Cobalamin (Vitamin B₁₂) Biosynthetic Genes of Salmonella typhimurium

RANDALL M. JETER†* AND JOHN R. ROTH

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

Received 12 January 1987/Accepted 7 April 1987

The enteric bacterium Salmonella typhimurium synthesizes cobalamin (vitamin B₁₂) de novo only under anaerobic growth conditions. We initiated a genetic analysis of the cobalamin biosynthetic (cob) gene cluster. Stable cob::lac operon fusions were generated by insertions of a transposition-defective derivative of bacteriophage Mu dI (Ap lac) into the cob genes. β-Galactosidase synthesis was repressed in the presence of exogenously supplied cyanocobalamin, demonstrating that the cobalamin biosynthetic pathway was regulated by end-product repression. Transcriptional polarity studies showed that the cob genes responsible for synthesis of the corrinoid intermediate cobinamide (branch I of the pathway) were organized into a single operon. Genes for the synthesis of 5,6-dimethylbenzimidazole (branch II) and the final assembly of the complete cobalamin molecule (branch III) were organized into two or more additional operons. All of the known cob genes (in branches I, II, and III) were transcribed in a counterclockwise direction relative to the S. typhimurium genetic map. These genes are located at 41 map units and near the his operon. No essential genes lie between the his and cob operons. Mutants that carried deletions extending from the his genes into the cob region were isolated and characterized. By using these mutants, a deletion map of the branch I cob operon was constructed and the order of genes (his-cobl-cobIII-cobII) was inferred.

The enteric bacterium Salmonella typhimurium synthesizes cobalamin (vitamin B₁₂) de novo only when the cells are grown anaerobically (22). Biochemical studies with several different cobalamin-producing microorganisms have established a general outline of the pathway (4, 15, 16, 28). This outline is based on the identification of several key intermediates, but the exact number and sequence of steps remain unknown (Fig. 1). We estimate from the chemical modifications required that as many as 20 enzymes may be directly involved in the synthesis of cobalamin. Characterization of the pathway by using biochemical methods has been hampered by the fact that many of the intermediates are structurally complex and some are sensitive to light or oxygen or both in vitro (5). Recognition that S. typhimurium produces cobalamin makes it possible to use genetic techniques to study the pathway, since this organism is genetically manipulatable (29).

Genetic analysis of cobalamin biosynthesis promises to reveal answers to several questions. Why is cobalamin made by S. typhimurium only under anaerobic culture conditions? To our knowledge, this example of a biosynthetic pathway that is conditionally expressed in the absence of oxygen is unique. The molecular mechanism that underlies this conditional expression has not been identified. During aerobic growth, synthesis of the intermediate cobinamide (branch I of the pathway) is specifically impaired; S. typhimurium has no difficulty making cobalamin if cobinamide is provided in the culture medium (22). One possibility is that one or more of the intermediates or enzymes in branch I may be sensitive to oxygen and not sufficiently protected in vivo to prevent destruction. Alternatively, the branch I biosynthetic genes are known to be repressed by oxygen (13, 21). This may block the aerobic synthesis of cobinamide, thus limiting the production of cobalamin. Oxygen repression of these genes may indicate that cobalamin plays a particularly important role in anaerobic metabolism, or it might simply reduce the costs of a futile attempt to make cobinamide aerobically if branch I enzymes or intermediates are oxygen sensitive.

What is the role of cobalamin in the metabolism of S. typhimurium? This organism is known to possess two enzymes that require cobalamin as a cofactor, i.e., tetrahydropteroylglutamate-homocysteine methyltransferase (EC 2.1.1.13) and ethanolamine ammonia lyase (EC 4.3.1.7) (8, 9). The first is a methionine biosynthetic enzyme (encoded by the metH gene) for which a cobalamin-independent alternative (encoded by the metE gene) exists (10). The second is required only if the cells are utilizing ethanolamine as a carbon or nitrogen source. Neither of these enzymes is required for growth under routine culture conditions. Cobalamin may also be a cofactor in additional, unrecognized enzymes that are expressed only in the absence of oxygen. By understanding the biosynthetic pathway and its control, we hope to be able to approach these questions. This paper presents initial genetic studies of the cobalamin biosynthetic (cob) genes of S. typhimurium.

(A preliminary account of some of this work has been reported previously [R. M. Jeter and B. M. Olivera, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, H225, p. 143].)

MATERIALS AND METHODS

Bacterial and bacteriophage strains. The genotypes of the bacterial strains used in this study are given in Table 1. All strains were derived from S. typhimurium LT2. The bacteriophage used for all transductional crosses, P22 H105/1 int201, is an integration-defective derivative of a high-frequency generalized transducing mutant of bacteriophage P2 (1). Mu da (formerly Mu dI-8) is a multiply mutated derivative of the Mu dI phage of Casadaban and Cohen (7); Mu da transposes only in strains that also carry an amber suppressor (19).

Culture media and growth conditions. The E medium of
TABLE 1. S. typhimurium strains used

| Strain | Genotype* | Parent strain |
|--------|-----------|---------------|
| TR6583 | metE205 ara-9 |
| TT7227 | pncA207::Mu dA |
| TT7690 | hisF9951::Mu dA |
| TT7893 | hisF9954::Mu dA |
| TT8388 | recA1 zeo-609::TnlO 128 zff-1066::Mu dA |
| TT9818 | metE205 ara-9 cob-11::Tn10 |
| TT10440 | metE205 ara-9 supD10 zeo-609::Tn10 |
| TT10441 | metE205 ara-9 cob-12::Tn10 |
| TT10442 | metE205 ara-9 cob-13::Tn10 |
| TT10443 | metE205 ara-9 cob-14::Tn10 |
| TT10687 | metE205 ara-9 DEL646([hisF9951]*Mu dA*(cob-21)) |
| TT10688 | metE205 ara-9 DEL647([hisF9951]*Mu dA*(cob-22)) |
| TT10689 | metE205 ara-9 DEL648([hisF9954]*Mu dA*(cob-42)) |
| TT10690 | metE205 ara-9 DEL649([hisF9954]*Mu dA*(cob-54)) |
| TT10691 | metE205 ara-9 DEL650([hisF9951]*Mu dA*(cob-26)) |
| TT10292 | metE205 ara-9 DEL651([hisF9951]*Mu dA*(cob-62)) |
| TT10693 | metE205 ara-9 DEL652([hisF9951]*Mu dA*(cob-27)) |
| TT10694 | metE205 ara-9 DEL653([hisF9951]*Mu dA*(cob-64)) |
| TT10695 | metE205 ara-9 DEL654([hisF9951]*Mu dA*(cob-66)) |
| TT10726 | metE205 ara-9 DEL655([hisF9954]*Mu dA*(cob-61)) |
| TT10727 | metE205 ara-9 DEL657([hisF9954]*Mu dA*(cob-63)) |
| TT10728 | metE205 ara-9 DEL658([hisF9954]*Mu dA*(cob-63)) |
| TT10729 | metE205 ara-9 DEL659([hisF9954]*Mu dA*(cob-67)) |
| TT10927 | metE205 ara-9 zeb-1845::Tn10 |
| TT10928 | metE205 ara-9 sphps-204 |
| TT10930 | metE205 ara-9 sphps-204 hisO1242 |
| TT10989 | metE205 ara-9 sphps-205 |
| TT10991 | metE205 ara-9 sphps-205 hisO1242 |
| TT11019 | metE205 ara-9 sphps-206 |
| TT11021 | metE205 ara-9 sphps-206 hisO1242 |
| TT11022 | metE205 ara-9 sphps-207 |
| TT11024 | metE205 ara-9 sphps-207 hisO1242 |
| TT11031 | metE205 ara-9 DEL681([his-phs-cob]) |
| TT11035 | metE205 ara-9 DEL683([his-phs-cob]) |
| TT11036 | metE205 ara-9 DEL686([his-phs-cob]) |
| TT11037 | metE205 ara-9 DEL687([his-phs-cob]) |
| TT11043 | metE205 ara-9 DEL683([his-phs-cob]) |

* All strains listed in Table 1 were either already present in the culture collection of J. Roth or were constructed during the course of this study. All cob::Mu dA insertion strains isolated in this study are listed in Table 2.

* DEL numbers are used for chromosomal rearrangements that affect a series of loci with different individual designations. The affected loci are indicated in parentheses following the DEL number. Asterisks indicate the presence of a transposable element at the join point of the rearranged chromosomal sequences (20).

Vogel and Bonner (33) with 0.2% D-glucose as the carbon source was used as the minimal medium. Nutritional supplements were added as needed to the following final concentrations: amino acids, purines, and pyrimidines, 0.1 to 0.5 mM; cobinamide dicyanide and cyanocobalamin, 20 µg/liter; and 5,6-dimethylbenzimidazole (DMBI), 50 mg/liter. Trace metals were added as suggested by Balch et al. (3), including cobalt chloride at a concentration of 1 µM. Nutrient broth (8 g/liter; Difco Laboratories) with added NaCl (5 g/liter) was used as the complex medium. Solid medium contained agar (15 g/liter; Difco). Antibiotics were added to the following final concentrations in minimal and complex media, respectively: tetracycline hydrochloride, 10 or 20 mg/liter; kanamycin sulfate, 125 or 50 mg/liter; and sodium ampicillin, 15 or 30 mg/liter. For anaerobic growth in minimal medium, the concentration of ampicillin was reduced to 1 mg/liter. Hydrogen sulfide production was scored on Kligler iron agar (Difco). Organic chemicals and biochemicals were supplied by Aldrich Chemical Co., Inc., ICN Nutritional Biochemicals, and Sigma Chemical Co. Inorganic chemicals were supplied by Mallinckrodt, Inc., and J. T. Baker Chemical Co.

Unless otherwise specified, all cultures were grown at 37°C. Aerobic liquid cultures were grown in an incubator rotary shaker (New Brunswick Scientific Co., Inc.) at 200 rpm. Anaerobic cultures were grown in an anaerobic chamber (model 1024; Forma Scientific Co.) under an atmosphere of 3% hydrogen–7% carbon dioxide–90% nitrogen.

Transductional methods. P22 lysates were prepared on appropriate donor strains as described by Davis et al. (12). Recipient cells were grown to maximum stationary phase (ca. 2 × 10⁹ CFU/ml) and then were mixed with phage at a multiplicity of infection of about 0.05 for 30 min. Samples (0.1 ml) were spread onto selective medium and incubated for growth. Transductant colonies were restreaked onto the same selective medium for purification and then streaked nonselectively onto green indicator medium to test for phage sensitivity (32). Individual clones were verified to be phage-free by cross-streaking liquid samples of each strain with P22 H5 (clear-plaque mutant) phage.

Mutant isolation. Tn10 insertion mutants with a Cob− phenotype were isolated by transducing a metE parent strain (TR6583) with bacteriophage grown on pooled Tn10 insertion mutants and by screening Tetr transductants for loss of the ability to synthesize cobalamin anaerobically. The metE mutation makes cells cobalamin dependent for the synthesis
of methionine (using the metH enzyme) (22). Thus, metE single mutants are Met+ anaerobically. When cob mutations are introduced into this genetic background, they confer an anaerobic Met− phenotype, but the cells can grow if they have an exogenous supply of either methionine or cobalamin. The pool of insertion mutants included over 10,000 S. typhimurium LT2-derived clones, each carrying a random chromosomal insertion of Tn10 (12, 25). Tetracycline-resistant colonies were selected aerobically and screened for the loss of cobalamin synthesis by testing their ability to grow anaerobically on minimal plates. The branch of the cobalamin biosynthetic pathway (I, II, or III) that was blocked by the insertion was identified by the phenotypic response of each mutant to the addition of cobinamide and DMBI as previously described (22).

Since Mu dA is conditionally defective for transposition (19), Mu dA insertion strains were isolated by transducing a metE mutant recipient strain (TT9818) that also carries an amber suppressor (supD) with a P22 lysate grown on a donor strain that possesses an F' plasmid carrying a Mu dA insertion (TT8388). The complete Mu dA element was introduced into the recipient strain as a consequence of a two-fragment transduction event (20). In the suppressor background, transposition occurs into random chromosomal sites. Transductant colonies were selected on ampicillin-containing complex medium and scored for simultaneous loss of anaerobic cobalamin synthesis as described above. P22 lysates grown on purified mutant clones were used to transduce the Mu dA element into a nonsuppressor background to stabilize the insertion (19).

β-Galactosidase activity in Mu dA insertion strains. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) is enzymatically hydrolyzed by β-galactosidase to form a nondiffusible blue pigment (30). The presence or absence of β-galactosidase in the insertion mutant was qualitatively tested by visually assessing the intensity of blue color produced within individual colonies on plates containing 25 mg of X-Gal per liter (25 mg dissolved in 1 ml of N,N-dimethylformamide prior to addition to culture medium). The medium was an NCe (no carbon E) minimal medium base (12) with sodium succinate (0.25%) added as a sole carbon source. Succinate was used because cobalamin synthesis in S. typhimurium is subject to catabolite repression (13); therefore, cob gene expression is improved on a medium with a poor carbon source.

Generation of duplication and deletion mutations by recombination between Mu d prophages. This method is described in greater detail by Hughes and Roth (20). P22 lysates were grown on S. typhimurium TT7690 and TT7693 (hisF::Mu dA insertions in opposite chromosomal orientations) and on a number of strains with cob::Mu dA insertions in one of the three branches of the cobalamin biosynthetic pathway (chromosomal orientations unknown). Equal concentrations of phage (PFU per milliliter) from individual his and cob mutant lysates were mixed. Antibiotic-resistant transductants of a metE recipient strain (TR6583) were selected on ampicillin-containing complex medium. Each transductant colony displayed one of four possible phenotypes: His+ Cob− (cob::Mu dA insertion inherited), His− Cob+ (hisF::Mu dA insertion inherited), His+ Cob+ (duplication of genetic material between hisF and cob generated by recombination between his::Mu dA and cob::Mu dA prophages), or His− Cob− (deletion of genetic material between hisF and cob generated by recombination between his::Mu dA and cob::Mu dA prophages). (See Results for an explanation of the origin of these recombinant types.) The phenotype of each isolated colony was determined by replica plating to the appropriately supplemented minimal medium.

Selection of deletion mutants on high-salt medium. A derivative of a metE mutant (TR6583) was constructed which carries a Tn10 insertion near but not in the branch I cob genes (TT1027). A positive selection for tetracycline sensitivity provides a means of isolating Tn10-generated deletion mutants of this strain (6, 24, 26). We isolated several Tet+ derivatives that retain the His+ Cob+ phenotype. P22 transducing lysates were grown on each isolate and used as donors to transduce a his cob double insertion mutant to His+ Cob+. If a donor simultaneously repaired the double mutant with high efficiency, the donor was identified as carrying a deletion that removed most of the genetic material intervening between the his operon and the branch I cob genes. The his and cob regions are too far apart to be cotransduced by P22 with wild-type donors. These deletion strains (TT10928, TT10989, TT11019, and TT11022) were also found to be deficient for phs gene function(s), the anaerobic production of hydrogen sulfide from thiosulfate. This confirms the deletion phenotype, since the phs genetic locus is situated just counterclockwise from the his operon (29).

Double mutants were constructed that carried one of these deletions and the hisOl242 mutation, which is a small deletion of the his attenuator that causes constitutive expression of the his operon (23). A method for selection of his mutants from a strain constitutive for his operon expression has been described by Fink et al. (14). This method was modified by the use of high-salt conditions. Nutrient broth-grown cultures of these strains (TT10930, TT10991, TT11021, and TT11024) were diluted, and samples were plated onto E medium that had a threefold-concentrated base. Histidine and methionine were provided as nutritional supplements. Strains which express high levels of the his biosynthetic genes are unable to grow on this high-salt medium. Thus, this medium can be used to select his mutants in which the critical hisH and hisF enzyme activities are reduced or eliminated (M. Schmid, personal communication). Individual colonies were scored for the appropriate (His− Cob−) phenotype. These putative Cob− deletion mutants were isolated, purified, and retested for phenotype. Each mutant was tested aerobically for growth on medium supplemented with either cobinamide alone or cobinamide and DMBI to determine whether the defect was limited to branch I cob functions or also included the branch II or III cob functions.

Deletion mapping experiments. Individual cob::Tn10 and cob::Mu dA insertion strains were initially tested by spreading nutrient broth-grown cells (0.1 ml) onto E minimal medium plates and then individually spotting donor P22 lysates (5 × 10^6 PFU) onto the recipient cells. The plates were incubated anaerobically for 4 days. Cob+ transductants appearing within a spotted area were interpreted as a positive test (recombination between donor DNA and the recipient chromosome had occurred). All spot tests that gave negative results were repeated on an entire plate with 10^6 cells and 10^5 donor phage to improve the sensitivity of detection. In positive-control crosses with a wild-type donor under the same conditions, a minimum of 10,000 transductants per plate was detected.

RESULTS

Phenotypes of mutants that carry Mu dA insertions in the cob genes. The defective bacteriophage Mu d1(Ap lac) of
FIG. 1. Outline of the cobalamin biosynthetic pathway. Branch I represents cobinamide synthesis, branch II represents DMBI synthesis, and branch III represents cobalamin synthesis from these precursors. Interconnections with the pyridine nucleotide, flavin, and heme biosynthetic pathways are depicted. Abbreviations: ALA, 5-aminolevulinic acid; Cby, cobyric acid; DMBI-RP, 1-o-t-d-ribofuranosido-DMBI; H2SHC, dihydroxyhydrochlorin; NaMN, nicotinic acid mononucleotide; PBG, porphobilinogen; SAM, S-adenosylmethionine; Succ CoA, succinyl coenzyme A; URO III, uroporphyrinogen III.

Casadaban and Cohen (7) has proven to be an extremely useful genetic tool in several respects. First, it can generate null mutations by inserting into a coding region. These insertions generally have a polar effect on transcription of downstream genes in the same operon. More importantly, insertions of Mu d can generate fusions of the target operon to the lac genes within the element. These fusions can be used to assess the rate of transcription and therefore regulation of the target operon (7, 27, 30). We used these properties of Mu d for analysis of the cob genes. For mutagenesis of the cob genes, we used a derivative of the original Mu d1 that contains nonsense mutations in several genes required for transposition and does not transpose unless an amber suppressor mutation is also present in the genetic background (19). In a strain that lacks the suppressor, these transposition-defective insertions are stable. We refer to this element as Mu dA. (In the paper originally describing this element, it was called Mu d1-8.)

The cob::Mu dA insertion mutants were isolated as described in Materials and Methods. All of these mutants were stabilized by transducing them into a suppressor-free strain (TR6583) that carries a metE mutation. As described previously, aerobically grown cells that lack the metE enzyme activity are auxotrophic for methionine on minimal medium (22). The alternative metH I enzyme functions only when cobalamin is present. Thus, metE mutants are prototrophic under anaerobic culture conditions (when cobalamin is synthesized). Double metE cob mutants fail to make cobalamin and thus require methionine to grow under anaerobic conditions. All of the cob mutants that were isolated fell into one of three phenotypic classes: (i) those that made cobalamin when supplied with cobinamide and are inferred to be blocked in the synthesis of the corrinoid intermediate cobinamide (branch I mutants), (ii) those that made cobalamin when fed DMBI and are inferred to be defective in the synthesis of DMBI (branch II mutants), and (iii) those that did not make cobalamin even when fed cobinamide and DMBI and are inferred to be blocked in the ability to incorporate cobinamide and DMBI into cobalamin (branch III mutants) (Fig. 1). A complete list of the cob::Mu dA isolates and their phenotypic classifications (I, II, or III) is given in Table 2.

Mu dA insertions were Lac+ if the lac genes were oriented such that they could be transcribed as part of the target operon. To check the orientation of the cob::Mu dA insertions, we transduced each one into the wild-type strain of S. typhimurium (LT2) to remove the insertion from the metE background and eliminate any possible effects it might have on regulation. The resulting strains were screened for β-galactosidase production on succinate minimal medium containing the chromogenic β-galactosidase substrate X-Gal (see Materials and Methods). On this medium, about half of the cob::Mu dA mutants formed blue colonies, indicating that operon fusion had occurred and that the lac genes were transcribed from a cob promoter. The remaining mutants formed white colonies; it was assumed that these Mu dA elements had inserted into the cob genes in the wrong orientation to generate operon fusions. This assumption was later shown to be correct (see below). The Lac phenotype of each cob::Mu dA insertion mutant is listed in Table 2.

Cobalamin repression of cob::Mu dA fusions. The operon fusion (blue-colony-forming) strains were tested to determine whether the cob genes in S. typhimurium are subject to end-product repression. All of these mutants were plated on
indicator medium with or without the addition of cobalamin to test its effect on expression of the cob::lac fusions. After growth under aerobic conditions, the isolated colonies displayed a less intense blue color in the presence of cobalamin than what they did in its absence. By contrast, cobalamin did not prevent the reduction of a strain that carries an operon fusion in a gene unrelated to cobalamin metabolism (TT227; pncA207::Mu dA). This result implies that cobalamin does not directly inhibit color development.

The repression of β-galactosidase synthesis by cobalamin was most severe in strains with branch I cob::Mu dA insertions. Repression of cob::Mu dA insertions in genes of branch II and III was also detected, but it was not as pronounced as for fusions in the branch I genes. These qualitative observations were verified and extended in β-galactosidase assays performed by Escalante-Semerena and Roth (13).

### Table 2. Mu dA insertions in the S. typhimurium cob genes

| Strain   | Genotype          | Branch | Phenotype |
|----------|-------------------|--------|-----------|
| TT10324  | metE205 ara-9 cob-21 | I      | Lac*      |
| TT10325  | metE205 ara-9 cob-22 | I      | Lac*      |
| TT10326  | metE205 ara-9 cob-23 | I      | Lac*      |
| TT10327  | metE205 ara-9 cob-24 | I      | Lac*      |
| TT10328  | metE205 ara-9 cob-25 | I      | Lac*      |
| TT10329  | metE205 ara-9 cob-26 | I      | Lac*      |
| TT10330  | metE205 ara-9 cob-27 | I      | Lac*      |
| TT10331  | metE205 ara-9 cob-28 | I      | Lac*      |
| TT10332  | metE205 ara-9 cob-29 | I      | Lac*      |
| TT10333  | metE205 ara-9 cob-30 | I      | Lac*      |
| TT10334  | metE205 ara-9 cob-31 | I      | Lac*      |
| TT10335  | metE205 ara-9 cob-32 | I      | Lac*      |
| TT10336  | metE205 ara-9 cob-33 | I      | Lac*      |
| TT10337  | metE205 ara-9 cob-34 | I      | Lac*      |
| TT10338  | metE205 ara-9 cob-35 | I      | Lac*      |
| TT10339  | metE205 ara-9 cob-36 | I      | Lac*      |
| TT10340  | metE205 ara-9 cob-37 | I      | Lac*      |
| TT10341  | metE205 ara-9 cob-38 | I      | Lac*      |
| TT10342  | metE205 ara-9 cob-39 | I      | Lac*      |
| TT10343  | metE205 ara-9 cob-40 | I      | Lac*      |
| TT10344  | metE205 ara-9 cob-41 | I      | Lac*      |
| TT10345  | metE205 ara-9 cob-42 | I      | Lac*      |
| TT10346  | metE205 ara-9 cob-43 | I      | Lac*      |
| TT10347  | metE205 ara-9 cob-44 | I      | Lac*      |
| TT10348  | metE205 ara-9 cob-45 | I      | Lac*      |
| TT10349  | metE205 ara-9 cob-46 | I      | Lac*      |
| TT10350  | metE205 ara-9 cob-47 | I      | Lac*      |
| TT10351  | metE205 ara-9 cob-48 | I      | Lac*      |
| TT10352  | metE205 ara-9 cob-49 | I      | Lac*      |
| TT10353  | metE205 ara-9 cob-50 | I      | Lac*      |
| TT10356  | metE205 ara-9 cob-53 | I      | Lac*      |
| TT10357  | metE205 ara-9 cob-54 | I      | Lac*      |
| TT10358  | metE205 ara-9 cob-55 | I      | Lac*      |
| TT10359  | metE205 ara-9 cob-57 | I      | Lac*      |
| TT10360  | metE205 ara-9 cob-59 | I      | Lac*      |
| TT10361  | metE205 ara-9 cob-60 | I      | Lac*      |
| TT10362  | metE205 ara-9 cob-61 | I      | Lac*      |
| TT10363  | metE205 ara-9 cob-62 | II     | Lac*      |
| TT10364  | metE205 ara-9 cob-63 | II     | Lac*      |

### Table 3. Expression of lac genes in cob::Tn10 cob::lac double mutants

| Branch I cob::Tn10 insertion | Lac phenotype of branch I cob::Mu dA insertion with (and without) cobalamin |
|------------------------------|--------------------------------------------------------------------------|
|                              | cob-21 | cob-51 | cob-23 | cob-39 | cob-40 | cob-22 |
| None                         | (+)   | (+)   | (+)   | (+)   | (+)   | (+)   |
| cob-13                       | (-)   | (-)   | (-)   | (-)   | (-)   | (-)   |
| cob-11                       | (+)   | (+)   | (+)   | (+)   | (+)   | (+)   |
| cob-16                       | (+)   | (+)   | (+)   | (+)   | (+)   | (+)   |
| cob-12                       | (+)   | (+)   | (+)   | (+)   | (+)   | (+)   |
| cob-14                       | (+)   | (+)   | (+)   | (+)   | (+)   | (+)   |

* The phenotypes scored are blue (+) versus white (−) colonies on minimal medium containing X-Gal with (or without) a supplement of cyanocobalamin. Symbols in parentheses indicate the results when cobalamin was not present.

### Operon organization of the genes for cobalamin biosynthesis

A transposable element inserted into an operon does not influence expression of insertion-proximal (upstream) genes, but usually does block the transcription of insertion-distal (downstream) genes in the same operon (25). Mu dA elements that are inserted within an operon can be used as probes to detect the transcriptional polarity of a second transposable element inserted in the same operon. We constructed a series of double mutants that carried in the cob genes both a Tn10 and a Mu dA insertion that singly showed a Lac' phenotype. If a particular Tn10 prevented the synthesis of β-galactosidase in the double mutant, we interpreted this to mean that Tn10 was inserted in a gene upstream of the Mu dA insertion in the same operon. If a particular Tn10 did not block the synthesis of β-galactosidase, we concluded that either the Tn10 was inserted downstream of the Mu dA element in the same operon or the two transposable elements were inserted in different operons altogether.

We tested 30 double mutants, all of which carried both a Tn10 and a Mu dA insertion in the branch I cob genes. The test results are presented in Table 3. From the overall pattern of transcriptional polarity on β-galactosidase synthesis, we inferred that all of the branch I cob genes are organized into a single operon in S. typhimurium. The critical observation is that one Tn10 (cob-13:Tn10) had a polar effect on all branch I cob::lac insertions. We also noted that several of the Tn10 insertions did not block transcription of the lac genes but left β-galactosidase synthesis in the double mutant nonrepressible by exogenously supplied cobalamin. We interpreted this to mean that the Tn10 insertion was upstream of the Mu dA insertion and that the lac genes were being transcribed from a promoter located within the Tn10 element itself, a phenomenon which has been observed previously (11). Depending upon the location of a Tn10 insertion vis-à-vis polarity sites in the region, the element can block transcription of a large operon but can provide for constitutive expression of downstream genes by using the outward promoter (31; M. S. Ciampi and J. R. Roth, submitted for publication). The conclusions we drew from these polarity tests are diagrammed in Fig. 2. The map order of insertions inferred from these results was confirmed by deletion mapping (see below).

Similar polarity studies were done by using branch II and III insertions. On the basis of previous cotransduction experiments, branch II and III cob mutations appear to constitute a separate cluster located near the branch I cob genes (22). No branch II cob::Tn10 appeared to be polar on
any branch III cob::lac fusion, and conversely, no branch III cob::Tnl0 had a polar effect on any branch II cob::lac fusion (data not shown). This suggests that the branch II and III genes lie in at least two different operons that are distinct from the branch I cob operon. This conclusion was also supported by deletion mapping results for the branch II cob genes, which showed that the branch II and III genes are clustered into two contiguous groups and are not interspersed (J. Escalante-Semerena, manuscript in preparation).

Insertions in the branch I cob genes have complicated effects on the expression of Mu dA fusions in the branch II and III operons. None of the Tnl0 insertions included in Fig. 2 were absolutely polar on branch II and III cob::lac fusions, also suggesting that the branch II and III cob operons are distinct from the branch I operon. Regulation of branch II and III cob::lac fusions by cobalamin was lost when certain branch I cob::Tnl0 insertion mutations were present in the same genetic background. However, other branch I cob::Tnl0 mutations had no discernible effect on the expression and regulation of the branch II and III cob::lac fusions (data not shown). We interpret these results to mean that the branch I cob operon is distinct from the operons of branches II and III but that a regulatory gene for the branch II and III operons may be located within the branch I operon (J. Escalante-Semerena and T. Doak, personal communications).

Isolation of his-cob deletion mutants. We isolated a series of mutants that carried deletions reaching from the his operon into the cob region for use in genetic mapping of the cob genes. Initially, we selected for strains in which the chromosomal material between but not including the his and cob genes was eliminated. This was done by looking for mutants in which these two sets of genes were cotransducible (see Materials and Methods). We isolated four such mutants (TT10928, TT10989, TT11019, and TT11022; Table 1), and all retained a His+ Cob+ phenotype. Removal of this intervening region increased the frequency of his deletions that extend into the cob genes. Thus, we used these strains as inocula in a positive selection for His+ mutants (see Materials and Methods). Deletion mutants were identified as having simultaneously acquired a His+ Cob+ phenotype.

These deletion mutants fell into two general classes with respect to the Cob phenotype: (i) those with defects in cobalamin synthesis only (which can synthesize cobalamin if provided with cobinamide) and (ii) those which were unable to synthesize cobalamin even when provided with both cobinamide and DMBI. Thus, the former class was defective only in branch I of the pathway, while the latter was defective in branch III. This second class of deletions appears to remove all of the branch I cob operon and to extend into the branch III cob gene cluster or beyond. These strains could not be tested by nutritional supplementation for the functions of branches I and II. No deletion mutants were recovered that lacked branch I and II but retained branch III functions.

From these phenotypes, we infer that the branch I cob operon lies closest to the his region and that genes for branch II and III enzymes lie farther from his in a counterclockwise direction (to the right in Fig. 2). Since no deletion caused the loss of branch I and II functions only, leaving branch III intact, it appears that the branch III genes are located to the right of the branch I cob operon and that the branch II genes lie immediately to the right of the branch III genes.

Deletion mapping. Strains that carry Tnl0 and Mu dA insertions in the branch I cob genes were used as recipients in transductional crosses. Five of the high-salt-selected deletion mutants with defects that extended from his into the branch I cob genes were used as donors. These mutants were used as donors rather than recipients because the deletions they carried were so large that they could not be repaired even by using a wild-type donor. Initial deletion mapping results were obtained in a series of spot tests; those crosses that did not yield Cob+ transductants were then repeated by using full plate tests (see Materials and Methods). The final results of all crosses are diagrammed in Fig. 2 and are consistent with the order of branch I insertion mutations deduced from the polarity-mapping data.

Direction of transcription of the cob genes. Genetic crosses involving Mu dA insertions can be used to determine the direction of gene transcription (20). P22 packages a maximum of 44 kilobase pairs of chromosomal DNA into transducing particles. Mu dA is 38 kilobase pairs long. In theory, P22 should be able to package a complete Mu dA prophage. In practice, however, inheritance of Mu dA in a P22 transduction cross appears to require the introduction of two separate chromosomal fragments that carry overlapping right- and left-hand portions of the Mu dA element (18, 20). Homologous recombination between the two fragments regenerates the intact element; this composite donor fragment can then recombine with the chromosome to allow inheritance of the entire Mu dA element. In a suppressor-free recipient strain, inheritance of Mu dA is entirely dependent on homologous recombination.

Chromosomal rearrangements could occur when two lysates, each carrying Mu dA at a different site, were mixed and used for transduction. In these crosses, recombination between fragments of different donor Mu dA elements could yield a hybrid Mu dA with flanking DNA sequences from the
FIG. 3. Mu dA-induced chromosomal rearrangements between the his operon and the cob genes. The two donor strains carry different chromosomal insertions of Mu dA. P22 transduces fragments of both Mu dA insertions into a single recipient cell, where homologous recombination regenerates an intact Mu dA element. Recombination between donor and recipient DNA then produces chromosomal duplications (from donor fragments A and B') or deletions (from donor fragments A' and B) as depicted. Letter designations for cob genes are included only to indicate DNA sequences that are involved in recombination.
Table 4. Orientation of cob::Mu dA insertions

| Characteristics of donor strain carrying Mu dA insertion | No. of transductant colonies with phenotype (n = 100) | Inferred relative orientation of Mu dA elements |
|----------------------------------------------------------|----------------------------------------------------|-----------------------------------------------|
|                                                          | Parental (Cob') | Parental (His') | Duplication (Cob' His') | Deletion (Cob' His') | |
| hisF9951 (Lac⁻)                                          | 0              | 99              | 1                       | 0                    | Same |
| hisF9954 (Lac⁺)                                          | 0              | 100             | 0                       | 0                    | Same |
| cob-21 (I, Lac⁺)                                         | 100            | 0               | 0                       | 0                    | Same |
| cob-22 (I, Lac⁺)                                         | _b             | 0               | _b                      | 0                    | Opposite |
| cob-23 (I, Lac⁺)                                         | 100            | 0               | 0                       | 0                    | Opposite |
| cob-25 (I, Lac⁺)                                         | 100            | 0               | 0                       | 0                    | Opposite |
| cob-29 (I, Lac⁺)                                         | 100            | 0               | 0                       | 0                    | Opposite |
| cob-32 (I, Lac⁻)                                         | 100            | 0               | 0                       | 0                    | Opposite |
| cob-42 (Lac⁻)                                            | 100            | 0               | 0                       | 0                    | Opposite |
| cob-54 (Lac⁻)                                            | 100            | 0               | 1                       | 0                    | Opposite |
| cob-21 + hisF9951                                        | 22             | 27              | 48                      | 3                    | Same |
| cob-22 + hisF9951                                        | _b             | 39              | _b                      | 21                   | Same |
| cob-23 + hisF9951                                        | 40             | 9               | 10                      | 41                   | Same |
| cob-25 + hisF9951                                        | 39             | 29              | 14                      | 18                   | Same |
| cob-29 + hisF9951                                        | 21             | 24              | 49                      | 6                    | Same |
| cob-32 + hisF9951                                        | 68             | 32              | 0                       | 0                    | Opposite |
| cob-42 + hisF9951                                        | 73             | 27              | 0                       | 0                    | Opposite |
| cob-54 + hisF9951                                        | 80             | 20              | 0                       | 0                    | Opposite |
| cob-21 + hisF9954                                        | 58             | 42              | 0                       | 0                    | Opposite |
| cob-22 + hisF9954                                        | _b             | 41              | _b                      | 0                    | Opposite |
| cob-23 + hisF9954                                        | 89             | 11              | 0                       | 0                    | Opposite |
| cob-25 + hisF9954                                        | 57             | 43              | 0                       | 0                    | Opposite |
| cob-29 + hisF9954                                        | 55             | 45              | 0                       | 0                    | Opposite |
| cob-32 + hisF9954                                        | 27             | 20              | 53                      | 0                    | Same |
| cob-42 + hisF9954                                        | 38             | 25              | 22                      | 15                   | Same |
| cob-54 + hisF9954                                        | 33             | 19              | 47                      | 1                    | Same |
| cob-26 (II, Lac⁺)                                        | 100            | 0               | 0                       | 0                    | Same |
| cob-62 (II, Lac⁺)                                        | 100            | 0               | 0                       | 0                    | Same |
| cob-61 (II, Lac⁻)                                        | 100            | 0               | 0                       | 0                    | Same |
| cob-26 + hisF9951                                        | 31             | 11              | 4                       | 54                   | Same |
| cob-62 + hisF9951                                        | 32             | 16              | 1                       | 51                   | Same |
| cob-61 + hisF9951                                        | 52             | 48              | 0                       | 0                    | Opposite |
| cob-26 + hisF9954                                        | 78             | 22              | 0                       | 0                    | Opposite |
| cob-62 + hisF9954                                        | 75             | 25              | 0                       | 0                    | Opposite |
| cob-61 + hisF9954                                        | 20             | 19              | 2                       | 59                   | Same |
| cob-27 (III, Lac⁺)                                       | 100            | 0               | 0                       | 0                    | Same |
| cob-64 (III, Lac⁺)                                       | 99             | 0               | 1                       | 0                    | Same |
| cob-66 (III, Lac⁺)                                       | 99             | 0               | 1                       | 0                    | Same |
| cob-63 (III, Lac⁻)                                       | 100            | 0               | 0                       | 0                    | Same |
| cob-65 (III, Lac⁻)                                       | 100            | 0               | 0                       | 0                    | Same |
| cob-67 (III, Lac⁻)                                       | 100            | 0               | 0                       | 0                    | Same |
| cob-27 + hisF9951                                        | 23             | 15              | 3                       | 59                   | Same |
| cob-64 + hisF9951                                        | 40             | 10              | 2                       | 48                   | Same |
| cob-66 + hisF9951                                        | 41             | 11              | 4                       | 44                   | Same |
| cob-63 + hisF9951                                        | 60             | 39              | 1                       | 0                    | Opposite |
| cob-65 + hisF9951                                        | 21             | 78              | 0                       | 0                    | Opposite |
| cob-67 + hisF9951                                        | 28             | 72              | 0                       | 0                    | Opposite |
| cob-27 + hisF9954                                        | 32             | 67              | 1                       | 0                    | Opposite |
| cob-64 + hisF9954                                        | 82             | 18              | 0                       | 0                    | Opposite |
| cob-66 + hisF9954                                        | 80             | 20              | 0                       | 0                    | Opposite |
| cob-63 + hisF9954                                        | 20             | 23              | 2                       | 55                   | Same |
| cob-65 + hisF9954                                        | 11             | 44              | 4                       | 41                   | Same |
| cob-67 + hisF9954                                        | 13             | 38              | 3                       | 46                   | Same |

* Individual cob alleles are designated as Lac⁺ (cob: lac operon fusions) or Lac⁻, and the branch of the cobalamin pathway that is blocked is given. The strain numbers that correspond to the cob alleles are listed in Table 2.
+ The cob-22 allele conferred a leaky phenotype such that the parental Cob⁺ prototrophic phenotypes could not be reliably distinguished under the conditions of this experiment.
different parental insertion sites. Such hybrids recombined with the bacterial chromosome and generated duplications and deletions if the two parental Mu dA prophages had the same orientation in the chromosome (Fig. 3). Such rearrangements did not occur if the two parental Mu dA prophages were in opposite orientations. If the orientation of one Mu dA insertion relative to the genetic map is known, the orientation of a second Mu dA insertion can be deduced by detecting the presence or absence of induced chromosomal duplications and deletions. By determining the orientation of Mu dA prophages that are inserted into the cob genes in this manner, we can infer the direction of transcription of these operons.

We tested 34 combinations of his::Mu dA insertions with cob::Mu dA insertions in all three branches of the cobalamin biosynthetic pathway. The phenotypes of the ampicillin-resistant colonies that resulted from the transduction crosses are given in Table 4. To summarize the data, a Lac+ his::Mu dA insertion formed duplications and deletions when used with Lac- cob::Mu dA insertions as donors in the crosses. Conversely, a Lac- his::Mu dA insertion formed duplications and deletions when used with Lac+ cob::Mu dA insertions. However, pairs of donors with the same Lac phenotypes, his::Mu dA (Lac+) with cob::Mu dA (Lac-) and his::Mu dA (Lac-) with cob::Mu dA (Lac+), did not generate such chromosomal rearrangements. (Occasional Cob+ His+ transductants that were seen by using a single donor Mu dA prophage were probably due to Mu dA insertions into spontaneous chromosomal duplications in the recipient [2].)

Thus, his::Mu dA and cob::Mu dA insertions that had different Lac phenotypes were in the same chromosomal orientation; his::Mu dA and cob::Mu dA insertions that had the same Lac phenotype were in opposite chromosomal orientations. The his operon is known to be transcribed in the clockwise direction (1, 17). Thus, we infer from our results that the cob genes in all three branches of the pathway are transcribed in the opposite direction to that of the his operon, that is, in a counterclockwise direction relative to the S. typhimurium genetic map.

Deletions generated from these crosses are useful in mapping. Each deletion has an endpoint at the site of a cob::Mu dA insertion. The properties of these deletions also give information regarding the general arrangement of the cob genes. We isolated 13 different mutants with Mu dA-generated deletions that terminated in the cob region (Table 1). The four deletions from his to the branch I cob genes (in strains TT10687, TT10688, TT10689, and TT10690) retained the functions of branches II and III. The six deletions from his to the branch III cob genes (TT10693, TT10694, TT10695, TT10727, TT10728, and TT10729) were CobIII−, but the CobI and CobII phenotypes of these strains could not be tested. The remaining three deletions from his to the branch II cob genes (TT10691, TT10692, and TT10726) also conferred a CobIII− phenotype. As was the case with the salt-selected his-cob deletions described above, these results suggest that branch III cob functions lie between the branch I operon and the branch II genes.

DISCUSSION

The results presented give a general description of the genetic region encoding the cobalamin (vitamin B12) biosynthetic pathway. Several conclusions can be drawn from these results. (i) All known cob mutations are located just counterclockwise from the his operon and near 41 map units on the S. typhimurium chromosome (22). (ii) The order of the genes is his-phs-cobl-cobIII-cobII. (iii) The cob genes that encode the enzymes of branch I (cobinamide synthesis) appear to constitute a single operon, on the basis of the polarity effects of Tn10 insertions. The number of operons encoding the other branches of the pathway is uncertain, but at least two additional operons appear to be present. (iv) All cob::lac fusions that were tested were repressed by exogenously supplied cobalamin. (v) All cob genes that were tested were transcribed in a counterclockwise direction, opposite to the direction of transcription of the his operon.

(vi) The material between the his and cob loci in the Salmonella chromosome is not essential, since deletions of this region are viable and permit genetic mapping of the cob region. This intervening region includes the gene(s) for anaerobic thiosulfate reduction (phs).

With this knowledge of the genetic structure of the region, we are assessing the number of genes involved in this extensive biosynthetic pathway and examining further how these genes are regulated in response to environmental conditions.

ACKNOWLEDGMENTS

We thank Baldomero M. Olivera and Kelly T. Hughes for helpful discussions.

This research was supported by Public Health Service grant GM34804 to J.R.R. from the National Institute of Health.

LITERATURE CITED

1. Anderson, R. P., and J. R. Roth. 1978. Tandem chromosomal duplications in Salmonella typhimurium: fusion of histidine genes to novel promoters. J. Mol. Biol. 119:147-166.
2. Anderson, R. P., and J. R. Roth. 1981. Spontaneous tandem genetic duplications in Salmonella typhimurium arise by unequal recombination between rRNA (rrn) cistrons. Proc. Natl. Acad. Sci. USA 78:3113-3117.
3. Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe. 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. 43:260-296.
4. Battersby, A. R., and E. McDonald. 1982. Biosynthesis of the corrin macrocycle. p. 107-144. In D. Dolphin (ed.), B12, vol. 1. John Wiley & Sons, Inc., New York.
5. Battersby, A. R., and L. A. Reiter. 1984. Synthetic studies relevant to biosynthetic research on vitamin B12. Part 3. An approach to isobacteriochlorins via nitrones. J. Chem. Soc. Perkins Trans. 1 12:2743-2749.
6. Bochner, B. R., H.-C. Huang, G. L. Schieven, and B. N. Ames. 1980. Positive selection for loss of tetracycline resistance. J. Bacteriol. 143:926-933.
7. Casabdan, M. J., and S. N. Cohen. 1979. Lactose genes fused to exogenous promoters in one step using a Mu-lac bacteriophage: in vivo probe for transcriptional control sequences. Proc. Natl. Acad. Sci. USA 76:4530-4533.
8. Cauthen, S. E., M. A. Foster, and D. D. Woods. 1966. Methionine synthesis by extracts of Salmonella typhimurium. Biochem. J. 98:630-635.
9. Chang, G. W., and J. T. Chang. 1975. Evidence for the B12-dependent enzyme ethanolamine deaminase in Salmonella. Nature (London) 254:150-151.
10. Childs, J. D., and D. A. Smith. 1969. New methionine structural gene in Salmonella typhimurium. J. Bacteriol. 100:377-382.
11. Ciampi, M. S., M. B. Schmid, and J. R. Roth. 1982. Transposon Tn10 provides a promoter for transcription of adjacent sequences. Proc. Natl. Acad. Sci. USA 79:5016-5020.
12. Davis, R. W., D. Botstein, and J. R. Roth. 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. Escalante-Semerena, J. C., and J. R. Roth. 1987. Regulation of cobalamin biosynthetic operons in Salmonella typhimurium. J. Bacteriol. 169:2251-2258.
14. Fink, G. R., T. Klopotowski, and B. N. Ames. 1967. Histidine regulatory mutants in Salmonella typhimurium. IV. A positive selection for polar histidine-requiring mutants from histidine operator constitutive mutants. J. Mol. Biol. 30:81–95.

15. Friedmann, H. C. 1975. Biosynthesis of corrinoids, p. 75–109. In B. Babior (ed.), Cobalamin: biochemistry and pathophysiology. John Wiley & Sons, Inc., New York.

16. Friedmann, H. C. 1979. Straight approaches to the nucleotide loop, p. 331–343. In B. Zagalak and W. Friedrich (ed.), Vitamin B12. Walter de Gruyter, Inc., New York.

17. Hartman, P. E., C. Rusgis, and R. C. Stahl. 1965. Orientation of the histidine operon in the Salmonella typhimurium linkage map. Proc. Natl. Acad. Sci. USA 53:1332–1335.

18. Hughes, K. T., B. M. Olivera, and J. R. Roth. 1987. Rec dependence of Mu transposition from P22-transduced fragments. J. Bacteriol. 169:403–409.

19. Hughes, K. T., and J. R. Roth. 1984. Conditionally transposition-defective derivative of Mu dl(Amp Lac). J. Bacteriol. 159:130–137.

20. Hughes, K. T., and J. R. Roth. 1985. Directed formation of deletions and duplications using Mud(Ap, lac). Genetics 109:263–282.

21. Jeter, R., J. C. Escalante-Semerena, D. Roof, B. Olivera, and J. Roth. 1987. Synthesis and use of vitamin B12, p. 551–556. In F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), Escherichia coli and Salmonella typhimurium: cellular and molecular biology. American Society for Microbiology, Washington, D.C.

22. Jeter, R. M., B. M. Olivera, and J. R. Roth. 1984. Salmonella typhimurium synthesizes cobalamin (vitamin B12) de novo under anaerobic growth conditions. J. Bacteriol. 159:206–213.

23. Johnston, H. M., W. M. Barnes, F. G. Chumley, L. Bossi, and J. R. Roth. 1980. Model for regulation of the histidine operon of Salmonella. Proc. Natl. Acad. Sci. USA 77:508–512.

24. Kleckner, N., K. Rechardt, and D. Botstein. 1979. Inversions and deletions of the Salmonella chromosome generated by the translocatable tetracycline resistance element Tn10. J. Mol. Biol. 127:89–115.

25. Kleckner, N., J. R. Roth, and D. Botstein. 1977. Genetic engineering in vivo using translocatable drug-resistance elements: new methods in bacterial genetics. J. Mol. Biol. 116:125–159.

26. Maloy, S. R., and W. D. Nunn. 1981. Selection for loss of tetracycline resistance by Escherichia coli. J. Bacteriol. 45:1110–1112.

27. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

28. Renz, P., J. Hörig, and R. Wurm. 1979. On the biosynthesis of the 5,6-dimethylbenzimidazole moiety of vitamin B12. In B. Zagalak and W. Friedrich (ed.), Vitamin B12. Walter de Gruyter, Inc., New York.

29. Sanderson, K. E., and J. R. Roth. 1983. Linkage map of Salmonella typhimurium, edition VI. Microbiol. Rev. 47:410–453.

30. Silhavy, T. J., and J. R. Beckwith. 1985. Uses of lac fusions for the study of biological problems. Microbiol. Rev. 49:398–418.

31. Simons, R. W., B. C. Hoopes, W. R. McClure, and N. Kleckner. 1983. Three promoters near the termini of IS10: pIN, pOUT, and pII1. Cell 34:673–682.

32. Smith, H. O., and M. Levine. 1967. A phage P22 gene controlling integration of prophage. Virology 31:207–216.

33. Vogel, H. J., and D. M. Bonner. 1956. Acetylornithinase of Escherichia coli: partial purification and some properties. J. Biol. Chem. 218:97–106.