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Mutations Affecting Regulation of Cobinamide Biosynthesis in *Salmonella typhimurium*

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Transcription of the genes for cobalamin biosynthesis is reduced during aerobic growth. We isolated and characterized mutants that showed a 2- to 90-fold increase in aerobic expression of the cobinamide biosynthesis (CobI) genes, depending on the particular mutation and growth conditions. Four different classes of mutations were characterized. All mutations (CobRI through CobRIV) were *cis*-acting, dominant mutations that mapped near the promoter end of the CobI operon. Two of these classes of mutations (III and IV) caused an increase in anaerobic as well as aerobic transcription of the CobII and CobIII operons; this led to increased biosynthesis of cobalamin under anaerobic growth conditions. A recessive mutation (*cobF*) mapping far from the CobI operon increased anaerobic CobI operon expression by about fourfold.

Vitamin B_{12} (cobalamin) is synthesized by *Salmonella typhimurium* only under anaerobic growth conditions (15); expression of the biosynthetic genes is reduced in the presence of oxygen. Neither the physiological significance nor the mechanism of this pattern of regulation is understood.

Cobalamin is used as a cofactor in four known reactions in *S. typhimurium*. First, it is required by one of the two methyltransferases (*metE* and *metH* gene products) that independently can catalyze methylation of homocysteine to form methionine (19, 24, 25, 27). Second, it is required for the cleavage of ethanolamine to acetaldehyde and ammonia, providing both a carbon and a nitrogen source (6, 21). Third, Frey et al. (11) showed that B_{12} is involved in formation of the nonessential hypermodified Q base found at position 34 in the anticodon of $tRNA^{Asp,Asn,His,Tyr}$; B_{12} is needed for conversion of epoxyqueuosine to queuosine. Finally, it was recently shown (R. M. Jeter, submitted for publication) that *S. typhimurium* can use 1,2-propanediol as a carbon source under aerobic growth conditions only if vitamin B_{12} is provided. The pathway for breakdown of propanediol by *S. typhimurium* has not been characterized but probably includes the enzyme propanediol dehydratase, which is known to require B_{12} in other organisms (28). None of the known cobalamin-dependent reactions is essential for cell growth under most conditions, and none of them has so far been shown to be of special significance during anaerobic growth.

Previous genetic analysis (15, 16) has established that most of the cobalamin biosynthetic (Cob) genes map near the *his* operon at 41 min on the chromosome. The genes appear to comprise three different operons; the CobI operon is involved in cobinamide biosynthesis, the CobII operon is involved in dimethylbenzimidazole (DMB) biosynthesis, and the CobIII operon encodes functions that join cobinamide and DMB to form cobalamin. All three operons are transcribed counterclockwise with repect to the *Salmonella* genetic map.

Previous experiments established three exogenous factors that affect transcription of the Cob operons (10). The end product of the pathway, cobalamin, reduces transcription, and cyclic AMP (cAMP) serves to activate these genes. Finally, transcription of the cobalamin biosynthetic genes is strongly reduced by the presence of oxygen, implying the existence of a specific regulatory mechanism that responds directly or indirectly to oxygen.

In the accompanying paper (1) we present experiments directed at how cells sense oxygen levels and how that information might be conveyed to the transcriptional apparatus of the CobI operon. In this paper we report *cis*-dominant mutations, mapping adjacent to the CobI operon, that increase CobI gene expression in the presence of oxygen. We also describe a recessive mutation that maps far from the CobI operon and causes increased expression of CobI under both aerobic and anaerobic conditions.

MATERIALS AND METHODS

Bacterial and bacteriophage strains. The genotypes of bacterial strains are given in Table 1. All bacterial strains used are derivatives of *S. typhimurium* LT2. Two transposition-defective derivatives of the specialized transducing phage Mu d1(Amp^r *lac* cts) of Casadaban and Cohen (3) were used. The derivatives, Mu dI1734 Kan^r (4) and Mu dI8Amp^r (13) are referred to as Mu dJ and Mu dA, respectively. Two transposition-defective derivatives of Tn*10* were used; Tn*10del16del17*Tet^r (30) and Tn*10d*Cam (9). Tn*10del16del17*Tet^r is referred to as Tn*10d*Tet. Finally, a Kan^r derivative of Tn*10* (18) was used and is referred to as Tn*10*Kan. We thank Charles Miller, Erica Barrett, Chris Higgins, respectively, for providing *oxrA oxrB pepT*, *hyd fhl*, and *oxrC* strains.

Throughout the paper, three *lac* operon fusions to the Cob operons were used; all were formed by insertion of a Mu dJ element. Fusion *cob-24*::Mu dJ is an insertion within the CobI operon; fusions *cob-62*::Mu dJ and *cob-66*:: Mu dJ are insertions in the CobII and CobIII operons, respectively. We will refer to these insertions as CobI::*lac*, CobII::*lac*, and CobIII::*lac*; it should be kept in mind that CobI, -II, and -III refer to operons and phenotypic classes and not to particular genes.

Culture media and growth conditions. Difco nutrient broth (0.8%) containing NaCl at a final concentration of 0.5% was used as a complex medium. The E medium of Vogel and Bonner (29) was used as a minimal medium; with carbon sources other than glucose, no-citrate E medium was used.

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TABLE 1. Strains of *S. typhimurium* LT2 used in this study

Strain	Genotype
TR6583	*metE205 ara-9*
TT9531	*pyrC2688*::Mu dA
TT10285	DUP519[(*pncX242*)*Mu dA*(*pyrC2688*)]
TT10399	DUP602[(*pmi-51**Mu dA* (*purB1883*)]
TT10427	LT2(pNK972)
TT10852	*metE205 ara-9 cob-24*::Mu dJ
TT10857	*metE205 ara-9 cob-62*::Mu dJ
TT10858	*metE205 ara-9 cob-66*::Mu dJ
TT10927	*metE205 ara-9 zeb-1845*::Tn*10*
TT12242	*hisD9953*::Mu dA(*lacZ950*::Tn*10*)
TT14676	*metE205 ara-9 cob-24*::Mu dJ *cobR1*
TT14677	*metE205 ara-9 cob-24*::Mu dJ *cobR2*
TT14678	*metE205 ara-9 cob-24*::Mu dJ *cobR3*
TT14679	*metE205 ara-9 cob-24*::Mu dJ *cobR4*
TT14680	*metE205 ara-9 cob-24*::Mu dJ DUP519[(*pncX242**Mu dA(*lacZ950*::Tn*10*)*(*pyrC2688*)]
TT14681	*metE205 ara-9* DEL299(*his phs cob*)
TT14682	*metE205 ara-9 cob-24*::Mu dJ *zcd-3677*::Tn*10d*Cam^r *cobF1*
TT14685	*his2236 zeb-1845*::Tn*10*Kan^r *cob-4*::Tn*10*/F'128 Pro$^+$ Lac$^+$ (pNK972)
TT14686	*metE205 ara-9* DEL(*cob-217*) *his22 rpsL1142 recA1*
TT14687	*metE205 ara-9* DEL(*cob-217*) *his22 rpsL1142 recA1*/ F'128 Pro$^+$ Lac$^+$ IS*10*::Cob1$^+$::IS*10*
TT14688	*metE205 ara-9 cob-401*::Tn*10d*Cam^r/F'128 Pro$^+$ *lacZ950*::Tn*10* IS*10*::CobI$^+$::IS*10*
TT14691	*metE205 ara-9 cob-24*::Mu dJ *cobF1*
TT14692	*metE205 ara9 cob-24*::Mu dJ *zeb-1845*::Tn*10 cobR1*
TT14693	*metE205 ara-9 cob-24*::Mu dJ *zeb-1845*::Tn*10 cobR2*
TT14694	*metE205 ara-9 cob-24*::Mu dJ *zeb-1845*::Tn*10 cobR3*
TT14695	*metE205 ara-9 cob-24*::Mu dJ *zeb-1845*::Tn*10 cobR4*
TT14696	*metE205 ara-9 zeb-1845*::Tn*10 cobR1*
TT14697	*metE205 ara-9 zeb-1845*::Tn*10 cobR2*
TT14698	*metE205 ara-9 zeb-1845*::Tn*10 cobR3*
TT14699	*metE205 ara-9 zeb-1845*::Tn*10 cobR4*
TT14700	*zcd-3678*::Tn*10d* Tet^r *cobF1*
TT14705	*metE205 ara-9 cob-62*::Mu dJ *zeb-1845*::Tn*10 cobR3*
TT14706	*metE205 ara-9 cob-62*::Mu dJ *zeb-1845*::Tn*10 cobR4*
TT14707	*metE205 ara-9 cob-66*::Mu dJ *zeb-1845*::Tn*10 cobR3*
TT14708	*metE205 ara-9 cob-66*::Mu dJ *zeb-1845*::Tn*10 cobR4*
TT14709	*metE205 ara-9* DEL1011(*his phs zeb-1845*::Tn*10*)
TT14710	*metE205 ara-9 cob-24*::Mu dJ *pyrC2688*::Mu dA
TT14711	*metE205 ara-9 cob-24*::Mu dJ *purB1883*::Mu dA

Solid media contained 15 g of Bacto-Agar (Difco) per liter. Carbon sources and electron acceptors were present at the following concentrations: glucose (10 mM), glycerol (10 mM), and fumarate (20 mM). Auxotrophic supplements were added at the concentrations recommended elsewhere (8). The final concentrations of antibiotics in complex media were 20 μg of tetracycline, 50 μg of kanamycin, 20 μg of chloramphenicol, and 30 μg of ampicillin per ml. The final concentrations of antibiotics in minimal medium were 10 μg of tetracycline, 125 μg of kanamycin, 5 μg of chloramphenicol, 15 μg of ampicillin, and 500 μg of streptomycin per ml. When added to plates, lactose analogs were used at final concentrations of 40 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 0.12 g of phenylethyl-β-D-thiogalactoside (PETG) per liter.

Cultures were grown aerobically in 0.5-ml volumes in 10-ml tubes at a speed setting of 8 in a Gyrotory shaker (New Brunswick Scientific Co.). Anaerobic cultures were grown under completely anoxic conditions as described previously (10). Cell density was monitored with a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., Inc.).

Transductional and conjugational methods. All transduction crosses were performed with the high-frequency, generalized transducing phage mutant P22 HT105/1 *int-201* (23) as previously described (5). Transductants were purified and made phage free by picking light-colored single colonies from nonselective green plates (5). All conjugational crosses were performed by mixing liquid cultures (0.1 ml each) of the donor and recipient strains for 1 h. This mixture was plated on selective medium.

Assays. β-Galactosidase was assayed as described by Miller (17) by using $CHCl_3$ and sodium dodecyl sulfate to permeabilize the cells. All values presented are Miller units (17). Cobalamin was assayed by using a commercial radioisotope dilution assay (Quanta phase; Bio-Rad Laboratories) as recommended by the manufacturer.

Mutagenesis. Diethyl sulfate (DES) mutagenesis was performed as described previously (8), except that the time of mutagenesis was increased to 45 min. Hydroxylamine (NH_2OH) mutagenesis was by the method of Hong and Ames (12). Phage lysates were mutagenized to a 0.1% survival rate.

Generation of a CobI-carrying F' plasmid. A composite transposon consisting of the CobI operon, a part of the CobIII operon, and two flanking Tn*10* elements was used to introduce the CobI genes onto an F' plasmid by transposase-mediated transposition. The general method of construction has been described previously (22). To construct F'CobI, the CobI region was first flanked by derivatives of Tn*10*. The Tn*10*Kan element (18) was placed outside the promoter end, and the *cob-4*::Tn*10* insertion (in CobIII) was placed at the distal end, of the CobI region. This constructed a composite CobI transposon capable of moving into a plasmid. An F'128 *pro*$^+$ *lac*$^+$ plasmid and a transposase-producing plasmid (pNK972) were introduced the strain bearing the composite Tn*10* CobI$^+$ Tn*10*. From this final strain (TT14685) the F' plasmid was transferred by conjugation into recipient strain TT14686 (*metE cobI217 recA1 rpsL1142*), selecting for Lac$^+$ exconjugants, which were replica printed to identify strains that had received the (unselected) Cob$^+$ phenotype with the F' plasmid that conferred the (selected) Lac$^+$ phenotype. One such Cob$^+$ exconjugant strain that had not acquired resistance to tetracycline or kanamycin (strain TT14687) was saved; the F' plasmid of this strain carries the CobI operon, which is presumably flanked only by copies of IS*10* derived from the original Tn*10* elements. This F' plasmid was shown to contain the CobI operon and part of the CobIII operon by its ability to complement and repair (by recombination) point and insertion mutations. A *lacZ950*::Tn*10* insertion from strain TT12242 was crossed into the Lac operon of the final F' plasmid to abolish β-galactosidase production. This final plasmid (in strain TT14688) was used as a donor of F' CobI$^+$ for the dominance tests of the four *cobR* mutations.

Dominance tests. (i) CobR. The CobI-containing F' plasmid from strain TT14688 was introduced into strains TT14676 through TT14679 (*cob-24*::Mu dJ *cobR*) by conjugation. Cells were plated on minimal glucose-tetracycline-kanamycin plates, anaerobically selecting for Cob$^+$. These exconjugants were purified nonselectively and assayed for β-galactosidase.

(ii) CobF. Strain TT14680, containing a *cob-24*::Mu dJ fusion and a chromosomal duplication extending from *pyrC* to *pncX*, was used as a recipient in transduction crosses. Donor phage grown on strain TT14682 (*zcd-3677*::Tn*10d*Cam *cobF1*) introduced the *cobF1* mutation. Transductants were selected (Cam^r) on minimal plates containing glucose, X-gal, PETG, and chloramphenicol. All transductant colonies were light blue, indicating that the *cobF* mutation, which causes

dark blue colonies in haploid strains, is recessive to *cobF*$^+$. To confirm the structure of each merodiploid, six transductants were purified, and a segregation test was performed. Cells were grown overnight in a nonselective complex medium and plated on rich plates. These colonies were replica printed to rich plates with tetracycline, ampicillin, or chloramphenicol or to minimal E plates with X-gal and PETG to score for antibiotic resistance and color (i.e., the expression level of the CobI::*lac* fusion). This demonstrates the structure of the duplication and the presence of both the *cobF1* and *cobF*$^+$ alleles.

RESULTS

Isolation of mutants with increased aerobic CobI operon transcription. Spontaneous and chemically induced mutants with increased aerobic expression of a *lac* fusion to the CobI operon were isolated. The level of β-galactosidase produced by the parental CobI::*lac* fusion (strain TT10852) in the presence of oxygen was sufficiently high that these strains formed dark blue colonies on X-gal plates and were able to grow on minimal lactose plates. To screen and select for mutants with increased aerobic CobI::*lac* transcription, the β-galactosidase inhibitor PETG was added to the plates to inhibit β-galactosidase activity. The concentration of PETG used (0.12 g/liter) caused the parent strain to form light blue colonies under aerobic conditions and prevented growth of the parent strain on lactose as the carbon source.

Hydroxylamine-induced mutants were isolated after local mutagenesis of the CobI region. A phage lysate was grown on strain TT10927, which carries a Tn*10* insertion outside the promoter end of the CobI operon; this phage lysate was used to transduce the CobI::*lac* fusion strain TT10852 (*cob-24*::Mu dJ), selecting for kanamycin (Mu dJ) and tetracycline (Tn*10*) resistance and screening for dark blue colonies on plates containing X-gal and PETG. Such mutants were found at a frequency of approximately 10^{-3}. DES-induced mutants were isolated after mutagenesis (8) of cells of the CobI::*lac* fusion strain (TT10852). After DES treatment, cells were plated on minimal glucose plates containing X-gal and PETG. Dark blue colonies were found at a frequency of 10^{-5} to 10^{-4}. To assure that all mutants are independent, only one colony was picked from each mutagenized culture. Spontaneous mutants were isolated after a positive selection for growth on lactose. The CobI::*lac* fusion strain (TT10852) was plated on lactose minimal plates containing PETG, and large Lac$^+$ colonies were picked. This type of mutant was found at a frequency of 10^{-7} to 10^{-6}.

Classification of regulatory mutants. These mutants were classified according to the magnitude of their effect on CobI::*lac* expression. Four different classes of *cobI*-linked mutants were found and given phenotype designations CobRI through -IV. We isolated and initially characterized nine mutants of class I, three mutants of class II, seven mutants of class III, and four mutants of class IV. From each class, one typical mutant was characterized in detail (strains TT14676 through 9, respectively). The chosen mutations, representing classes I through IV, were designated *cobR1* through *-4*, respectively. After either hydroxylamine or DES mutagenisis, strains with mutations that were unlinked to the CobI operon were isolated, and the mutations were designated *cobF*, since all proved to affect a single locus. These mutants were found at a frequency of 10^{-5} to 10^{-4} (without mutagenesis) and 10^{-3} (after DES treatment of cells). We isolated nine mutants with mutations at this locus, and the DES-induced mutant *cobF1* (strain TT14691) was studied in detail.

All regulatory mutations were transduced into strain TR6583 and were thereby separated from the CobI::*lac* insertion in the parent strain. In all cases, the regulatory mutants were able to synthesize cobalamin de novo under anaerobic growth conditions (data not shown). Therefore none of these mutations disrupt any vitamin B_{12} biosynthetic functions. Despite higher aerobic expression of the CobI operon, none of the mutations permitted B_{12} biosynthesis in the presence of oxygen.

To determine whether the mutations affect any general metabolic functions, we tested ability of these mutants to grow on glucose, glycerol-fumarate, and glycerol-nitrate both aerobically and anaerobically (data not shown). The mutants belonging to classes CobRI through IV (strains TT14696 through 9, respectively) were indistinguishable from the parent strain (TT10927) under all conditions tested. In contrast, *cobF* mutants grew about twofold slower than the wild type on glucose under anaerobic conditions, which suggests that they are impaired for fermentation; under all other conditions tested the *cobF* mutants showed growth behavior similar to that of wild-type cells (data not shown).

Effect of *cobR* and *cobF* mutations on CobI transcription. Table 2 shows the effect of the *cob* mutations on the expression of β-galactosidase from the CobI::*lac* fusion under a variety of conditions. The *cobR1* mutant, which was isolated after hydroxylamine mutagenesis, showed increased aerobic expression of the CobI::*lac* fusion but had very little effect on expression under anaerobic conditions.

The DES-induced mutant, *cobR2*, showed its major effect when cells were grown on glycerol aerobically. This mutant also showed a slight increase in CobI transcription anaerobically on glucose.

The *cobR3* mutant, found after DES mutagenesis, showed an increase in CobI::*lac* transcription when cells were grown aerobically on either glucose of glycerol. Under anaerobic conditions, CobI::*lac* expression was increased during fermentation of glucose but not during anaerobic respiration to glycerol-fumarate.

The spontaneous mutant (*cobR4*) was the most extreme type, showing a large increase in CobI expression during aerobic growth on either glucose or glycerol. This mutant showed a smaller increase during fermentation of glucose. Like the *cobR1*, *-2*, and *-3* mutants, it showed no increase in CobI::*lac* transcription compared with the wild type during anaerobic growth on glycerol-fumarate.

All four CobR mutants still showed transcription control in response to external cobalamin (Table 2), similar to the wild type (10). Furthermore, externally added cAMP stimulated CobI::*lac* transcription of the mutants as well as that of the wild type (Table 2). This suggests that the mutations affect only oxygen control and do not interfere with the other mechanisms that regulate the operon (10).

The *cobF1* mutation, unlinked to the CobI operon, showed its major regulatory effect during anaerobic fermentation of glucose. The mutation had minimal effects on expression under the other conditions tested. The behavior of *cobF* mutants appears to contradict the manner in which these mutants were isolated. The *cobF* mutants were identified under aerobic conditions as colonies that showed high expression of a CobI::*lac* fusion, judged by X-gal hydrolysis. Nevertheless, when *cobF* mutants were grown aerobically in liquid medium, β-galactosidase assays showed very little increase compared with results with the wild type (Table 2). The major effect of *cobF* on CobI expression was seen anaerobically. The mutants were probably detected by virtue of the increased β-galactisidase produced by cells within

TABLE 2. Effect of the *cobR1* through *-4* and *cobF* regulatory mutations on CobI::*lac* transcription

Strain	Genotype	β-Galactosidase activity[a] under the indicated conditions[b]					
		Aerobic				Anaerobic	
		Glucose			Glycerol (NA)	Glucose	Glycerol-fumarate
		NA	$+B_{12}$[c]	+cAMP[d]			
TT10852	CobI::*lac*	3	2	14	5	16	820
TT14676	CobI::*lac cobR1*	14	2	104	40	26	1,100
TT14677	CobI::*lac cobR2*	4	2	22	20	22	1,066
TT14678	CobI::*lac cobR3*	40	3	177	73	130	838
TT14679	CobI::*lac cobR4*	270	5	399	340	310	720
TT14691	CobI::*lac cobF1*	5	ND[e]	ND	5	60	812

[a] The numbers are Miller units (17) and represent the means of three separate measurements.
[b] The cells were grown as described in Materials and Methods. NA, No addition.
[c] Cobalamin was added to a final concentration of 1.5×10^{-7} M.
[d] cAMP was added to a final concentration of 5×10^{-3} M.
[e] ND, Not determined.

the colonies, which had limited access to oxygen and were in fact growing anaerobically. In *cobF* mutants these anaerobic cells make a larger contribution to the β-galactosidase levels in the colony than they would in a $cobF^+$ colony. To exclude the possibility of a nonspecific effect of the *cobF1* mutation on Lac expression, we tested the effect of the *cobF1* mutation on Lac fusions to the *cysG*, *thr*, *trp*, *metE*, *hisD*, *bio*, and *gal* genes. The *cobF1* mutation had no effect on the expression of any of these operon fusions (data not shown).

To look for interactions, we constructed double mutants carrying both the *cobF1* mutation and one of the *cobR* mutations (*cobR1* through *-4*). When grown aerobically on glucose, the *cobF-cobR* double mutants showed 1.3- to 2-fold higher CobI expression than did the parental *cobR* mutant (data not shown). Thus the effects of these two mutation types are at least partially additive.

We examined the effect of the *cobF1* mutation on the expression of genes that are induced anaerobically and known to be controlled by the oxygen-regulatory genes *oxrA*, *-B*, and *-C* (14, 26). (The *oxrA* gene of *S. typhimurium* is equivalent to the *fnr* gene of *Escherichia coli*.) A *pepT7*::*lac* (14, 26) fusion (*oxrA*, *-B*, and *-C* dependent), a *hyd-4*::*lac* (14) fusion (*oxrC* dependent), and an *fhl-5*::*lac* (14) fusion (*oxrC* dependent) were all unaffected by the *cobF1* mutation (data not shown). Finally, we found that *oxrA*, *-B*, and *-C* mutations had no effect on expression of a CobI::*lac* fusion under either aerobic or anaerobic conditions (data not shown).

Mapping of regulatory mutations. The CobI-linked regulatory mutations were mapped with respect to a Tn*10* element upstream of the CobI operon (*zeb-1845*::Tn*10*) and a Mu dJ insertion within the CobI operon (*cob-24*::Mu dJ). To determine linkage between *cobR* mutations and the Mu dJ insertion, strains TT14676 through TT14679 were used as donors to transduce the *cobR1* through *-4* mutations, respectively, with the Mu dJ insertion into strain TR6583; kanamycin-resistant transductant colonies were selected (Mu dJ) and screened to identify recombinants that form dark blue colonies aerobically on plates containing X-gal and PETG. The cotransduction frequency of the Mu dJ and *cobR1* through *-4* mutations was widely variable; the frequencies varied from 22 to 60% cotransduction, suggesting that the *cobR* mutations map far apart or that particular alleles have a strong effect on transduction frequencies.

To determine whether the regulatory mutations were promoter proximal or distal to Mu dJ, we determined the linkage of the regulatory mutations to the Tn*10* located outside the promoter end of the operon. Phage grown on strains TT14696 through TT14699 (*zeb-1845*::Tn*10* *cobR1* through *-4*) was used to transduce strain TT10852 (*cob-24*::Mu dJ), selecting for tetracycline and kanamycin resistance (to maintain the recipient Mu dJ) and screening for light blue ($cobR^+$) and dark blue (*cobR1* through *-4*) colonies. The cotransduction frequency between the Tn*10* element and *cobR1* through *-4* mutations in this cross was between 83 and 91%. The linkage between the *zeb-1845*::Tn*10* and *cob-24*::Mu dJ insertions (determined in other crosses) was 27%, suggesting that the *cobR1* through *-4* mutations were promotor proximal to the Mu dJ element and very close to the *zeb-1845*::Tn*10* insertion.

To determine the exact order of *zeb-1845*::Tn*10*, *cobR1* through *-4*, and *cob-24*::Mu dJ, we performed crosses with a strain (TT14709) that contains the deletion DEL1011 (*his phs*) extending from the *zeb-1845*::Tn*10* insertion clockwise (away from the CobI operon) into the *his* operon. This deletion leaves the CobI operon intact. Because of its large size, this deletion cannot be repaired by a single transduced fragment. Donor P22 phage was grown on strains with *zeb-1845*::Tn*10* and *cob-24*::Mu dJ and one of the *cobR* mutations (strains TT14692 through TT14695); this phage was used to transduce the deletion strain to kanamycin resistance, screening for dark blue colonies. If the regulatory mutations were on the left side of the Tn*10* element (nearer the *his* locus; Fig. 1), they could not be coinherited with the Mu dJ insertion because there is no homology available for recombination. If the mutations were located on the right side of the Tn*10* element, between Tn*10* and the Mu dJ insertion, they would show linkage to the Mu dJ, since recombination could occur between the Tn*10* insertion site and the regulatory mutations. All four mutations showed 3 to 5% cotransduction with Mu dJ; the inferred gene order is therefore *zeb-1845*::Tn*10*, *cobR1* through *-4*, *cob-24*::Mu dJ (Fig. 1), which suggests that the *cobR1* through *-4* mutations are located at the promotor-proximal end of the CobI operon.

The *cobF1* mutation was initially mapped by Hfr crosses. We first isolated a Tn*10d*Tet element (9) that was linked to the *cobF1* mutation and performed Hfr mapping on this strain (TT14700) by the method of Chumley et al. (7). These crosses suggested that the *cobF* mutation was located between 22 and 34 min (data not shown).

To refine the mapping of the *cobF1* mutation, we transduced the linked *xcd-3678*::Tn*10d*Tet into two strains (TT10285 and TT10399) that carry different duplications, one

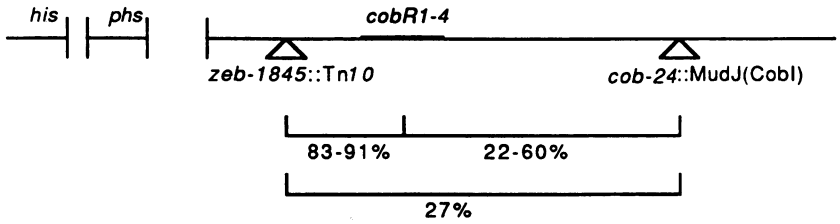

FIG. 1. Genetic mapping of the *cobR1* through *-4* regulatory mutations. The values represent cotransduction frequencies between the different markers. The crosses were performed as described in Results.

extending from *pyrC* to *pncX* (23 to 27 min) and the other extending from *purB* to *pmi* (25 to 31.5 min). If the *zcd-3678*::Tn*10d*Tet element were located in the duplicated region, transductants inheriting the Tn*10* element would be unstable due to the segregation of the duplication, and tetracycline-sensitive derivatives would be found at a low frequency. Unstable Tet^r transductants were found with the recipient carrying a duplication extending from *pyrC* to *pncX*, suggesting that *cobF1* was located between 23 and 27 min on the chromosome. The other duplication strain (25 to 31.5 min) gave stable transductants. These results placed the *cobF* mutation between 23 and 25 min.

We further mapped the *cobF* mutation by determining cotransduction frequencies between the *zcd-3678*::Tn*10d*Tet element and Mu dA insertions in this area. No cotransduction was detected between this Tn*10* element and insertions in the *put* or *purB* genes. Weak linkage (3% cotransduction) was seen to the *pyrC2688*::Mu dA insertion (TT9531). The *cobF1* mutation showed not linkage to either *pyrC* or *purB* mutations. From this data, we infered a gene order of *pyrC*, *zcd-3678*::Tn*10d*Tet, *cobF*, *purB* (Fig. 2).

Dominance tests. To determine whether the *cobR1* through *-4* mutations were dominant or recessive, we constructed an F′ plasmid including the CobI operon (see Materials and Methods). The F′ plasmid was then transferred (from strain TT14688) into strains TT14676 through TT14679 (*cobR1* through *-4 cob-24*::Mu dJ), and the β-galactosidase expression of these merodiploids was determined. Expression of CobI::*lac* in these strains was similar to that in the haploid mutants (data not shown), indicating that the *cobR1* through *-4* mutations are dominant to the wild type.

To determine whether the mutations are *cis* or *trans* acting, we introduced the CobI::*lac* fusion into the F′ plasmid, which was mated into the *cobR1* through *-4* strains (TT14696 through TT14699, respectively). There was no effect of the chromosomally located *cobR1* through *-4* mutations on the CobI::*lac* fusion located in the F′ plasmid; thus all four regulatory mutations are *cis* dominant. A dominance test performed with duplications of this region gave similar results (data not shown).

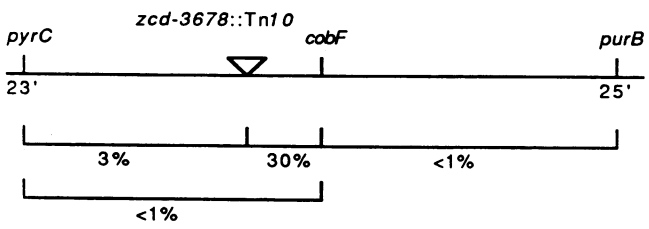

FIG. 2. Genetic mapping of the *cobF1* mutation. The values shown are cotransduction frequencies between the different genetic markers, which were determined as described in Results.

Recessivity of the *cobF* mutations was demonstrated by using chromosomal duplications. The *cobF1* mutation was introduced into duplication strain TT10285 by using the 40% linked *zcd-3677*::Tn*10d*Cam insertion (TT14682) (Fig. 3). Since the *cobF1* mutation is only 40% cotransducible with the *zcd-3677*::Tn*10d*Cam insertion, we examined six transductants. None of the potential *cobF1*/*cobF*$^+$ merodiploid strains showed increased CobI::*lac* expression under conditions that retained the duplication, suggesting that, if the *cobF1* mutation were inherited, it must be recessive to the wild-type allele. The presence of the *cobF1* mutation was shown in two different ways. When duplications were allowed to segregate (Fig. 3), three of the six strains yielded ampicillin- and tetracycline-sensitive segregants that formed dark blue colonies on X-gal medium. We conclude that these merodiploid transductants had received the *cobF1* mutation. It was also possible to recover the *cobF* mutation by transduction crosses from these three merodiploid strains. We conclude that the *cobF*$^+$/*cobF1* merodiploids are not constitutive for CobI::*lac* expression and therefore the *cobF1* mutation is recessive to the wild-type allele.

Effect of the *cobR3* and *cobR4* mutations on CobII and CobIII transcription. The presence of the *cobR3* mutation, which increased CobI expression 13-fold, caused about a 2-fold increase in the expression of Lac fusions to the CobII and CobIII operons (Table 3). Similarly, the *cobR4* mutation, which increased CobI::*lac* expression 90-fold, stimulated CobII and CobIII transcription approximately 10-fold. This result could be explained by transcriptional readthrough from the CobI operon into the CobII and CobIII operons; alternatively the CobII and CobIII operons could have independent promoters stimulated by a protein encoded within CobI. We designed two different tests to examine these possibilities.

We constructed a duplication of the Cob region that carried a *cobR* mutation in one copy and a CobIII::*lac* fusion in the other. The duplication was arranged such that transcription from the CobR mutant control region did not proceed toward the CobIII fusion; thus the effects of CobR mutations on CobIII would have to be in *trans*. Under these conditions, both the *cobR3* and *cobR4* mutations had only a minor effect (about twofold) on expression of the CobIII::*lac* fusion; this small effect was seen for both *cobR* mutations even though *cobR3* stimulated CobI expression 10-fold and *cobR4* stimulated CobI expression 90-fold. We conclude that the major effect of *cobR* mutations on CobIII acts in *cis*. The minor *trans* effect was also seen when the fusion was placed on an F′CobI plasmid and the *cobR* mutations were placed in the chromosome.

Cobalamin production in the wild type and *cobR3* and *cobR4* regulatory mutants. The *cobR3* and *cobR4* mutations increased aerobic transcription levels of the CobI operon so that it approached the level attained in wild-type cells during

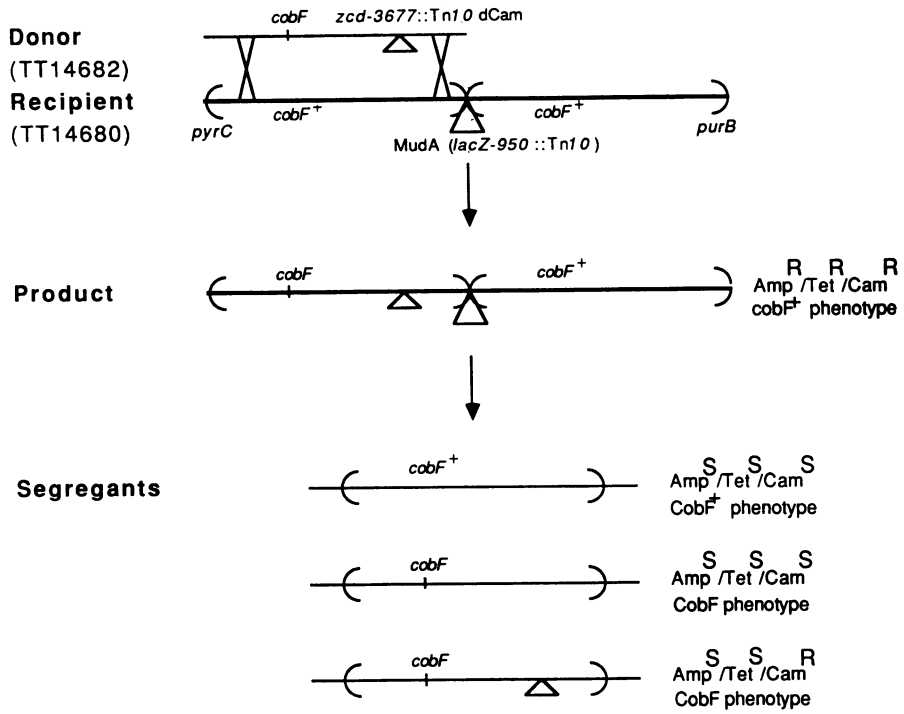

FIG. 3. Dominance test of the *cobF1* mutation. The genetic crosses were performed as described in Results. The thick lines represent duplicated material.

anaerobic respiration (Table 2). Despite these high levels of Cob operon transcription, the regulatory mutants (like wild-type cells) were unable to produce B_{12} aerobically. Table 4 shows the levels of cobalamin determined by using a radioactive dilution assay (see Materials and Methods). The same quantitative results were obtained by using an alternative ^{57}Co labeling assay (data not shown). The first thing to notice is the complete absence of vitamin B_{12} in aerobically grown wild-type cells. Second, a low amount of cobalamin (about 100 molecules per cell) was synthesized in the wild-type cells during anaerobic growth. Even though the *cobR3* and *cobR4* mutants transcribed the CobI, -II, and -III operons at high rates aerobically, they did not synthesize B_{12} under these conditions. However, anaerobically the *cobR3* and *cobR4* mutants synthesized two- to threefold more cobalamin than the wild type. When the vitamin B_{12} precursor DMB was added anaerobically, the wild type synthesized ca. 400 molecules per cell and the *cobR4* mutant synthesized almost 2,000 molecules per cell.

TABLE 3. Effect of regulatory mutations *cobR3* and *cobR4* on transcription of CobII::*lac* and CobIII::*lac* fusions

cobR allele[a]	β-Galactosidase level[b] of strains carrying the indicated operon fusion:		
	CobI::*lac*	CobII::*lac*	CobIII::*lac*
cobR$^+$	3	30	20
cobR3	40	63	51
cobR4	270	377	244

[a] The following strains were assayed: *cobR*$^+$ strains TT10852 (CobI::*lac*), TT10857 (CobII::*lac*), and TT10858 (CobIII::*lac*); *cobR3* strains TT14678 (CobI::*lac*), TT14705 (CobII::*lac*), and TT14706 (CobIII::*lac*); and *cobR4* strains TT14679 (CobI::*lac*), TT14707 (CobII::*lac*), and TT14708 (CobIII::*lac*).

[b] The numbers presented are Miller units (17) and represent the means of three separate measurements. The cells were grown aerobically in glucose minimal medium as described in Materials and Methods.

An in vivo test also indicated an increased production of B_{12} in the *cobR3* and *cobR4* mutants. We tested the anaerobic growth rate of a *metE* mutant in which methionine production (and cell growth) was entirely dependent on the B_{12}-dependent MetH enzyme. Under these conditions the presence of *cobR3* and *cobR4* mutations (strains TT14698 and TT14699, respectively) led to a significantly faster growth rate (10 to 20%) compared with that of the wild type (strain TT10927), indicating an increased production of cobalamin in the mutants. Externally supplied cobalamin was transported and accumulated to high levels (approximately 5,000 molecules per cell) (Table 4). This high cobalamin level had no detrimental effect on cell growth either aerobically or anaerobically (data not shown).

DISCUSSION

We isolated regulatory mutants that showed an increase in aerobic expression of the cobinamide biosynthetic genes

TABLE 4. Cobalamin production in the wild type and the *cobR3* and *cobR4* mutants

Strain	Description	Cobalamin levels in cells grown under indicated conditions[a]:				
		Aerobic		Anaerobic		
		−DMB	+DMB	NA	+B_{12}	+DMB
TR6583	Wild type	<5	<5	124	5,122	420
TT14681	DEL299 (Cob$^-$)	<5	<5	<5	ND	<5
TT14698	*cobR3*	<5	<5	335	ND	ND
TT14699	*cobR4*	<5	<5	237	ND	1,944

[a] The cobalamin levels are expressed as molecules per cell and represent the means of duplicate measurements. DMB and cobalamin were added to a concentration of 50 and 0.2 μg/ml, respectively. The cells were grown in minimal glucose medium. To estimate B_{12} molecules per cell, B_{12} levels were determined for a volume of culture of known cell titer; all B_{12} was shown to be cell associated. NA, No addition; ND, not determined.

(CobI). These mutations increased aerobic CobI expression from 4- to 90-fold, depending on the mutant allele and the carbon source used for growth (Table 2). The *cobR1* through *-4* mutations were all located at the promotor-proximal end of the CobI operon (Fig. 1). It is unlikely that these mutations create fusions to foreign promotors, since the regulatory profile of the mutants was otherwise similar to that of the wild type; the mutants were still subject to transcriptional regulation of the CobI operon by externally added cobalamin and cAMP, and they continued to show partial anaerobic induction of CobI transcription (Table 2).

In dominance tests, all CobI-linked mutations were dominant to the wild type and only acted on the CobI operon when located in *cis*. These characteristics would be expected for mutations affecting a promotor, operator, or attenuator; *cobR* mutants could even affect a strongly *cis*-acting regulatory protein. The transductional linkage data and preliminary physical mapping suggest that the mutations are distributed across a large region. The exact location and nature of these mutations are currently being determined.

The *cobR1* through *-4* mutants are Cob^+ as determined phenotypically by their ability to synthesize methionine anaerobically via the B_{12}-dependent MetH enzyme. For the two mutants with the highest increase in CobI::*lac* expression, we measured cobalamin production under both aerobic and anaerobic growth conditions. Neither the *cobR3* nor the *cobR4* mutant could synthesize vitamin B_{12} aerobically (Table 4), even though their transcription levels approached those of the wild type grown anaerobically (Table 2). This suggests that aerobic cobalamin biosynthesis is limited by more than transcription of the Cob operons. It is conceivable that oxygen-sensitive intermediates or enzymes are involved in this pathway; oxygen-sensitive intermediates have been found in vitro in other organisms (2). Alternatively, there may be essential steps in B_{12} biosynthesis that are carried out by proteins encoded by genes outside of the Cob operons whose expression is not increased by the *cobR3* and *cobR4* mutations. Anaerobically, however, the *cobR3* and *cobR4* mutants were able to synthesize significantly more (two- to threefold) cobalamin than was the wild type (Table 4). When one of the intermediates in the pathway (DMB) was added, both the wild type and the *cobR4* mutant showed an increase (three- and eightfold, respectively) in synthesis of B_{12}. This result indicates that the amount of B_{12} synthesized anaerobically is limited by the amount of DMB synthesized by the CobII operon. This situation has been reported for B_{12} synthesis by other organisms (20).

We determined the effect of the *cobR3* and *cobR4* mutations on the expression of Lac fusions to the CobII and CobIII operons (Table 3). Both mutations led to an increase in expression of the CobII and CobIII operon when present in *cis*, which is mainly the result of transcriptional readthrough from the CobI operon into CobII and CobIII (Tom Doak, personal communication). The small *trans* effect (about twofold) noted here could be due to a transcriptional activator, encoded in the CobI operon, which acts on the CobII and CobIII operons. Alternatively, increased biosynthesis of cobinamide or its precursors could have an indirect stimulatory effect on CobII and CobIII transcription.

One class of regulatory mutations proved to be unlinked to the CobI operon. These mutations, designated *cobF*, mapped near the *pyrC* gene at about 24 min on the chromosome (Fig. 2). The *cobF1* mutation increased CobI::*lac* transcription 1.5- to 4-fold, depending on whether oxygen was present (Table 2). Double mutants (*cobF1* and *cobR1*, *-2*, *-3*, or *-4*) showed a small increase in aerobic CobI transcription, compared with that of either of the single mutants alone, which suggests that the effects of *cobF1* and *cobR1* through *-4* on CobI::*lac* expression are independent and additive. The *cobF1* mutation seems to be specific for the CobI operon and did not increase the expression of any other genes tested. A dominance test showed that the mutation was recessive to the wild type (Fig. 3). These results are compatible with the *cobF* gene encoding a CobI-specific *trans*-acting repressor. However, since *cobF* mutants showed impaired growth under fermentative conditions, we suspect that these mutations owe their effect to secondary consequences of abnormal metabolism. We have isolated plasmids complementing the *cobF* mutation and are determining the nature of the products encoded by this plasmid.

Finally, we showed that mutations in the *oxrA*, *-B*, or *-C* genes (which affect other oxygen-controlled genes in *S. typhimurium*) have no effect on CobI transcription either aerobically or anaerobically. (The *oxrA* gene of *S. typhimurium* is equivalent to the *fnr* gene of *E. coli*.). These results imply that expression of the cobalamin biosynthesis genes is controlled by a mechanism distinct from those identified for other genes regulated in response to oxygen.

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