



Participation of genes involved in the process of anaerobic respiration of infection in chickens by *Salmonella* Typhimurium.

Yuli M. S. Arguello, Jacqueline B. de Paiva, Rafael A. C. Penha Filho, Ângelo Berchieri Junior

Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, FCAV/Unesp
Via de acesso Paulo Donato Castellane, s/n
14.884-900. Jaboticabal, SP, Brazil

Corresponding author: Yuli M. S. Arguello. E-mail. yuli_melisasierra@yahoo.com

Submitted September 9th 2009, Accepted November 20th 2009

Abstract

Intestinal pathogens are exposed to various stress conditions during their infectious cycle. Anaerobiosis, one of such hostile condition, is offered by the host within gut and intestinal lumen, where survival, multiplication and entry into intestinal epithelial cells are priority for the invasion of the pathogen. The fumarate reductase (*frdABCD*), dimethyl sulfoxide (DMSO)-trimethylamine *N*-oxide (TMAO) reductase (*dmsABC*), and nitrate reductase (*narGHIJ*) operons in *Salmonella* Typhimurium (STM) encode enzymes involved in anaerobic respiration to the electron acceptors fumarate, DMSO, TMAO, and nitrate, respectively. They are regulated in response to nitrate and oxygen availability and changes in cell growth rate. Vitamin B₁₂ (cobalamin) is synthesized by *Salmonella* Typhimurium only under anaerobic growth conditions used as a cofactor in four known reactions. The deletion of *cobS* and *cbiA* genes prevent any form of cobalamin production. In the present study we evaluate the infection of birds by mutants of STM, with the anaerobic respiratory system committed by mutations in the genes: *narG*, *napA*, *cobS*, *cbiA*, *frdA*, *dmsA*, and *torC*. Virulence was assessed by oral inoculation of groups of one-day-old broilers with 0.1 mL of culture contained 10⁸ colony forming units (CFU)/mL or diluted at 10⁻³ and 10⁻² of strains mutants of *Salmonella* Typhimurium. Clinical signs and mortality were recorded over a period of 21 days. In general, the symptoms of chickens infected with the mutant strains were similar to those presenting by control birds. Except for STMNaI^r Δ*cbiA*, all showed reduced capacity to cause mortality in comparison with the original strain. The mortality of group of chickens infected with STMNaI^r Δ*narG*, STMNaI^r Δ*frdA*, STMNaI^r Δ*dmsA* and STMNaI^r Δ*cobS*Δ*cbiA* showed significant decrease in mortality compared to control group (p<0.05).

Key Words: *Salmonella* Typhimurium, respiration, anaerobic, gene.

Introduction

Salmonellae are significant not only as threat to public health worldwide, but also as a model system for the study of fundamental mechanisms of bacterial pathogenesis. During *Salmonella* infection, the growth phase and growth conditions of the organism are important in attachment, invasion, and the regulation of many virulence genes (17, 33, 32).

Cells grown under limited oxygen concentrations are more invasive and adhere better to cells than do aerobically grown or stationary-phase cells (33). As the pathogen enters and moves through its animal host, it encounters a series of unique environments (41). During infection, serovar Typhimurium must adapt to changes in oxygen

encountered in the gastrointestinal tract of the host (44). Exposure of STM to anaerobic conditions has been observed to enhance its virulence (52), indicating anaerobiosis to be an important environment for intracellular survival of the bacterium (20). *Salmonella* Typhimurium can respire either aerobically or anaerobically by using one of the alternative electron acceptors: oxygen, TMAO, DMSO, or fumarate. Depending on the availability of these respiratory substrates, the cell synthesizes one or more of the terminal enzymes of the electron transport pathways. DMSO and TMAO reductase, encoded by *dmsABC*, exhibit a broad substrate specificity for reducing TMAO, DMSO, and others amine-*N*-oxides (10, 60, 47). Fumarate reductase (*frd*) is encoded by the *frdABCD* operon. It catalyzes the interconversion of

fumarate and succinate and is a key enzyme for the anaerobic functioning of many organisms respiring with fumarate as terminal electron acceptor, although under physiological conditions, it is thought to perform the reductive reaction primarily during anaerobic growth (1, 57, 28).

Under different growth conditions, *Salmonella* expresses three different nitrate reductase activities converting nitrate to nitrite during anaerobic cell growth (8). The periplasmic nitrate reductase (*nap*) is expressed primarily during anaerobic growth in the presence of very low concentrations of nitrate (14). The inducible nitrate reductase A (NRA), encoded by the *narGHJI* operon, is a membrane-bound enzyme containing three subunits α , β and γ . The active site of the membrane-associated α subunit, *narG*, is a molybdopterin guanine dinucleotide cofactor located in the cytoplasm (34, 29, 45). It couples the oxidation of physiological substrates, especially formate, to nitrate reduction generating a proton electrochemical gradient (7, 13). The third nitrate reductase, encoded by the *narZYWV* operon, is structurally very similar to nitrate reductase A (11, 12), but it is expressed extremely weakly during both aerobic and anaerobic growth (27).

The synthesis of vitamin B₁₂ is a complex anaerobic process that requires approximately 25 different enzymes (21). Cobalamin is a known cofactor for numerous enzymes mediating methylation, reduction, and intramolecular rearrangements (49, 18). These enzymes are as follows: (i) Homocysteine methyltransferases, (ii) Ethanolamine ammonia lyase, (iii) Propanediol dehydratase (Havemann et al, 2003), (iv) Queuosine synthetase (49).

Material and Methods

Bacterial strain and culture media. *Salmonella enterica* serovar Typhimurium produces gastroenteritis and intestinal lesions (31). For ease of enumeration spontaneous nalidixic acid-resistant (25 μ g/mL) mutant derivatives of this strain were used. The mutants were constructed from *Salmonella* Typhimurium F98 NaI^r strain. *S. enterica* serovar Typhimurium F98 is a wild-type strain that is virulent for chickens and colonizes the chicken gut efficiently (5, 61, 56).

Broth cultures consisted of 10mL volumes of LB broth (Invitrogen No 12780-052) incubated for 24h/37° C in a shaking incubator (100 rpm). The broth culture contained approximately 1.0 x10⁸ (*S. Typhimurium*) CFU/mL.

Mutant construction. *Salmonella* Typhimurium mutants were constructed defective in anaerobic respiration. 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 *Kpn*I or *Bam*HI sites facilitating insertion of a kanamycin (*cbiA*, *torC*, *narG* genes) or spectinomycin (*napA*, *frdA*, *dmsA* e *cobS* genes) cassette. Initial cloning was into pGEM T Easy (Promega) and then into the suicide

vector pJCB12 in *Escherichia coli* (*E.coli*) S.17.1 λ pir, which was used for conjugation into *Salmonella* Typhimurium F98. Mutants were selected by resistance to kanamycin or streptomycin sensitivity and were checked for their *Salmonella* O-serotype 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.

Transduction. Mutants of *Salmonella* Typhimurium with double deletions were obtained by transduction using bacteriophage P22 (\emptyset P22) following standard protocols (6, 50). Transductants were plated on LB agar containing spectinomycin or kanamycin. After incubation at 37°C/24h, they were checked again by PCR.

Virulence assays.

Experiment 1. Assessment of mortality

Virulence was assessed by oral inoculation of groups of one-day-old broilers with 0.1 mL of culture containing 10⁸ CFU/mL or diluted at 10⁻³ of strains mutants of *Salmonella* Typhimurium. Nine groups of 20 birds were formed. Inocula were prepared for STM, strains STMNaI^r Δ *dmsA* Spc^r, STM NaI^r Δ *cobS* Spc^r, STMNaI^r Δ *frdA* Spc^r, STMNaI^r Δ *napA* Spc^r, STMNaI^r Δ *cbiA* Can^r, STMNaI^r Δ *narG* Can^r, STMNaI^r Δ *torC* Can^r Δ *dmsA* Spc^r, STMNaI^r Δ *cobS* Spc^r Δ *cbiA* Can^r and original strain STM NaI^r (control group). Clinical signs and mortality were recorded over a period of 21 days.

Experiment 2. Assessment of systemic infection

Systemic infection was assessed by oral inoculation of groups of one-day-old broilers with 0.1 mL of the mutant or wild culture containing 10⁸ CFU/mL or diluted at 10⁻² of strains mutants of *Salmonella* Typhimurium. Six groups of 20 birds were formed. Inocula were prepared for STM, strains STMNaI^r Δ *dmsA* Spc^r, STM NaI^r Δ *cobS* Spc^r, STMNaI^r Δ *frdA* Spc^r, STMNaI^r Δ *napA* Spc^r, STMNaI^r Δ *cbiA* Can^r, STMNaI^r Δ *narG* Can^r, STMNaI^r Δ *torC* Can^r Δ *dmsA* Spc^r, STMNaI^r Δ *cobS* Spc^r Δ *cbiA* Can^r and original strain STM NaI^r (control group). Clinical signs and mortality were recorded over a period of 21 days. Twenty one day post-infection (dpi), half of the birds were euthanized by cervical dislocation, and then, necropsied. Liver and cecal contents were collected to determine the number of *Salmonella* using Brilliant Green Agar plates containing 25 μ g/mL nalidixic acid and 40 μ g/mL novobiocin (VBNal/Nov), as described by Smith *et al.* (53). Swabs were streaked directly onto VBNal/Nov plates and then kept into a tube containing 2 mL selenite broth. Both plates and tubes were incubated at 37°C/24h. In case of no growth, the swab was plated again onto VBNal/Nov and incubated at 37°C/24h (5).

Statistical analysis

Non parametric Qui-square test with significance level of 5% was performed to analyze mortality; *P* values of <0.05 were regarded as statistically significant (25).

Results

In experiment 1, it was assessed the mortality caused by mutants of *Salmonella Typhimurium* with defectives genes (*narG*, *napA*, *cobS*, *cbiA*, *frdA*, *dmsA*, and *torC*). After infection, the birds were daily observed. Clinically, from the 3rd dpi, the chicks showed drowsiness, apathy, weakness and anorexia. Around the cloaca, there was accumulation of feces, sometimes brown-greenish. Mortality started from 4 dpi. Incoordination was observed from 7 dpi, with swelling of tibiotarsal and humeroradial joints with claudication. There were also shaking of head and neck

and some animals showed blindness (unilateral or bilateral). After 16 dpi, the chicks began to recover. It was noticed an increase in consumption of food and water. In general, the symptoms displayed by the birds infected with the mutant strains were similar to those presented by the birds of control group.

The results about mortality are depicted in tables 1 and 2. In Table 1, the birds were challenged with diluted culture (10^{-3}) of STM. Mortality was lower in the group challenged with STMNaI^f *narG* strain (*p* <0.05). A new experiment was conducted by using inoculums diluted at 10^{-2} . This experiment was done with the mutants of STM that provoked less mortality. The results are in Table 2. Tested STM strains were less virulent in some degrees. Nonetheless among the five mutants tested (STMNaI^f $\Delta narG$, STMNaI^f $\Delta cbiA\Delta cobS$, STMNaI^f $\Delta cbiA$, STMNaI^f $\Delta frdA$, STMNaI^f $\Delta dmsA$), only mortality provoked by STMNaI^f $\Delta cbiA$ strain was not significant (*p* > 0.05).

Table 1. Mortality of one- day-old broilers in experimental infection with diluted culture (10^{-3}) of strains of STM with defective genes related to anaerobic respiration

Defective gene	Cumulative mortality in days-post-infection																	Total		Swabs (+)		
	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	N./Total	(%)	CE#	(%)
<i>narG</i>				1							2								2/20*	10		100%
<i>frdA</i>			1		4	5				6									7/20	35		100%
<i>dmsA</i>			1		2	5		6	7		8	9							10/20	50		100%
<i>cbiA</i>			1	5	6		7	8		9	10								10/20	50		100%
<i>cobS</i>		1	2		3	4	5	6											6/20	30		100%
<i>napA</i>	1		4			5		6	7										7/20	35		100%
<i>cobS cbiA</i>			1	2					3		4								4/20	20		100%
<i>torCdmsA</i>		1	2		5		6						7	8					8/20	40		100%
**C		1	3	4	5			6	7	8									8/20	40		99,9%

**C: Control group (wild strain). #Ce: Cecum.
 * Statistically significant difference compared to the control group (χ^2 , *p* < 0.05).

Table 2. Mortality of one-day-old broilers in experimental infection with diluted culture (10^{-2}) of strains of STM with defective genes related to anaerobic respiration.

Defective gene	Cumulative mortality in days-post-infection																	Total %		Swabs (+)	
	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Núm. Total	(%)	#L %	#CE %
<i>narG</i>	5	14	20	21	22	23			24	27	28	29		30				30/60*	50	31	100
<i>frdA</i>	3	10	15	19	21	22	24	25		26	29							29/60*	48	40	100
<i>dmsA</i>	2	13	17	21	23	24	25	26	27		28	29	30					30/60*	50	39	100
<i>cbiA</i>	3	12	16	21	25				26	30	32	33	35					35/60	58	33	100
<i>cobS cbiA</i>	2	14	19	23	26	27	28	29		30								30/60*	50	25	100
**C	3	11	15	19	24			28	32	36	41	43	45		46			46/60	77	33	100

**C: Control group (wild strain). #Ce: Cecum. #L: Liver.
 * Statistically significant difference compared to the control group (χ^2 , *p* < 0.05).

Discussion and conclusion

The present work was an attempt to begin to understand in a more detailed way the nature of the interaction between colonising *Salmonella* strains and the host, during the process of anaerobic respiration. To respire anaerobically, *Salmonella* produces enzymatic complexes according to the available substrate into the host cell. Sometimes, under certain circumstances, the enzymatic complexes could inhibit the action of each other. In this work, mutants of STM containing defective genes related to anaerobic respiration were prepared. These mutants were inoculated orally in 1-day-old chickens susceptible to clinical fowl paratyphoid. In general, the symptoms of chickens infected with the mutant strains were similar to those presented by control birds. The mortality of the group infected with STMNaI^r $\Delta narG$, STMNaI^r $\Delta frdA$, STMNaI^r $\Delta dmsA$ and STMNaI^r $\Delta cobS\Delta cbiA$ showed significant lower mortality in comparison to the wild strain ($p < 0.05$). Within the experimental adopted procedure, it was not possible to establish the reasons why the mutant strains became less pathogenic. However, there are studies that seek to clarify these mechanisms (42).

Under anaerobiosis, nitrate is the most favorable electron acceptor (45). *Salmonella Typhimurium* survives and proliferates within macrophages, where it withstands anti-microbial responses such as the production of reactive oxygen species (ROS) (58), and reactive nitrogen species (RNS), including nitric oxide (NO). Mills *et al.* (2005) suggested that *Salmonella* serovars contain multiple enzymes that are capable of detoxifying NO under both oxic and anoxic conditions. *Salmonella Typhimurium* grown anaerobically with nitrate is capable of generating detectable NO after nitrite (NO₂) addition (Fang, 2004). According to Gilberthope *et al.* (2008), mutant cells lacking the membrane-bound nitrate reductase, *narGHI*, and membranes derived from these cells are unable to produce NO, demonstrating that, in wildtype STM, this enzyme is responsible for NO production. Enterobacteria possess several NO detoxifying mechanisms. This process is part of the inhibition mechanism of nitric oxide action, completing the reduction of NO₃, producing a less toxic substance (3, 22, 24, 50, 51, 38). According to Pawaria *et al.* (2007), during the infectious cycle, the intracellular pathogen *Mycobacterium bovis* uses mechanisms of bacterial protection against the toxic effects of nitric oxide similar to the ones presented by STM.

There is evidence that in *E coli* and STM, the fumarate plays an important role in obtaining energy in conditions of anaerobiosis (28, 57). With the inhibition of production of the enzyme fumarate reductase, delays may occur during bacterial growth (55, 28). As the results obtained in this study, we found that the strain STMNaI^r $\Delta frdA$ caused lower mortality of birds in comparison to results for the original strain. The decrease in mortality due to inactivation of *frdA* gene could be justified by the role of fumarate and its metabolites as factors involved in activation of flagellar

direction and rotation. This mechanism is essential for understanding of the process of chemotaxis, because the direction of flagellar rotation determines the mode of swimming (4). Motility is usually considered virulence factor of pathogenic bacteria (16).

Dimethyl sulfoxide (DMSO) is a component of the sulfur cycle in anaerobic conditions. Several microorganisms have the ability to reduce DMSO into dimethyl sulfide (DMS), but this mechanism is unknown (9, 60, 37). The expression of the enzyme DMSA is well characterized in the virulence of *Actinobacillus pleuropneumoniae*, etiologic agent of pleuropneumonia in pigs. The deletion of the gene DMSA was shown to attenuate the clinical symptoms of acute infection (2).

The decrease in mortality of birds inoculated with the mutant $\Delta cobS\Delta cbiA$ can be correlated to the fact that cobalamin is a cofactor of several enzymes that act in the process of anaerobic respiration. Cobalamin is important for normal development of STM. The synthesis of cobalamin is limited to a few representatives of bacteria and archaea (36, 48, 15, 50). Several derivatives of vitamin B₁₂ act as cofactors of reactions responsible for the anaerobic catabolism of carbon sources (35). Under Price-Carter *et al.* (2001), mutant defects in the synthesis of B₁₂ have impaired anaerobic growth. This is in agreement with an expansive view of virulence determinants which include not only the factors that cause disease, but also functions that contribute to bacterial survival and multiplication in the host (38, 26). Further research on bacterium physiology should be carried out to elucidate the events described in this research and to assess the mutant.

Since we now believe that interaction between enteric pathogens and the avian mucosa plays an integral role in determining the level of colonization, it is important in designing such an experiment to include a selection of bacterial pathogens which have different colonization phenotypes. In summary, the pattern of gene expression indicated specific gene associated with growth and nutrient acquisition. Taken together, these data lead us to propose that *narG*, *frdA*, *dmsA* and *cobScbiA* activity may be crucial in the respiratory and gastrointestinal tracts, by providing either energy by anaerobic respiration or important metabolic intermediates or both of the above. 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.

Acknowledgement

To FAPESP - Fundação de Amparo a Pesquisa do Estado de São Paulo, by financial supports.

References

1. ACKRELL BA., JOHNSON MK., GUNSALUS RP., CECCHINI G. Structure and function of succinate dehydrogenase and fumarate reductase. In

Chemistry and Biochemistry of Flavoproteins, ed. Muller F, 1990, 3, 229–297.

2. BALTES N., HENNIG-PAUKA I., JACOBSEN I., GRUBER AD. GERLACH GF. Identification of dimethyl sulfoxide reductase in *Actinobacillus pleuropneumoniae* and its role in infection. *Infect. Immun.*, 2003, 71, 6784-92.
3. BANG IS., AUDIA JP., PARK YK., FOSTER JW. Autoinduction of the *ompR* response regulator by acid shock and control of the *Salmonella enterica* acid tolerance response. *Mol. Microbiol.*, 2002, 44, 1235-50.
4. BARAK R., EISENBACH M. Fumarate or a fumarate metabolite restores switching ability to rotating flagella of bacterial envelopes. *J. Bacteriol.* 1991, 174, 643-645.
5. BARROW PA., HUGGINS MB., LOVELL M., SIMPSON J. Observations on the pathogenesis of experimental *Salmonella* Typhimurium infection in chickens. *Rev. Vet. Sci.*, 1987, 42, 194–199.
6. BARROW PA. Immunity to experimental fowl typhoid in chickens induced by a virulence plasmid-cured derivative of *Salmonella* Gallinarum. *Infect. Immun.*, 1990, 58, 2283-88.
7. BERG B., STEWART V. Structural genes for nitrate-inducible formate dehydrogenase in *Escherichia coli* K-12. *Genet.*, 1990, 125, 691-702.
8. BERTERO M., ROTHERY R., PALAK M., HOU C., LIM D., BLASCO F., WEINER J. Insights into the respiratory electron transfer pathway from the structure of nitrate reductase A. *Nat. Struc. Biol.*, 2003, 10, 681-687.
9. BILOUS PT., WEINER JH. Dimethyl sulfoxide reductase activity by anaerobically grown *Escherichia coli* HB101. *J. Bacteriol.*, 1985, 162, 1151–55.
10. BILOUS PT., WEINER JH. Molecular cloning and expression of the *Escherichia coli* DMSO reductase operon. *J. Bacteriol.*, 1988, 170, 1511-18.
11. BLASCO F., IOBBI C., RATOUCHEJAK J., BONNEFOY V., CHIPPAUX M. Nitrate reductase of *Escherichia coli*: sequence of the second nitrate reductase and comparison with that encoded by the *narGHJI* operon. *Mol. Gen. Genet.*, 1990, 222, 104-111.
12. BONNEFOY V., RATOUCHEJAK J., BLASCO F., CHIPPAUX, M. Organization of the *nar* genes at the *chlZ* locus. *FEMS Lett.*, 1997, 147, 147–149.
13. CHÉNEBY D., HALLET S., MONDON M., MARTIN-LAURENT F., GERMON JC., PHILIPPOT L. Genetic characterization of the nitrate reducing community based on *narG* nucleotide sequence analysis. *Microb. Ecol.*, 2003, 46, 113–121.
14. CLEGG SJ., JIA W., COLE JA. Role of the *Escherichia coli* nitrate transport protein, NarU, in survival during severe nutrient starvation and slow growth. *Microbiol.*, 2006, 152, 2091–2100.
15. CROFT MT., LAWRENCE AD., RAUX-DEERY E., WARREN MJ., SMITH AG. Algae acquire vitamin B₁₂ through a symbiotic relationship with bacteria. *Nat.* 2005, 438, 90–93.
16. DIBBA-FULLER MP., WOODWARD MJ. Contribution of fimbriae and flagella of *Salmonella enteritidis* to colonization and invasion of chicks. *Avian Pathol.*, 2002, 9, 295– 304.
17. ERNST R., DOMBROSKI D., MERRICK J. Anaerobiosis, type I fimbriae and growth phase are factors that affect invasion of HEp-2 cells by *Salmonella* Typhimurium. *Infect. Immun.*, 1990, 58, 2014-16.
18. ESCALANTE-SEMERENA J. Conversion of cobinamide into denosylcobinamide in bacteria and archaea. *J. Bacteriol.*, 2007, 189, 4555–60.
19. FANG FC. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat. Rev. Microbiol.*, 2004, 2, 820–832.
20. FINK R., EVANS M., PORWOLLIK S., VAZQUEZ-TORRES A., JONES-CARSON J., TROXELL B., HASSAN H. FNR is a global regulator of virulence and anaerobic metabolism in *Salmonella enteric* serovar Typhimurium. *J. Bacteriol.*, 2007, 189, 2262–73.
21. FRESQUET V., WILLIAMS L., RAUSHEL FM. Mechanism of cobyrinic acid a,c-diamide synthetase from *Salmonella Typhimurium* LT2. *Biochim.*, 2004, 43, 10619-27.
22. GARDNER, AM.; HELMICK, RA.; GARDNER, PR. Flavorubredoxin, an inducible catalyst for nitric oxide reduction and detoxification in *Escherichia coli*. *J. Biol. Chem.*, 2002, 277, 8172-77.
23. GILBERTHORPE N., POOLE R. Nitric oxide homeostasis in *Salmonella* Typhimurium: roles of respiratory nitrate reductase and flavohemoglobin. *J. Biol. Chem.*, 2008, 283, 11146-54.
24. GOMES CM., GIUFFRÉ A., FORTE E., VICENTE J. B., SARAIVA LM., BRUNORI M., TEIXEIRA M. A novel type of nitric-oxide reductase. *Escherichia coli* flavorubredoxin. *J. Biol. Chem.*, 2002, 277, 25273- 76.
25. GREENWOOD PS., NIKULIN MS. In: WILEY, J. A Guide to Chi-Squared Testing. New York, 1996, 280.
26. GROISMAN EA., SAIER MH. *Salmonella* virulence: new clues of intramacrophage survival. *Trends. Biochem. Sci.*, 1990, 15, 30-33.
27. IOBBI C., SANTINI C., BONNEFOY V., GIORDANO G. Biochemical and immunological evidence for a second nitrate reductase in

- Escherichia coli* K12. *Eur. J. Biochem.*, 1987, 168, 451-459.
28. IVERSON TM., LUNA-CHAVEZ CL., CECCHINI, G., REES DC. Structure of the *Escherichia Coli* Fumarate Reductase Respiratory Complex. *Sci.*, 1999, 284, 1961-1966.
29. JONES RW., GARLAND PB. Sites and specificity of the reaction of bipyridylum compounds with anaerobic respiratory enzymes of *Escherichia coli*. *J. Biochem.*, 1977, 164, 199-211.
30. JONES HM., GUNSALUS RP. Transcription of the *Escherichia coli* fumarate reductase genes (*fir*ABCD) and their coordinate regulation by oxygen, nitrate and fumarate. *J. Bacteriol.*, 1985, 164, 1100-1109.
31. KAISER P., ROTHWELL L., GALYOV E., BARROW PA., BURNSIDE J., WIGLEY P. Differential cytokines expression in avian cells in response to invasion by *Salmonella* Typhimurium, *Salmonella* Enteritidis and *Salmonella*. *Microbiol.*, 2000, 146, 3217-26.
32. KHULLAR M., DEEP R., SMRITI M., KUMAR N. Anaerobiosis-induced virulence of *Salmonella enterica* subsp. *enterica* serovar Typhimurium: role of phospholipase C γ signalling cascade. *J. Med. Microbiol.*, 2003, 52, 741-745.
33. LEE C., FALKOW S. The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. *Proc. Natl. Acad. Sci.*, 1990, 87, 4304-4308.
34. LUND K., DEMOSS JA. Association-dissociation behaviour and subunit structure of heat-released nitrate reductase from *Escherichia coli*. *J. Biol. Chem.*, 1976, 251, 2207-16.
35. MAGGIO-HALL LA., ESCALANTE-SEMERENA, JC. *In vitro* synthesis of the nucleotide loop of adenosylcobalamin by *Salmonella* Typhimurium enzymes. *Proc. Natl. Acad. Sci.*, 1999, 96, 11798-11803.
36. MARTENS JH., BARG H., WARREN MJ., JAHN D. Microbial production of vitamin B₁₂. *Appl. Microbiol. Biotechnol.*, 2002, 58, 275-285.
37. MATSUZAKI M., KUBOTA K., SATOH T., KUNUGI M., BAN S., IMURA S. Dimethyl sulfoxide-respiring bacteria in Suribati Ike, a hypersaline lake, in Antarctica and the marine environment. *Polar Biosci.*, 2006, 20, 73-81.
38. MEKALANOS JJ. Environmental signals controlling expression of virulence determinants in bacteria. *J. Bacteriol.* 1992, 174, 1-7.
39. MILLS PC., RICHARDSON DJ., HINTON JCD., SPIRO S. Detoxification of nitric oxide by the flavorubredoxin of *Salmonella enterica* serovar Typhimurium. *Biochem. Soc. Transact.*, 2005, 33, 198-199.
40. MILLS PC., ROWLEY G., SPIRO S., HINTON JC., RICHARDSON DJ. A combination of cytochrome c nitrite reductase (NrfA) and flavorubredoxin (NorV) protects *Salmonella enterica* serovar Typhimurium against killing by NO in anoxic environments. *Microbiol.*, 2008, 154, 1218-28.
41. OHL ME., MILLER SI. *Salmonella*: a model for bacterial pathogenesis. *Annu. Rev. Med.*, 2001, 52, 259-274.
42. PAIVA, J.; CASARIN, RA.; SIERRA, Y.; BERCHIERI, A.; FRANCO, MV.; BARROW, P. 2009. A DEFECTIVE MUTANT OF *Salmonella enterica* Serovar Gallinarum IN COBALAMIN BIOSYNTHESIS IS AVIRULENT IN CHICKENS. *Braz. J. Microbiol.* 40, 495-504.
43. PAWARIA S., RAJAMOHAN G., GAMBHIR V., LAMA A., VARSHNEY GC., DIKSHIT KL. Intracellular growth and survival of *Salmonella enterica* serovar Typhimurium carrying truncated hemoglobins of *Mycobacterium tuberculosis*. *Microb. Pathol.*, 2007, 42, 119-128.
44. PLYM FL., WIERUP M. *Salmonella* contamination: A significant challenge to the global marketing of animal food products. *Rev. Sci. Tech.*, 2006, 25, 541-554.
45. POTTER L. MILLINGTON P. THOMAS GH., ROTHERY R. Novel growth characteristics and high rates of nitrate reduction of an *Escherichia coli* strain, LCB2048, that expresses only a periplasmic nitrate reductase. *FEMS Microbiol. Lett.*, 2000, 185, 51-57.
46. PRICE-CARTER M., TINGEY J., BOBIK T., ROTH J. The alternative electron acceptor tetrathionate supports B₁₂-dependent anaerobic growth of *Salmonella enterica* Serovar Typhimurium on ethanolamine or 1,2-propanediol. *J. Bacteriol.*, 2001, 183, 2463-75.
47. RAY N., OATES J., TURNER RJ., ROBINSON C. DmsD is required for the biogenesis of DMSO reductase in *Escherichia coli* but not for the interaction of the DmsA signal peptide with the Tat apparatus. *FEBS Lett.*, 2003, 534, 156-160.
48. RODIONOV DA., VITRESCHAK AG., MIRONOV AA., GELFAND MS. Comparative genomics of the vitamin B₁₂ metabolism and regulation in prokaryotes. *J. Biol. Chem.*, 2003, 278, 41148-59.
49. ROTH JR., LAWRENCE JG., RUBENFIELD M., KIEFFER-HIGGINS S., CHURCH GM. Characterization of the cobalamin (vitamin B₁₂) biosynthetic genes of *Salmonella* Typhimurium. *J. Bacteriol.*, 1993, 175, 3303-3316.
50. SANTOS F., VERA J., VAN DER HEIJDEN R., VALDEZ G., VOS W., SESMA F., HUGENHOLTZ J. The complete coenzyme B₁₂

- biosynthesis gene cluster of *Lactobacillus reuteri* CRL1098. *Microbiol.*, 2008, 154, 81–93.
51. SAMBROOK J., RUSSEL DW. *Molecular cloning. In: A laboratory manual.* Cold Spring Harbor Laboratory Press. 2001.
52. SINGH DR., KHULLAR M., GANGULY NK. Role of anaerobiosis in virulence of *Salmonella* Typhimurium. *Mol. Cell. Biol.*, 2000, 215, 39-46.
53. SMITH HW., TUCKER JF. The virulence of *Salmonella* for chickens; their excretion by infected chickens. *J. Hy.*, 1980, 84, 479-488.
54. STEVANIN TM., POOLE RK., DEMONCHEAUX EA., READ RC. Flavohemoglobin Hmp protects *Salmonella* enterica serovar Typhimurium from nitric oxide-related killing by human macrophages. *Infect. Immun.*, 2002, 70, 4399-4405.
55. STEVANIN TM., READ RC., POOLE RK. The *hmp* gene encoding the NO-inducible flavohaemoglobin in *Escherichia coli* confers a protective advantage in resisting killing within macrophages, but not in vitro: links with swarming motility. *Gen.*, 2007, 398, 62-68.
56. TURNER A., LOVELL M., HULME S., ZHANG-BARBER L., BARROW PA. Identification of *Salmonella* Typhimurium Genes Required for Colonization of the Chicken Alimentary Tract and for Virulence in Newly Hatched Chicks. *Infect. Immun.*, 1998, 66, 2099–2106.
57. VAN HELLEMOND JJ., TIELENS AG. Expression and functional properties of fumarate reductase. *J. Biochem.*, 1994, 304, 321-331.
58. VAZQUEZ-TORRES A., FANG FC. Oxygen-dependent anti-*Salmonella* activity of macrophages. *Trends Microbiol.*, 2001, 9, 29–33.
59. WEINER JH., CAMMACK R., COLE ST., CONDON C., HONORÉ N., LEMIRE BD., SHAW G. A mutant of *Escherichia coli* fumarate reductase decoupled from electron transport. *Proc. Natl. Acad. Sci. U S A.*, 1986, 83, 2056-60.
60. WEINER, JH., MACISAAC DP., BISHOP RE., BILOUS PT. Purification and properties of *Escherichia coli* dimethyl sulfoxide reductase, an iron-sulfur molybdoenzyme with broad substrate specificity. *J. Bacteriol.*, 1988, 170, 1505–10.
61. ZHANG-BARBER L., TURNER AK., MARTIN G., FRANKEL G., DOUGAN G., BARROW PA. Influence of genes encoding proton-translocating enzymes on suppression of *Salmonella* Typhimurium growth and colonization. *J. Bacteriol.*, 1997, 179, 7186-90.