THE CONTRIBUTION OF GENES REQUIRED FOR ANAEROBIC RESPIRATION TO THE VIRULENCE OF SALMONELLA ENTERICA SEROVAR GALLINARUM FOR CHICKENS

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ABSTRACT

Salmonella enterica serovar Gallinarum (SG) is an intracellular pathogen of chickens. To survive, to invade and to multiply in the intestinal tract and intracellularly it depends on its ability to produce energy in anaerobic conditions. 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. In this study mortality rates of chickens challenged with mutants of Salmonella Gallinarum, which were defective in utilising anaerobic electron acceptors, were assessed in comparison to group of bird challenged with wild strain. The greatest degree of attenuation was observed with mutations affecting nitrate reductase (napA, narG) with additional attenuations induced by a mutation affecting fumarate reductase (frdA) and a double mutant (dmsA torC) affecting DMSO and TMAO reductase.

Key words: Salmonella Gallinarum, anaerobic genes, poultry, mutations.

INTRODUCTION

Fowl typhoid is caused by Salmonella enterica serovar Gallinarum (SG), a very severe disease of worldwide economic significance and where the mortality may reach up to 80%. Disease is most severe where environmental conditions and management systems do not allow hygienic control measures to be introduced. Although a live vaccine (9R) is commercially available and has been used extensively it harbours undefined attenuations and retains a degree of virulence (20, 21, 40). Although additional strains have been described which have more defined mutations (4, 6, 7, 23, 49) none of these combine the high level of protection of the 9R strain, with has ability to stimulate high level of circulating specific antibody. There thus remains considerable scope for further exploration of the basis of virulence in this pathogen.

The mechanisms whereby bacterial pathogens generate energy during infection are poorly understood. The Enterobacteriaceae are facultative anaerobes with a modular...
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respiratory system and which, in the absence of any externally supplied electron acceptors, may generate energy by substrate level phosphorylation. There is a strong hierarchy in the choice of electron acceptors, which are used, and with different bioenergetic efficiencies for the different donor/acceptor combinations.

Respiration in *Escherichia coli* and *Salmonella enterica* has been reviewed by Gennis & Stewart (19) and Richardson (36). A variety of electron donors, including NADH, formate, lactate, glycerol-3-phosphate, succinate and pyruvate donate electrons to the quinone membrane pool which then pass the electrons to final acceptors including oxygen, nitrate, nitrite, DMSO, TMAO, fumarate and thiosulphate. At the first and final stages energy may be expended enabling protons to be translocated across the membrane creating an electrochemical tension from the proton gradient. The proton gradient generated by electron transport is used by the F$_0$F$_1$ proton-translocating ATPase for ATP synthesis, flagella rotation, and nutrient uptake (12, 30). During fermentative growth, the bacterial F$_0$F$_1$ proton-translocating ATPase hydrolyzes ATP to generate the proton gradient.

Despite the extensive understanding of bacterial electron transport and proton translocation, little is known about the relative contribution of the relevant proton-translocating and related enzymes to the growth and survival of pathogens in the host in either the gut or the intracellular environment. The contribution of NADH dehydrogenase I, cytochrome o- and d-oxidase and the F$_0$F$_1$ ATPase to the virulence of serovars Typhimurium and Gallinarum in chickens and of serovar Dublin in mice have been determined through straight mutation exercises. Mutations crucially affecting enzyme activity, including *nuoG*, which codes for a key polypeptide of the NADH dehydrogenase I complex (12, 16), *cyoA* and *cydA*, which code for subunits of cytochrome o and cytochrome d oxidases, respectively (19), and *atpB* and *atpH*, which code for polypeptides of the F$_0$ and F$_1$ components, respectively, of the F$_0$F$_1$ proton-translocating ATPase (24) were all found to contribute to virulence to different degrees. These studies indicated that different proton translocating proteins contributed to the virulence of *S. enterica* serovars, considering different pathotypes and host specificities in different ways. Mutations in *atpB* or *atpH* were attenuating in *S. Typhimurium* (ubiquitous), *S. Dublin* (mammalian specific causing typhoid in mice and severe disease in cattle) and SG (avian specific causing typhoid in chickens) while mutations in *nuoG* (NADH oxidoreductase) and *cydA* and *cyoA* (cytochrome o and d oxidase respectively) were attenuating in *S. Gallinarum* they were less attenuating in Typhimurium and Dublin infections in mice (46).

The proton translocating enzymes above all use oxygen as electron acceptor. No information is currently available on the contribution of electron acceptors other than oxygen to virulence in *Salmonella*. Given that for most of these serovars infection is by the faecal oral route and the intestinal colonisation is an important initial phase of infection, even for those typhoid serovars that do not colonise the intestine extensively, it seems likely that metabolism and energy generation/respiration under conditions of reduced oxygen tension will be important at some stage of the infection process. In addition the work of Turner et al., (46) indicated that mutations in *cyoA* were less attenuating than those in *cydA* indicating that growth at lower oxygen tension may be significant since cytochrome d is generally expressed at lower oxygen tensions (17). We therefore decided to assess the contribution of nitrate, fumarate, DMSO and TMAO to the virulence of SG in chickens.

**MATERIAL AND METHODS**

**Bacterial strain and culture media**

*Salmonella* Gallinarum strain 9 produces typical systemic fowl typhoid with high mortality (34, 42). For ease of enumeration spontaneous nalidixic acid-resistant (25μg/mL) mutant derivates of this strain were used. This has been found previously not to affect their virulence (43). The mutants were constructed from *Salmonella* Typhimurium F98 Nal$^+$ strain. The DNA homology was such as to permit use the same inactivated serovar Typhimurium sequences in...
serovar Gallinarum. The construction of the mutants, conjugation and transduction followed Sambrook & Russel (39) and Turner et al. (46).

Unless otherwise stated, broth cultures consisted of 10mL LB broth (Invitrogen) incubated for 24h/37ºC in a shaking incubator (100 rpm). The broth culture contained approximately 1x10^8 (S. Gallinarum) and 1.2 x10^9 (S. Typhimurium) CFU/mL.

**Mutant construction**

Mutants were constructed in an identical way to the proton translocating-deficient mutants described previously (46). In brief, four primers were designed for each gene to be mutated such that two fragments close to the 5' and 3' ends (Table 1) of the gene could be amplified leaving a central deletion in the amplified gene and incorporating KpnI or BamHI sites facilitating insertion of a kanamycin or spectinomycin cassette. Initial cloning was into pGEM T Easy (Promega) and then into the suicide vector pJCB12 in E.coli S.17.1Δ pir, which was used for conjugation into Salmonella Gallinarum 9 Nal'. Mutants were selected by resistance to kanamycin or streptomycin and ampicillin 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. For the S. Gallinarum narZ and narG mutants an additional primer was used for the PCR derived from the deleted part of the gene (primer 5).

Transduction. Mutants of Salmonella Gallinarum with double deletions were obtained by transduction using bacteriophage P22 following standard protocols (5, 39). Transductants were plated on LB agar containing spectinomycin or kanamycin and after incubation at 37C/24h were again checked by PCR.

### Table 1. Primers used for mutant construction.

| Gene | Primer 1 | Primer 2 | Primer 3 | Primer 4 |
|------|----------|----------|----------|----------|
| DmsA | cgtctagaatgaaactaagatcctga | aagatgggatcccatgaaagtggtaagttg | tcagggatccactttaacacagctggacc | actctagaacggaggttctgtatgttg |
| TorC | actctagaatgcgaaaactctggagagc | aaagcagggtacccaatcccttggagctg | ggattgggtaccctgttctgaagtgaaagtc | catctagattcattgttctccctat |
| NarG | cgtctagaatcgcacccgggtctgtga | aagaaaggtaccecaatcagcaagagaata | gatctagatgttgctgctgtcagtaat | cgtctagaatcgcacccgggtctgtga |
| NarZ | atggcgggtacctttttataacgcggtta | gcctggtacccgctgtcagtaat | tacccttacccgggtctgtg | tttgatattctcgactgg |
| NapA | aatctagatcaacccaggggctcaactg | accttctggatccatgacgagtaagttct | acacaggggacctgggttgctcttctg | gatctagatgttgctgctgtcagtaat |
| FrdA | cgtctacaagttttcaagcgcctttggt | cagctcggtacctcttggtgctgaaggggaaac | aacacaggatcgagctgctcttgctcttctg | gatctagatgttgctgctgtcagtaat |

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Virulence assays

Mortality rates were assessed by oral inoculation of five-day-old brown commercial layers. This variety of chicken was chosen because it is susceptible to the clinical fowl typhoid (10, 18). Groups of 30 birds were challenged with 0.5 mL of either an undiluted culture contained $10^8$ CFU/mL or diluted at $10^{-2}$ to contained $10^6$ CFU/mL. Mortality was recorded over a period of 30 days. Mortality in groups of birds inoculated with mutants of SG were compared to mortality in group inoculated with wild strain of SG using Chi-Square test ($p<0.05$) (22).

RESULTS

A Km$^r$ insertion cassette was made in genes torC, narG, and narZ and a Spc$^r$ insertion cassette was made in genes napA, frdA, dmsA. The effects of the virulence of the SG mutants in the brown variety of layers are present in tables 2 and 3. Mortality shown in Table 2 did not differ among groups of birds inoculated with the SG mutant strains than that provoked by the wild strain of SG ($\chi^2$, $p > 0.05$), except to SG$\Delta$frdA that at lower dose provoked less mortality than the wild strain of SG ($\chi^2$, $p < 0.05$). The mortality was higher in groups inoculated with diluted cultures of SG $\Delta$dmsA strain compared with the diluted culture of wild SG strain ($\chi^2$, $p < 0.05$). Table 3 presents the results related to the remaining mutants. Both doses produced less mortality in birds inoculated with SG $\Delta$narG and SG $\Delta$napA strains in comparison with the wild SG strain ($\chi^2$, $p < 0.05$). The difference was significant only to the higher dose to SG $\Delta$narG$\Delta$napA ($\chi^2$, $p < 0.05$) and the lower dose to $\Delta$torC$\Delta$dmsA ($\chi^2$, $p < 0.05$).

Table 2. Mortality of 5-day-old brown commercial layers due to experimental Salmonella Gallinarum strains being defective in any gene related to anaerobic respiration.

| $\Delta$   | CFU/mL | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | Total# | %     |
|------------|--------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|      |
| dmsA       | $10^8$ | 12 | 22 | 27 | 28 | 29 | 30 |   |   |   |   |   |   |   |   |   |    |   |    |   |   |   |    | 28 | 28 |    |  a  | 100  |
|            | $10^6$ |    | 2  | 3  | 4  | 6  | 7  | 11 | 12 | 14 | 21 | 23 | 25 |   | 28 |  28 | b  |    |   |    |   |    |    |  a  | 93.33|
| torC       | $10^8$ | 7  | 22 | 25 | 26 | 27 | 28 | 29 | 30 |    |    |    |    | 10 | 12 | 14 | 15 | 17 | 20 | 21 | 21 |    | 21 | b  | 70  |
|            | $10^6$ | 1  | 2  | 4  | 5  | 6  | 10 | 12 | 14 | 15 | 17 | 20 | 21 | 21 | 21 |  26 |    | 26 | a  | 86.67|
| narZ       | $10^8$ | 3  | 13 | 24 | 25 |    | 26 |    |    |    |    |    |    |    |    |    |    |    |  26 | a  | 86.67|
|            | $10^6$ | 1  | 8  | 13 | 14 | 15 | 17 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  17 | ab  | 56.67|
| frdA       | $10^8$ | 5  | 12 | 16 | 18 | 19 | 21 | 22 | 23 | 25 | 26 | 27 | 28 |    |    |    |    |    |    |    |    |    |    |    |    |    |  28 | a  | 93.33|
|            | $10^6$ | 4  | 9  | 10 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  11 | ab  | 36.67|
| None*      | $10^8$ | 9  | 21 | 25 | 26 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  26 | a  | 96.30|
|            | $10^6$ | 5  | 6  | 9  | 10 | 11 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  12 | a  | 96.30|
|            |        |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  18 | 20 | 20 b| 64.52|

*Group of birds infected with the original strain of SGNa$t^r$; # Different letters to the same dilution between mutant and wild strains are significant by Chi-Square test ($p<0.05$).

DISCUSSION AND CONCLUSION

Fowl typhoid is a systemic disease of chickens caused by SG, an intracellular anaerobic facultative parasite. The disease is very severe and during its course the bacterium persists intracellularly. The virulence factors are responsible for the development and evolution of the illness (47). In addition, the bacterium also utilized other resources to survive intracellularly. 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. According to Mahan et al. (29), there is direct association among expression of genes related to

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anaerobic process and expression of genes related to invasion process. This would provide to the bacterium better favorable energetic way to survive and to growth. The virulence of STM decreases in a strain with altered genes encoding enzymes of the metabolic pathway of respiratory process (31). Therefore, genes responsible for the respiratory enzymes production could be regulated in the same process that encodes invasiveness (13, 41).

Table 3. Mortality of 5-day-old brown commercial layer due to experimental Salmonella Gallinarum strains being defective in any gene related to anaerobic respiration.

| CFU/mL | Cumulative mortality at any day post infection (dpi) | Total# | % |
|--------|---------------------------------------------|--------|---|
|        | CFU/mL | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 18 | 20 | 21 | 22 | 23 | 24 | 25 | Total# |
| **narG** | 10⁸ | 1 | 3 | 5 | 10 | 11 | 14 | 15 | 20 | 21 | 22 | 23 | 24 | 24 b | 80 |
|         | 10⁶ | 1 | 3 | 5 | 7 | 8 | 9 | | | | | | | | | | | | | | | | | 9 a | 30 |
| **napA** | 10⁸ | 1 | 9 | 15 | 16 | 19 | 20 | 21 | 22 | | | | | | | | | | | | | | | 22 b | 73.33 |
|         | 10⁶ | 1 | 2 | 3 | 4 | | | | | | | | | | | | | | | | | | | | | 5 | 16.67 |
| **torC/dmsA** | 10⁸ | 1 | 4 | 7 | 12 | 17 | 22 | 23 | | | | | | | | | | | | | | | | 24 25 | 25 a | 83.33 |
|         | 10⁶ | 2 | 4 | 9 | | | | | | | | | | | | | | | | | | | | | | | | 9 a | 30 |
| **narG/napA** | 10⁸ | 2 | 5 | 11 | 17 | 20 | 21 | 22 | | | | | | | | | | | | | | | | | | | | 23 | 24 | 24 b | 80 |
|         | 10⁶ | 1 | 4 | 8 | 11 | 12 | | | | | | | | | | | | | | | | | | | | | | | | 14 b | 46.67 |
| **narZ/napA** | 10⁸ | 3 | 11 | 19 | 22 | 25 | 26 | 27 | 28 | 29 | | | | | | | | | | | | | | | | | | | 29 a | 96.67 |
|         | 10⁶ | 1 | 5 | 8 | 10 | 14 | | | | | | | | | | | | | | | | | | | | | | | | | 15 | 15 b | 50 |
| **None*** | 10⁸ | 2 | 4 | 10 | 18 | 21 | 24 | 27 | 28 | 29 | | | | | | | | | | | | | | | | | | | 29 30 | 30 a | 100 |
|         | 10⁶ | 4 | 12 | 15 | 17 | 18 | | | | | | | | | | | | | | | | | | | | | | | | | | | 18 b | 60 |

*Group of birds infected with the original strain of SGNal; Different letters to the same dilution between mutant and wild strains are significant by Chi-Square test (p<0.05).

In the present work mutants of SG containing defective genes related to anaerobic respiration were prepared. These mutants were inoculated orally in 5-day-old chickens susceptible to clinical fowl typhoid. The results are reported in tables 2 and 3. The mortality in groups of birds inoculated with SG ΔnarG, SG ΔfrdA, SG ΔnapA, SG ΔtorCΔdmsA a strain was lower in comparison with that inoculated with the wild SG strain (p < 0.05%). Conversely, SG ΔdmsA provoked higher mortality in comparison to the wild strain (p < 0.05%).

When the defective mutant did not alter the course of the disease it is possible to speculate in two ways: the activity of the altered gene did not interfere in the enzyme performance or the enzyme, if inactivated, is not essential either because the microorganism may produce other enzyme with similar activity or the host cell has several substrates available. According to Moreno-Vivían & Ferguson (32) the enzymes may have distinct activity under different metabolic conditions. The assimilatory and dissimilatory vias could be connected to facilitate the rapid adaptation during the changing from aerobic to anaerobic ambient, increasing the chance of surviving in the host. Perhaps, for this reason, some mutants continued expressing the same profile even after the insertion of the defective gene.

Members of the Enterobacteriaceae express three different nitrate reductases (3, 71) encoded by narGHJI (Nitrate reductase A), narUZYWV (15) that appears to be expressed at low levels and a periplasmic reductase encoded by napFDAGHBC with NapA as the catalytic unit (33, 44). The mid-point potentials (E_m,7) of electron donor and acceptor couples to nitrate reductase have been determined. Thus E_m,7 for NO₃⁻/NO₂⁻ is +433 mV (45). This is likely to provide a crude estimation of the redox conditions at the site of multiplication within the macrophage, which is thought
generally to be the acidified phagolysosome (1). Thus, in splenic macrophages and Kupfer cells the redox conditions are quite high, which must reflect the conditions experienced by most bacterial pathogens that multiply in this site in the chicken, such as *Mycobacterium avium*. This may not be the case for other animals including mammals and this may represent a basis for determining the host specificity of different *Salmonella enterica* pathovars.

The mid-point potential for the other electron donor/acceptor couples involved are +30 mV fumarate/succinate (45), +160 mV for \((\text{CH}_3)_2\text{SO}/(\text{CH}_3)_2\text{S}\) (48) and +130 mV for \((\text{CH}_3)_3\text{N}/(\text{CH}_3)_2\text{NH}^+\) (3, 25, 26). These values are considerably lower than the value for nitrate/nitrite above or for oxygen (+818 mV, 45). This suggests that respiration within the intracellular environment of the macrophage, where cytochromes o- and d-oxidase and now nitrate reductase are actives and mutation of which attenuates virulence, occurs under redox conditions which are considerably more positive than the environments where fumarate, DMSO and TMAO or related compounds are likely to be active. This suggests that these may be active in the intestinal phase of the infection which, despite the fact that typhoid serovars such as SG colonise the gut poorly in the absence of clinical disease, do have an active early intestinal phase to systemic infection (38). The redox conditions are highly likely to be much more negative and further investigations to evaluate their role would be of value. Despite of these enzymes are not so efficient in regard to catabolism like nitrate reductases, the lower mortality would be due the decrease in the production of essential substances. In *Wolinella succiogenes* fumarate reductase enzyme is implicated in generation of ATP (35), to create protons gradient through citoplasmatic membrane (11, 27). Also the fumarate would be the most important source of succinate in *Bacterioides fragilis* (8, 28) and finally, bacterium like *E. coli* requires succinate for succinyl-coA synthesis. Succinyl-coA is an essential cofactor in biosynthesis of methionine, diaminopimelic acid and lysine (9, 14).

According to the experimental model adopted when the challenge was done with diluted culture of the strain containing single mutation in *narG*, *frdA* and *napA* genes or double mutation in *torC* and *dmsA* the mortality was lower than that observed in control group. On another hand single deletion in *narZ*, *dmsA e torC* genes did not interfere in the relation between SG and the birds.

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