

A DEFECTIVE MUTANT OF *Salmonella enterica* Serovar Gallinarum IN COBALAMIN BIOSYNTHESIS IS AVIRULENT IN CHICKENS

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ABSTRACT

Salmonella enterica serovar Gallinarum (SG) is a fowl typhoid agent in chickens and is a severe disease with worldwide economic impact as its mortality may reach up to 80%. It is one of a small group of serovars that typically produces typhoid-like infections in a narrow range of host species and which therefore represents a good model for human typhoid. The survival mechanisms are not considered to be virulent mechanisms but are essential for the life of the bacterium. Mutants of *Salmonella* Gallinarum containing defective genes, related to cobalamin biosynthesis and which *Salmonella* spp. has to be produced to survive when it is in an anaerobic environment, were produced in this study. *Salmonella* Gallinarum is an intracellular parasite. Therefore, this study could provide information about whether vitamin B₁₂ biosynthesis might be essential to its survival in the host. The results showed that the singular deletion in *cbiA* or *cobS* genes did not interfere in the life of *Salmonella* Gallinarum in the host, perhaps because single deletion is not enough to impede vitamin B₁₂ biosynthesis. It was noticed that diluted SG mutants with single deletion produced higher mortality than the wild strain of SG. When double mutation was carried out, the *Salmonella* Gallinarum mutant was unable to provoke mortality in susceptible chickens. This work showed that B₁₂ biosynthesis is a very important step in the metabolism of *Salmonella* Gallinarum during the infection of the chickens. Further research on bacterium physiology should be carried out to elucidate the events described in this research and to assess the mutant as a vaccine strain.

Key words: *Salmonella* Gallinarum, cobalamin biosynthesis, attenuated strain, *cbiA* and *cobS* genes

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INTRODUCTION

Salmonella enterica serovar Gallinarum (SG) is the fowl typhoid agent in chickens, a very severe disease with worldwide economic impact and with which mortality may reach up to 80% (5, 9). It is one of a small group of serovars that typically produces typhoid-like infections in a narrow range of host species and which therefore represents a good model for human typhoid. The disease is most severe where environmental conditions and management systems do not allow hygienic control measures to be introduced (36). Although a live vaccine (SG9R strain) is commercially available and has been used extensively, it harbor undefined attenuations and retains a degree of virulence (18, 19, 28, 48). Despite of additional strains having been described of having more defined mutations (5, 7, 8, 22, 56) none of these combine the high level of protection of the 9R strain with its ability to stimulate a specific circulating antibody (55). There thus remains considerable scope for further exploration of the virulence basis of this pathogen.

To survive intracellularly, SG uses anaerobic respiration to obtain its energy (20). As part of its ability to adapt to different growth conditions, SG may alter the composition of its respiratory system (23, 39). Different components can be substituted in the membrane in place of or in addition to other components as they are needed and can become fully functional parts of the entire system (32, 33). There are three types of respiratory components. (i) Substrate-specific dehydrogenases, which carry out the oxidation of organic substrates and feed electrons into the mobile quinone pool; (ii) quinones, which deliver reducing equivalents to the terminal oxidoreductases; and (iii) terminal oxidoreductases, which prevent the terminal electron acceptors from being used (17). The amount of each component is strictly regulated to optimize the respiratory chain according to the

substrates present and the physiological needs of the cell (34). Bacteria can use substrates other than NADH, such as succinate, pyruvate, or lactate as primary electron donors and a number of alternative electron acceptors, such as dimethyl sulfoxide, fumarate, and nitrate, in addition to oxygen (17). Despite of the extensive understanding of bacterial electron transport and proton translocation, little is known about the relative contribution of the relevant proton-translocation enzymes to the growth and survival of pathogens in the host in either the gut or the intracellular environment.

Virtually all *Salmonella* isolates synthesize vitamin B₁₂ (cobalamin or CBL) *de novo* under anaerobic conditions alone. The synthesis can take place under aerobiosis only if the bacterium is provided by precursor substances such as cobinamide. (13, 24, 27). The ability to synthesize and import B₁₂ requires more than 35 known genes (42). This means 1% of the microorganism genome (46), in addition to an extra 1% related to metabolic activity involving cobalamin. This large genetic investment in vitamin B₁₂ suggests that a B₁₂-dependent metabolism is important to natural populations of *Salmonella* (53). Mutations that eliminate B₁₂ synthesis from otherwise wild-type strains cause no growth defect under the standard aerobic or anaerobic lab conditions used so far. The CBL biosynthetic operon of *Salmonella* Typhimurium is made up of 20 putative genes. These genes are organized in a single operon and can be separated into two groups: (i) genes involved in corrin ring synthesis, designated *cbi* (the operon contains 17 *cbi* genes, *cbiA* being the first) and (ii) genes involved in nucleotide loop assembly, designated *cob* (the operon contains three genes: *cobU*, *cobS* and *cobT*) (37).

The biologically active forms of vitamin B₁₂ are required in methylation, reduction and intramolecular rearrangements reactions (13, 47, 54). A low redox potential generated during the anaerobic growth, increases expression of the *cob*

operon 4 to 100 fold (2, 3, 14).

Cobalamin is used as a cofactor in four known reactions in STM: (i) it is required by one methyltransferase (methH gene product) that catalyzes the last step in the methionine B₁₂ dependent biosynthesis (43); (ii) it is required for the degradation of ethanolamine to acetaldehyde and ammonia providing both a carbon and a nitrogen source (12, 42); (iii) B₁₂ is involved in the formation of the nonessential hypermodified Q base found in position 34 in the anticodon of RNAt^{Asp,Asn,His,Tyr} (16, 35).

The main use of B₁₂ in *Salmonella* appears to be a cofactor for propanediol dehydratase, the first enzyme in propanediol degradation. This conclusion is based on the fact that the *cob* operon, is induced by propanediol and is co-regulated with the *pdu* operon, which are encoding genes for propanediol degradation (11, 41). This control pattern suggests that perhaps propanediol could be used as a useful carbon and energy source which is frequently valuable and available under anaerobic conditions, because it is made as a by-product during degradation of common plant sugars (29, 53). Mutants blocked in B₁₂ synthesis show harmed anaerobic growth in carbon poor sources (38). The propanediol supports anaerobic growth when tetrathionate (not another acceptor) is used as an alternative electron acceptor (38, 53).

CbiA (*Cobyrinic acid a,c-diamide sintetase*) is the first glutamina aminotransferase in the anaerobic pathway of vitamin B₁₂ and catalyzes reactions in many sites of the substrate. CbiA is an indirect response by *cob* operon repression (40). CobS enzyme (*Adenosylcobalamin-5'-P synthase*) is bound to the cellular membrane and catalyzes the second to last step of the Ado-CBL production (30). The cobalamin biosynthesis is completely dependent on this enzyme (1, 31). The deletion of *cobS* and *cbiA* genes prevent any form of cobalamin production (Adenosyl-, Cyano-,

Methyl-, Hidroxi-B₁₂) under anaerobic conditions by *Salmonella* mutants (40). However, the exact relation is unknown.

Studies to understand the pathogenicity of *Salmonella* look for virulence genes. Although, the survival mechanisms are not considered to be virulent mechanisms, they are essential for the life of the bacterium. In this study, mutants of SG containing defective genes related to cobalamin biosynthesis were produced. Since SG is an intracellular parasite, the study might provide information about whether vitamin B₁₂ biosynthesis could be essential to its survival in the host.

MATERIAL AND METHODS

Bacterial strain and culture media: *Salmonella enterica* serovar Gallinarum strain 9 produces typical systemic fowl typhoid with high mortality (36, 49). For ease of enumeration, spontaneous nalidixic acid-resistant (25µg/mL) mutant derivatives of this strain were used. The mutants were constructed from *Salmonella* Typhimurium F98 Na^r strain. The DNA homology was such as to permit using the same inactivated serovar Typhimurium sequences in serovar Gallinarum. The construction of the mutants, conjugation and transduction followed Sambrook & Russel (44) and Turner *et al.* (51).

Broth cultures consisted of 10mL volumes of LB broth (Invitrogen N° 12780-052) incubated for 24h/37°C in a shaking incubator (100 rpm). The broth culture contained approximately 1x10⁸ (*S. Gallinarum*) and 1.2 x10⁹ (*S. Typhimurium*) CFU/mL.

Mutant construction: Mutants were constructed in cobalamin biosynthesis deficiency. In brief, four primers were designed for each gene to be mutated, so that two

fragments close to the 5' and 3' ends of the gene could be amplified leaving a central deletion in the amplified gene and incorporating *KpnI* (Gibco 15232) or *BamHI* (Gibco 15201) sites facilitating the insertion of a kanamycin (*cbiA* gene) or spectinomycin (*cobS* gene) cassette. Initial cloning was carried out in pGEM T Easy vector (Promega L2001) and then in the pJCB12 suicide vector in *E.coli* S.17.1λ pir, which was used for conjugation into SG 9 Na^r. Mutants were selected based on their resistance to kanamycin or on streptomycin and ampicillin sensitivity and were checked for *Salmonella* O-antigens by slide agglutination with antisera and for smoothness by absence of agglutination with acriflavin (0.001%). The integrity of the constructs was checked by PCR using the same primers (Figure 1).

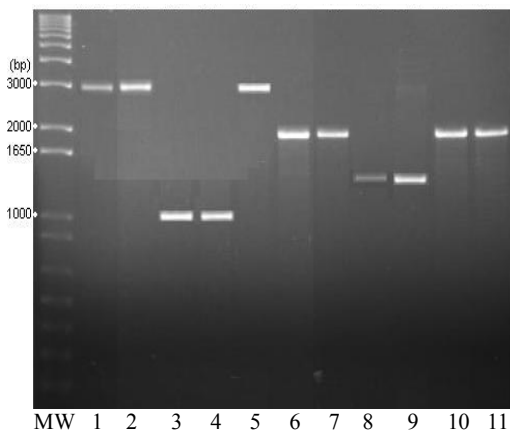


Figure 1: Agarose gel electrophoresis of the PCR products. DNA from isolated bacteria colonies. MW: molecular weight (1Kb plus DNA Ladder). Lanes 1-2: $\Delta cobS$ Spec^r fragment from SG9Na^r $\Delta cobS \Delta cbiA$ (2964bp). Lanes 3-4: *cobS* fragment (wild) from SG9Na^r (994bp). Lane 5: Positive control with *Salmonella* Typhimurium F98 $\Delta cobS$ Spec^r (2964bp) fragment. Lanes 6-7: $\Delta cbiA$ Kan^r fragment from SG9Na^r $\Delta cobS \Delta cbiA$ (1930bp). Lanes 8-9: *cbiA* fragment from wild SG9Na^r (1360bp). Lanes 10-11: Positive control with *Salmonella* Typhimurium F98 $\Delta cbiA$ Kan^r (1930pb).

Primers designed:

cobS:

Primer 1: gagatctagaacgaatctgctgtttgcgct

Primer 2: cagcagggtacctagcggataccacaccag

Primer 3: ccgctaggtaccctgctgaccgggtgtttca

Primer 4: tctagaacagaccagcagaaagatc

cbiA:

Primer 1: catctagaaggcatcacgcatttattc

Primer 2: ccgtaaggtaccaatggcattttgaggagct

Primer 3: gccattggtaccatacggatgttaaaacat

Primer 4: tgtctagacagccagtgtgcaacattt

Transduction: Mutants of SG with double deletions were obtained by transduction using bacteriophage P22 following standard protocols (4, 44). Transductants were plated on LB agar (Invitrogen N° 22700-025) containing spectinomycin or kanamycin and after incubation at 37C/24h were again checked by PCR.

Virulence assays

Experiment 1. Assessment of mortality: Virulence was assessed by oral inoculation of groups of five-day-old brown commercial layers with 0.5 mL of either an undiluted culture containing 10⁸ CFU/mL or diluted at 10⁻² to contain 10⁶ CFU/mL of mutant and wild cultures of *Salmonella* Gallinarum. This variety of commercial brown chickens was chosen because it is susceptible to clinical fowl typhoid (9, 15). Mortality was recorded over a period of 28 days. Data were assessed by Qui-square test (p<0.05) (21).

Experiment 2. Assessment of systemic infection: The systemic infection was assessed by oral inoculation of groups of five-day-old brown commercial layers with 0.5 mL of the mutant or wild culture containing 10⁸ CFU/mL. The bacteriological analysis was carried out as described by

Barrow & Lovell (6) with some modification. At 2, 5, 7, 14, 21 and 28 days post-inoculation (dpi), samples from the spleen and liver were collected and diluted (1:10) in phosphate-buffered saline, pH 7.4 (PBS). The organ samples were macerated in a pestle and mortar. The viable count of SGNaI^f $\Delta cobS\Delta cbiA$ and SGNaI^f in the samples were estimated by plating aliquots of decimal dilutions on BGA NaI/Nov incubated overnight at 37°C. The first dilution of the sample was added to an equal volume of double-concentrated SN. This was incubated at 37°C overnight and plated on BGA NaI/Nov agar plate when there was no growth from the viable count. Data of viable counts were transformed logarithmically (\log_{10}) and subjected to analysis of variance. Media were compared statistically by Tukey test ($p < 0.05$).

RESULTS

In experiment 1, the mortality caused by SG $\Delta cobS$ or/and $\Delta cbiA$ genes was assessed (Table 1). In groups where the birds were challenged with neat culture of the strains containing single mutation, the mortality was similar to group of birds receiving the wild strain of SGNaI^f, and there was no mortality in birds inoculated with SG NaI^f $\Delta cobS\Delta cbiA$ ($p < 0.05$). At lower dose, however, SG $\Delta cobS$ and SG $\Delta cbiA$ provoked mortality higher than SGNaI^f ($p < 0.05$).

Figure 2 shows the results on the presence of SG NaI^f $\Delta cobS\Delta cbiA$ in the liver and spleen of birds over a period of 28 days post-infection (dpi) in comparison with the infection of birds by the wild SG strain. Five birds in each group were inspected at 2, 5, 7, 14, 21 and 28 dpi. There was always a smaller quantity of the SG NaI^f $\Delta cobS\Delta cbiA$ strain in the liver and spleen than there was of the SGNaI^f strain ($p < 0.05$). Mortality by SGNaI^f strain started around seven dpi and all of remaining birds died before 14dpi whereas no mortality was seen in group of birds infected with SG NaI^f $\Delta cobS\Delta cbiA$.

DISCUSSION AND CONCLUSION

Fowl typhoid is a systemic disease in chickens caused by *Salmonella Gallinarum*, an intracellular anaerobic facultative parasite. It is a very severe disease and during its course, the bacterium persists intracellularly. The virulence factors are responsible for the development and evolution of the illness (52). The bacterium also uses other resources to survive intracellularly. To breathe anaerobically, *Salmonella* produces enzymatic complexes according to the available substrate in the host cell. Sometimes, under certain circumstances, enzymatic complexes could inhibit each other's action.

In the present work, mutants of SG containing defective genes related to cobalamin biosynthesis were prepared. These mutants were inoculated orally in 5-day-old chickens susceptible to clinical fowl typhoid. For SGNaI^f $\Delta cobS$ and SGNaI^f $\Delta cbiA$, the lower dose provoked higher mortality in comparison to the wild strain ($p < 0.05$), while there was no mortality in groups of birds inoculated with the strain with double mutation (SGNaI^f $\Delta cbiA\Delta cobS$). These results were similar to findings of Sampson & Gotschlich (45) that discussed the possibility of the *Salmonella Typhimurium cob* mutant strain being more virulent than the wild strain in mice. Sampson & Gotschlich (45) suggested that the inability to produce vitamin B₁₂ might be the factor, which is responsible for increasing its virulence. However, the strain of SG containing double defective genes, *cbiA* and *cobS*, was completely attenuated, provoking no mortality. There is also the fact that the bird's life span is shorter than that of the wild strain of SG. There is no explanation why the single mutation in *cbiA* and *cobS* genes might increase the virulence and why the double mutant loses it. The explanation given by Sampson & Gotschlich (45) cannot be accepted since works carried out later demonstrated that the wild STM strain used

Table 1. Mortality of 5 day-old brown commercial layers due to experimental infection with SG strains with defective genes related to pathway of cobalamin biosynthesis ($\Delta cobS$ and/or $\Delta cbiA$).

Challenge strains	Dose*	Cumulative mortality in days-post-infection (dpi)																												Total	
		5	6	7	8	9	10	11	12	13	15	17	19	20	21	22	23	24	25	26	27	28	29	30	N°/total	%					
SG $\Delta cobS$	N																											0/30a	0		
$\Delta cbiA$	10 ⁻²																											0/30a	0		
SG $\Delta cbiA$	N	10	25	26	28	29					30																30/30b	100.00			
	10 ⁻²	9	14	18	19	21	23	24	25	26	27	28															28/30b	93.33			
SG $\Delta cobS$	N	6	18	20	23	24	25	26	29																		29/30b	96.67			
	10 ⁻²	1	6	9	10	10	11	12	14	15	16	19	22	24	25	25	27	29										29/30a	96.97		
SG NaI ^f	N	9	21	25	26																						26/27b	96.30			
	10 ⁻²	5	6	9	10	11	12																				20/31b	64.52			

N= neat culture containing 10⁸ CFU/mL and diluted at 10⁻² (10⁶ CFU/mL). Number of dead birds followed by different letters in the same column regarding the dose of inoculum indicating significant difference (χ^2 , p<0.05).

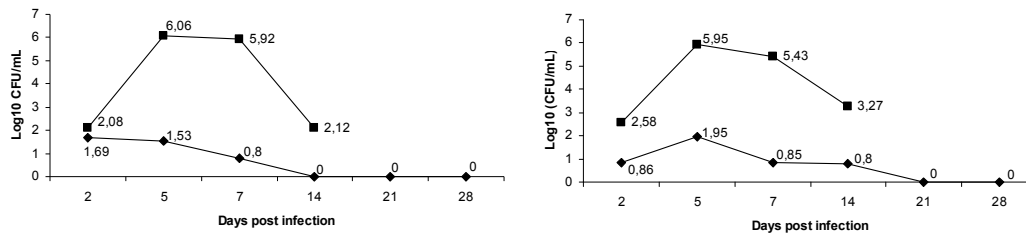


Figure 2. Media of the viable number (Log₁₀ CFU/mL) of *Salmonella Gallinarum* NaI^r Δ*cobS* Δ*cbiA* (◆) and *S. Gallinarum* NaI^r (■) in the liver and spleen of five brown commercial layers experimentally infected at 5 days old. In group infected with *S. Gallinarum* NaI^r the remaining birds died before 21 dpi.

for comparison with *cobS* mutant was naturally attenuated (10).

According to Jeter *et al.* (24), *Salmonella* Typhimurium may be unable to synthesize cobalamin for three reasons: (i) - the phenotypically defective strain in branch I (*cob*) synthesizes cobalamin only when cobinamide is supplied; (ii) - the defective strain in branch II (*cob*) synthesizes cobalamin only when dimethylbenzimidazole (DMB) is supplied; and (iii) - the defective strain in branch III (*cob*) fails to make cobalamin even when provided with both precursors (cobinamide or DMB). Therefore, the SGNal^r Δ*cobS* strain which has altered branch III, and SGNal^r Δ*cbiA* which has altered branch I should be incapable of synthesizing cobalamin (vitamin B₁₂). However, in this study, single mutation exacerbated the virulence of SG, and only when the bacterium was defective in two genes did the strain become attenuated. Hence, it is not possible to understand the role that cobalamin plays in the virulence of the bacterium. Perhaps, when the mutant has a single alteration, additional pathway takes place in the bacterium while the double alteration cannot be overcome. According to Roth *et al.* (43), the *cbiA* gene is located in part I of the *cob* operon and it is the first place to start the transcription for the biosynthesis of cobalamin in *S. Typhimurium*, which codifies an amidase

that acts later in the biosynthesis via. Thus, the double mutant has defective genes that act in branch I (*cbiA*) and branch III (*cobS*).

It is possible that vitamin B₁₂ within the bacterium is capable of regulating a gene or a group of genes which directly or indirectly control any virulence factor. Another possibility is that somehow it was sensed by the host. This detection might conceivably cause an altered response by the host resulting in less virulent infection.

Huang *et al.* (23) showed that *cob* operon expression is induced during the infection of pigs by STM, where according to them possible environmental signals exist specifically in the lymphoid tissues that induce the *cob* operon, either as a life requirement in this tissue itself or as a prelude to passage into the intestinal tract. Previous work has shown that the invasion of epithelial cells by *Salmonella* is co-regulated with propenodiol and ethanolamine catabolism including *cob* expression (25, 26). It is possible that environmental cues encountered by *Salmonella* are regulated by both metabolic and virulent functions in the host.

The results obtained showed that the singular deletion in *cbiA* or *cobS* genes did not interfere in the life of SG in the host, perhaps because single deletion is not enough to impede

vitamin B₁₂ biosynthesis. It was noticed that diluted SG mutants with single deletion produced higher mortality than the wild strain of SG did. When double mutation was carried out, the mutant of SG was unable to provoke mortality in susceptible chickens. Further work towards bacterium physiology should be carried out to elucidate the events described in this research.

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RESUMO

Mutante de *Salmonella enterica* serovar Gallinarum duplo defectivo na biossíntese de cobalamina é avirulento para aves.

Salmonella enterica serovar Gallinarum (SG) é o agente do tifo aviário, doença severa que provoca mortalidade em até 80% do plantel de aves. SG encontra-se entre os poucos sorotipos de *Salmonella* que são agentes etiológicos de enfermidade específica, à semelhança de *Salmonella* Typhi em seres humanos podendo, portanto, servir de modelo experimental para outras salmoneloses hospedeiro específicas. Além dos mecanismos de virulência, a bactéria utiliza mecanismos de sobrevivência para permanecer no hospedeiro. A ativação desses mecanismos pode ou não estar associada à ativação dos mecanismos de virulência. Entre os mecanismos fisiológicos, está a produção de vitamina B₁₂ que *Salmonella* spp. realiza em ambientes anaeróbicos, como quando encontra-se intracelularmente no organismo

hospedeiro. Neste estudo, analisou-se a infecção de aves por cepas de SG, que tiveram genes alterados que participam da biossíntese de vitamina B₁₂. Foram produzidos mutantes de SG contendo os genes *cbiA* e *cobS* alterados e um terceiro, contendo ambos os genes alterados. A sobrevivência e a ação patogênica de SG não foi modificada pela alteração simples de um dos genes, mas tornou a cepa de SG completamente atenuada quando os dois foram modificados. A mortalidade provocada pela cepa selvagem de SG foi de 64,52%, enquanto que não observou-se mortalidade alguma no grupo de aves infectadas com SG Δ *cbiA* Δ *cobS*. Estudos futuros deverão ser realizados para elucidar este processo fisiológico bacteriano e para avaliar a utilização desta cepa de SG como cepa vacinal.

Palavras-chaves: *Salmonella* Gallinarum, cobalamina, mutantes de *Salmonella*, atenuação de genes, *cbiA*, *cobS*.

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