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Adaptation to the chicken intestine in *Salmonella* Enteritidis PT4 studied by transcriptional analysis

A.A. Dhawi [1], A. Elazomi, M.A. Jones, M.A. Lovell, H. Li, R.D. Emes, P.A. Barrow [*]

School of Veterinary Medicine and Science, University of Nottingham, Sutton Bonington, Leicestershire LE12 5RD, UK

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ABSTRACT

The transcriptional changes that occurred in *Salmonella enterica* serovar Enteritidis during colonization of the alimentary tract of newly hatched chickens were studied. A whole genome oligonucleotide microarray was used to compare the expression pattern with that from bacteria cultured in nutrient broth *in vitro*. Amongst other changes Salmonella Pathogenicity Island (SPI)-1, SPI-2 and SPI-5 genes were up-regulated *in vivo* suggesting a close association with the mucosa during colonization. Particular attention was paid to genes associated with metabolism of dicarboxylic acids and to responses to high osmolarity. Association between the colonization phenotype and gene mutations indicated that the latter was more important as a contribution to the colonization phenotype.

1. Introduction

The alimentary tract of animals is a major site for microbial colonization and interaction with the host immune system. At birth and hatching the gut of mammals and birds respectively is sterile but is very rapidly colonized by aerobic and facultatively anaerobic bacteria including members of the Enterobacteriaceae and lactic acid bacteria including streptococci and lactobacilli (Smith, 1965). A succession of waves of colonization occurs resulting in the establishment of and numerical predominance by obligate anaerobes (Smith, 1965). The exact site of localisation by particular groups of bacteria depends on diet and associated anatomical specialisation. Thus the rumen of some herbivores is the major site of colonization whereas in most mono-gastric animals it is the caeca and colon (Drasar and Barrow, 1985).

The presence of anaerobes in the caecal lumen of monogastric animals and poultry at microbial densities of greater than Log_{10} 11 per gram contents implies reduced availability of oxygen and nutrients for other bacteria. This suggests that for enteric pathogens such as *Escherichia coli*, *Salmonella enterica* or *Campylobacter jejuni* to establish and colonize the gut of animals which already have an established gut flora these organisms must either employ completely different metabolic pathways which do not compete with the predominant anaerobes or they must colonize a separate niche not colonized by the anaerobes. *C. jejuni* appears to do the former since it is able to colonize at relatively high bacterial densities of between Log_{10} 9–10 per gram (Jones et al., 2004) and the patterns of gene expression *in vivo* indicate much greater flexibility in electron transport utilisation in comparison with the Enterobacteriaeae (Woodall et al., 2005). In contrast it seems likely that organisms such as *Salmonella* and *E. coli* preferentially colonize the region of the gut close to the mucosa where nutrients and oxygen may be present at higher concentrations as a result of which obligate anaerobes are unable to colonize (Poulsen et al., 1995; Barrow et al., unpublished data). Their densities in gut contents are generally much lower than during colonization of new-born animals (Barrow et al., 1988).

The exact mechanism whereby enteric pathogens colonize the gut of livestock is still relatively poorly

* Corresponding author. Tel.: +44 115 951 6428; fax: +44 115 961 6415.
E-mail address: paul.barrow@nottingham.ac.uk (P.A. Barrow).
[1] Present address: Faculty of Veterinary Medicine, Alfateh University, Tripoli, Libya.

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understood although a number of studies using mutational analysis have indicated a role for interactions between the pathogens and the host through the involvement of fimbriae and Salmonella Pathogenicity Island (SPI) genes and patterns of metabolism which are different to those expressed *in vitro* (Turner et al., 1998; Morgan et al., 2004; Pullinger et al., 2008). Other adaptations to the gut likely to be important include the response to a different osmotic environment (Mishra et al., 2003; Liu et al., 2009), response to temperature stress and a flexibility in utilisation of available electron acceptors, indicated by the complex modular arrangement in respiration in these bacteria (Gennis and Stewart, 1996). The importance of defined carbon sources, including gluconate, during colonization has been shown by Conway and his colleagues (Chang et al., 2004; Fabich et al., 2008).

Modelling intestinal colonization by enteric pathogens generally involves oral inoculation of animals with individual strains or pools of mutants. Studies on gene expression with this model are more intractable since expression by a numerically inferior microbial species will be confused by RNA from more dominant members of the flora, unless the GC ratio of the genome is sufficiently different such that interference during hybridisation does not occur, as with *C. jejuni* (Woodall et al., 2005). For this reason colonization of new-born mammals or newly hatched chickens has also been used as a model for investigating gene expression (Barrow et al., 1987; Zhang-Barber et al., 1997; Lovell and Barrow, 1999) which, although the conditions present do not reflect those in the adult gut as a result of the absence of a mature, complex gut flora, it does represent one aspect the situation that occurs in the real world, namely the establishment of pathogens in the gut of new-born animals or in poultry hatcheries where rapid establishment and extensive dissemination amongst the population can occur sometimes with severe clinical repercussions.

In this study we have investigated colonization of the chicken alimentary tract by *Salmonella* Enteritidis P125109 (www.sanger.ac.uk/Projects/Salmonella) using a whole genome microarray. Colonization of newly hatched chickens was used and the role of selected genes in this model studied by site-directed gene inactivation. Arrays are being increasingly and successfully used to monitor microbial population structure and host and pathogen behaviour during infection (Eriksson et al., 2003; Snyder et al., 2004; Bower et al., 2009; Rajilić-Stojanović et al., 2010; Nielsen et al., 2010).

2. Materials and methods

Chick colonization and sample collection. One hundred fertile commercial broiler eggs were hatched in pre-fumigated incubators. The chickens were infected within 12 h of hatching, orally by gavage with 0.1 ml of the *S.* Enteritidis PT4 culture grown for 18 h in nutrient broth (NB) at 37 °C in a shaking incubator (150 rpm) and diluted to contain 10^7 CFU/ml. At 16 h post-infection the birds were killed individually and the caecal contents from three birds were collected separately and stored on ice for viable count estimations. The caecal contents from the remaining birds were removed and mixed with RNA Protect (Qiagen), followed by pooling and centrifuging at $5000 \times g$ for 10 min at 20 °C prior to RNA extraction using RNeasy mini columns (Qiagen).

The experiment was repeated three times. Viable count estimations were made by plating decimal dilutions on MacConkey agar to allow the presence of any contaminating colonies amongst the predominant non-lactose fermenting *Salmonella* to be detected.

2.1. In vitro culture

For *in vitro* control cultures, 2 ml of an overnight NB culture of *S.* Enteritidis was inoculated into 200 ml of pre-warmed NB and incubated with shaking (150 rpm) for 2 h at 37 °C. Cultures were pre-treated with RNAProtect before being centrifuged at $5000 \times g$ for 10 min at 20 °C prior to RNA extraction.

2.2. RNA processing and transcriptional analysis

The extracted and purified RNA from the 3 pooled *in vivo* samples and 3 *in vitro* samples was amplified using the MessageAmp II kit (Ambion) incorporating aminoallyl UTP labelling using Cy5 (Amersham). RNA extracted from *in vitro*-cultured bacteria was amplified as above with labelling with Cy3.

Changes in intestinal bacterial gene expression were evaluated using whole genome oligonucleotide DNA microarrays using a commercially available array design system (Agilent).

The fully annotated sequence of *S.* Enteritidis P125109 (Thomson et al., 2008) formed the basis of our custom microarray design. All the ORFs predicted previously were regarded as potentially transcribed genes, thus were designated for probe design. For designing microarray probes on the transcriptome sets created, the web-based Agilent eArray system (Agilent Technologies, https://earray.chem.agilent.com/earray/) was used with the following settings during the microarray probe design: Tm (70 °C) matching methodology, 60-mer probe length, 3 probes/target. The protocol, experimental setup, RNA extraction, amplification, labelling and hybridisation are described in detail at http://www.ebi.ac.uk/arrayexpress/ (Roy et al., 2007). Data analysis was done using GeneSpring GX 10.0 (Agilent).

Quanititative RT-PCR. Gene expression was measured by quantitative real-time PCR (qRT-PCR) using the Light Cycler 480 System (Roche Applied Science, UK) for 96 well plates. The sense and anti-sense primers of these genes (Table 1) were designed using the universal probe library assay design centre (Roche Applied Science, UK) available at: https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000. RT-PCR was performed using the Light Cycler 480 Probes Master kit (Roche Applied Science, UK) with the following cycle profile: one cycle at 95 °C for 10 min, 45 cycles at 95 °C for 10 s, 60 °C for 30 s and 72 °C for 1 s and one cycle at 40 °C for 30 s. The RT-PCR experiment contained three no-template controls and test samples, the *in vitro* sample was considered as control and the *in vivo* as treatment with the experiment being

Table 1
Primers used in quantitative real-time-PCR.

Gene ID	Gene symbol		Primer sequence
SEN 1762	*gap*A	Sense	CGTATCGGTCGCATTGTT
		Antisense	TTCGTCCCATTTCAGGTT
SEN 1304	*yci*E	Sense	CATTTCTCGTTCGGTGTT
		Antisense	AATTGTTCCGTTGTCTGC
SEN 0215	*cda*R	Sense	CGGCGAACCAGAGCATCT
		Antisense	ATCCCAGCGACCAAACGA
SEN 2725	*sip*C	Sense	GTCTTCCAGTGCCGTTGC
		Antisense	GTGGCTTTCAGTGGTCAGTTTA
SEN 0073	*cai*B	Sense	GAAACGGGTAAAGGTGAAAG
		Antisense	GCAACCAGCGTAGTAGGG
SEN 0221	*map*	Sense	CCGAAGTGCTGGAAATGA
		Antisense	GAGGTATCGCCGTGGAAT
SEN 0375	*rdg*C	Sense	AGATTCCCTGAAGGATGAAGT
		Antisense	AACCCTGAGCGACCGTAC
SEN 2278	*ais*	Sense	GTGCTGGCATTTACCCTA
		Antisense	GGCGGAATAACACGACTA
SEN 2079	*udg*	Sense	TGGTGGCGTTAGACATTG
		Antisense	CACGATACGGGAGGGATA

carried out three times. A standard $\log_{10}$ dilution (10, 100 and 1000) series were performed for each combination of probes and primers for generation of standard curves and determination of PCR efficiencies. Normalized values were determined using the advanced relative quantification method (Pfaffl et al., 2002) using Light Cycler 480 analysis software.

2.3. Mutation method

The significance of these genes in colonization was tested by site directed mutagenesis using the lambda-Red system (Datsenko and Wanner, 2000). Mutations were further transduced to a fresh parental background using P22 HT*int* (Barrow et al., 1990) to ensure single loci were mutated.

2.4. In vivo colonization assay in chicken gut

For the colonization studies groups of 10 chickens were inoculated orally within 24 h of hatching with 0.1 ml of a 24 h NB culture of a nalidixic acid-resistant (Nal^R) mutant of the metabolic mutant to be tested. Chickens were then given access to water and food. One day later 3 birds were killed and the viable numbers of the inoculated strain were estimated by decimal dilution and plating aliquots on Brilliant Green agar containing 20 μg/ml sodium nalidixate to ensure that they had colonized sufficiently. Simultaneously, the remaining 7 birds were inoculated orally with Log_{10} 5 in 0.1 ml of a spectinomycin-resistant (Spc^R) mutant of the parent strain from which the metabolic mutant was derived. Two days later all birds were killed and both Spc^R and Nal^R mutant were counted on Brilliant Green containing either spectinomycin (50 μg/ml) or sodium nalidixate (20 μg/ml). Two additional control groups were included in which the challenge strain alone was inoculated or in which the first strain was a Nal^R mutant of the parent challenged with a Spc^R mutant of the parent strain.

3. Results

In the three chicken experiments the numbers of *Salmonella* were between Log_{10} 8.95 and 10.20 per gram caecal contents and lactose fermenters or other colony types were not detected ($<\log_{10} 2$ per gram). It was thus clear that any signals from the array analysis would be derived solely from the *Salmonella* organisms.

Changes in expression in general and virulence genes. The pattern of gene transcription was very different in the intestine compared with broth culture. The results represent the mean of the results obtained by comparing the 3 *in vivo* samples with the 3 *in vitro* samples. Of the 4380 genes represented on the microarray 1467 (33%) genes showed a significant change in the level of expression greater than 2 fold with 2863 genes (67%) showing no change in expression or no expression at all. 714 genes were significantly up-regulated by a factor of greater than 2 fold and 753 down-regulated in the intestine. The genes were grouped by COGs classification and are shown in Fig. 1. This overarching classification indicated major changes occurring from adaptation to the caecal environment with up-regulation of genes required for energy generation, carbohydrate metabolism and transport, protein turnover, including chaperones and signal transduction and down-regulation of amino acid and nucleotide metabolism, translation, replication and cell wall biogenesis.

There were indications of a mixed metabolism with a number of carbon sources including glucose as indicated by the large number of genes associated with the PTS system, and genes associated with C4 carbohydrates, fumarate, gluconate, fucose, sialic acid, ethanolamine and 1,2-propanediol and unusual carbon sources such as allantoin (*allPBCD*), in addition to peptidases (*pepT*, *ptrB*) although this may also be a sources of raw amino acids.

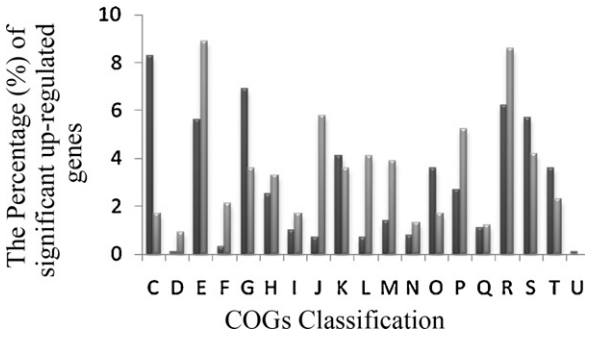

Fig. 1. The percentage of significantly up-regulated genes (fold change > 2) of *S.* Enteritidis during colonization *in vivo* (black columns) and *in vitro* (grey columns) classified according to the Clusters of Orthologous Groups of genes/proteins (COGs) functional categories. C, Energy production and conversion; D, cell cycle control, mitosis and meiosis; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; J, translation; K, transcription; L, replication, recombination and repair; M, cell wall/membrane biogenesis; N, cell motility; O, posttranslational modification, protein turnover, chaperones; P, inorganic ion transport and metabolism; Q, secondary metabolites biosynthesis, transport and catabolism; R, general function prediction only; S, function unknown; T, signal transduction mechanisms.

Associated with these last two carbon sources was up-regulation of genes associated with cobalamin biosynthesis (15 *cbi* genes) and tetrathionate as an electron acceptor (*ttrSABC*) in addition to other electron acceptors including nitrate (*narHIJKV*, *napABDFGH*) thiosulphite (*phsABC*), hydrogen (*hyaA-E*, *hybA-G*) and DMSO (*dmsABC*) although a number of oxidoreductases both putative and *cyd* genes were also up-regulated.

A number of genes associated with stress including higher temperature *clpABCP*, *hscC*, *dnaK*, *csiE*, *rpoS*, *rpoH*, *uspAB*, *ibpAB* and *mopB* were up-regulated in addition to cold shock genes *cspCDE*. A number of toxin efflux/inactivation systems were also operational including the multiple antibiotic resistance genes *marRAB*, thioredoxin (*trxC*) and glutathione S-transferases. Surprisingly, given the likely low redox state in the caeca, increased-expression of oxidative stress response elements (*soxRS*, *katE*, *sodC*) and phage encoded genes was observed.

Many surface antigens, including OMPs (*ompS*, *nmp*), LPS synthesis enzymes (*rfaBCGIJKLYZ*), flagellar components (*fliBDMNR*, *flgDG*) and certain fimbrial proteins (*bcfABH*, *stiB*, *stfC*, *safAB*, *stbADE*, *lpfDE*, *sthBDE*), were significantly down-regulated.

Amongst the dedicated virulence genes SPI1, SPI2 and SPI5 (Salmonella Pathogenicity Island) genes were up-regulated 3–17, 3–20 and 9–12 fold respectively compared with *in vitro* levels of expression whilst SPI3 genes were down-regulated and SPI4 genes were unchanged.

Summary tables containing the normalized GE data of these functional groups can be found in the Supplemental material (Tables S1–S3).

3.1. Microarray validation

The array data was validated by quantitative RT PCR of 8 selected genes which showed a wide range of expression from the microarray results. The correlation between the two sets of data was very good (Fig. 2). R^2 was 0.95 again indicating a good fit.

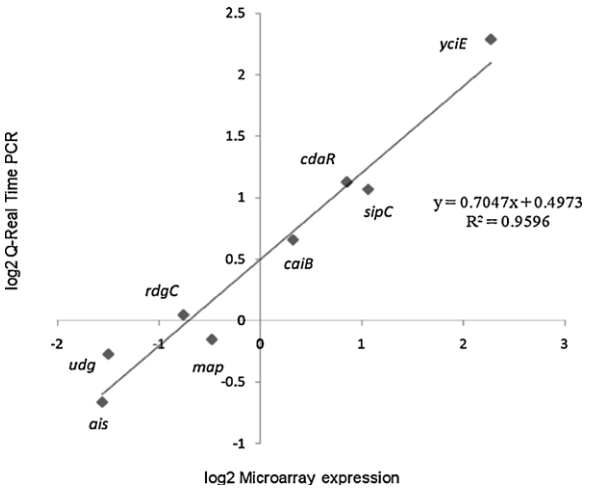

Fig. 2. Correlation between microarray and real-time RT-PCR expression values. Log 2-transformed expression values for 8 genes from total bacterial RNA extracted from day old chick cecal contents in triplicate. The best-fit linear regression line is shown together with the R^2 value and calculated equation for slope.

3.2. Adaptation to high osmolarity is a more important factor in intestinal colonization than availability of individual electron acceptors

Two areas of particular interest were carbon source utilisation and respiration and the effect of osmotic pressure on colonization. These are both likely to be amongst the factors present in the intestine which modulate gene expression and to which bacteria are required to adapt to be able to colonize the intestinal niche.

The genes associated with metabolism of dicarboxylate units and related functions with the pattern of changes in transcription *in vivo* compared with that *in vitro* are shown in Table 1. There was consistent up-regulation of expression in the intestine with the greatest changes observed with genes associated with respiration using fumarate as terminal electron acceptor (*frdABCD*) with increases in expression of between 13 and 17 fold.

The significance of these genes in colonization was tested by site directed mutagenesis. Mutants were assessed for their ability to colonize the gut of newly hatched chickens and for their ability to inhibit the establishment in the gut of the parent strain inoculated 24 h later. All mutants tested were able to colonize the gut well with viable bacterial numbers in the caeca of $>\mathrm{Log}_{10}8$ per ml caecal contents. The viable numbers of the challenge strain in the caeca of birds which had been previously inoculated with mutants defective in particular enzymes associated with the TCA cycle are shown in Fig. 3. It is clear that all mutants inhibited growth of the parental challenge strain to the same extent that the parent strain itself did. The chickens inoculated with broth only before challenge (negative controls) showed no detectable Nal^R *Salmonella* (which is literally counts of less than Log 2 per gram contents, which is the limit of quantification with this method, as shown in Fig. 3 and in the experiment shown in Fig. 4).

Similar experiments were carried out using mutations in genes which are known to affect the ability to respond to

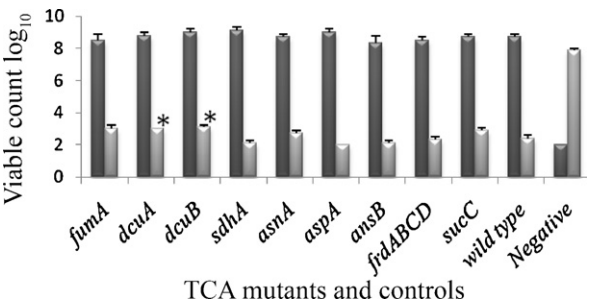

Fig. 3. The effect of intestinal pre-colonization of newly hatched chicks with wild type *S.* Enteritidis (Nal^R) or individual TCA-generated mutants on the caecal colonization by a subsequent challenge with wild type parent given orally (1.8×10^5 cells) 24 h later. Counts were made 48 h post-challenge; pre-colonized strain (black columns); challenge (grey columns). One asterisk (*) indicates significant difference between mutants ($P < 0.05$) and the wild type by Student's *t* test.

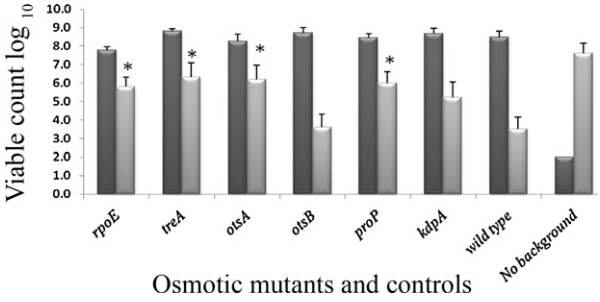

Fig. 4. The effect of intestinal colonization of newly hatched chicks with wild type *S.* Enteritidis (NaI^R) or individual osmotic-mutants on the caecal colonization by wild type parent inoculated orally (1.8×10^5 cells) 24 h later. Readings were taken 48 h post-challenge; pre-colonized strain (black columns); challenge (grey columns). Mean and SE of 7 birds. One asterisk (*) indicates a significant difference between mutants ($P \leq 0.05$) and the wild-type by Student's *t* test.

changes in osmotic pressure. It was clear that several of these genes were highly significantly up-regulated in the chicken caeca indicating a significant role in colonization. When tested in the *in vivo* competition experiment the parent strain and *otsB* mutant both inhibited the challenge strain whereas the remaining mutants were less able to do so (Fig. 4).

4. Discussion

It was clear that *S.* Enteritidis behaved very differently in the caeca of the newly hatched chicken in comparison with a mid log phase nutrient broth culture. The caeca are the main site of colonization, where bacterial numbers are highest in chickens of any age and it is tempting to associate up-regulation of sets of genes with a role in colonization.

The *Salmonella* organisms were harvested largely from the caecal lumen although it is highly probable that a mixed population was present in the sample taken, including bacteria which had been in close proximity to the mucosa where oxygen levels, antimicrobial peptides and selected nutrients will be present in higher concentrations.

We were content that the array data represented true levels of expression since there was an excellent correlation with levels of expression quantitated by qRT-PCR with a handful of genes selected which spanned the range of expression levels.

Despite the fact that the sample may have represented a slightly heterogeneous population, colonization of the caeca was clearly associated with a number of changes in metabolism with a wide range of carbon sources, some of which (sialic acid, ethanolamine, propanediol) will have resulted from degradation of cellular material from the host mucosa in addition to a range of electron acceptors ranging from oxygen, probably at low redox values (Jones et al., 2007), perhaps occurring in bacteria closer to the mucosa but also nitrate, thiosulphite and tetrathionate which may result from sulphurous material arising from the yolk sac which remains as a source of nutrients for a few days after hatching. The involvement of *sucABCD* suggests that the TCA cycle was showing anaerobic behaviour and acting in a non-cyclic manner supporting the contention that the caeca is a low redox environment even in newly hatched chickens.

Peptidases were also up-regulated although this may be a requirement for nitrogen from the amino acids themselves rather than a source of carbon. Allantoin was an interesting carbon source, in all probability derived from the yolk sac. This has been found to contribute to colonization and to virulence in *S.* Typhimurium for the chicken and mouse (Matiasovicova et al., 2011).

It is hardly surprising that with a body temperature of 41.5 °C, heat shock proteins indicating increased protein turnover should be more highly expressed. This is interesting because it does suggest that in evolutionary terms, birds and especially the domestic fowl is not a natural host for *S.* Enteritidis. It would be interesting to determine whether this is also true for the poultry adapted typhoid serovars, *S.* Gallinarum and *S.* Pullorum.

The involvement of SPI genes confirms previous mutational studies (Turner et al., 1998; Morgan et al., 2004) which suggested an intimate relationship with the mucosa during colonization. How this explains *in vivo* behaviour is difficult to see. Up-regulation of SPI-1 genes in the gut is not surprising since temperatures above environmental are known to increase expression of secreted SPI-1 effector proteins (Wood et al., 1996) although the presence of bile salts are thought to suppress SPI-1 gene expression (Papezova et al., 2007) and other signals present in the gut, including pH, osmolarity and oxygen tension also affect expression (Altier, 2005). Expression of SPI-5 genes is intimately associated with SPI-1 and suggests that in the same way that some of the SPI-5 effector proteins are associated with enteritis in mammals this may also contribute to the general diarrhoea in birds infected with Salmonella. Increased expression of SPI-2 genes was unexpected since these are expressed intracellularly, inside the macrophage-monocyte series and induced by factors including stationary-phase conditions, low pH, and Mg^{2+} and phosphate concentrations (Coombes et al., 2004; Papezova et al., 2007; Hensel, 2000; Xu and Hensel, 2010; Fass and Groisman, 2009). There was some evidence of exposure to oxidative stress although this itself was unexpected in an environment which is thought to have a low redox value. However, we speculate that a pre-requisite for colonization of the chicken caeca is a close association with the caecal tonsil and perhaps even involving an intracellular phase. The caecal tonsil has close and tight control over the material and bacteria that enter and exit the caeca and an early intracellular phase as a pre-requisite to caceal colonization may occur.

Up-regulation of genes associated with fumarate metabolism and response to higher osmolarity suggest that these characteristics could be important in colonization of the caeca. Assessment of colonization ability was difficult since many microorganisms, which are normally unable to colonize the gut of the adult birds possessing a full and complex gut flora, are nevertheless able to colonize the gut of the newly hatched chicken, presumably because the inhibitory floral components which suppress growth are absent in the newly hatched bird (Barrow et al., 1988). However, we have used microbial competition between

related bacterial strains in the intestine of the newly hatched chicken as a model to determine colonization fitness in this niche (Zhang-Barber et al., 1997; Methner et al., 2011). It was clear that the genes affecting dicarboxylate metabolism did not compromise the ability of *Salmonella* to colonize the caeca of the chick nor their ability to inhibit the establishment of the parental challenge strain. Given the amount of redundancy in carbon source utilisation also suggested by the use of a wide range of carbon sources in the gut from the array data and from suggestions from others that this is a characteristic of colonization ability (Fabich et al., 2011) it is perhaps not surprising that mutation of genes affecting a single set of carbon sources does not markedly affect colonization and it is difficult to know how much colonization would be affected by mutation affecting any one carbon source.

A number of genes associated or thought to be associated with responses to high osmolarity were both highly up-regulated *in vivo* and reduced competitiveness *in vivo*. Osmolarity has been found previously (Dorman et al., 1989; Ni Bhriain et al., 1989; Bower et al., 2009) to be important in gut colonization although this has not previously been reported for the chicken. The RpoE Sigma factor regulates a number of microbial characteristics associated with normal physiology including flagellation (Du et al., 2011; McMeechan et al., 2007), starvation and cold shock (McMeechan et al., 2007) and also virulence gene expression (Osborne and Coombes, 2009). The turgor control model for osmotic regulation suggests that turgor loss induces both the Kdp transport operon and the proline-glycine betaine transport system which would affect both *kdpA* and *proP* (Balaji et al., 2005). Mutants which are *treA* are unable to catabolise trehalose under conditions of high osmotic stress indicating a role for trehalose catabolism under the conditions found in the gut (Giaever et al., 1988). However, trehalose appears to be involved not only in the osmotic stress response but also in stationary-phase thermotolerance (Hengge-Aronis et al., 1991; Strøm and Kaasen, 1993). The phenotypes of *otsA* and *otsB* (trehalose phosphate synthase), which exist in a single operon, are thought to be identical in their osmotic sensitivity in glucose mineral medium (Giaever et al., 1988) but there are many environmental signals in the intestine which may induce these genes and their expression under the complex conditions in the intestine would be worth further study.

Although we have been able to present no more than a snapshot here, it is clear that the pattern of gene expression *in vivo* was very different to that observed in a mid-log phase NB culture. This is hardly surprising given the differences in temperature, osmolarity, proximity of host tissue with its many innate immune factors, plus availability of very different panoply of carbon nitrogen sources and other nutrients. This has been found previously both for the gut and other host environments during infection (Nielsen et al., 2010; Bower et al., 2009; Eriksson et al., 2003).

In the intestine different forms of competition between bacterial strains have been shown to take place between related and unrelated bacterial genera (Barrow et al., 1987; Berchieri and Barrow, 1991) and although the use of stationary phase cultures has been used to study the contribution of respiration genes to colonization it has been found to be inadequate (Zhang-Barber et al., 1997) and suggests that more detailed models both of bacterial competition and of colonization per se are required for more effective modelling.

Conflict of interest statement

There are no conflicts of interest in the work presented in this manuscript.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2011.07.013.

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