Evolution of Coenzyme B_{12} Synthesis Among Enteric Bacteria: Evidence for Loss and Reacquisition of a Multigene Complex

Jeffrey G. Lawrence and John R. Roth

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

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ABSTRACT

We have examined the distribution of cobalamin (coenzyme B_{12}) synthetic ability and cobalamin-dependent metabolism among enteric bacteria. Most species of enteric bacteria tested synthesize cobalamin under both aerobic and anaerobic conditions and ferment glycerol in a cobalamin-dependent fashion. The group of species including *Escherichia coli* and *Salmonella typhimurium* cannot ferment glycerol. *E. coli* strains cannot synthesize cobalamin *de novo*, and *Salmonella spp.* synthesize cobalamin only under anaerobic conditions. In addition, the cobalamin synthetic genes of *Salmonella spp.* (*cob*) show a regulatory pattern different from that of other enteric taxa tested. We propose that the cobalamin synthetic genes, as well as genes providing cobalamin-dependent diol dehydratase, were lost by a common ancestor of *E. coli* and *Salmonella spp.* and were reintroduced as a single fragment into the Salmonella lineage from an exogenous source. Consistent with this hypothesis, the *S. typhimurium cob* genes do not hybridize with the genomes of other enteric species. The Salmonella *cob* operon may represent a class of genes characterized by periodic loss and reacquisition by host genomes. This process may be an important aspect of bacterial population genetics and evolution.

COBALAMIN (coenzyme B_{12}) is a large evolutionarily ancient molecule (GEORGOPAPADAKOU and SCOTT 1977; ESCHENMOSER 1988; SCOTT 1990, 1993) that participates as a cofactor in both prokaryotic and eukaryotic metabolism (SCHNEIDER and STROÏNSKI 1987; STROÏNSKI 1987). Cobalamin is derived from uroporphyrinogen III (Uro III), a precursor in the synthesis of heme, siroheme, cobamides and chlorophylls (in photosynthetic organisms). As detailed in Figure 1, the conversion of Uro III to the complex coenzyme B_{12} intermediate cobinamide is referred to as part I of the biosynthetic pathway (hereafter denoted CobI). Part II of the pathway (CobII) entails the biosynthesis of dimethylbenzimidazole (DMB) from probable flavin precursors (HÖRIG and RENZ 1978; JOHNSON and ESCALANTE-SEMERENA 1992; LINGENS *et al.* 1992; CHEN *et al.* 1995). The covalent joining of cobinamide, DMB, and a phosphoribosyl group donated by nicotinate mononucleotide is achieved by part III of the pathway (CobIII). Among the enteric bacteria, both *Salmonella typhimurium* (JETER *et al.* 1984) and *Klebsiella pneumonia* (ALBERT *et al.* 1980) are known to synthesize cobalamin *de novo* under anaerobic conditions. These organisms can perform all three parts of the biosynthetic pathway. *Escherichia coli* synthesizes cobalamin only when provided with cobinamide (VOLCANI *et al.* 1961); therefore, *E. coli* performs only parts II and III of the cobalamin biosynthetic pathway.

Corresponding author: John Roth, Department of Biology, University of Utah, Salt Lake City, UT 84112.
E-mail: roth@bioscience.utah.edu

The cobalamin biosynthetic genes have been characterized in *S. typhimurium;* most genes are located in a large 20-gene cluster at minute 41 on the genetic map (JETER and ROTH 1987; ROTH *et al.* 1993). Within the cluster, genes encoding the enzymes for the three parts of the pathway are clustered (Figure 1). Mutations conferring $CobI^-$, $CobIII^-$, and $CobII^-$ phenotypes are localized, in that order, within the *cob* operon (JETER and ROTH 1987; ESCALANTE-SEMERENA *et al.* 1992; O'TOOLE *et al.* 1993; ROTH *et al.* 1993). Additional genes outside this cluster encode both biosynthetic enzymes for corrinoid adenosylation (ESCALANTE-SEMERENA *et al.* 1990) and synthesis of aminopropanol, the linker between the corrin ring and the lower ligand (GRABAU and ROTH 1992); transport of cobalamin is also encoded by genes unlinked to the *cob* operon (RIOUX and KADNER 1989; RIOUX *et al.* 1990; BRADBEER 1991). Despite this large genetic investment in cobalamin synthesis and acquisition, entailing nearly 1% of the *S. typhimurium* chromosome, only four known, and one likely, cobalamin-dependent reactions have been characterized among the enteric bacteria:

1. Homocysteine methyltransferase catalyzes the final step in methionine biosynthesis. The *metE* and *metH* genes encode cobalamin-independent and cobalamin-dependent methyltransferases, respectively (SMITH and CHILDS 1966; CHILDS and SMITH 1969), in *S. typhimurium.* These genes are also found in *E. coli* (DAVIS and MINGIOLI 1950).

2. Ethanolamine-ammonia lyase converts ethanolamine into acetylaldehyde and ammonia and is encoded by the *eutBC* genes in *S. typhimurium;* the enzyme

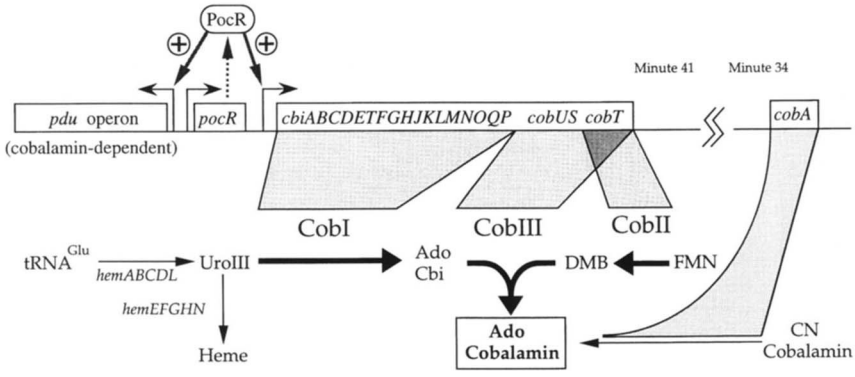

FIGURE 1.—The metabolism of tetrapyrroles in *Salmonella typhimurium*. Heavy arrows denote reactions specific for cobalamin biosynthesis. Gray arrows denote regulatory interactions. Abbreviations are as follows: Ado, adenosyl; Cbi, cobinamide; CN, cyano; DMB, dimethylbenzimidazole; FMN, flavin mononucleotide; Uro III, uroporphyrinogen III. The CobI, CobII, and CobIII pathways are discussed in the text. The *hem* genes are described by XU *et al.* (1992); the *cbi* and *cob* genes are described by ROTH *et al.* (1993) and CHEN *et al.* (1995).

is also present in strains of *E. coli* and *K. aerogenes* (CHANG and CHANG 1975; SCARLETT and TURNER 1976; ROOF and ROTH 1988, 1989).

3. Propanediol dehydratase catalyzes the conversion of 1,2-propanediol to propionaldehyde and is encoded in the *pdu* operon in *S. typhimurium* (JETER 1990; BOBIK *et al.* 1992); the *pdu* operon maps adjacent to the *cob* operon described here. In this paper, the general term propanediol will always refer to the 1,2 isomer. Cobalamin-dependent diol dehydratases, active with 1,2 ethanediol and propanediol, have also been described in Citrobacter and Klebsiella species (ABELES and LEE 1961; FORAGE and FOSTER 1979; TORAYA *et al.* 1979, 1980; OBRADORS *et al.* 1988).

4. Glycerol dehydratase is an enzyme distinct from diol dehydratase (TORAYA and FUKUI 1977), allowing anaerobic fermentation of glycerol in species of Klebsiella and Citrobacter (SCHNEIDER *et al.* 1970; FORAGE and FOSTER 1979, 1982); this enzyme has not been described among strains of Salmonella or *E. coli*.

5. Queuosine synthetase catalyzes the final step in the synthesis of queuosine, a hypermodified base present in four tRNAs and has been reported to be a cobalamin-dependent enzyme in *E. coli* (NOGUCHI *et al.* 1982; FREY *et al.* 1988).

The known cobalamin-dependent reactions in *S. typhimurium* do not obviously justify this organism's large genetic investment in cobalamin biosynthesis and transport. The cobalamin-dependent methionine synthetase is redundant, and the queuosine synthetase is apparently nonessential under laboratory conditions. Propanediol utilization appears to be the primary use for cobalamin in *S. typhimurium* since the adjacent cobalamin biosynthetic genes (*cob*) and the propanediol degradative operon (*pdu*) are coregulated by the same protein (PocR) and are both induced in the presence of propanediol (BOBIK *et al.* 1992; CHEN *et al.* 1994). A paradox is evident in that propanediol serves as a sole carbon and energy source only in the presence of oxygen while cobalamin is synthesized *de novo* only under anaerobic growth conditions.

To understand the large investment made by *Salmonella spp.* in cobalamin metabolism despite its nonobvious utility, we have examined the distribution of cobalamin synthesis and cobalamin-dependent metabolism among enteric bacteria. In this manner, the selective influences operating on cobalamin synthetic genes may be elucidated. We suggest that the Salmonella *cob* and *pdu* operons were acquired by horizontal transfer after the loss of these genes by a common ancestor of both *E. coli* and *Salmonella spp.* The evolution of the cobalamin synthesis and utilization may provide a general model for understanding the evolution of functions and phenotypes under selection that is temporally or spatially heterogeneous.

MATERIALS AND METHODS

Bacterial strains and culture conditions: Strains of enteric bacteria employed in this study are listed in Table 1. Strains of the SARA collection (BELTRAN *et al.* 1991) of natural isolates of *Salmonella spp.* were kindly provided by K. SANDERSON and H. OCHMAN. Strains of the ECOR (OCHMAN and SELANDER 1984) and SARB (BOYD *et al.* 1993) collections of natural isolates of *E. coli* and *Salmonella spp.* were kindly provided by H. OCHMAN. An *E. coli* strain harboring a *metE*::Tn*10* insertion was provided by G. STAUFFER; the wild-type *K. aerogenes* strain M5a1 was provided by G. ROBERTS. Additional species of Salmonella and other species of enteric bacteria were obtained from laboratory collections. The rich medium used was LB; defined media included E medium (VOGEL and BONNER 1956) as well as its derivative NCE, which lacks carbon compounds. MacConkey indicator agar was used with the addition of specific carbon sources to 1% concentrations. For anaerobic growth on solid medium, cultures were grown under an atmosphere of 89% N_2, 6% CO_2, and 5% H_2. Liquid culture media were prepared, degassed for 24 hr, inoculated, and sealed in tubes with aluminum caps under the anoxic atmosphere described. Headspace gas was exchanged three times with 100% N_2 under a pressure of 2 atmospheres. Loss of pressure in a sealed tube during incubation indicated a possible decay of anoxic conditions and the experiment was repeated.

Cobalamin synthesis bioassay: To assay ability to synthesize cobalamin, strains were grown to stationary phase on a defined medium under appropriate conditions. Cells were recovered from a volume of 1.5 ml (aerobic) or 4 ml (anaerobic) of growth medium by centrifugation, were rinsed with a solution of 50 mM Tris pH 8.0 and 100 mM NaCl, recovered by centrifugation, and resuspended in the same solution at 5 μl/mg cell weight. The cells were lysed by boiling for 15 min; cell debris was removed by centrifugation. Sterile filter disks were soaked with 10 μl aliquots of the appropriate dilutions of this supernatant and were placed on solid mini-

TABLE 1

Strain list

Species	Strain(s)[a]	Phenotype
Citrobacter freundii	TR7173	Natural isolate
Escherichia blattae	ATCC 29907, 33429, 33430	
E. coli	ECOR 1–72	Natural isolates[b]
	TR7177 (W3100; K-12)	Wild-type laboratory strain
	TT17196	*pro*-3 Δ*lac*-6 *supE*44 *xyl*-5 *his*-218 *ilvC*7 *metB*1 *rpsL*109 *eutA*403*metE*:163:Tn*10*
E. fergusonii	ATCC 35469–35473	Clinical isolate
E. hermannii	ATCC 33650–33652	Clinical isolate
E. vulneris	ATCC 29943, 33821, 33832	Clinical isolate
Enterobacter cloacae	TR7174 (E482)	Natural isolate
Klebsiella aerogenes	TR7176 (M5a1)	Laboratory strain
Klebsiella pneumonia	TR7175 (LD119)	Natural isolate
Salmonella typhimurium	TT10858	*metE*205 *ara*-9 *cob*-66::Mu*dJ*
	TT11855	*metE*205 *ara*-9 DEL299 (his^-, cob^-)
	TR7172	*met*A53
	TT14305	*metE*2113::Mu*dJ* *trp*-101
Salmonella sp.	SARA 1–72	Natural isolates[c]
	SARB 1–72	Natural isolates[d]
S. seminole	TR6332	Natural isolate
Serratia ficari	ATCC 33105	
S. fonticola	ATCC 29844	
S. grimesii	ATCC 14460	
S. liquefaciena	ATCC 27592	
S. marcesens	ATCC 13880	
S. odorifera	ATCC 33077	
S. plymuthica	ATCC 183	
S. proteamaculans	ATCC 19323	
Shigella dysenteriae	ATCC 13313	
S. flexnerii	ATCC 29508	
S. sonnei	ATCC 29930	

[a] All laboratory *S. typhimurium* strains are derived from strain LT2. Sources of non-Salmonella species are described in LAWRENCE *et al.* (1991). Alternate strain names are in parentheses.

[b] The ECOR collection is described by OCHMAN and SELANDER (1984).

[c] The SARA collection is described by BELTRAN *et al.* (1991).

[d] The SARB collection is described by BOYD *et al.* (1993).

mal medium seeded with 10^6 cells of *S. typhimurium* indicator strain TT10858 or TT11855. A mutation in the *metE* gene of these strains requires them to utilize the cobalamin-dependent MetH protein for methionine synthesis, and the CobIII mutations present in these indicator strains preclude cobalamin biosynthesis. Growth of the indicator strain on minimal medium requires an exogenous source of either methionine or cobalamin. Growth of the indicator strain around the filter disk indicated that the cell lysate contained either cobalamin or methionine. None of the cell lysates tested provided sufficient free methionine to allow growth of the *metA* defective strain TR7172, indicating that all positive responses indicated the presence of cobalamin in the lysate. The radius of the growth ring for various dilutions of the lysate were compared to a standard curve. Positive results are seen with >0.5 ng cobalamin present in the aliquot applied to the disc.

Assays of cobalamin usage: To determine if propanediol and ethanolamine were degraded in a cobalamin-dependent fashion, MacConkey plates were supplemented with either carbon source to 1% final concentration. Acid excretion only upon the addition of cobalt or cobalamin is recorded as a cobalamin-dependent change in color. Strains capable of cobalamin-dependent fermentation of glycerol (as a sole carbon and energy source) were able to grow anaerobically on NCE minimal glycerol medium when provided cobalt or cobalamin; strains unable to ferment glycerol required fumarate or nitrate as an electron acceptor. Minimal medium and MacConkey indicator agar provide insufficient cobalt to allow *de novo* synthesis of cobalamin under aerobic conditions by most of the species of bacteria tested. Therefore, when a positive result on either medium (growth on minimal medium, color change on MacConkey) was dependent on either cobalt *or* cobalamin, that result was judged cobalamin-dependent. All strains showing a positive result upon cobalt addition also showed a positive result upon cobalamin addition. Therefore, it is unlikely that cobalt-dependence reflected a cobalt-dependent enzyme. Only one such enzyme, methionine aminopeptidase, is known among enteric bacteria (RODERICK and MATTHEWS 1993). In all strains that showed a cobalt-dependent growth response, the cobalt-dependent synthesis of cobalamin was verified by the bioassay described above.

DNA hybridization: DNA fragments for use as radioactively labeled probes in Southern hybridizations were prepared from the *S. typhimurium pocR* regulon as follows. The CobI gene fragments were isolated as the following restriction fragments from plasmids p41-1, p53-3, pJE1, and pJE2 (plasmid descriptions and sequence coordinates from ROTH *et al.* 1993): (1) a 2490-bp *Mlu*I fragment (bp 1218–3705) carrying

portions of the *cbiA*, *cbiB*, and *cbiC* genes, (2) a 900-bp *Mlu*I fragment (bp 3705–4607) carrying portions of the *cbiC* and *cbiD* genes, (3) a 4360-bp *Hpa*I fragment (bp 2115–6476) carrying portions of the *cbiA*, *cbiB*, *cbiC*, *cbiD*, *cbiE*, and *cbiT* genes, (4) a 920-bp *Hpa*I fragment (bp 6476–7399) carrying portions of the *cbiT*, *cbiF*, and *cbiG* genes, (5) a 4490-bp *Hpa*I fragment (bp 7793–12080) carrying portions of the *cbiG*, *cbiH*, *cbiJ*, *cbiK*, *cbiL*, and *cbiM* genes, (6) a 780-bp *Hpa*I fragment (bp 12080–12857) carrying portions of the *cbiM*, *cbiN*, and *cbiQ* genes, and (7) a 2150-bp *Hpa*I fragment (bp 12857–15006) carrying portions of the *cbiQ*, *cbiO*, and *cbiP* genes. The CobIII/II gene fragment was isolated as a 2180-bp *Cla*I/*Bst*EII restriction fragment from plasmid pJE2 bearing portions of the *cbiP*, *cobU*, *cobS*, and *cobT* genes. Additional CobIII DNA was amplified via PCR (SAIKI *et al.* 1985, 1988). The *pocR* gene fragment was isolated as a 1005-bp (bp 213–1218) *Mlu*I fragment from plasmid p51-3.

Chromosomal DNA was isolated from bacterial strains and quantified by spectrophotometry as described (SAMBROOK *et al.* 1989). Plasmid DNA was prepared from Qiagen columns according to the manufacturer's instructions. DNA was digested by restriction endonucleases, and DNA fragments were separated by electrophoresis, transferred to nylon membranes, and bound by UV irradiation using conventional methods (SAMBROOK *et al.* 1989). Two membranes were prepared from the same DNA samples by a bidirectional transfer method. Following electrophoresis and denaturation of the DNA within the agarose gel, nylon membranes were placed on both sides of the gel. Both membranes were covered with Whattman 1MM filter paper, and the gel/membrane assembly was placed between two stacks of paper toweling. In this manner, equal amounts of DNA were transferred to each of the two membranes. One membrane from each membrane-pair was consistently used as a DNA hybridization control. The DNA fragments described above were labeled to high specific activity with ^{32}P-dATP and hybridized to the nylon-bound DNA as described (FEINBERG and VOGELSTEIN 1983).

Genetic methods: Transduction crosses mediated by bacteriophage P22 were performed as described (DAVIS *et al.* 1980) using the high transducing mutant P22 HT *int*-205. Bacteriophage P1 lysates were prepared using the high transducing mutant P1 *vir*, kindly provided by P. HIGGINS. Fresh overnight cultures of *E. coli* W3110 were diluted to 1×10^7 cfu/ml in LB, 5 mM $CaCl_2$, 0.2% glucose and grown for 30 min at 37°. Bacteriophage P1 was added to a final concentration of 1×10^7 pfu/ml, and the cultures were grown for an additional 4 hr, or until the culture cleared. Cell debris was removed by centrifugation and lysates were stored over three drops of chloroform at 4°. For bacteriophage P1-mediated transductional crosses, fresh overnight cultures of *E. coli* W3110 were diluted to 1×10^7 cfu/ml in LB and grown for 2 hr at 37°. Cells were recovered by centrifugation and resuspended in an equal volume of 5 mM $CaCl_2$, 10 mM $MgSO_4$. A volume of 100 μl cells was added to an equal volume of bacteriophage P1 lysate and incubated for 30 min at 30°. The sample was added to 300 μl 1 M sodium citrate and plated on the appropriate medium.

RESULTS

Distribution of cobalamin synthesis among *Salmonella spp.*: The laboratory strain *S. typhimurium* LT2 synthesizes cobalamin *de novo* under anaerobic conditions, but the distribution of this function among other Salmonella species was unclear. A cobalamin bioassay (described in MATERIALS AND METHODS) was employed to ascertain the distribution of cobalamin synthesis among

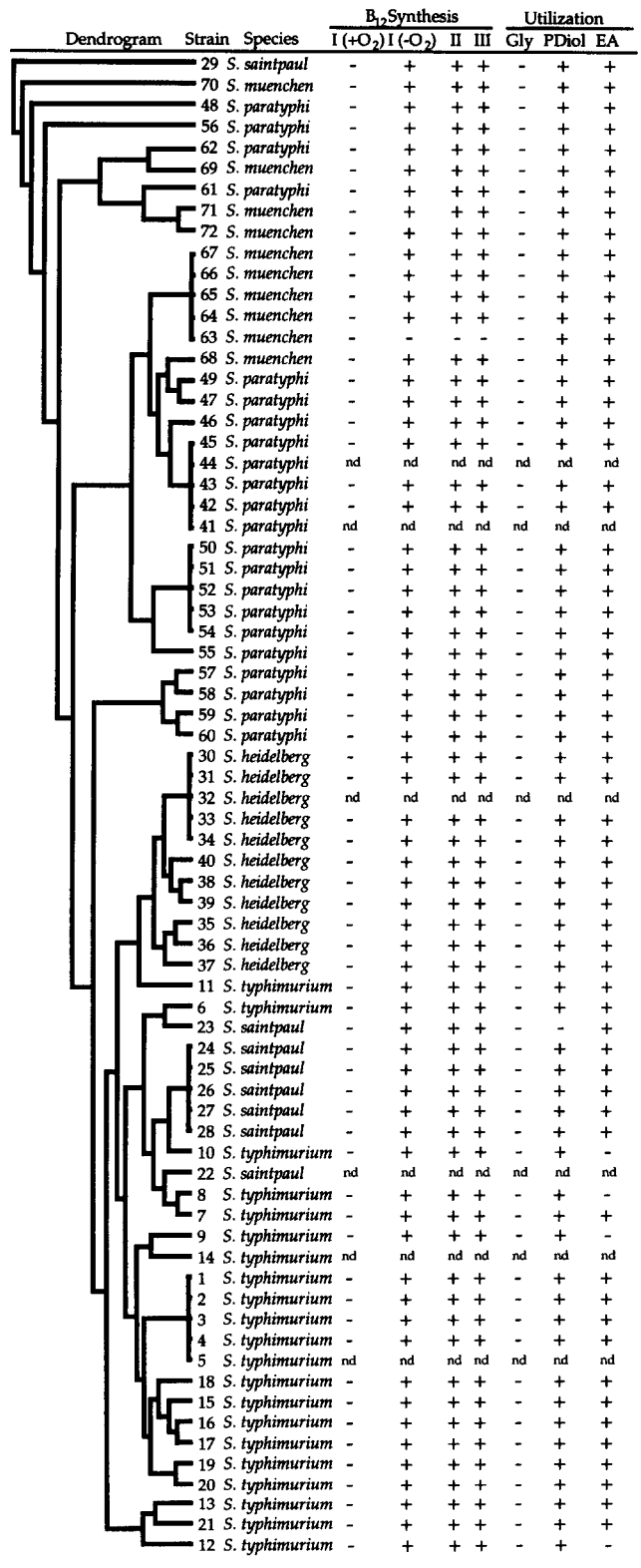

FIGURE 2.—Cobalamin phenotypes among strains of the Salmonella SARA collection (BELTRAN *et al.* 1991). For coenzyme B_{12} synthesis, I, II, and III refer to the CobI, CobII, and CobIII portions of the biosynthetic pathway (see text). CobI function was assayed under aerobic (denoted $+O_2$) and anaerobic (denoted $-O_2$) conditions. The abilities to ferment glycerol (Gly), utilize propanediol (PD), and utilize ethanolamine (EA) in cobalamin-dependent fashions were tested as described in the text. nd, not done.

the Salmonellae. Natural isolates of certain Salmonella subspecies collected by R. K. SELANDER and colleagues (BELTRAN *et al.* 1991; BOYD *et al.* 1993), as well as additional subspecies (see Table 1) were tested for cobalamin production and use (Figures 2 and 3). Nearly all of the Salmonella strains tested were capable of cobalamin synthesis. In all cases, *de novo* cobalamin synthesis occurred only under anaerobic growth conditions. Among the 138 SARA and SARB strains tested, four strains (2.9%) did not synthesize cobalamin. Since these four strains are not phylogenetically related (Figures 2 and 3), these biosynthetic deficiencies are likely to represent four independent losses of cobalamin synthetic capacity.

The cobalamin biosynthetic genes of *S. typhimurium* LT2 are induced by propanediol and high levels of the Crp/cAMP complex via the PocR regulatory protein (ESCALANTE-SEMERENA and ROTH 1987; ANDERSSON and ROTH 1989a,b; BOBIK *et al.* 1992; RONDON and ESCALANTE-SEMERENA 1992; AILION *et al.* 1993). To determine the generality of this regulatory model, cobalamin production was quantitated among even-numbered SARB strains grown on a variety of substrates. All strains produced the greatest amount of cobalamin (3–15 ng/μg wet weight cells) during anaerobic respiration on pyruvate/propanediol/fumarate. A reduced amount of cobalamin was produced during growth on glucose/fumarate (<0.1 ng/μg), pyruvate/fumarate (<0.1 ng/μg), or glucose/fumarate/propanediol (<0.1 ng/μg). These results show that the pattern of regulation seen for *S. typhimurium* LT2, requiring both propanediol and high levels of the Crp/cAMP complex for induction, appears to be generalized among Salmonella species.

Distribution of cobalamin-dependent metabolism among *Salmonella spp.*: Like *S. typhimurium* LT2, numerous natural isolates of Salmonella employed cobalamin for the degradation of propanediol and ethanolamine; no strain was found to degrade either compound in a cobalamin-independent fashion. Among the 138 isolates tested, 11 strains of Salmonella had lost the ability to degrade ethanolamine. Inspection of the phylogeny reveals that these strains are likely to represent at least seven independent losses of this ability. Nine strains have lost the ability to degrade propanediol; these strains are likely to represent at least four losses of this ability. No strain of Salmonella was found to ferment glycerol, that is, grow anaerobically on minimal glycerol medium without an electron acceptor. For these assays, cobalamin was added to the growth medium. These results show that the patterns of cobalamin utilization found in the laboratory strain *S. typhimurium* LT2 can be generalized to include strains representing the diversity of the Salmonellae. Moreover, although the sample size is small, ethanolamine degradation was found to be lost nearly twice as often as propanediol degradation. The loss of neither phenotype was correlated with loss of the other or with loss of cobalamin synthetic capacity.

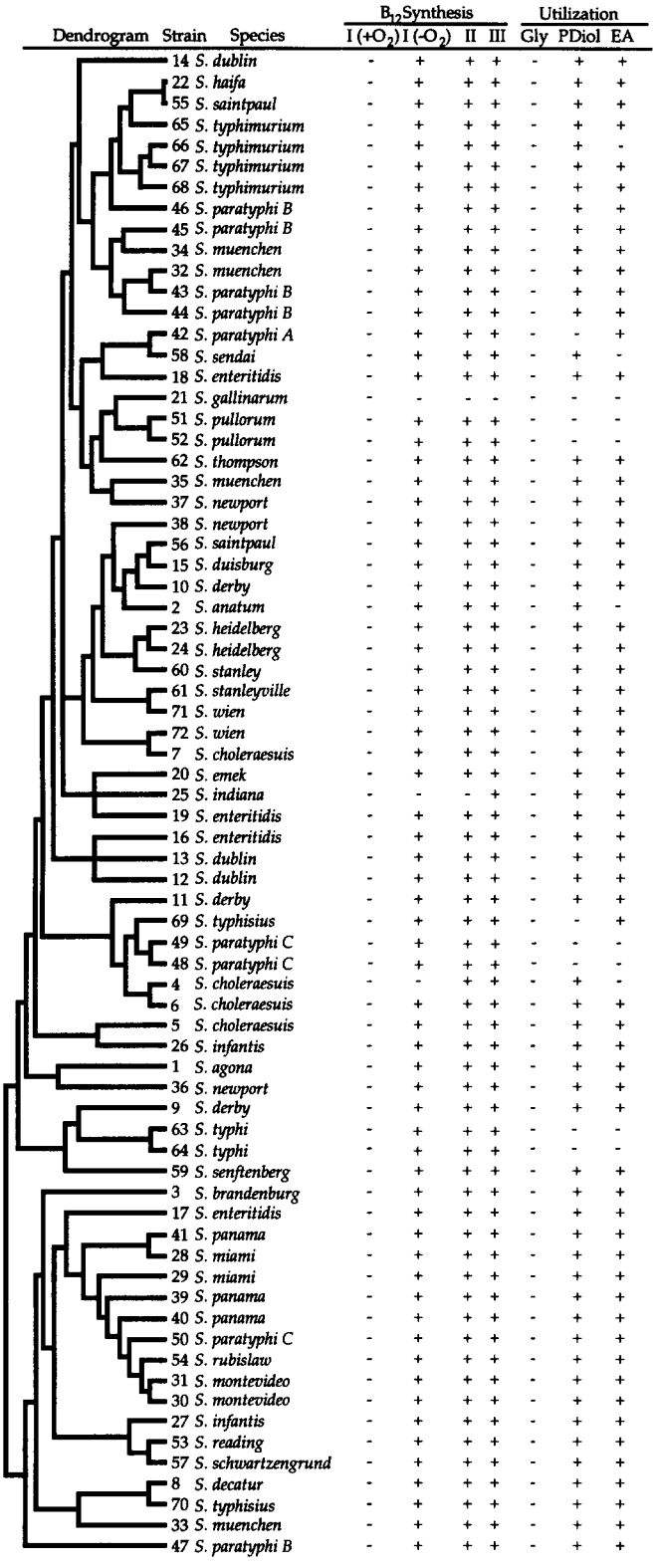

FIGURE 3.—Cobalamin phenotypes among strains of the Salmonella SARB collection (BOYD *et al.* 1993). See the legend for Figure 2 for abbreviations for phenotypic characters.

Distribution of cobalamin synthesis among *E. coli*: In contrast to Salmonella isolates, no strain of *E. coli* could

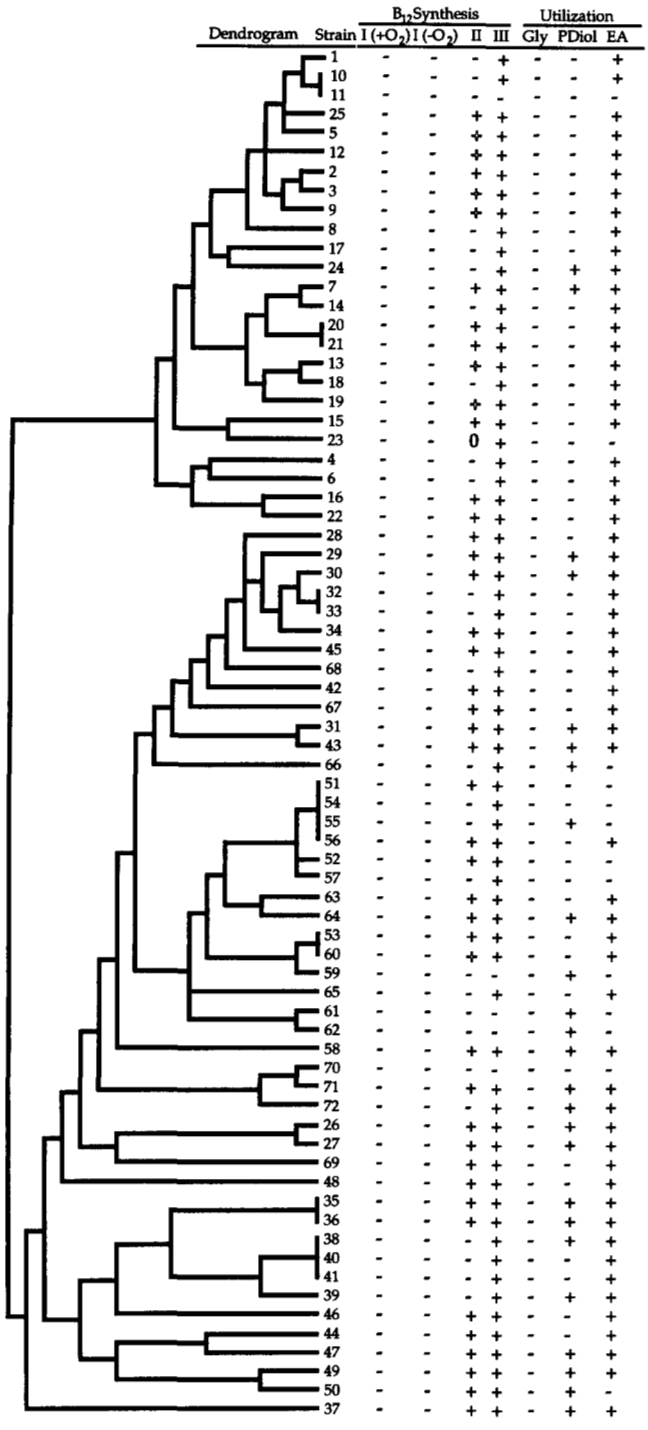

FIGURE 4.—Cobalamin phenotypes among *E. coli* ECOR strains. Strain numbers refer to the ECOR collection (OCHMAN and SELANDER 1984); dendrogram is after SELANDER *et al.* (1987). See the legend for Figure 2 for abbreviations for phenotypic characters. 0, no growth. Propanediol is utilized in a cobalamin-independent fashion among these strains (see text).

synthesize cobalamin *de novo* (Figure 4). Yet most strains retained the capacity to synthesize cobalamin when provided with complex precursors; that is, most retain CobIII gene functions (Figure 4, see also Figure 1). Since all strains of *E. coli* require cobinamide to synthesize cobalamin, all strains are phenotypically CobI$^-$. Unlike the characterized laboratory strains, 38% of natural isolates of *E. coli* require exogenous DMB in addition to cobinamide for cobalamin biosynthesis. Therefore, not all strains bear CobII gene functions. We cannot formally eliminate the possibility that *E. coli* strains harbor CobI and CobII gene functions that remain inactive under test conditions. The 27 strains lacking CobII function do not form a monophyletic group (Figure 4); rather, they are likely to represent $\geq$15 independent losses of this pathway among *E. coli* lineages. Current evidence suggests that the CobII function is provided by a single gene, *cobT* (CHEN *et al.* 1995).

Unlike the regulated coenzyme B_{12} biosynthesis observed in Salmonella species, the levels of cobalamin produced from cobinamide in *E. coli* strains were independent of growth conditions, including carbon source (Crp/cAMP levels) and the presence of propanediol; similar amounts of cobalamin were synthesized in strains grown in minimal glucose medium and in succinate/propanediol medium (~5ng/μg cells). These data suggest that regulation of the few cobalamin synthetic genes of *E. coli* differs from that of the *S. typhimurium cob* operon.

Distribution of cobalamin-dependent metabolism among *E. coli*: When assayed for cobalamin-dependent functions, only ethanolamine degradation was found among tested *E. coli* isolates. No strain of *E. coli* was found to degrade either glycerol or propanediol in a cobalamin-dependent fashion under either aerobic or anaerobic growth conditions. Although most *E. coli* isolates could degrade ethanolamine in a cobalamin-dependent fashion, 13 strains could not. These strains are likely to represent at least eight independent losses of this ability. Among strains unable to degrade ethanolamine, the majority (9/12) had lost CobII function as well, twice as many (75%) as expected (38%). Although the sample size is small, these data suggest that the loss of *eut* function may influence the maintenance of the CobII gene functions among *E. coli* strains. These results show that the patterns of both cobalamin synthesis and use differ substantially between strains of *E. coli* and *Salmonella spp.*

Although some 20% of *E. coli* isolates could degrade propanediol, they did so only anaerobically and in a cobalamin-independent fashion (Table 2). Lactaldehyde can be converted to lactate in *E. coli* by the *ald* gene product. Since propanediol is converted to lactaldehyde anaerobically by the *fucO* gene product, constitutive expression of the *fucO* gene could give the observed phenotype. Such phenotypes have been observed among mutant isolates of laboratory strains (SRIDHARA *et al.* 1969; HACKING *et al.* 1978; OBRADORS *et al.* 1988). In contrast, no Salmonella isolate degraded propanediol in a cobalamin-independent fashion. *S. typhimurium* lacks the *ald* gene and cannot convert lac-

TABLE 2

Phenotypes of selected enteric bacteria on MacConkey-propanediol indicator agar

Species	Strain	Phenotype[a]	Aerobic addition[b]			Anaerobic addition		
			–	Co^{2+}	B_{12}	–	Co^{2+}	B_{12}
E. coli	TR7177	Wild type	W	W	W	W	W	W
E. coli	ECOR 36	$FucO^c$	W	W	W	R	R	R
K. pneumoniae	TR7176	Wild type	W	R	R	W	R	R
S. typhimurium	LT2	Wild Type	W	W	R	W	R	R
S. typhimurium	TT10858	Cob^-	W	W	R	W	W	R
S. typhimurium	TT11855	Cob^-Pdu^-	W	W	W	W	W	W

[a] Relevant phenotype of strain; full genotypes are presented in Table 1. The $FucO^c$ phenotype of *E. coli* strain ECOR 36 was inferred by analogy to the behaviour of *E. coli* K12 mutants having the same phenotype (see text).

[b] Additions to MacConkey-propanediol agar medium were made as described. Colors of colonies were either red (R) or white (W). Red colony color indicated that propanediol was consumed and acid was excreted as a consequence.

taldehyde into lactate (A. LIMÓN and J. AGUILAR, personal communication).

Distribution of cobalamin synthesis among enteric bacteria: Biosynthesis of cobalamin is evident among numerous species of enteric bacteria (Figure 5). *K. pneumonia, K. aerogenes, C. freundii,* and *E. vulneris, E. hermannii, E. blattae* and *Enterobacter cloacae* all synthesize cobalamin *de novo* under anaerobic growth conditions. Unlike *Salmonella spp.*, cobalamin synthesis under aerobic conditions in these taxa was facilitated by the addition of excess cobalt (10 μM). Strains were grown in a variety of different media; growth conditions were varied with respect to the presence of ethanolamine, glucose, glycerol, oxygen, propanediol, pyruvate, and succinate. These growth conditions included those known to induce or repress cobalamin synthesis in *S. typhimurium* (BOBIK *et al.* 1992). Comparable levels of synthesis of coenzyme B_{12} were detected among all non-Salmonella species regardless of growth conditions. In most cases, multiple strains of each species were tested (Table 1); results were congruent among conspecific strains.

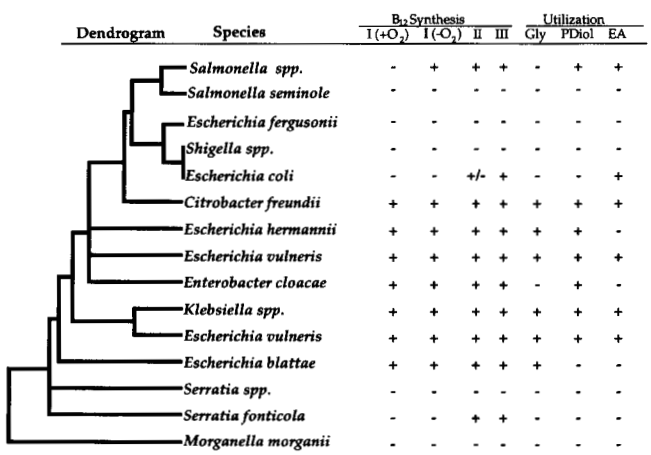

FIGURE 5.—Cobalamin phenotypes among enteric bacteria. Dendrogram is after LAWRENCE *et al.* (1991). The species of Shigella, Salmonella, Klebsiella, and Serratia tested are listed in Table 1 and Figures 2 and 3. See the legend for Figure 2 for abbreviations for phenotypic characters.

However, we cannot formally exclude the possibility that our failure to observe cobalamin synthesis in some species was due to inappropriate growth conditions.

Unlike strains of Salmonella, however, cobalamin production among other enteric species was unaffected by growth medium and oxygen levels. Strains grown on glucose or succinate/propanediol synthesized comparable amounts of cobalamin (3–15 ng per μg cells). Since neither Crp/cAMP levels nor the presence of propanediol affects cobalamin synthesis among non-Salmonella enteric species, these data suggest a mode of regulation of cobalamin synthesis among non-Salmonella enteric bacteria that differs fundamentally from that observed in Salmonella. The only factor strongly influencing the production of cobalamin among non-Salmonella species was the addition of exogenous cobalt. It was inferred that the levels of cobalt in the local water supply typically supplied insufficient cobalt to allow *de novo* cobalamin synthesis under aerobic conditions for non-Salmonella species. However, the cobalt levels in water were typically adequate for *de novo* cobalamin synthesis under anaerobic conditions for all species. From these data, we inferred that the expression of cobalt transport mechanisms may be induced under anaerobic growth conditions.

Species of Serratia, including *S. ficari, S. grimesii, S. liquefaciena, S. marcesens, S. odorifera, S. plymuthica, S. proteamaculans,* were found to lack cobalamin synthetic capacity. One species, *S. fonticola,* showed CobII and CobIII gene functions (Figure 5). These taxa are among the enteric species tested most distantly related to *Salmonella spp.* and are generally considered to be soil-resident bacteria. The relationship between the lack of cobalamin synthesis and the ecology of Serratia species is unclear.

Distribution of cobalamin-dependent metabolism among enteric bacteria: Cobalamin-dependent use of propanediol was widespread among enterics, as was use of ethanolamine. Both functions were absent from strains of *E. blattae; E. cloacae* also failed to utilize ethanolamine in a cobalamin-dependent fashion. Cobala-

TABLE 3

Phenotypes of selected enteric bacteria on defined glycerol medium

Species	Strain	Anaerobic addition[a]					Aerobic[b]
		–	Co^{2+}	B_{12}	Fumarate	Glucose	
E. coli	TR7177	–	–	–	+	+	+
K. pneumoniae	TR7176	–	+	+	+	+	+
S. typhimurium	LT2	–	–	–	+	+	+

[a] Additions to NCE glycerol medium were made as described. +, growth on the supplemented NCE glycerol medium under anaerobic conditions.

[b] +, indicate growth on NCE glycerol medium under aerobic conditions.

min-dependent fermentation of glycerol was found in most species of enteric bacteria tested, including *E. blattae* (see also Table 3). The clade comprising strains of *Salmonella spp., E. coli, Shigella spp.,* and *E. fergusonii* did not ferment glycerol even when provided with coenzyme B_{12}. Therefore, the capacity to ferment glycerol correlated best with a strain's ability to synthesize cobalamin. All strains that synthesized cobalamin *de novo* aerobically and anaerobically also fermented glycerol anaerobically. Species of Serratia, which failed to synthesize cobalamin, did not participate in any of the cobalamin-dependent metabolism tested.

Verification of bioassays: To verify that the bioassay was providing an accurate assessment of a strain's ability to synthesize cobalamin, *metE* mutations were transduced via bacteriophage P22 (among *Salmonella spp.*) or via bacteriophage P1 (among *E. coli* strains) into otherwise wild-type backgrounds. Introduction of these mutations resulted in strains dependent on cobalamin for methionine synthesis; in this manner, the cobalamin synthetic capacity of the strain was tested directly. Twelve strains of Salmonella from the SARB and laboratory collections and 12 strains of *E. coli* from the ECOR collection were tested. In all cases, strains determined to produce cobalamin anaerobically by the bioassay were found to produce cobalamin by the genetic test in that they could grow without exogenous methionine under anaerobic conditions, but not under aerobic conditions (data not shown). Growth under aerobic conditions was restored by the addition of exogenous cobalamin. Those strains shown not to produce cobalamin by the bioassay required exogenous cobalamin or methionine for growth on defined medium under both aerobic and anaerobic conditions. We conclude that the bioassay is an accurate measure of a strain's ability to synthesis cobalamin and is applicable in organisms lacking suitable tools for genetic testing.

Distribution of sequences homologous to the *S. typhimurium cob* operon among enteric bacteria: To assay the distribution of homologues of the *S. typhimurium cob* operon among related bacteria, DNA fragments were isolated from the *pocR* regulatory gene and from various parts of the *cob* operon as described in MATERIALS AND METHODS. Chromosomal DNA was isolated from enteric bacteria, cleaved with restriction endonucleases, size fractionated by agarose gel electrophoresis, and transferred to nylon membranes. The DNA fragments detailed above were employed to detect potential homologues among the enteric bacterial chromosomes.

Figure 6 shows the hybridization of a DNA fragments bearing *S. typhimurium cbiD* and *cbiE* genes, encoding CobI enzymes; the *cobU, cobS,* and *cobT* genes, encoding CobIII and CobII enzymes; and the *cobA* gene. The *cobA* gene is unlinked to the *S. typhimurium cob* operon and is homologous to the *E. coli btuR* gene (Figure 1). The CobA and BtuR proteins adenosylate corrins during both biosynthesis and transport of cobalamin. The *S. typhimurium cobA* gene hybridizes to *S. arizonae, S. seminole, E. coli,* and *K. pneumonia.* For the *cbiDE* and *cobUST* probes strong hybridization is shown by *S. arizonae. S. arizonae* is distantly related to *S. typhimurium* and is placed in a separate genus by some taxonomists. The *cobA, cbiDE* and *cobUST* probes hybridize strongly to all cobalamin-synthesizing Salmonella (data not shown). The *cbiDE* and *cobUST* gene probes do not hybridize to

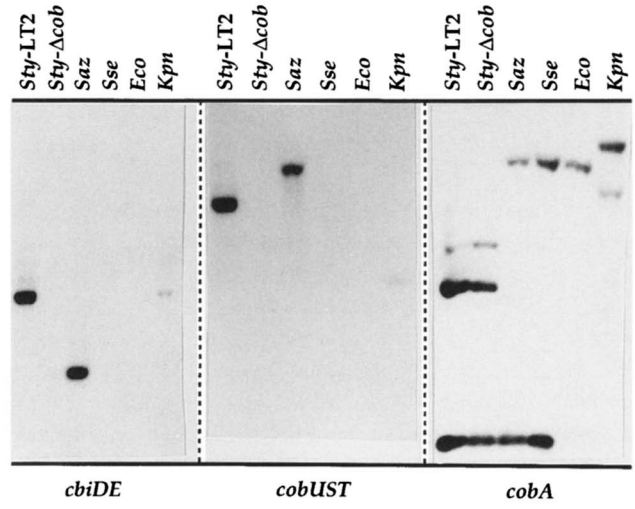

FIGURE 6.—DNA hybridization using probes from the *S. typhimurium cbiD* and *cbiE* genes; *S. typhimurium cobU, cobS,* and *cobT* genes; and *S. typhimurium cobA* gene. Abbreviations are as follows: *Sty*-LT2, *S. typhimurium* LT2; *Sty*-D*cob, S. typhimurium* TT11855, which contains a deletion of the *cob* operon; *Saz, S. arizonae* TR6331; *Sse, S. seminole* TR6332; *Eco, E. coli* TR7177; *Kpn, K. pneumonia* TR7176.

S. seminole, which fails to synthesize cobalamin under laboratory conditions. More importantly, the *cbiDE* and *cobUST* genes do not hybridize with *E. coli* or *K. pneumonia.* Yet both taxa contain *cobUST* gene functions and *K. pneumonia* also shows *cbiDE* gene functions. Similar results were obtained for all DNA fragments isolated from the *cob* operon and the *pocR* regulatory gene; in no case did DNA from the *S. typhimurium cob* operon hybridize with a non-Salmonella strain. Control hybridizations with DNA fragments from the *S. typhimurium cobA, gap, ompA,* and *trp* genes all detected homologues as strongly hybridizing bands among all strains tested (data for the *cobA* gene are shown in Figure 6).

These results indicate that the genes encoding cobalamin synthetic enzymes of *Salmonella spp.* are not closely related to the analogous of other enteric species tested. The DNA hybridization conditions employed detect DNA sequences >70% identical to the DNA sequences. Typical chromosomal loci shared among the enteric bacteria listed in Table 1 are >70% identical (LAWRENCE *et al.* 1991). As expected, hybridizations with the Salmonella *cobA, gap, ompA,* and *trp* loci detected homologues among enteric genomes. Therefore, we conclude that the *cob* operon of *Salmonella spp.* differs significantly at the nucleotide level from the genes encoding cobalamin synthetic genes among other enteric bacteria.

DISCUSSION

Although most species of enteric bacteria synthesize and use cobalamin to some extent, it is clear that species of Salmonella differ from other enterics in several respects. First, Salmonella species synthesize cobalamin *de novo* only under anaerobic conditions. In contrast, all other species of enteric bacteria capable of *de novo* synthesis do so under both anaerobic and aerobic conditions; aerobic synthesis of cobalamin by these organisms invariably required exogenous cobalt (see Table 2). Supplementation with exogenous cobalt did not allow aerobic cobalamin biosynthesis in Salmonella species. Therefore, synthesis of cobalamin in *Salmonella spp.* resembles cobalamin biosynthesis in *Propionibacterium spp.* in that pathways in both organisms function most efficiently under anaerobic growth conditions (MENON and SHEMIN 1967). In contrast, the cobalamin biosynthetic pathway among other enterics resembles the *Pseudomonas denitrificans* pathway in that these pathways function well under aerobic growth conditions (LAGO and DEMAIN 1969). For reviews of cobalamin biosynthesis, see BATTERSBY (1994) and SCOTT (1990, 1993).

In *S. typhimurium* the cobalamin biosynthetic operon, *cob,* and the propanediol utilization operon, *pdu,* are immediately adjacent on the genetic map and are coordinately controlled by regulatory proteins. The PocR protein induces the *cob* and *pdu* operons in the presence of propanediol; both the ArcAB (anaerobic growth conditions) and the Crp/cAMP (growth on poor carbon sources) complexes are required for induction of both operons (BOBIK *et al.* 1992; AILION *et al.* 1993). As described, the cobalamin biosynthetic genes among other Salmonella isolates appear to be regulated similarly. While it is known that the diol and glycerol dehydratases of *Klebsiella spp.* are regulated by Crp/cAMP and propanediol levels (RUCH *et al.* 1974; TORAYA *et al.* 1978; FORAGE and FOSTER 1982), cobalamin synthesis in this and other species of enteric bacteria remains unaffected by propanediol or by CRP/cAMP levels. In addition, the compounds pyrroloquinoline quinone (PQQ) and aspartic acid have been shown to stimulate cobalamin synthesis in Klebsiella (OHSUGI *et al.* 1989); no stimulation of cobalamin synthesis by these compounds was observed in Salmonella (data not shown). This is a second major difference in the manner by which cobalamin synthesis is regulated in Salmonella species as compared with other species of enteric bacteria.

Cobalamin-dependent utilization of ethanolamine, propanediol, and glycerol is widespread among enterics (Figure 5). However, glycerol fermentation is absent from the clade including *E. coli, Shigella spp., E. fergusonii* and *Salmonella spp.* (Figures 2–5). In viewing these phenotypes parsimoniously, one may speculate that the ability to ferment glycerol was lost by the common ancestor of all these taxa.

These phenotypic data strongly suggest that the cobalamin synthetic genes in Salmonella taxa differ substantially from those present in other enteric bacteria. When tested by DNA hybridization, it was found that the *S. typhimurium cbiDE* and *cobUST* genes did not hybridize with the genomes of closely related bacteria except those of other Salmonella species (Figure 6). Under identical conditions, the unlinked *S. typhimurium cobA* gene hybridized strongly with homologues in these same taxa (Figure 6). Yet all of these genes participate in the production of the coenzyme adenosylcobalamin and should be subject to similar selective constraints. From these results, we conclude that the genes of the *cob* operon of *Salmonella spp.* differs genotypically from the analogous genes in other enteric bacteria.

Two possible explanations for these data arise: (1) The *S. typhimurium cob* operon has undergone rapid evolutionary change, resulting in both an altered mode of expression and a significantly altered DNA sequence. (2) The *cob* operon has been introduced into the Salmonella genome from an exogenous source as a single fragment that included the *pdu* operon and the shared regulatory mechanism. The case of rapid evolution seems unlikely for several reasons, aside from the *ad hoc* necessity for locally increased substitution rates.

1. DNA fragments from the *S. typhimurium cob* operon hybridize as strongly with the genomes of sister Salmonella species as do DNA fragments from other chromosomal genes. If the rate of nucleotide substitution were accelerated for the *S. typhimurium cob* operon, one

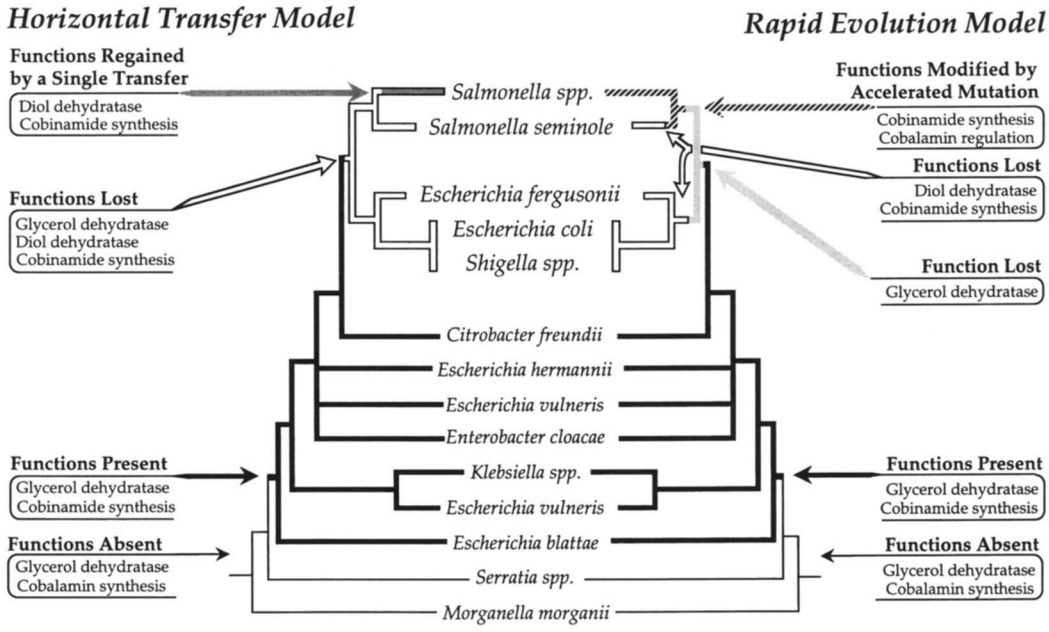

FIGURE 7.—Models for the evolution of cobalamin synthesis and use among enteric bacteria. Solid bars denote species capable of both glycerol fermentation and aerobic cobalamin synthesis. Open bars denote the lack of these phenotypes. Shaded bars denote species with anaerobic cobalamin synthesis and diol dehydratase phenotypes. Striped bars denote species with modified cobinamide synthetic genes.

would have expected divergence and weak hybridization, among at least the most divergent of the Salmonella sublineages.

2. The *S. typhimurium cob* operon bears numerous aberrant properties atypical of other chromosomal genes, including a high %G + C content (~59%) and an unusual codon bias (ROTH *et al.* 1993; LAWRENCE and ROTH 1995). Unusual codon biases are unlikely to represent accelerated rates of nucleotide substitution, but rather are likely to represent the codon bias of a foreign donor genome. The unusual features of the *cob* operon sequences are shared by known genes of the *pdu* operon (T. BOBIK, personal communication).

3. The genes encoding the CobII and CobIII genes of *E. coli* have been cloned, and their nucleotide sequences have been determined (LAWRENCE and ROTH 1995). Comparisons of the DNA sequences of homologous *E. coli* and *S. typhimurium cob* genes show numbers of synonymous substitutions in excess of those expected even if nucleotide substitutions had accumulated without selection (LAWRENCE and ROTH 1995). These data suggest that the divergence of these gene sets predated the divergence of the *E. coli* and *S. typhimurium* genomes.

4. A comparison of the DNA sequences of the *cobU, cobS,* and *cobT* genes of *S. typhimurium* and their homologues in *E. coli* reveals a bias toward higher %G + C in excess of 12% at synonymous sites among the *S. typhimurium cob* genes (LAWRENCE and ROTH 1995). It is unlikely that an accelerated rate of evolution would lead to a direction bias in genic G + C content.

5. The proposed loss of cobalamin synthetic capacity in the *E. coli*/Salmonella ancestor coincides with the loss of the glycerol dehydratase and oxygen-sensitive diol dehydratases present in all other cobalamin-synthesizing enteric species.

Model for the evolution of cobalamin synthesis and use among enteric bacteria: We propose the following model for the evolution of cobalamin synthesis and use among enteric bacteria (Figure 7). Cobalamin synthesis is maintained among most enteric bacteria for use in glycerol dehydratase. The ancestor of *E. coli* and *Salmonella spp.* lost the glycerol dehydratase and diol dehydratase functions and subsequently lost *de novo* cobalamin synthetic capacity. *Salmonella spp.* reacquired the adjacent *pdu* and *cob* operons, which provide cobalamin synthetic capacity and propanediol degradation functions. These genes were received from an organism that synthesized B_{12} only during anaerobic growth. *Salmonella spp.* maintain cobalamin synthetic capacity via selection for the degradation of propanediol. This model is consistent with the coregulation of the *S. typhimurium cob* and *pdu* operons by the PocR protein (BOBIK *et al.* 1992; AILION *et al.* 1993).

While this model is consistent with our data, the differences between the *S. typhimurium cob* operon and the cobalamin synthetic genes of other enteric species may reflect internal evolutionary processes. If so, the genes encoding CobI and diol dehydratase functions may have been lost from the ancestor of *E. coli* and *E. fergusonnii* (Figure 7). The *cob* genes of extant *Salmonella spp.* must have evolved to be functional only under anaerobic conditions, regulated by Crp/cAMP, induced by propanediol, high in %G + C, and twice as divergent

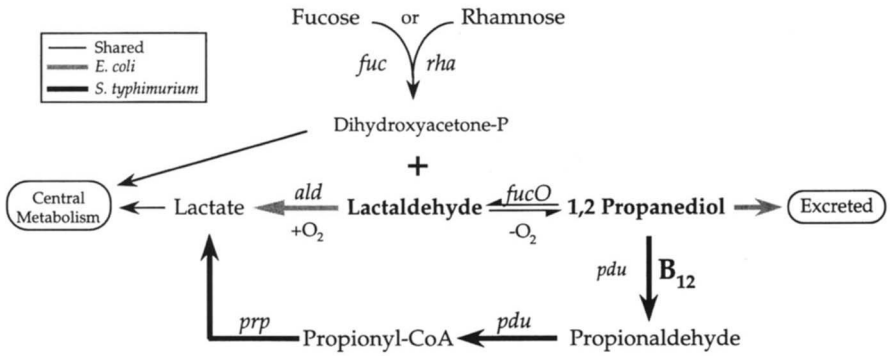

FIGURE 8.—Metabolism of methylpentoses in *E. coli* and *S. typhimurium*. Genes encoding relevant enzymes are noted. The heavy arrows represent metabolism unique to *S. typhimurium*. The shaded arrows represent metabolism unique to *E. coli*.

from their *E. coli* homologues as typical chromosomal genes (LAWRENCE and ROTH 1995). As is the case for all examples of horizontal transfer, we cannot formally exclude this possibility, but we find the necessary assumptions to be highly unlikely.

Intrinsic to these analyses is the use of the phylogeny shown in Figure 7. The conclusions discussed here rely upon the statistical confidence of the *E. coli/E. fergusonii*/Salmonella clade. Overwhelming genetic and molecular data divide *E. coli* and *Salmonella spp.* into two distinct groups. Although the relationships of these species to other enteric bacteria are diagrammed precisely in Figures 5 and 7, the confidence of every node of the dendrogram has not been determined. Rather, the clade comprising *E. coli, E. fergusonii,* and *S. typhimurium* was shown to be a statistically significant monophyletic group (LAWRENCE *et al.* 1991). *K. pneumonia, C. freundii, E. hermannii, E. vulneris, E. blattae, E. cloacae,* and *Serratia spp.* were shown to lie outside this group. The models presented here rely only upon the established statistical confidence of the distinction between the Salmonella and *E. coli* groups and the distinction of their common clade from all other enteric species tested.

Cobalamin synthesis and use is notably absent among enteric taxa distantly related to *Salmonella spp.*, such Serratia. Species of Serratia differ from the other enteric species analyzed here in that they are isolated primarily from soil and grow well at low temperatures (26°). In contrast, the other enteric taxa are found primarily as gut inhabitants of mammals, avians, reptiles, and insects. The significance of these environmental differences and their potential contribution to the maintenance of cobalamin synthesis and use remain unclear. Serratia species may have lost the ability to synthesize cobalamin that was present in the ancestor of all the enteric bacteria discussed here. Alternatively, the ancestor of cobalamin-synthesizing enteric bacteria may have gained this ability after the divergence of the lineage containing Serratia species.

Propanediol metabolism in *Salmonella spp.* and *E. coli*: Consistent with the horizontal transfer model are the substantial differences in the ways Salmonella species and *E. coli* metabolize propanediol. Likely sources of propanediol for enteric bacteria include the methylpentoses rhamnose and fucose, whose degradation can yield the diol as a byproduct (Figure 8) (for a review see LIN 1987). Both sugars are catabolized by *E. coli* (EAGON 1961) and by *S. typhimurium* (OLD and MORTLOCK 1977; AKHY *et al.* 1984) to produce dihydroxyacetone phosphate (DHAP) and lactaldehyde (Figure 8). The DHAP enters central metabolism as a glycolytic intermediate in both taxa, but the fate of the lactaldehyde differs between the two species (Figure 8).

In *E. coli* lactaldehyde can be converted to lactate aerobically by the *ald* gene product (CABALLERO *et al.* 1983). Under anaerobic conditions, *E. coli* converts lactaldehyde into propanediol and excretes it into the medium. Mutant strains of *E. coli* K12 (SRIDAHARA *et al.* 1969; COCKS *et al.* 1974; HACKING *et al.* 1978; OBRADORS *et al.* 1988) and 20% of natural isolates (Figure 4) can metabolize propanediol anaerobically without coenzyme B_{12}. It is deduced that propanediol induces the FucO enzyme, which converts propanediol into lactaldehyde, which, in turn, is metabolized by the Ald enzyme. In this manner, propanediol is metabolized in a cobalamin-independent fashion. This function was observed for several other species of enteric bacteria, including *E. vulneris* and *S. liquefaciena* (data not shown).

Salmonella isolates, in contrast, lack the *ald* gene product (A. LIMÓN and J. AGUILAR, personal communication). As expected, no strain of Salmonella tested in this study was able to utilize propanediol in a cobalamin-independent fashion. It is unclear, however, whether the *ald* gene function seen in *E. coli* has been lost from the Salmonella lineage or was acquired by the *E. coli* lineage. Rather, *Salmonella spp.* employ the cobalamin-dependent enzyme encoded by the *pdu* operon (JETER 1990) to convert propanediol to propionyl-CoA (Figure 8). Propionyl-CoA can be disproportionated by *prp*-encoded enzymes to yield either propanol and ATP, or pyruvate (J. TITTENSOR, personal communication). *E. coli* species lack the *pdu* operon.

We view the Salmonella *pdu* operon and the *E. coli ald* gene as alternative pathways for lactaldehyde dissimilation during the oxidation of methylpentoses. *E. coli* employs the *ald* gene product directly during aerobic growth to convert lactaldehyde to lactate and support growth (Figure 8). During anaerobiosis, propanediol is

created and excreted. Among *Salmonella spp.*, no *ald* gene function exists and all lactaldehyde produced must be converted to propanediol. To use the propanediol, Salmonella employs the cobalamin-dependent propanediol dehydratase in the *pdu* operon (Figure 8). Since *Salmonella spp.* synthesize coenzyme B_{12} only under anaerobic conditions, we presume that it catabolizes propanediol mainly under these conditions. The large genetic investment that Salmonella commits to this function may indicate that propanediol is unusually important in Salmonella biology. A recent method for the discrimination of Salmonellae from other enteric bacteria exploits Salmonella's ability both to synthesize cobalamin and to degrade propanediol (RAMBACH 1990).

Use of dimethylbenzimidazole in *E. coli*: Also of interest is the curious lack of CobII gene function among numerous isolates of *E. coli* (Figure 4). Assuming that the strain ancestral to the ECOR strains bore CobII function, inspection of Figure 4 reveals that these functions have been lost no fewer than 15 times from the set of 72 strains analyzed here. The absence of CobI gene function from $CobIII^+$ *E. coli* isolates is not surprising. This function requires 17 genes in *S. typhimurium* and therefore represents a large target for mutation. The complex precursor of coenzyme B_{12}, cobinamide, is transported well (58% the rate of vitamin B_{12} uptake) in *E. coli* K-12 (BRADBEER *et al.* 1978; KENLEY *et al.* 1978); cobinamide transport was curiously lacking, however, in *E. coli* B (TAYLOR *et al.* 1972). It is reasonable to posit that the uptake of complex corrinoid precursors from the growth environment allows cobalamin synthesis from this complex intermediate and provides selection for the observed maintenance of CobIII functions and cobalamin-dependent ethanolamine degradation. In contrast, the lack of CobII functions in strains of *E. coli* maintaining CobIII functions is enigmatic.

From a naïve standpoint, one may conclude that these strains employ the CobIII pathway to synthesize cobalamin only when both exogenous cobinamide *and* DMB are present. We find it unlikely that these conditions arise with sufficient frequency in natural environments to maintain CobIII gene functions in these strains. Rather, it seems more likely that *E. coli* employs alternative α-ligands to DMB in the synthesis of cobamides or uses exogenous non-DMB bearing cobamides directly.

Strains of *E. coli* with functional CobII and CobIII pathways, *e.g.*, *E. coli* 113-3, will readily synthesize alternate cobamides when provided with cobinamide plus excess exogenous compounds such as benzimidazole, 5-methylbenzimidazole, aminobenzene, and adenine (FORD *et al.* 1955). These compounds result in the formation of cobamides that differ structurally from coenzyme B_{12}. Therefore, *E. coli* is able to synthesis non-DMB-bearing cobamides under laboratory conditions. Moreover, natural isolates of *E. coli* have been shown to harbor numerous nontraditional cobamides whose α-ligands comprise compounds such as 5-hydroxybenzimidazole, 5-methylbenzimidazole, adenine, naphthimidazole, 2-methyladenine, and 2-methylmercaptoadenine (FRIEDRICH 1975). When tested in various bioassays, these compounds provided measurable function as coenzymes (FRIEDRICH 1975). While the functions of these compounds in *E. coli* are unclear; they may serve as coenzymes in the cobamide-dependent methionine synthase MetH (DAVIS and MINGIOLI 1950) or in the ethanolamine ammonia lyase encoded by the *eut* operon (SCARLETT and TURNER 1976), or they may play roles in an as-yet-undiscovered cobalamin-dependent metabolism.

We have provided evidence that the cobalamin synthetic pathway of *S. typhimurium* represents a case of the evolutionary loss of a biological function and the reacquisition of that function by horizontal transfer from a foreign donor. We suggest that this process may frequently affect functions under weak selection. The resulting turnover of genes within genomes may play an important role in bacterial evolution.

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