ABSTRACT

Salmonella enterica serovar Typhimurium synthesizes cobalamin (vitamin B_{12}) only during anaerobiosis. Two percent of the S. Typhimurium genome is devoted to the synthesis and uptake of vitamin B_{12} and to B_{12}-dependent reactions. To understand the requirement for cobalamin synthesis better, we constructed mutants of Salmonella serovars Enteritidis and Pullorum that are double-defective in cobalamin biosynthesis (ΔcobSΔcbiA). We compared the virulence of these mutants to that of their respective wild type strains and found no impairment in their ability to cause disease in chickens. We then assessed B_{12} production in these mutants and their respective wild type strains, as well as in S. Typhimurium ΔcobSΔcbiA, Salmonella Gallinarum ΔcobSΔcbiA, and their respective wild type strains. None of the mutants was able to produce detectable B_{12}. B_{12} was detectable in S. Enteritidis, S. Pullorum and S. Typhimurium wild type strains but not in S. Gallinarum. In conclusion, the production of vitamin B_{12} in vitro differed across the tested Salmonella serotypes and the deletion of the cbiA and cobS genes resulted in different levels of alteration in the host parasite interaction according to Salmonella serotype tested.

Key words: Salmonella, cobalamin, gene deletion, chickens.

INTRODUCTION

Cobalamin (vitamin B_{12}) is a large, evolutionarily conserved cofactor and is one of the most structurally complex biomolecules described (12, 39, 40). Vitamin B_{12} is derived from uroporphyrinogen III (Uro III), which is a common precursor in the synthesis of heme, siroheme, cobnamides, and chlorophylls, the latter playing a functional role functioning in photosynthetic organisms. Salmonella enterica serovar Typhimurium synthesizes cobalamin de novo during anaerobiosis (20), and researchers have speculated that this is carried out by all Salmonella
isolates (24, 32). Nearly 1% of the S. Typhimurium genome is devoted to the synthesis and uptake of cobalamin. An additional 1% is involved in metabolic pathways that require cobalamin (36). Only four vitamin B₁₂-dependent reactions are known to exist in Salmonella Typhimurium. First, B₁₂ is required by one methyltransferase, the product of the gene methH, which catalyzes the methylation of homocysteine to form methionine (31). Second, B₁₂ is required for the cleavage of ethanolamine into acetaldehyde and ammonia, providing both a carbon and a nitrogen source (34, 35). Third, B₁₂ is involved in the formation of the nonessential hyper-modified Q base found in the anti-codon of tRNA^Asp,Asn,His,Tyr (15). Finally, the main use of B₁₂ appears to be as a cofactor for propanediol dehydratase, the first enzyme in the propanediol degradation. This finding is based on evidence showing that the cob operon, which encodes the B₁₂ biosynthetic genes, is induced in response to propanediol and is co-regulated with the pdu operon, which encodes genes that are required for propanediol degradation. This regulatory pattern suggests that propanediol might be a useful carbon and energy source under anaerobic conditions (18, 6). Interestingly, these B₁₂-dependent reactions are not necessary for the laboratory cultivation of Salmonella in either aerobic or anaerobic conditions. This large genetic investment in the production and utilization of vitamin B₁₂ suggests that B₁₂-dependent metabolism might be important during Salmonella infection. Therefore, vitamin B₁₂ biosynthesis might be important under specific conditions, such as during Salmonella growth within its animal hosts. Furthermore, the selective pressure to maintain B₁₂ synthesis might vary with the lifestyle of the organism, as well as in the host-parasite relationship (36).

The genus Salmonella can be divided in two groups based on bacterial pathogenesis and infection biology. The first group includes the majority of the recognized serovars and infects a broad range of host species. Bacteria from this group colonize the gastrointestinal tracts of poultry, resulting in no clinical symptoms in chickens but causing gastroenteritis in humans. The bacteria contaminate the infected animal carcasses during processing, thereby accessing the human food chain and leading to enteric fever, gastroenteritis, bacteremia and systemic infection. This group of bacteria includes the Salmonella enterica serovar Enteritidis in poultry. The second group includes a few serovars that produce systemic typhoid-like diseases in healthy, immunologically mature adult animals and derive from a limited range of host species. This second group includes the Salmonella enterica serovars Pullorum and Gallinarum, which are host-specific for poultry. Bacterial multiplication mainly takes place in the cells of the reticuloendothelial system (10). Although infection is typically introduced via the fecal-oral route, these Salmonella strains do not colonize the digestive tract. Salmonella Typhimurium is one of the leading causes of food poisoning in both humans and mice.

We recently demonstrated that the double mutant S. Gallinarum ΔcobSΔcbiA is avirulent in chickens and that the mortality of chickens infected by S. Typhimurium ΔcobSΔcbiA is reduced to half that induced by the wild type strain (30,41).

In the present work, S. Enteritidis and S. Pullorum ΔcobSΔcbiA mutants were generated. Their virulence was compared to the virulence of their respective wild type strains, and assays to detect cobalamin production in the wild type and mutant strains were performed. The S. Typhimurium ΔcobSΔcbiA and S. Gallinarum ΔcobSΔcbiA strains were included in the latter assays.

**MATERIALS AND METHODS**

**Bacterial strains and culture media**

The designation and source of each bacterial strain used in this study are provided in Table 1. Cultures for transductions and inoculums were grown in 10 mL of Luria Bertani broth (LB) (Invitrogen Nº 12780-052) and incubated for 24 hours at 37°C in a shaking incubator (100 rpm). The aerobic broth cultures contained approximately 5.0x10⁸ CFU/mL. The selection of mutants following transduction was performed on
Luria Bertani agar (Lennox L 22700-025 Invitrogen) supplemented with nalidixic acid (25 µg/mL), kanamycin (30 µg/mL), or spectinomycin (50 µg/mL). Cultures for the detection of the B_{12} that was produced by the S. Enteritidis and S. Typhimurium strains were prepared in 10 mL of M9 glucose minimal medium supplemented with CoC_{2}H_{2}O (1.2 mg/L) and covered with a layer of Vaseline. To detect B_{12} that was produced by the S. Gallinarum and S. Pullorum strains, the M9 minimal medium was supplemented with vitamin-free casaminoacid (2%) and vitamin B_{1}. This medium was then boiled to eliminate any remaining oxygen. Anaerobic cultures were incubated at 37°C for 24 hours.

**Table 1. Salmonella strains used in this study.**

| Strain         | Relevant characteristics                  | Reference or source         |
|----------------|------------------------------------------|------------------------------|
| S. Gallinarum 9Nal' | Isolate from laying hens with Fowl Typhoid | IAH, Compton, UK             |
| S. Gallinarum 9Nal' ΔcobsΔcbiA | Isogenic cobs and cbiA deletion mutant of S. Gallinarum 9Nal' Spec'Kan' | 30                           |
| S. Enteritidis Na'l | Isolate from laying hens                 | IAH, Compton, UK             |
| S. Enteritidis Na'l ΔcobsΔcbiA | Isogenic cobs and cbiA deletion mutant of S. Enteritidis Na'l Spec'Kan' | This study                   |
| S. Pullorum 449/87 Na'l | Isolate from laying hens with Pullorum disease | IAH, Compton, UK             |
| S. Pullorum 449/87 Na'l ΔcobsΔcbiA | Isogenic cobs and cbiA deletion mutant of S. Pullorum 449/87 Na'l Spec'Kan' | This study                   |
| S. Typhimurium F98 Na'l | Isolate from broiler chickens with Paratyphoid infection | IAH, Compton, UK             |
| S. Typhimurium F98 Na'l | Isogenic cobs and cbiA deletion mutant of S. Typhimurium F98 ΔcobsΔcbiA | NA                           |

Na'l nalidixic acid resistant; Spec' spectinomycin resistant; Kan' kanamycin resistant.

**Mutant construction**

Double ΔcobsΔcbiA mutants for S. Enteritidis and S. Pullorum were constructed from single S. Gallinarum ΔcobsSpec' and S. Gallinarum ΔcbiAKan' mutants (30). Gene transference was carried out using the bacteriophage P22 followed by transduction according to standard protocols (37).

**Birds**

Virulence was assessed by oral inoculation of one-day-old Hy-line® commercial layers with 0.1 mL of culture containing 10^8 CFU/mL of double mutant or wild type strains. We used the variety of Hy-line® Isa Brown layers for experiments conducted with Salmonella Pullorum and both Hy-line® varieties W36 and Isa Brown for the Salmonella Enteritidis assays. These birds were chosen because they are susceptible to Pullorum diseases and to Salmonella Enteritidis infection (3, 4, 13).

**Virulence assays**

**Experiment 1. Assessment of mortality:** This experiment was performed only with S. Pullorum because the adopted S. Enteritidis strain does not cause mortality (4). Each group contained 20 birds, and mortality was recorded over a period of 28 days. Data were assessed using the Chi-square test (p < 0.05).

**Experiment 2. Fecal excretion:** This assay was performed only with Salmonella Enteritidis because this serovar extensively colonizes the gastrointestinal tract. The
assay was carried out as previously described with some modifications (1). The challenge was performed as described in Experiment 1. Cloacal swabs were placed in selenite broth (CM0395 and LP0121A; Oxoid) containing 40 mg/mL of novobiocin (SN/Nov) and were directly plated onto Brilliant Green Agar (BGA) (CM0263; Oxoid) containing 100 mg/mL of nalidixic acid and 0.04% novobiocin (BGA Nal/Nov). The cultures were incubated at 37°C for 24 hours. In the absence of growth, the appropriate enriched swab culture was streaked out onto fresh plates of BGA Nal/Nov.

**Experiment 3. Assessment of systemic infection:** The bacteriological analysis was performed as previously described with some modifications (1). At 2, 5, 7, 14, 21, and 28 days post-inoculation (dpi), samples from the spleen, liver, and cecal content were collected and diluted (1:10) in phosphate-buffered saline, pH 7.4 (PBS). The organ samples were macerated using a mortar and pestle, and the cecal content was homogenized. A viable count for the number of mutant and wild type strains in the samples was estimated by plating aliquots of decimal dilutions onto BGA Nal/Nov and then incubating the cultures overnight at 37°C. In the absence of growth on the BGANal/Nov, an equal volume of double-concentrated SN/Nov was added to the first dilution of the samples that was incubated at 37°C overnight and plated on BGANal/Nov. The plates were incubated at 37°C overnight. Data for the viable counts were transformed logarithmically (Log_{10}), and their variance was analyzed (ANOVA). For media comparison Tuckey’s test was used (p < 0.05).

**RESULTS**

**Virulence assays**

**Experiment 1. Mortality rates:** No difference was observed between mortality rates for birds that were challenged with *S. Pullorum* ΔcobSΔcbiA and *S. Pullorum* Nal^r^ (p>0.05; data not showed).

**Experiment 2. Fecal excretion:** Figure 1 shows the number of *S. Enteritidis*-positive cloacal swabs. Intestinal colonization by the *S. Enteritidis* ΔcobSΔcbiA strains was increased in both Hy-line® varieties W36 and Isa Brown layers compared to the *S. Enteritidis* Nal^r^ strain (p>0.05). Similar to the data obtained for fecal excretion, higher counts of *S. Enteritidis* ΔcobSΔcbiA, as
compared to *S. Enteritidis* Nafr, were observed in the cecal contents of both varieties of layers that were analyzed. However, at 5 and 14 dpi, a difference was detected in the brown variety of the layer (p > 0.05) (Figure 2).

Figure 3 shows the results obtained for infection with *S. Pullorum*. Similar counts of *S. Pullorum* ΔcobSΔcbiA and *S. Pullorum* Nafr were observed in the liver, spleen, and cecal contents (p > 0.05).

**Figure 1.** Recovery of *Salmonella* Enteritidis from cloacal samples from experimentally infected one-day-old white commercial layers and brown commercial layers; * and ** statistically significant (χ² p < 0.05).

**Figure 2.** Media of viable number (Log₁₀ CFU/mL) of *Salmonella* Enteritidis ΔcobSΔcbiA and *S. Enteritidis* Nafr in liver, spleen, and cecal contents of birds experimentally infected at one day of age; * and ** statistically significant (Tuckey’s test p < 0.05).
Paiva, J.B. et al. Requirement for cobalamin by Salmonella during infection

Vitamin B₁₂. Detection and quantification: S. Pullorum ΔcobSΔcbiA, S. Enteritidis ΔcobSΔcbiA, and S. Typhimurium ΔcobSΔcbiA strains do not synthesize cobalamin because the double mutation abrogates the cobalamin synthetic pathway. These strains produced uncolored colonies on MacConkey agar due to their inability to degrade propanediol without cobalamin, and their B₁₂ levels were below the inferior limit of detection in the Immulite assay. S. Pullorum Naḷ, S. Enteritidis Naḷ and S. Typhimurium Naḷ were positive for cobalamin production in both the MacConkey agar and the Immulite assays (Table 2). S. Gallinarum ΔcobSΔcbiA and S. Gallinarum Naḷ did not synthesize cobalamin in either of the in vitro tests (Table 2).

Table 2. Production of cobalamin by Salmonella strains.

| Strain               | Cobalamin production a (pg mL⁻¹)b | Phenotype on MacConkey 1,2Pd indicator medium c |
|----------------------|-----------------------------------|-----------------------------------------------|
|                      | Media (pg mL⁻¹)                   |                                                |
|                      |                                    |                                                |
| S. Enteritidis Naḷ  | 362                               | Red                                           |
|                     | 352                               |                                                |
|                     | 418                               |                                                |
| S. Enteritidis ΔcobSΔcbiA | <150     | White                                         |
|                     | <150                              |                                                |
|                     | 262                              |                                                |
| S. Typhimurium Naḷ | 261                               | Red                                           |
|                     | 241                               |                                                |
|                     | 200                               |                                                |
| S. Typhimurium ΔcobSΔcbiA | <150     | White                                         |
|                     | <150                              |                                                |
|                     | 152                              |                                                |
| S. Pullorum Naḷ    | 150                               | Red                                           |
|                     | 154                               |                                                |
|                     | 160                               |                                                |
| S. Pullorum ΔcobSΔcbiA | <150     | White                                         |
|                     | <150                              |                                                |
|                     | <150                              |                                                |
| S. Gallinarum Naḷ | <150                              | White                                         |
|                     | <150                              |                                                |
| S. Gallinarum ΔcobSΔcbiA | <150     | White                                         |
|                     | <150                              |                                                |
|                     | <150                              |                                                |

a An automated immunoassay with quinololuminescent-labeled cobalamin was used to determine the amounts of unlabeled cobalamin present in the samples.
b 150 pg/mL is the inferior limit of detection of the test.
c 1,2-PD = 1,2-propanediol.
d MacConkey 1,2-Pd indicator medium detects acid production from 1,2-Pd. Strains that can degrade 1,2-Pd are red on the medium, and strains that are unable to degrade 1,2-Pd are white.
DISCUSSION

The cobalamin biosynthetic genes have been characterized in Salmonella Typhimurium. Most genes are located in a large 20-gene cluster 17 genes in cbi A-P, and 3 genes cob UST. Within this cluster, genes that encode the enzymes that are required for the three parts of the cobalamin synthesis pathway are grouped together. Mutations in these genes confer three phenotypes: CobI (cbi genes), CobIII (cobU and cobS genes) and CobII ~ (cobT gene) (19, 11). CobIII mutants are unable to synthesize cobalamin, even if all of the precursor substrates are provided. In S. Typhimurium, the CbiA enzyme catalyzes the first step of adenosylcobnamide synthesis, and the CobS enzyme catalyzes the last step of adenosylcobnamide synthesis (14, 27). Thus, Salmonella ΔcobSΔcbiA strains are unable to synthesize cobalamin. This phenotype was verified in our analysis of B12 production using either the MacConkey 1,2-Pd agar or the Immulite assays.

Salmonella Typhimurium synthesizes cobalamin de novo under anaerobic conditions (20). Many researchers have suggested that all isolates of Salmonella possess this ability (36, 32). Using two applied assays, we have demonstrated in the present study that Salmonella Gallinarum does not synthesize cobalamin under in vitro anaerobic conditions. However, S. Gallinarum appears to require this cofactor during infection in chickens. S. Gallinarum ΔcobSΔcbiA caused no mortality, but the wild type strain killed 80% of the chickens (30). Therefore, induction of the cob operon seems to be active only during in vivo conditions, such as during an infection. Alternatively, cob genes are never induced, but their products directly target virulence genes. This hypothesis is supported by genome sequencing data for Salmonella Gallinarum 287/91, demonstrating a loss of function of genes that are related to cobalamin biosynthesis (pocR, cobD, cbiD, cbiC, cbiO,) and propanediol utilization by Salmonella Gallinarum (pdbG and pdbO) as well as, independently, by S. Typhi (42).

The known cobalamin-dependent reactions in S. Typhimurium do not clearly justify this organism’s large genetic investment in cobalamin biosynthesis and transport. The enzyme methionine synthetase is redundant, and the enzyme queosine synthetase is apparently nonessential. Propanediol utilization appears to be the primary use for cobalamin in S. Typhimurium (18). Propanediol is a useful and readily available carbon and energy source in the gastrointestinal tract of birds and mammals; because it is produced during the anaerobic catabolism of two common pentose sugars, rhamnose and fucose, and it is found in glycoconjugates present in the intestinal epithelium (29). In the presence of oxygen, propanediol serves as the sole carbon and energy source, but cobalamin is synthesized de novo under anaerobic growth conditions (18). Tetrationate is able to act as an alternative electrons acceptor, and its availability for S. Typhimurium in the host was demonstrated, recently (46). Tetrationate is product of gut inflammation, trigger by Salmonella, and the luminal sulphur compounds, the ability to use this new electron acceptor produce a growth advantage for S. Typhimurium over the competing luminal microbiota (46). Also, the ability to degrade 1,2-Pd confers a selective advantage in anaerobic niches, such as the intestinal tract of host animals, and within macrophages (26, 29). Evidence suggests that 1,2-Pd plays a role in Salmonella pathogenesis. In vivo expression technology (IVET) indicates that 1,2-Pd degradation is important for S. Typhimurium growth in host tissues, and competitive index studies in mice indicate that pdu but not cob confers a virulence defect in S. Typhimurium (9, 16). The pdu locus is positioned adjacent to the cob operon, and both operons are controlled globally by the same systems in both aerobic and anaerobic conditions. Furthermore, both operons are induced by propanediol (5, 9). Deficiencies in invasion by cob-cbi-pdu cluster-mutated S. Typhimurium have been observed in mice, demonstrating that the cob-cbi-pdu gene cluster increases the intracellular fitness of Salmonella (23). The ΔcobSΔcbiA deletion in S. Typhimurium partially reduces the pathogenicity of the bacteria in broiler chickens (41).

As previously observed for S. Typhimurium that contain
defective genes related to vitamin B₁₂ biosynthesis (38, 5), the ΔcobSΔcbiA S. Enteritidis and ΔcobSΔcbiA S. Pullorum mutants exhibited normal growth under both aerobic and anaerobic conditions. Both S. Enteritidis and S. Pullorum maintained the ability to colonize the avian intestinal tract and cause systemic infection. In addition, fecal excretion and cecal colonization by S. Enteritidis ΔcobSΔcbiA was higher than that induced by the wild type S. Enteritidis strain (p < 0.05). S. Pullorum and S. Gallinarum are avian host-specific pathogens, whereas S. Enteritidis has a broad range of hosts. Although these three serovars belong to the same monophyletic group, they differ in their pathogenicity characteristics (25). The primary importance of cobalamin biosynthesis for these three serovars remains unclear and might be variable. The survival of S. Enteritidis in chickens does not appear to require cobalamin. Although they are responsible for causing distinct diseases, S. Pullorum and S. Gallinarum have been historically considered to be very similar based on their relationship with birds and their phenotypic behavior. They are also easily distinguished from S. Enteritidis (2). However, in terms of vitamin B₁₂ requirement, S. Enteritidis and S. Pullorum appear to be more similar to each other and differ from S. Gallinarum. The identification of the genetic and environmental factors that regulate specific in vivo-induced (ivi) genes expression, as well as the host site(s) in which these genes are expressed, can provide clues about the intracellular environment and possible functions for ivi genes in these specific host tissues. The functions of some of these genes might change depending on the context of the animal, organ, cell type, or sub-cellular compartment that the organism inhabits. In the present study, such clues proved to be useful for understanding S. Gallinarum but not the other Salmonella serotypes (16). Signals present specifically in pig tonsils induce the cob operon of S. Typhimurium, either as a requirement for survival in the tonsil or as a prelude to migration into the intestinal tract (17).

Several comparative studies between S. Gallinarum and other Salmonella serotypes have demonstrated differences in the cellular mechanisms that might be responsible for the specificity and adaptability of S. Gallinarum to the avian host (43, 45, 22, 44, 8). For example, glycogen has a complex role in survival and, therefore, in prolonging the infectivity of broad-host-range Salmonella outside of the host. It also plays a minor role in Salmonella virulence and colonization (28). S. Gallinarum and S. Pullorum do not accumulate glycogen under test conditions (21). The loss of glycogen synthesis is an example of pseudogene accumulation by host-restricted serovars and is indicative of functions that are no longer required due to niche specialization. Host adaptation is often associated with extensive gene deletion (7) and complex nutritional requirements, as observed in other host-adapted serovars, such as S. Typhi.

Salmonella enterica is a bacterial species that includes examples of both promiscuous and host-adapted pathotypes. There is a consensus among researchers that the selection pressure to maintain B₁₂ synthesis varies with the lifestyle of the organism. Escherichia coli does not maintain a complete cob operon and seems to fill a niche that does not require complete de novo cobalamin synthesis. Conversely, the ability of S. Enteritidis and S. Typhimurium to synthesize B₁₂ must be strongly selected for as these organisms maintain the full operon. If we identify the precise moment at which the cob operon is induced (if at all) in S. Enteritidis, S. Typhimurium, S. Gallinarum, and S. Pullorum, we might develop a better understanding of its importance for colonization, multiplication, survival in macrophages, and immune evasion.

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