The CobII and CobIII Regions of the Cobalamin (Vitamin B₁₂) Biosynthetic Operon of *Salmonella typhimurium*

JORGE C. ESCALANTE-SEMERENA,^{1*} MICHAEL G. JOHNSON,¹ AND JOHN R. ROTH²

¹Department of Bacteriology, University of Wisconsin, 1550 Linden Drive, Madison, Wisconsin 53706,
and ²Department of Biology, University of Utah, Salt Lake City, Utah 84112

Received 15 July 1991/Accepted 20 October 1991

A detailed deletion map of the CobII and CobIII regions of the cobalamin biosynthetic (*cob*) operon of *Salmonella typhimurium* LT2 has been constructed. The CobII region encodes functions needed for the synthesis of lower ligand 5,6-dimethylbenzimidazole (DMB); CobIII encodes functions needed for the synthesis of the nucleotide loop that joins DMB to the corrin macrocycle. The genetic analysis of 117 deletion, insertion, and point mutations indicates that (i) the CobII and CobIII mutations are contiguous—that is, they are grouped according to function; (ii) the CobII region is composed of four complementation groups (*cobJKLM*); (iii) *cobM* mutations do not complement mutations in any of the other three CobII groups; and (iv) CobIII mutations include three complementation groups that correspond to the *cobU*, *cobS*, and *cobT* genes.

Early studies of cobalamin (CBL) biosynthesis in *Salmonella typhimurium* reported the isolation of four phenotypic classes of mutants: (i) CobI mutants that are blocked in the synthesis of the corrin macrocycle and make CBL only if the precursor cobinamide (CBI) is provided; (ii) CobII mutants that are blocked in the synthesis of the base 5,6-dimethylbenzimidazole (DMB); (iii) CobIII mutants that are blocked in the synthesis of the nucleotide loop that joins DMB to the corrin ring; and (iv) *cobA* mutants that are unable to adenylate an endogenous de novo CBL intermediate or exogenous complete or incomplete corrinoids (14, 20). The structure of CBL is shown in Fig. 1.

Except for *cobA* mutations (34 min), all *cob* mutations map at 41 min on the linkage map of *S. typhimurium* and are part of a single operon including about 26 genes. Within this operon, CobI mutations map at the promoter-proximal end and CobIII and CobII mutations map distally in the operon (11, 14, 20).

In this paper, we report that CobII and CobIII mutations are located in two contiguous blocks within the *cob* operon. Genetic studies indicate that the CobII region is composed of four complementation groups. Correlation of the genetic map with available DNA sequence data for the CobI and CobIII regions of the operon suggests that three open reading frames are included in the CobIII region (5, 7–9, 11).

MATERIALS AND METHODS

Bacteria, culture media, and growth conditions. All bacterial strains used are derivatives of *S. typhimurium* LT2 and are listed in Table 1. Nutrient broth (Difco, Detroit, Mich.) (0.8%) containing 85 mM NaCl was used as complex medium. E minimal medium (33) supplemented with glucose (11 mM) or glycerol (22 mM) was used as chemically defined medium. When added, sodium nitrate was 20 mM. Other supplements were as reported elsewhere (1, 10). The following concentrations of antibiotics (in micrograms per milliliter) were added to the complex medium: tetracycline, 20; ampicillin, 30; kanamycin, 50; chloramphenicol, 20; and streptomycin, 2,000. The following concentrations of antibiotics (in micrograms per milliliter) were added to the minimal

medium: tetracycline, 10; ampicillin, 15; kanamycin, 125; chloramphenicol, 5; and streptomycin, 2,000. Solid media contained 15 g of BactoAgar (Difco) per liter. Cyano (CN)-CBL or dicyano [(CN)₂]-CBI was added at 15 nM, and DMB was added at 300 μM. Anoxic culture media were prepared as described elsewhere (13). All chemicals were from commercial sources.

Genetic techniques. Transductional crosses were performed with phage P22 HT 105/1 *int-201* (30, 31) as described elsewhere (10, 13).

Localized chemical mutagenesis. P22 lysates were mutagenized with hydroxylamine by the procedure of Hong and Ames (16) as described by Davis et al. (10).

Generation of pools of insertion mutations. The method of Elliott and Roth (12) was used to generate mutant pools with insertions of the transposition-defective derivative of Tn10, Tn10DEL16DEL17 (Tc) [hereafter referred to as Tn10d(Tc)] (36). The transitory *cis* complementation method (19) was used to isolate a pool of independent Mu dI1734 (6) (hereafter referred to as Mu dJ) mutants with fusions of *lacZ* to Cob promoters.

Isolation of Tn10d(Tc) and Mu dJ insertions with a CobII phenotype. Phage grown on a pool of approximately 50,000 independent Mu dJ(Km) mutants was used to transduce *cob-4::Tn10* (TT7573) to kanamycin resistance. Km^r transductants displaying Tc^s, CobIII⁺, and CobII[−] phenotypes were freed of phage and further classified by their phenotype on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plates.

A similar strategy was used to isolate insertions of the Tn10d(Tc) element. Phage lysate grown on a pool of approximately 32,000 mutants was used to transduce *cob-27::Mu dI-8* (TT10330) to tetracycline resistance. Recombinants with Ap^s, Tc^r, CobIII⁺, and CobII[−] phenotypes were purified and used in mapping studies.

Isolation of insertions *cob-236::Tn10d(Tc)* and *zea-1872::Tn10d(Tc)*. Two strains with insertions of Tn10d(Tc) flanking the CobIII-CobII region were recovered from a pool of approximately 32,000 mutants. Phage grown on this pool was used as a donor to transduce JE50 (*cob-62::Mu dJ* insertion in the CobII genes) to tetracycline resistance. Tc^r transductants displaying Km^s, CobII⁺, and CobIII⁺ phenotypes were isolated and purified. Insertion *cob-236::*

* Corresponding author.

FIG. 1. Structure of cobalamin. The exact number of reactions for each pathway in *S. typhimurium* remains unknown. The parts of the molecule that are boxed are those proposed to be synthesized by CobII and CobIII functions. Adenosyl-CBL has a 5' deoxyadenosyl group attached to the cobalt ion as the upper (Co β) ligand through a covalent, photolabile C—Co bond.

Tn10d(Tc) (JE588) was mapped within the CobI region, promoter proximal to CobIII, and shown to have a CobI⁺ CobIII⁺ CobII⁺ phenotype. Insertion *zea-1872::Tn10d(Tc)* (JE761) was mapped downstream of CobII. Cells harboring this insertion displayed a Cob⁺ phenotype.

Deletion selection method. The method of Bochner et al. (3) as described by Maloy and Nunn (25) was used to generate deletions of the CobII and/or CobIII regions *in vivo*. Insertion *cob-236::Tn10d(Tc)* (JE588) was used to select deletions entering the region from the left, and insertion *zea-1872::Tn10d(Tc)* (JE761) was used to select deletions entering from the right (see Fig. 2). Fusaric acid dissolved in *N,N*-dimethyl-

formamide was added to the culture medium. Tc^s clones were screened for CobII[−] and CobIII[−] mutants.

Construction of F' CobII⁺ CobIII⁺ plasmids. Complementation studies were performed with F' CobII⁺ CobIII⁺ plasmids constructed by the method of Roof and Roth (28). Strain JE1476 was used as the donor of the F'T80 plasmid in a mating cross with strain JE215. The isolation of exconjugants with Tc^r, Str^r, CobII⁺, and CobIII⁺ phenotypes was genetic evidence for a successful transposition of the CobIII⁺-CobII⁺ region onto the plasmids. Strain JE1543 was used later in complementation crosses.

Placing *cob* point mutations onto the F'T80 CobII⁺ CobIII⁺ plasmid. Point mutations in CobIII and CobII were placed onto the F'T80 CobII⁺ CobIII⁺ plasmid (JE1543) by cotransduction with *cob-22::Mu* dJ(Km). All transduction events placed the drug resistance marker and the point mutation onto the F' plasmid, since the recipient strain carries a nontransducible chromosomal *his-cob* deletion (DEL299).

Complementation tests. Donors and recipients (0.1 ml of each) were mixed, spread on nutrient broth plates without antibiotics, and incubated overnight and then replica printed onto nutrient broth plates containing kanamycin to select inheritance of the F' plasmid and chloramphenicol to counterselect the donor strain. Exconjugants selected on these plates were replica printed to minimal medium containing CBI and incubated aerobically to test their CobII phenotypes.

β -Galactosidase activity assays. β -Galactosidase activity was assayed by the method of Miller (26) as described elsewhere (13), with CHCl₃-sodium dodecyl sulfate-permeabilized cells. Cultures with a cell density of about 0.3 A₆₅₀ units were routinely used for this purpose. A unit of activity is defined as the amount of enzyme that produces 1 nmol of *o*-nitrophenol per min per A₆₅₀ unit.

RESULTS AND DISCUSSION

Isolation of point mutations and deletions in the CobII and CobIII regions. A total of 73 point and deletion mutations in CobII and CobIII were isolated as described in Materials and Methods. Twenty-eight deletions entering the CobIII-CobII region from the left were derived from insertion *cob-236::Tn10d(Tc)*, and 21 deletions entering from the right were derived from insertion *zea-1872::Tn10d(Tc)* (Fig. 2). A total of 24 CobII and 24 CobIII point mutations were isolated by localized mutagenesis. Some CobII and CobIII amber mutations were identified by their suppressibility in a *supD* background (29). The *supD* allele from TT7759 was introduced into CobII and CobIII mutants by cotransduction to a nearby Tn10 element. As a positive control for suppression, the *supD* allele was also introduced into a bona fide *his*(Am) mutant (TR248). Alleles *cob-300* and *cob-308* (CobII) and *cob-324*, *cob-327*, *cob-332*, *cob-335*, *cob-336*, and *cob-337* (CobIII) were suppressed by *supD*.

The genetic map of the CobII and CobIII regions. A detailed deletion map was generated by transductional crosses with *cob* point mutants used as donors and deletion mutants used as recipients. To monitor CBL biosynthesis, a *metE* mutation was present in the genetic background of both donor and recipient strains. The *metE* mutation forces the methylation of homocysteine to methionine to occur via the CBL-dependent MetH methyltransferase (32). Thus, in the absence of endogenous or exogenous CBL, MetE mutants are auxotrophic for methionine.

To construct the genetic map, transductional crosses were

TABLE 1. Bacterial strains used^a

Strain	Genotype
JE588	 <i>metE205 ara-9 cob-236::Tn10d(Tc)</i>
JE761	 <i>metE205 ara-9 zea-1872::Tn10d(Tc)</i>
JE215	 <i>recA1 DEL299 strA1 metE205 ara-9</i>
JE1476	 <i>metE205 ara-9 cob-12::Tn10(Km) zea-1872::Tn10d(Tc)</i>
JE1543	 <i>DEL299 strA1 metE205 ara-9/F'T80 [IS10*<i>CobIII</i>⁺ <i>CobII</i>⁺ <i>Tn10d(Tc)</i>]</i>
TT7759	 <i>hisC9968::Mu d1-8 supD501 zeb-609::Tn10</i>

^a All strains are derivatives of *S. typhimurium* LT2, and all except TT7759 were constructed during the course of these studies.

FIG. 2. Genetic map of the *CobII* and *CobIII* regions. Thick solid lines represent the *CobII* and *CobIII* regions of the *cob* biosynthetic operon; $\square$, *Mu* d-*lacZ* operon fusions (arrows above operon fusion symbols indicate the direction of transcription, and rightward-pointing arrows indicate *Mu* d-*lacZ* fusions which produce β -galactosidase); ∇ , *Tn10*(Tc) element insertions. Numbers listed across the two regions represent point mutations. Deletion mutations are shown in the lower part of the figure, with the extent of the deleted material indicated by the length of the arrow. Nonsense amber mutations identified in each region were *cob*-300 and *cob*-308 in the *CobII* region and *cob*-324, *cob*-327, *cob*-332, *cob*-335, *cob*-336, and *cob*-337 in the *CobIII* region.

performed by selecting for Met^+ recombinants on medium containing CBI; this selection demands *CobII*⁺ and *CobIII*⁺ functions that convert CBI to CBL. Under standard mapping conditions, a control cross between a *cob* point mutant and a wild-type donor yielded approximately 15,000 *cob*⁺ recombinants. These crosses were scored as negative when no *cob*⁺ recombinants were found when the cross was performed in quintuplicate, a (7.5×10^4) -fold reduction in recombination.

Figure 2 shows the genetic map of the *CobII* and *CobIII* regions, with the direction of transcription of the *cob* operon occurring from left to right, a clockwise direction (21). Points and insertions are indicated by the *cob* allele numbers shown across the map. The arrow above each *Mu* d-*lacZ* fusion shows its direction of transcription. All rightward-pointing arrows indicate *Mu* d-*lacZ* fusions which produce β -galactosidase. Notice that all *Tn10* insertions in *CobIII* map at the downstream end of the region, which may suggest a *Tn10* hot spot. Deletions are shown in the lower part of Fig. 2, with arrows indicating the extent of genetic material missing in each case.

This genetic map resolves the *CobIII* and *CobII* region

into 24 deletion intervals and demonstrates that genes encoded by the two types of functions are contiguously grouped within the *cob* operon.

Evidence for internal promoters within the *cob* operon. *Tn10* insertions in *CobI* did not prevent expression of *CobIII-lacZ* fusions or *CobII-lacZ* fusions. They did, however, result in a low level of unregulated expression of the fusions (data not shown), suggesting that there exist weak internal promoters for the *CobIII* and *CobII* regions. An internal promoter for the *CobII* region was inferred from the results of similar experiments with strains carrying *Tn10* insertions in *CobIII* and *CobII-lacZ* fusions. These internal promoters are not regulated by oxygen (13) and are most likely responsible for the ability of *S. typhimurium* to synthesize CBL aerobically when CBI is provided. These internal promoters will be described in more detail elsewhere in conjunction with evidence that the entire *cob* gene cluster is a single operon (11).

The *CobIII* genetic map and its correlation with its DNA sequence. Two plasmids were recovered from a library of sized *S. typhimurium* chromosome fragments generated by partial *Sau3A* digestion of genomic DNA and cloned into

FIG. 3. Complementation analysis of the CobII region. Symbols: +, DMB⁺ merodiploid; -, DMB⁻ merodiploid. Allele numbers are arranged as in the genetic map (Fig. 2). In this figure, transcription proceeds from top to bottom and left to right. Alleles *cob-300* and *cob-308* are nonsense amber mutations. Solid lines enclose the proposed complementation groups *cobJ*, *cobK*, *cobL*, and *cobM*. Superscript a's indicate that results obtained from reciprocal crosses involving these alleles (in some crosses as donors, in others as recipients) were not reciprocal.

vector pBR328. This library was constructed and generously provided by Russell Maurer and Charles Miller (University of Illinois—Urbana).

Plasmid pJE1 recombinationally repaired mutation *cob-335* and all CobIII mutations upstream of it, including some CobI mutations located upstream of the CobIII region, but did not repair any CobIII mutation mapping downstream of *cob-335*. Hence, plasmid pJE1 contains enough CobIII DNA to recombinationally repair mutations that map within the promoter-proximal half of the genetic map (Fig. 2). A second plasmid, pJE2, recombinationally repaired all CobIII mutations and the *cob-300* allele of CobII but did not complement any CobII mutations.

Plasmids pJE1 and pJE2, each carrying an 8-kb insert,

were used with other plasmids in sequencing 17 kb of the *cob* operon (11). The sequenced portion of the operon extends from the main promoter-control region at the far left of the CobI region of the operon (as depicted in Fig. 2) through the CobIII region and includes all material in these two plasmids. The endpoints of the inserts carried by these plasmids are shown on the genetic map in Fig. 2. Beyond the last open reading frame (*cobT*), only 93 bp of the Cob sequence are included in pJE2. The initial portion of the first CobII gene (*cobJ*) must be included in these 93 bp, since the CobII amber mutation *cob-300* can be recombinationally repaired by this plasmid. A 2.2-kb *Bst*EII-*Aar*II fragment obtained from pJE2 has been shown by complementation to contain all three of the proposed CobIII genes (*cobU*, *cobS*, and

cobT) (27). The sequence of these genes is homologous with that of genes of *Pseudomonas denitrificans* that have been shown to encode enzymes catalyzing reactions involved in joining adenosyl-CBI and DMB (2, 4, 5, 9). These sequences and their analysis will be presented in detail elsewhere (11).

Complementation studies of the CobII region. The mutations in the genetic map were further classified by their ability to complement one another. Figure 3 shows the results obtained from complementation tests performed with point mutations within the CobII region. We identified three complementation groups (*cobJ*, *cobK*, and *cobL*) and a fourth group of mutations (*cobM*) that did not complement mutations in any of the other three groups. The failure of amber alleles *cob-300* and *cob-308* to complement any CobII mutations is probably due to polar effects on the expression of downstream genes, suggesting that there are no internal promoters within the CobII region.

Nutritional studies were used as an adjunct to complementation studies for the classification of CobII mutations. The CobII⁻ phenotype of *cobK* and *cobM* mutants was corrected only by exogenous DMB (22). However, the addition of the putative intermediate 4,5-dimethylphenylenediamine (DMPDA) to the culture medium corrected the CobII⁻ phenotype of all *cobJ* mutants and mutants carrying allele *cob-299*, *cob-301*, or *cob-303*. Since these alleles map downstream of the *cobK* alleles, they were designated *cobL*.

Lack of complementation in *cobM* mutants. It is worth noting the failure of *cobM* mutations to complement mutations in any other complementation group. This observation held true for all six point mutations (Fig. 3), two *Mu d-lacZ* operon fusions, and 3' deletions of *cobM* (*cob-285*, *cob-286*, and *cob-292*) (data not shown).

The failure of *cobM* mutations to complement all other promoter-proximal CobII mutations may not be due to a dominant defect, since all *cobM* mutations were recessive to a wild-type CobII region. It is also unlikely that the lack of complementation is due to polar effects, since *cobM* is the most promoter-distal group in the CobII region (Fig. 2).

The effects could be explained if the *cobM* gene product were required in *cis*. To address this possibility, two sets of *cobM cobJ* merodiploid strains carrying the *cobM* mutation in the genome or the F' plasmid were tested for their ability to grow on DMPDA. The *cobJ* alleles tested were *cob-316* and *cob-314*; the *cobM* alleles used were *cob-304* and *cob-309*. All the merodiploids tested were able to make DMB, and thus grow, when DMPDA was present in the medium. These results support the idea that *cobM* may act in *cis*.

These putative *cis* effects may also be explained if the CobII region encodes a single polypeptide with four functionally independent domains, with the *cobM* domain required for folding or for the activity of all portions of a single protein chain. Alternatively, *cobM* may be required to stabilize the CobII mRNA. Molecular analysis of *cobM* mutants is needed to understand these effects.

In summary, the pathway and biosynthetic precursors of DMB in *S. typhimurium* are poorly understood, but we are currently using the classification of CobII mutations described here to elucidate the sequence of reactions that lead to DMB synthesis in this bacterium. Our preliminary results suggest that *cobJ* and *cobL* mutants may be involved in the synthesis of DMPDA (or an internally derived compound) and that the *cobK* and *cobM* gene products convert DMPDA to DMB. This pathway appears to be different from the others found in aerobes and aerotolerant CBL producers, which derive DMB from flavin mononucleotide (15, 17, 18). The requirement for molecular oxygen in the latter pathway

makes it unlikely that this pathway is used by *S. typhimurium*, since this bacterium synthesizes DMB under both anaerobic and aerobic conditions. An alternative pathway has been proposed for the anaerobe *Eubacterium limosum*, which derives the benzene ring of DMB from glycine (C-8 and C-9) and erythrose-4-phosphate (C-4, C-5, C-6, and C-7), the methyl groups from S-adenosylmethionine, the N-1 of the imidazole ring from glycine, and N-3 from glutamine. The origin of the C-2 atom of the imidazole ring is unknown (15, 23, 24, 34, 35). The existence of this pathway in *S. typhimurium* is under investigation.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM40313 from the National Institutes of Health to J.C.E.-S. and Public Health Service grant GM34804 from the National Institutes of Health to J.R.R.

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