp*⁺ strain in either the presence or absence of molecular oxygen. When glycerol was used as the source of energy and fumarate was substituted for molecular oxygen as the final electron acceptor, a 44-fold increase in the expression of the fusion was recorded. As expected, strains containing *cya* or *crp* mutations failed to grow on glycerol. However, the strain carrying the *cobI-lac*, *crp*^{*}, and *cya::Tn10* mutations transcribed the fusion as efficiently as the *crp*⁺ *cya*⁺ *cobI-lac* strain during aerobic growth. The expression of fusions in strains harboring a *cobII-lac* or a *cobIII-lac* fusion and the above-mentioned cAMP-related functions was not affected at all (Table 4).

These results demonstrate that stimulation of transcription under conditions of anaerobic respiration is not mediated by variation of the cAMP levels. This conclusion is supported further by measurements of the expression of the fusions under anoxic conditions (Table 5). Our results show that, although exogenous cAMP greatly stimulates the expression of fusions in cells growing fermentatively on glucose or citrate, we could not achieve the levels seen for cells growing under conditions of anaerobic respiration of glycerol and fumarate. Assuming adequate transport of cAMP,

we would expect higher enzyme levels if the induction were mediated by cAMP. This can be seen when *S. typhimurium* ferments citrate. Under these conditions, the presence of exogenous cAMP (5 mM) stimulates the expression of all the *cob-lac* fusions, but even the most responsive *cobI* fusion reaches only 60% of the level measured when cells respire glycerol-fumarate. If the regulatory effect of oxygen were exerted through variations in the levels of cAMP alone, we would expect the addition of cAMP to result in higher levels of expression at a level near that seen for cells respiring glycerol-fumarate. These experiments do not rule out the possibility that oxygen represses the synthesis of the Crp protein. If this were the case, one might expect that anoxia would derepress Crp^{*} levels and could thus stimulate expression of a Crp-dependent operon even in a *cya* mutant background.

The results described in Table 4 suggested a requirement for cAMP for transcription of *cob* genes. Thus, it was predicted that strains of *S. typhimurium* with a *metE cob*⁺ genotype might require the *cya* and *crp* gene products to synthesize B₁₂ and show methionine-independent growth anaerobically via the MetH enzyme. This prediction was tested by growing appropriate strains carrying a *metE* mutation anaerobically in minimal medium; under these conditions cells must synthesize cobalamin to satisfy their methionine requirement (through use of the cobalamin-dependent MetH enzyme). The doubling times of anaerobically grown cultures are shown in Table 6. (i) With no additions to the culture medium all the strains grew. However, the *cya* and *crp* mutants grew 39 and 25% slower than the wild-type strain, respectively. (ii) Addition of methionine to the medium did not increase the growth rate of the *cya* and *crp* mutants. (iii) Exogenous cAMP increased the growth rate of the *cya* mutant to that of the *cya*⁺ strain. (iv) Exogenous cAMP had a deleterious effect on the growth rate of the *crp* mutant, resulting in a decrease of almost 50% in the growth rate of this strain under that measured for the isogenic *crp*⁺ strain. Thus, the basal level of transcription of *cob* genes seen in the absence of *crp* and *cya* functions appears to be sufficient to provide cobalamin for methionine synthesis.

TABLE 6. Effect of cAMP on B₁₂-dependent growth^a

Strain	Relevant genotype	Doubling time (min) anaerobically in the presence of:		
		No addition	L-Methionine	cAMP
TR6583	<i>metE205 cob</i> ⁺	60	65	62
TT10855	<i>metE205 cob</i> ⁺ <i>cya::Tn10</i>	98	102	62
TT10856	<i>metE205 cob</i> ⁺ <i>crp::Tn10</i>	80	85	115
TT11775	<i>metE205 cob</i> ⁺ <i>crp*-771 cya::Tn10</i>	60	60	62

^a Strains were grown low-oxygen in E medium supplemented with glucose (11 mM) as the energy source. The concentrations of added compounds in the culture medium: L-methionine (0.5 mM) and cAMP (5 mM). Growth conditions were described in Materials and Methods. Cell growth was monitored with a Klett-Summerson photocolormeter (540 nm). Doubling times were obtained from plots of Kett units versus time (minutes).

FIG. 1. Effect of O_2 on the expression of *cob-lac* fusions. Key: ■, *cob-24::lac* (*cobI*); □, *cob-66::lac* (*cobIII*); ▲, *cob-62::lac* (*cobII*); △, *pncB-252::lac*. All cultures were grown in E minimal medium supplemented with glycerol (22 mM) as the source of carbon and energy and fumarate (10 mM) as the final electron acceptor. The medium was made anoxic as described under Materials and Methods. L-Methionine was added to a final concentration of 0.5 mM. Oxygen (air) was injected into the sealed tubes after removal of an equivalent volume of gas from the headspace of the tubes prior to autoclaving. Each point is the average of duplicate measurements. Ideal gas behavior was assumed in determining the partial pressure of oxygen. The rate of decrease in the expression of the fusions as a function of the partial pressure of oxygen was (enzyme units per A_{650} unit per kilopascal of O_2): *cob-24::lac*, 150; *cob-62::lac*, 140; *cob-66::lac*, 135.

The slight effect of *cya* and *crp* mutations on the growth rate seem due to impairment of other metabolic functions required for anaerobic growth.

Regulation by molecular oxygen. Figure 1 illustrates the regulatory effect of oxygen on the of *cob-lac* fusions. Expression was exquisitely sensitive to oxygen. Providing oxygen at pressures greater than 2 kPa (<2% of 1 atm of pure O_2 , or about 10% of atmospheric oxygen) resulted in a sharp decrease in the transcription of the fusions. To investigate

whether this effect of oxygen was specific to fusions to *cob* operons, a control experiment was performed with a fusion to the gene encoding for nicotinic acid phosphoribosyl transferase (*pncB*). The results of this control experiment are also shown in Fig. 1. In this case, unlike the *cob-lac* fusions, increasing levels of oxygen resulted in slightly increased transcription of the *pncB-lac* fusion. The above results explain the variability in the maximum levels of expression of the same fusions that was seen in early experiments (Tables 2, 4, and 7). In later experiments (Tables 3 and 5), O_2 has been excluded completely by boiling the culture medium under oxygen-free nitrogen gas (see Materials and Methods); that is, if dissolved oxygen is removed to different degrees from the culture medium, the expression of the fusions will vary accordingly.

Regulation by cobalamin. To study the effect of cobalamin on the regulation of its biosynthetic pathway we measured the levels of beta-galactosidase synthesized during anaerobic growth (glycerol-fumarate) in the presence or absence of cyanocobalamin in the culture medium. Operon fusions in each of the three branches were tested (Table 7). Transcription of the fusion in *cobI* was reduced sixfold; expression of the *cobII* fusion was reduced approximately threefold; and the expression of the *cobIII* fusion was reduced twofold. Also shown in Table 7 is the effect of cobinamide, DMB, or cobinamide and DMB on the expression of *cob-lac* fusions. The expression of *cobI* remained unaffected by the presence of DMB, but it showed some reduction in the presence of cobinamide. The addition of cobinamide and DMB together resulted in a reduction of approximately 50%. This is interpreted to mean that B_{12} can be readily synthesized from these precursors and that repression of the *cobI* operon can occur. Practically no effect of cobinamide or DMB individually on fusions to branches II and III was recorded. Larger repressive effects of B_{12} have recently been seen (Dan I. Andersson, unpublished results) in cells grown anaerobically on glycerol-fumarate.

Role of biosynthetic intermediates in gene regulation. The regulatory interactions between branches of the pathway might be mediated by levels of particular biosynthetic intermediates. For this purpose strains were constructed which carried both the branch I *cob-24::lac* fusion and a *Tn10* element inserted in either branch II or III. No change was recorded in the amount of beta-galactosidase synthesized in either of the double mutants tested (strains TT10874 and TT12231) (data not shown). Similarly, we tested the effect of *cysG* mutations. A mutation in the *cysG* locus has a *CobI*⁻ phenotype (19), since the substrate for the branch I of the pathway is not made. Thus, in a *cysG* mutant neither cobinamide nor any of its precursors should be present. The regulation of fusions in any of the branches of the pathway remained unaffected when a *cysG* mutation was present in the background (strains TT10863, TT10864, and TT10865)

TABLE 7. Regulation by cobalamin of *cob-lac* fusions

Strain	Relevant genotype	Phenotype of fusion	Beta-galactosidase activity ^a in the presence of:				
			No addition	DMB	CBi	CBi + DMB	B_{12}
TT10852	<i>cob-24::lac</i>	<i>CobI</i> ⁻	280	240	210	130	50
TT10857	<i>cob-62::lac</i>	<i>CobII</i> ⁻	200	190	190	220	70
TT10858	<i>cob-66::lac</i>	<i>CobIII</i> ⁻	110	150	140	150	50

^a Values represent the average of two separate determinations. A unit of activity is defined under Materials and Methods. Concentrations: DBM (3×10^{-4} M), cobinamide (CBi) (1.8×10^{-8} M, and cyanocobalamin (B_{12}) (1.5×10^{-8} M). Cultures were grown under low-oxygen conditions on E medium containing glycerol (22 mM) as the source of energy, and fumarate (10 mM) as the electron acceptor. Enzyme assays were performed on early-log-phase cultures. All the strains carry a *metE205* mutation.

(data not shown), suggesting that intermediates of the pathway have subtle (if any) regulatory effects on the synthesis of B₁₂. The introduction of a mutation in the *oxrA* locus (20, 37a) into strains carrying *cob-lac* fusions had no effect on the transcription of the latter (data not shown).

DISCUSSION

Cobalamin biosynthesis in *S. typhimurium* is regulated by cobalamin and by molecular oxygen. Our findings indicate that the regulation of the pathway is primarily exerted on *cobI*, i.e., the biosynthesis of the corrin ring. We have shown that transcription of all the branches of the pathway is negatively controlled by cobalamin. Our data suggest that cobalamin, and not biosynthetic intermediates, is responsible for the regulation of the pathway. This conclusion is based on our results, which show that *cysG* mutants (which fail to make the substrate for branch I of the pathway) still show normal regulation of all the branches of the pathway.

Although it is largely unclear how the presence of molecular oxygen regulates the biosynthesis of this macromolecule, it is clear that the transcription of *cob* genes is strongly inhibited by even low levels of O₂ (Fig. 1). However, there are further complications to the regulatory effect(s) by molecular oxygen. This is clearly illustrated by our results, which show that anaerobiosis is most effective in stimulating the transcription of *cob* genes when the cell is respiring to an alternative electron acceptor instead of fermenting. This observation can be explained by suggesting that the transcription of *cob* genes is somehow entrained with the changes that occur when the cell shifts from fermentation to respiration, e.g., oxidative phosphorylation, membrane potential, reducing conditions, level of cAMP, etc. On the one hand, we have shown that exogenous cAMP stimulates transcription of *cob* genes when the cell is fermenting glucose or citrate. Although the addition of exogenous cAMP stimulates the transcription of the pathway when the cell is fermenting glucose or citrate, this increase is only a fraction, 14 and 60% respectively, of the levels observed under anaerobic respiration of glycerol-fumarate (Table 5). Thus, there must be other factors modulating the expression of this pathway.

Regulation in response to molecular oxygen is independent of intracellular cAMP levels. This is seen in a strain which carries an inactive adenylate cyclase and can therefore make no cAMP. This strain also carries a *crp** mutation which allows transcription of cAMP-regulated genes or operons in the absence of the nucleotide (13, 27, 28). In this strain, which should be unable to modulate its cAMP effects, molecular oxygen still shows the full range of regulation of all branches of the pathway.

Also, it should be noted that although exogenous cAMP stimulates substantial transcription of the pathway under aerobic conditions, growth fails to occur under conditions that demand the biosynthesis of cobalamin. In other words, functional B₁₂ molecules are not synthesized, not even the minimal amounts needed to satisfy the methionine auxotrophy through the function of the MetH enzyme. This suggests the existence of additional mechanisms whereby molecular oxygen prevents cobalamin biosynthesis or, alternatively, that anoxia is required to avoid the inactivation of O₂-labile intermediate or enzymes (or both) of the pathway.

It seems likely that the stimulatory effect of cAMP on the biosynthesis of cobalamin under anaerobic conditions is a secondary consequence of the global metabolic effect on metabolism caused by this nucleotide especially under

anaerobic conditions. Our results shown in Table 6 support this idea. The addition of methionine, which is the only auxotrophic requirement of strain TT10855 (which carries *cyat::Tn10*), fails to reduce the doubling time of the culture. Moreover, the sole addition of cAMP to the culture medium is enough to reduce the doubling time to wild-type behavior. As expected this stimulatory effect(s) of cAMP is mediated by the *crp* gene product (Table 6). The deleterious effect of cAMP on the anaerobic growth of a *crp* strain is not understood at this point. This result illustrates the importance of the role of cAMP in the anaerobic growth of *S. typhimurium*. Since all the strains deficient in the synthesis of cAMP or the Crp protein (strains TT10855, and TT10856) grew in the absence of exogenous cAMP or L-methionine, it follows that cAMP is not required for the synthesis of cobalamin. In other words, cAMP may contribute to establishing the physiological conditions that favor cobalamin biosynthesis, but may not participate in the process directly.

It is important to notice that transcription levels of the *cobI* operon are quite low (20 U of beta-galactosidase activity) under conditions of anaerobic fermentation of glucose, yet this low level is sufficient to satisfy the cells' methionine biosynthetic requirement. This suggests that rather little B₁₂ is required for methionine. This low level of transcription is also adequate to supply B₁₂ for use of ethanolamine as a nitrogen source (unpublished results). This raises the question of why transcription increases to such high levels (800 U of beta-galactosidase activity) under conditions of anaerobic respiration. We suppose that under conditions of anaerobic respiration some unidentified functions exist that require B₁₂. It seems that these functions are dispensable, since deletion mutants of *S. typhimurium* lacking the entire cobalamin biosynthetic pathway grow normally on glycerol-fumarate.

The results presented here suggest some clues to the metabolic importance of B₁₂. The biosynthetic genes are very highly transcribed under conditions of anaerobic respiration, suggesting that the major value of B₁₂ is realized under these conditions. Although it is not clear whether there is a direct correlation between anaerobic respiration and cAMP levels, our data show that if the levels of this cyclic nucleotide are increased by providing an excess of it during fermentative growth, the level of transcription of *cob* genes approaches that observed when the cell is respiring anaerobically. Assuming that cAMP signals a shortage of carbon source, under both aerobic and anaerobic conditions, the stimulatory effect of cAMP might be interpreted to mean that B₁₂ is particularly valuable under conditions of limiting carbon sources. We therefore suggest that the major value of B₁₂ may prove to be in the catabolism of poor carbon sources that can only be utilized under conditions of anaerobic respiration.

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