School *of* Veterinary Medicine and Science

Transcriptional Analysis of Intestinal Colonization by *Salmonella* Enteritidis PT4 in 1-Day Chickens using Microarray

By

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Abstract:

The recent association between *S.* Enteritidis PT4 and poultry products has caused a great deal of concern from adverse publicity and with resulting national and international requirements to control the major food-poisoning *Salmonella* serotypes at the breeder and layer levels in order to ensure that poultry products are *Salmonella*-free. The exact mechanism whereby these serotypes are able to colonise the intestine of chickens is still exactly unknown.

Indeed, there is increasing evidence that colonisation is not solely a metabolic function but that some form of physical association with cells or an organ in the gut is involved. Thus, invasion and fimbrial genes required for colonisation have been identified (Clayton *et al.,* 2008, Morgan *et al.,* 2004) suggesting physical contact was required.

An alternative approach would be to analyse the patterns of gene expression by microarray analysis at the site of colonisation (caeca). This has been done for a number of niches and is now being applied to intra-cellular infection but has not so far been applied to the intestine.

The *S.* Enteritidis transcriptome during the colonisation of the caeca of one day chicks was characterised by Agilent microarray. The microarray results were evaluated by real-time PCR with 96% compatibility. The pattern of gene transcription was different in the intestine compared with broth culture. Thirty four percent of the genes showed a significant change in level of expression. Major changes occured from adaptation to the caecal environment with up-regulation of genes required for energy generation and carbohydrate metabolism/transport, while amino acids and nucleotide metabolism, translation, replication and cell wall biogenesis genes were among the down-regulated genes.

Fumarate respiratory and osmotic response genes were selected from the up-regulated genes and were mutated and tested in the lab for their inhibitory effect and for competitive growth under anaerobic and osmotic environments showing variable responses. Association between chicken colonisation

phenotype and gene mutation indicated that genes associated with osmolarity was more important than tri carboxylic acid (TCA)-associated genes in their contribution to the colonisation phenotype.

There is considerable scope for improvement in inactivated vaccines through a more rational approach. An inactivated vaccine prepared by formalising *S.* Enteritidis harvested directly from the chicken caeca was thought to be more protective than bacteria grown *in vitro*. Unfortunately this was not the case. Expected reasons for this failure are explained, and alternative approach to producing a proper effective inactivated vaccine is suggested.

Declaration:

I declare that the work in this dissertation was carried out in accordance with the regulations of the University *of* Nottingham.

The work is original, except where indicated by special reference in the text and no part of the thesis has been submitted for any other academic award.

Name: *Abdulgader Dhawi Alfitouri*

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Abbreviations

Agilent spectrophotometer	AG
Amplified RNA	aRNA
Chloramphenicol	Cm
Coding sequences	CDs
Colony forming unit	cfu
Clusters of Orthologous Groups	COGs
Distilled water	DW
Escherichia coli	*E. coli*
Frequency of incorboration	FOI
Gastrointestinal tract	GI tract
Gene annotation list	Gal
Gene Expression	GE
In vitro Transcription	IVT
Kanamycin	Km
Lipopolysaccharide	LPS
Luria broth	LB
Minute (s)	min
NanoDrop	ND
Nutrient agar	NA
Nutrient broth	NB
Open reading frames	ORF
Overnight	o/n
Phosphate buffered saline	PBS
Quantitative Real time PCR	qRT-PCR
Room temperature	RT
Salmonella	*S.*
Salmonella containing vacuoles	SCV
Salmonella Pathogenicity Islands	SPI
Second (s)	sec
Tri carboxylic acid	TCA
Tris and EDTA	TE
Type 3 secretion system	T3SS

Chapter - 1**: General Introduction**

1.1 Overview on *Salmonella*

The *Salmonella* genus consists of the two species *S. enterica* and *S. bongori*. *S. bongori* is represented by 17 serotypes and incriminated in diseases of cold-blooded animals; while *S. enterica* contains more than 2,500 serotypes (Smith-Palmer *et al.,* 2003), which are implicated in a variety of diseases in warm-blooded animals such as typhoid-like diseases and gastroenteritis according to host species and the *Salmonella* serotype. The subspecies IIIa (*arizona*) of *S. enterica* is associated with diseases in cold blooded organisms, but rarely involved in human systemic infection (Wain *et al.,* 2001, Chan *et al.,* 2003, Blanc *et al.,* 1999).

Depending on *Salmonella* infection biology and pathogenesis, *Salmonella enterica* is divided into two groups of serotypes. One small group causes systemic typhoid-like diseases in a restricted range of host species for example *S.* Typhi in humans and *S.* Gallinarum or *S.* Pullorum in poultry (Table 1.1).

The second group comprises the remaining serotypes with a wide-host range. The best known ones are *S.* Enteritidis and *S.* Typhimurium, which are motile and capable of colonising the gut effectively. They rarely produce systemic or septicaemic disease in their host except in e.g. young chickens. Older chickens infected with *S.* Enteritidis often do not show any signs of disease at all. However *S.* Enteritidis and *S.* Typhimurium also produce typhoid in mice. Both *S.* Enteritidis and *S.* Typhimurium produce gastrointestinal infection in a range of hosts including humans (Uzzau *et al.,* 2000, Wigley *et al.,* 2001, Alokam *et al.,* 2002).

The *Salmonella* serovars Gallinarum, Pullorum and Enteritidis are of major economic significance regarding poultry disease. *S.* Enteritidis phage type 4 (PT4) together with *S.* Typhimurium definitive type 104 (DT 104) are the main cause of human zoonotic infections (Smith-Palmer *et al.,* 2003). The number of *Salmonella* infections that are of worldwide economic and health significance

has increased since the mid 1980s, and some EU countries reported a 20-fold increase in incidents during 1985-2000 (Smith-Palmer *et al.*, 2003).

S. Enteritidis is considered as a major cause of food-borne infection of man, with poultry and poultry products cited as major sources (Rampling *et al.*, 1989, Schmidt, 1995, Rabsch *et al.*, 2001, Gillespie *et al.*, 2005, de Jong and Ekdahl, 2006).

The UK national surveillance indicates that the number of human salmonellosis cases has declined since 1997. This decline is mainly attributed to the reduction in incidence of disease due to *S.* Enteritidis PT4 following the introduction of vaccines against *S.* Enteritidis in the majority of flocks in the UK egg industry (Cogan and Humphrey, 2003). In 2008, there were about 9800 reported cases of human salmonellosis in the UK, 4200 of which were associated with *S.* Enteritidis and 1800 were associated with *S.* Typhimurium (DEFRA, 2010).

1.2 Public Health Concern of non-host specific *Salmonella*

Human *Salmonella* infection continues to be a major concern, in terms of both morbidity and economic cost (Barnass *et al.*, 1989). In many countries, there was a marked increase in number of outbreaks of human salmonellosis between the mid-1980s and mid-1990s. The majority of this has been associated with *S.* Enteritidis, and because of its worldwide increase in human infection has been referred to as a pandemic (Rodrigue *et al.*, 1990). This serotype is mainly chicken-associated and the vehicles most commonly incriminated with human infection are contaminated chicken meat and eggs. There are about 50 phage types (PT) of *S.* Enteritidis. In the mid-1980s, when the pandemic salmonellosis with *S.* Enteritidis began among humans, there was a clear geographical distribution of these phage types. For example, *S.* Enteritidis phage type one (PT1) was common in Eastern Europe, *S.* Enteritidis phage type 4 (PT4) was dominant in Western Europe and *S.* Enteritidis phage type 8 or 13a (PT8 or PT13a) were the most common phage types in North America (Rodrigue *et al.*, 1990). Although *S.* Enteritidis PT4 continues to cause widespread infection among Western European countries (Schmidt,

1995), including England and Wales, it has spread to many countries other than those in Western Europe and it is becoming increasingly important in USA and Canada (Altekruse *et al.,* 1997). It is of interest that despite the widespread infection with *S.* Enteritidis PT4, the infection rate in different countries is variable (Anon, 2004).

Although *S.* Typhimurium DT104 has many general characteristics in common with *S.* Enteritidis PT4 (Humphrey, 1997) including highly invasive behaviour in chickens (Williams *et al.,* 1998, Leach *et al.,* 1999). *S.* Enteritidis PT4 is considered as the most significant *Salmonella* serovar as human and poultry pathogen. A study carried out in England and Wales showed that *S.* Enteritidis PT4 is more implicated in different varieties of food stuff than *S.* Typhimurium DT104 (Anon, 1997). The most common vehicles for *S.* Enteritidis PT4 in the period (1989-1996) were egg and egg dishes including desserts, poultry meat and their products, red meat and its products, fish and shellfish, salad/fruit/vegetables, sauces (again those sauces containing raw shell eggs) and milk and milk products. However, researchers (Garcia-Villanova Ruiz B, 1987) indicated that presence of *S.* Enteritidis in vegetables does not appear to constitute a major source of infection for humans. Nevertheless, the potential hazard of pathogenic bacteria in vegetables should not be underestimated especially in those eaten raw or only lightly cooked. In contrast the most common vehicles for *S.* Typhimurium DT 104 in the period (1992-1995) were poultry meat, red meat/meat products, milk/milk products and egg/egg dishes. Ministry of Agriculture, Fisheries and Food (MAFF) reports showed that infections with *S.* Enteritidis were uncommon in cattle and pigs (Anon, 1989).

In September 2009, the Department of Gastrointestinal, Emerging and Zoonotic infections at the Health Protection Agency (HPA) reported a marked increase in the number of non-travel human related outbreaks of infection with *S.* Enteritidis PT 14b with resistance to nalidixic acid and partial resistance to ciprofloxacin, most of which are linked to contaminated eggs (Janmohamed *et al.,* 2011). The investigations identified eggs imported from Spain used in the food-service sector as the main cause of increase (Gillespie, 2004, Fisher,

2004, Gillespie and Elson, 2005, (CDC). 2010, CDC., 2010). Before this marked increase of *S.* Enteritidis PT 14b infections (in Sep 2009), there had been other sustained increases in the incidence of *S.* Enteritidis non-PT4 infections in England and Wales between 2000-2004 (Gillespie, 2004, Gillespie and Elson, 2005).

The association between *S.* Enteritidis and table eggs has caused a great deal of concern from adverse publicity and with resulting national and international requirements to control the major food-poisoning *Salmonella* serotypes (*S.* Typhimurium and *S.* Enteritidis) at the breeder and layer levels in order to ensure that poultry products (meat and eggs) are *Salmonella*-free to avoid the risks associated with their consumption [Requirement of the Commission of European Communities "Directive 2003/99/EC, Regulation 2160/2003"; (Davies and Breslin, 2003)].

Most *Salmonella* food-poisoning outbreaks reported worldwide are the result of consumption of poultry products (meat and eggs) or their derivatives (Braden, 2006, Gantois *et al.*, 2009). This is because poultry represents a major source of cheap high quality protein for much of the world. The majority of reported information on infection is available from western countries and little is available from elsewhere (Khakhria *et al.*, 1983, Notermans and Hoogenboom-Verdegaal, 1992, Rabsch *et al.*, 2001, Daniels *et al.*, 2002, Cowden *et al.*, 2003, Lenglet, 2005).

In the USA, in the period between 1973-1987, 51% of human food-borne bacterial disease cases were caused by *Salmonella* (Bean *et al.*, 1990); while in England and Wales the percentage of *Salmonella* outbreaks that were associated with poultry meat consumption rose from less than 13% in the period between 1959-1962 to more than 32% in 1985-1991 (Humphrey, 2000). In Scotland, in the period 1980-1989, 84% of food-borne human illness was caused by *Salmonella* (Oboegbulem *et al.*, 1993).

In the EU *Salmonella* infects 160,000 individuals every year. The cost of food-borne *Salmonella* infections is estimated at up to €2.8 billion per year (EU, 2002). According to the USA Centres for Diseases Control and Prevention,

salmonellosis may affect as many as 1-5 million people annually in USA; about 20,000 hospitalizations and 500 deaths are associated with *Salmonella* and are reported annually (Potter, 1992). The Economic Research Services (ERS) of the US Department of Agriculture has reported the annual economic cost of *Salmonella* infections at up to $2.9 billion, and the number of cases infected with *Salmonella* is higher than EU countries as it was estimated up to 1.4 million cases during late 1990s (ERS, 2011). However, although many different measures have been recommended for the control of *Salmonella* in poultry, vaccination is likely to have a central role in the reduction of *Salmonella* in commercial poultry (Van Immerseel *et al.*, 2005). Research studies indicate that vaccines may reduce both the horizontal and vertical transmission of *Salmonella* (Hassan and Curtiss, 1990, Gast *et al.*, 1992, Gast *et al.*, 1993, Curtiss *et al.*, 1993, Hassan and Curtiss, 1994, van de Giessen *et al.*, 1994, Hassan and Curtiss, 1996, Curtiss and Hassan, 1996, Hassan and Curtiss, 1997, Woodward *et al.*, 2002, Holt *et al.*, 2003, Protais *et al.*, 2003, Nakamura *et al.*, 2004, Inoue *et al.*, 2008, Dorea *et al.*, 2010). Vaccination works by reducing the prevalence of *Salmonella* in breeder hens and their progeny (Gast *et al.*, 1992, Hassan and Curtiss, 1997, Inoue *et al.*, 2008) or by increasing the passive immunity of meat birds and blocking the horizontal transmission of *Salmonella* to broiler chickens (Truscott and Friars, 1972, Hassan and Curtiss, 1996, Inoue *et al.*, 2008). Therefore control programmes to limit *Salmonella* infections mainly in poultry are being developed in many countries (Hassan and Curtiss, 1990, Gast *et al.*, 1992, Curtiss *et al.*, 1993, Hassan and Curtiss, 1994, van de Giessen *et al.*, 1994, Hassan and Curtiss, 1996, Curtiss and Hassan, 1996, Van Immerseel *et al.*, 2002, Woodward *et al.*, 2002, Nakamura *et al.*, 2004).

1.3 Clinical Infections in Animals (Host-Specific *Salmonella*):

Salmonella enterica has the capacity to produce a variety of forms of systemic diseases, including typhoid-like infections, sometimes with disease-free tissue

carriage, septicaemia, abortion in addition to gastroenteritis and disease-free colonisation of the intestine.

A number of serotypes have been associated with abortion in farm animals, often without other obvious clinical signs in dams. The *Salmonella* serotypes of importance in domestic animals (most of which are host-associated serotypes) and the consequences of infection are indicated in Table 1.1.

Table 1.1: *Salmonella* serotypes of clinical importance and the consequences of infections

Salmonella Serotype	Hosts	Consequences of infection
S. Dublin	Cattle and sheep	Many disease conditions Enterocolitis and septicaemia
S. Choleraesuis	Pigs	Enterocolitis and septicaemia
S. Pullorum	Chicks	Pullorum disease (bacillary white diarrhoea)
S. Gallinarum	Adult birds	Fowl typhoid
S. Abortusovis	Sheep	Abortion
S. Enteritidis & Typhimurium	Poultry and mice	Systemic disease in young poultry Typhoid in mice

Host-specific serotypes are generally poor colonisers of the intestine, which may be related to the higher frequency of auxotrophy in these serotypes (Wilson *et al.*, 1964). They invade from the intestine and multiply in organs rich in macrophage-monocyte cell series such as liver and spleen. As disease progresses they re-enter the gut from the tissues in high numbers either through lymphoid tissue or by excretion from the gall bladder. Extensive colonisation is not a requirement for disease. Indeed, although serotypes such as *S.* Gallinarum and *S.* Typhi, in common with other serotypes, produce up to 12 different types of fimbriae, uptake is thought to be largely through the M cells of the Peyer's patches and related lymphoid tissue of the intestine, since *Salmonella* pathogenicity island (SPI) -1-mediated invasiveness is not a prerequisite for virulence in *S.* Gallinarum in chickens (Jones *et al.*, 2001) or for *S.* Typhimurium in mice (Murray and Lee, 2000). Nevertheless, fimbrial genes are known to be pre-requisite for the colonisation of the mucosa that does occur with these serotypes (Baumler *et al.*, 1997a, Rychlik *et al.*, 1998).

S. Gallinarum and Pullorum are the causative agents of two different disease syndromes, fowl typhoid and pullorum disease respectively which can be differentiated biochemically (Ryll *et al.*, 1996) and genotypically (Olsen *et al.*, 1996). These two diseases are mostly eradicated from the commercial poultry industry in many developed countries (Johnson *et al.*, 1992), but the occurrence of the disease in areas such as Eastern EU, Africa and South America, where the poultry industry is experiencing rapid expansion, remains high (Shivaprasad, 2000, Barrow and Freitas Neto, 2011). It is also re-appearing in those countries where extensive, free-range rearing is becoming more common. Fowl typhoid usually presents as septicaemia, affecting birds of all ages, mainly those over 3 months, while pullorum disease tends to be restricted to an enteric infection of birds under 6 weeks of age. The infection occurs via the faecal-oral route or by means of vertical transmission.

S. Abortusovis is also host-restricted since it only been recovered from ovine sources under natural conditions (Pardon *et al.*, 1988). It is one of the main causes of ovine abortions in Europe and western Asia (Jack, 1968.), where it represents a major pathological and economic problem in states with sheep-based economy. Ingestion of contaminated pasture with *S.* Abortusovis represents the most probable route of infection.

S. Choleraesuis and *S.* Dublin produce both enteritis and systemic infection mainly in pigs and cattle respectively. *S.* Choleraesuis colonisation of pig intestine and invasion of intestinal mucosa does not generally produce severe enteritis and it is followed by systemic dissemination (Roof *et al.*, 1992). *S.* Choleraesuis is defined as host-adapted on the basis that 99% of the incidents are associated with pigs. However, it does naturally infect other host species, including man, in which the infection can be severe (Huang and Lo, 1967).

S. Dublin is host-adapted and affects both young and adult cattle causing enteritis and/or systemic disease. Acute disease is characterised by fever, anorexia, and reduced milk yield followed by severe diarrhoea and high levels of mortality. Milder cases of disease sometimes occur resulting in pregnant cow abortion associated with diarrhoea. Other clinical manifestations of *S.*

Dublin may include typhoid-like illness, which often result in a chronic carrier state (Richardson, 1973). Natural infection with this serotype may also occur in other animals including man and, in particular, sheep and goats (Fierer and Fleming, 1983).

The molecular basis of host-restriction is poorly determined, and study in this topic is complicated by the complex pathogenesis of *Salmonella* infections.

1.4 Infection by non-host-specific serotypes

The exact mechanism whereby non host-specific serotypes such as *S.* Enteritidis, *S.* Typhimurium, *S.* Hadar, *S.* Infantis etc are able to colonise the intestine of chickens and pigs is unknown although recent studies have begun to identify genes associated with colonisation.

It is known that such serotypes, if inoculated experimentally orally into chickens, are excreted in relatively high numbers for several weeks before they are eliminated, with more invasive serotypes being cleared sooner than less invasive types, presumably as a result of a stronger immune response (Barrow *et al.*, 1988). Infection of adult birds results in excretion of smaller numbers of bacteria for shorter periods of time, whereas inoculation of very young birds results in very heavy excretion for much longer periods. The major reason for this age effect is the presence of a mature normal gut flora in adults which inhibits colonisation and forms the basis for protection through competitive exclusion (see section 1.9). It has been known for many years that the major sites of colonisation are the caeca (Sadler *et al.*, 1969, Brownell *et al.*, 1969). The reason for this is unclear. Earlier views were that adhesion to specific sites was important in preferential colonisation of this site, the localisation in the caeca by non-colonising microorganisms such as *Saccharomyces* sp., *Pseudomonas* sp. and *E. coli* K12 suggested a non-specific or host-related mechanism (Barrow *et al.*, 1988).

Searches for *Salmonella* genes required for colonisation have involved screening transposon mutant banks (Turner *et al.*, 1998, Morgan *et al.*, 2004).

The earlier of these exercises suggested regulatory genes (such as *dks*A) were involved, which suggested playing a role in the regulation of rRNA expression during stationary phase. In addition to a requirement for intact lipopolysaccharide (e.g. *rfa*Y), later work also suggested that a variety of metabolic and fimbrial genes are implicated. Indeed, there is increasing evidence that colonisation is not solely a metabolic function but that some form of physical association with cells or an organ in the gut is involved. Invasion genes such as *inv* and *sip*C genes, regulatory genes such as *dks*A, *clp*B (encoding for heat shock protein), and *hupA* are required for colonisation of *S.* Typhimurium in chickens (Porter and Curtiss, 1997, Turner *et al.*, 1998). *S.* Typhimurium defective in one of the fimbrial genes (*orf*7, *srg*A "*orf*8", *stb*, *fim*Z and *sth*) was attenuated in chicken intestinal colonisation (*orf*7, *srg*A "*orf*8", *stb*, *fim*Z and *sth*) compared to the wild type (Morgan *et al.*, 2004) suggesting physical contact might be required.

Fimbriae have been considered as obvious candidates for colonisation determinants but many studies have not found a clear association. Using experimental infections of epithelial cell lines and chicken and murine models researchers have found that *S.* Typhimurium possesses the ability to elaborate at least four kinds of fimbriae, including type 1 fimbriae, plasmid encoded fimbriae (*pef*), long polar fimbriae (*lpf*) and a curli orthologue (SEF17), each of which have been incriminated in the pathogenesis of chicken and mouse (Lockman and Curtiss, 1992a, Baumler *et al.*, 1996a, Baumler *et al.*, 1996b, Collinson *et al.*, 1996, van der Velden *et al.*, 1998). In addition a number of other gene clusters thought to encode fimbriae can be identified on *Salmonella* genome sequences (www.sanger.ac.uk/Projects/*Salmonella*). Type 1 fimbriae of *S.* Typhimurium are regarded as virulence determinants which have been found to play a role in colonisation of gut epithelia (Lockman and Curtiss, 1992b). *S.* Enteritidis has also been found to express many distinct fimbriae on its surface, including SEF21 "type 1 fimbriae" (Muller *et al.*, 1991), SEF14 (Thorns *et al.*, 1990), SEF17 (Collinson *et al.*, 1993), *stb* and *peg*A (Clayton *et al.*, 2008) besides possessing the genetic potential to elaborate a PEF analogue (Baumler and Heffron, 1995) and LPF (Baumler *et al.*, 1996c). The structure of

long polar fimbriae (*lpf*) has not been described in *S.* Enteritidis, while the serological response to plasmid-encoded fimbriae (*pef*) has been demonstrated *in vivo* (Woodward *et al.,* 1996). The *lpf* operon is thought to have been acquired by the genus *Salmonella* by way of horizontal gene transfer (Baumler, 1997). Furthermore, in other study it is shown that all *Salmonella* isolates that were able to cause lethal infection in mice possessed the *lpf* operon (Baumler *et al.,* 1997b). However, among 11 strains that were unable to cause lethal infection in mice, 7 also carried the *lpf* operon. These data show that like SPI 1, the *lpf* operon is present in, but is not limited to, mouse-virulent *Salmonella* serotypes. A clear association was also demonstrated between SEF21 (type 1 fimbriae) of *S.* Enteritidis, when expression of these fimbriae was induced prior to cell epithelia infection (Dibb-Fuller *et al.,* 1999); while a SEF14 mutant of the same bacterium showed no significant difference in either association or invasion compared to their respective wild type (Thorns *et al.,* 1996, Dibb-Fuller *et al.,* 1999).

Moreover, *S.* Enteritidis SEF14 and SEF17 fimbriae have been shown to adhere to even inanimate surfaces (Woodward *et al.,* 2000). SEF14, SEF 17 and SEF 21 (type 1 fimbriae) fimbrial proteins have been reported to mediate *S.* Enteritidis attachment by binding to the human epithelial cells (Muller *et al.,* 1991, Collinson *et al.,* 1993). SEF14 and SEF21 fimbriae have also been shown to contribute to the bacterial adherence and invasion into culture cells (Peralta *et al.,* 1994, Ogunniyi *et al.,* 1997, Dibb-Fuller *et al.,* 1999). However, the role of *S.* Enteritidis fimbriae (SEF) in poultry infection is ambiguous (Allen-Vercoe and Woodward, 1999a, Allen-Vercoe *et al.,* 1999, Allen-Vercoe and Woodward, 1999b, Dibb-Fuller *et al.,* 1999).

As for flagella, apart from motility, the role of *S.* Typhimurium *S.* Enteritidis flagella in pathogenesis is also ambiguous. Two studies reported that *S.* Typhimurium deficient in both flagella and SEF21 were greatly reduced in its ability to invade epithelial cells *in vitro* and persist in the liver and spleen of orally challenged chicks, while mutants defective for the elaboration of either flagella or fimbriae alone showed no significant effect (Barrow *et al.,* 1988,

Lee *et al.,* 1996). Aflagellate mutants of *S.* Enteritidis associated and invaded significantly less than motile bacteria (Dibb-Fuller *et al.,* 1999). This study also indicated that it is most likely of epithelial cells by enabling motility rather than providing adhesion. Others reported that the direction of flagellar rotation affected the ability of *S.* Typhimurium to invade cultured epithelial cells (Jones *et al.,* 1992). Flagella have been implicated in assisting survival of *S.* Typhimurium within murine macrophages (Weinstein *et al.,* 1984).

The mechanism of colonisation in calves (Morgan *et al.,* 2004) appears to require an association with *Salmonella* Pathogenicity Island 1 and 2 (SPI) genes, involving type 3 secretion systems (T3SS). These appear to be less important for colonisation of the chicken gut indicating the difference in patterns of colonisation in the two host species. Several genes on a novel *Salmonella*-specific genetic island (CS54 Island) were shown to be required for intestinal colonisation in mice (Kingsley *et al.,* 2000, Kingsley *et al.,* 2003). This was indicated by mutations in *shd*A, *rat*B and *siv*H that resulted in a reduced ability of *S.* Typhimurium to colonise intestinal tissues in mice.

A comparative study of mutants deficient in their ability to colonise human tissue culture, bovine and/or murine Peyer's patches and spleens demonstrated that different genes were also required for colonisation of these three species (Tsolis *et al.,* 1999b, Tsolis *et al.,* 1999a). Morgan and others (2004) reported that SPI 1 or SPI 2 genes were required for calf gut colonisation; in contrast minor genes were required for chicken caeca colonisation. Genes encoding the effector proteins PipB and $SopE_2$ were found to be required for chicken caeca colonisation but not for calf gut colonisation (Morgan *et al.,* 2004). This could be due to the difference in the environmental conditions, diets and bacterial flora that exists in chicken caeca and calf gut mucosa or to different digestive secretions between two hosts. A number of environmental factors such as nutrient deprivation, osmolarity and availability of oxygen, have been linked to *Salmonella* virulence modulation and its survival in nature and in the host (Arricau *et al.,* 1998, Barak *et al.,* 2005). Carbon and energy source mechanism is considered to be essential during the early stages of many bacterial infections

(Conway and Schoolnik, 2003). The majority of intestinal bacteria require a fermentable carbohydrate for growth, and fermentation is assumed to be the mode of metabolism used by most species (Salyers *et al.*, 1978). Facultative anaerobic bacteria (e.g. *Salmonella*) grow most rapidly when respiring oxygen and switch to anaerobic respiration in the absence of oxygen or to fermentation in the absence of alternative electron acceptors (Gennis and Stewart, 1996). However, the extent to which facultative anaerobes use oxygen to maximize their growth rate in the intestine is unknown. Intestinal pathogens such *E. coli* and *Salmonella* preferentially colonise the region of the gut close to the mucosa where nutrients and oxygen may be present at higher concentrations as a result of existing anaerobes are unable to colonise (Poulsen *et al.*, 1995) and (Barrow *et al.*,unpublished). Flora densities in gut contents are generally much lower than during colonisation of new born animals (Barrow *et al.*, 1988). The exact mechanisms by which enteric pathogens colonise the gut of livestock is still relatively poorly understood although a number of studies using mutational analysis have indicated a host-pathogen interactions role through the involvement of fimbrial, SPI, metabolic and regulatory genes, which are different from those expressed *in vitro* (Turner *et al.*, 1998, Morgan *et al.*, 2004, Pullinger *et al.*, 2008). Other adaptation to the gut likely to be important includes the response to a different osmotic environment (Mishra *et al.*, 2003, Liu *et al.*, 2009), response to temperature 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 colonisation has been shown by Conway and his colleagues (Chang *et al.*, 2004, Fabich *et al.*, 2008).

However, to gain a comprehensive picture and to understand the colonisation mechanism of Enterobacteriacae (e.g. *Salmonella*) in host intestinal tract, it is worthy to have an idea about how these bacteria live, gain their energy and counter the obstacles (such as osmotic stress) in host gut. Therefore a brief introduction about facultative anaerobic respiration/fermentation and osmoregulation of these bacteria are presented in the following sections.

1.5 Facultative anaerobic respiration and fermentation

Facultative anaerobic bacteria such as *Salmonella* and *Escherichia coli* are able to derive energy by respiration or fermentation. For most microorganisms respiration is preferred to fermentation as it is energetically more favourable (Ingledew and Poole, 1984, Iuchi *et al.*, 1986). Thirty six molecules of ATP are generated from aerobic respiration of 1 molecule of glucose; while only two molecules of ATP are generated by fermentation. The respiratory process involves membrane-associated, proton-translocating, electron transport pathways that couple substrate oxidation to the reduction of an electron acceptor such as oxygen, nitrate etc. (Stewart, 1988). On the other hand the fermentation process involves redox-balanced dismutations of the substrate and energy production is by substrate-level phosphorylation (Egami, 1973, Hasona *et al.*, 2004, Gonzalez *et al.*, 2008).

In the absence of oxygen (e.g. lower intestinal tract of mammals or birds), facultative anaerobic bacteria respond by replacing aerobic respiratory pathways with anaerobic respiratory or fermentative pathways, depending on the availability of different electron acceptors. Anaerobic growth of bacteria on non-fermentable carbon sources requires substances that can function as terminal electron acceptors for respiration [e.g. fumarate, nitrate, nitrite, trimethylamine *N*-oxide "TMAO", dimethylsulphoxide "DMSO] (Iuchi *et al.*, 1986, Stewart, 1988). The mechanisms regulating the expression of these pathways are organised in a hierarchical manner such that in a specific environment the most energetically-favourable process is used (Spiro and Guest, 1990).

1.6 Bacterial osmoregulation

When *Salmonella* are transferred to a hyperosmotic environment, as may occur on entry into the intestine, the bacterial cells lose internal water (plasmolysis) with loss of turgor and the bacterial cells may become shrunken. Respiration in *E. coli* becomes severely inhibited during hyperosmotic stress (Meury, 1994),

resulting in decreases in both the intracellular ATP concentration (Ohwada and Sagisaka, 1987) and the cytoplasmic pH (Dinnbier *et al.,* 1988). The bacterium aims to increase its internal osmotic pressure to preserve cell turgor. This is achieved by accumulation or synthesis of solutes that counter the external osmotic pressure but are tolerated by the whole cellular machinery, so-called "compatible solutes" (Sutherland et al., 1986, Brown, 1990). Sutherland and others (1986) reported that potassium ion is the solute most preferred by the bacteria for this purpose. The potassium (K^+) uptake system is encoded by *kdp*A (Altendorf *et al.,* 1992). As it shown in Figure 1.1, the KdpA system serves mainly to scavenge K^+ when the ion is present in low concentrations. If the medium or the environment is limited in potassium or if the osmotic pressure cannot be regulated by K^+ ions alone, *Salmonella* can up-regulate the uptake or biosynthesis of other neutral osmoprotectants such as trehalose, proline or betaine (Csonka, 1988, Rod *et al.,* 1988, Howells *et al.,* 2002).

A number of prokaryotes and eukaryotes accumulate/synthesise trehalose under osmotic, heat and/or desiccation stress (Rod *et al.,* 1988, Van Laere, 1989). Trehalose is a non-reducing disaccharide of glucose, which is synthesized by two enzymes encoded by the genes of the *ots*AB operon (Kaasen *et al.,* 1992, Giaever *et al.,* 1988). Trehalose is synthesized and accumulated by cells exposed to high osmotic stress as osmoprotectant and synthesis is dependent on the *ots*BA operon (Giaever *et al.,* 1988, Hengge-Aronis *et al.,* 1991). The *ots*A gene, trehalose-6-phosphate synthase, catalyzes the condensation of glucose-6-phosphate and UDP-glucose; while trehalose-6-phosphate phosphatase, encoded by *ots*B, produces free trehalose.

Under conditions of physiological osmolarity bacteria can utilize trehalose as a carbon source (Hengge-Aronis *et al.,* 1991), using an osmotically inducible periplasmic trehalase (*tre*A or *osm*A). Trehalase releases glucose, which is transported into the cytoplasm by the glucose phosphotransferase system (Boos *et al.,* 1987). It is observed that when bacterial cells are grown at high osmolarity, large amount of trehalose is synthesised (Strom *et al.,* 1986). It is reported that cytoplasmic synthesis of trehalose occurs independently of carbon

source present in the medium. The trehalose is synthesized in the cytoplasm and excreted from there into the bacterial periplasmic space where it is split into two molecules of glucose (Styrvold and Strom, 1991). Trehalose is more needed by bacteria during osmotic and 42^{o}C stress (Canovas *et al.*, 2001).

S. Typhimurium and *E. coli* have three independent proline transport systems: *put*P, *pro*P, and *pro*U (Milner *et al.*, 1988). The PutP system is not required for proline transport for osmoprotection at hyperosmolarity. It is mainly required to transport proline to be metabolised as a carbon or nitrogen source (Ratzkin *et al.*, 1978). However, the other two systems ProP and ProU are responsible for the accumulation of proline and glycine betaine to high levels under conditions of hyperosmotic stress (Milner *et al.*, 1988), as shown in Figure 1.1.

Occasionally the uptake of organic osmoprotectants such as glycine betaine is more effective in inducing cellular rehydration and growth than the uptake of K^+ and/or the biosynthesis of trehalose (Wood *et al.*, 1999). Osmoregulatory betaine uptake may promote growth in urine and colonization of the human urinary tract by *E. coli* (Chambers and Lever, 1996).

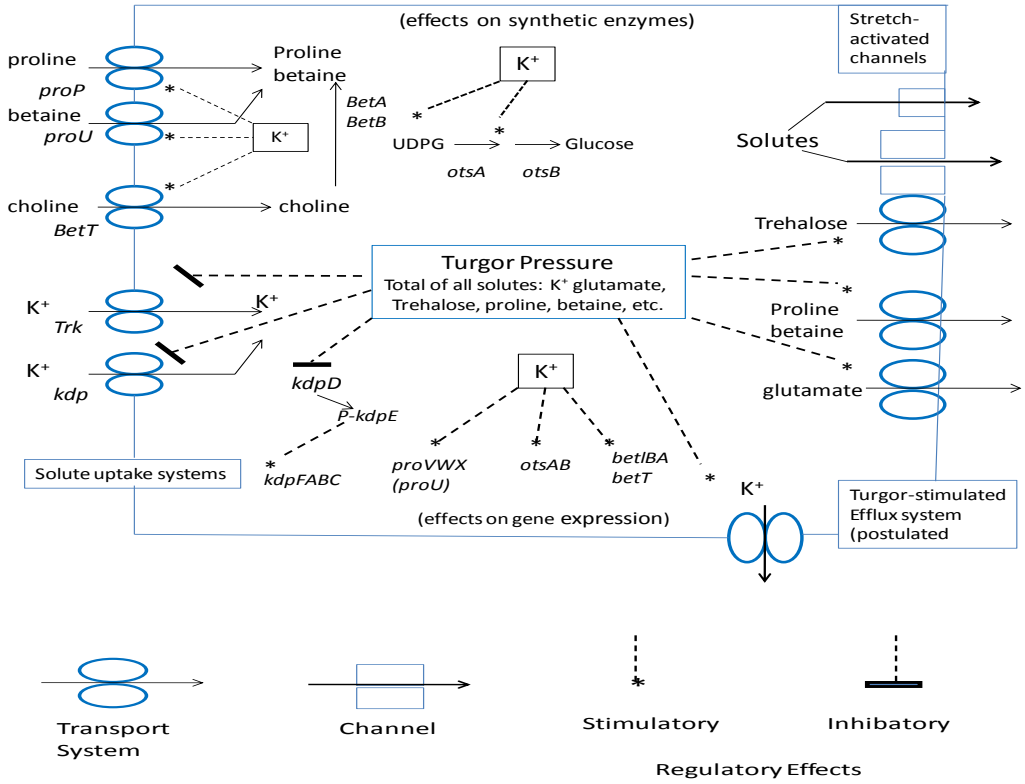

Figure 1.1: Suggested model for bacterial osmoregulation, in which turgor and cytoplasmic K+ are supposed to control the activity of transport systems, enzyme steps, and transcription of genes shown. Regulation of biosynthetic events are shown at the top, and effects on gene expression are shown at the bottom. Solid arrows show movements of solutes, biochemical steps, or phosphorylation of KdpE by KdpD and transcription of the *kdp*FABC operon. The proposed regulators and their targets are connected by dashed lines. Where increase in the regulator stimulates a process, the lines end at an asterisk (*); where increase in the regulator inhibits a process, the lines end at a bar (—). This figure is reproduced from Booth and Higgins (1990).

Figure 1.1 illustrates the movement of above mentioned solutes in and out of the bacterial cells under different osmotic conditions. The major transport systems that accumulate compatible solutes are shown on the left; while mechanisms for eliminating (efflux) of compatible solutes are at the right of the Figure 1.1.

1.7 Control of *Salmonella*

The economic requirements associated with the poultry industry make it more practical to control the *Salmonella* infection on the poultry farm than to control it at slaughter house (e.g. using vaccines). Treatment of table eggs is not permitted in many EU countries and control of *Salmonella* infection in layer farms is therefore needed (Anon, 1991, Anon, 1995).

Control of non host-specific *Salmonella* serotypes can be more difficult than host-specific serotypes since there are many potential reservoirs of infection other than the animals themselves. Many countries which have recently intensified their poultry industries have the disadvantage of high ambient temperatures with the reduced options for environmental control. Those countries and companies which have enclosed housing can control the entry of pathogens using HACCP analysis (Schmidt, 1995). Totally *Salmonella*-free poultry can be produced, but this requires enclosed poultry housing and strict control of feed quality, hygiene and management etc [Commission of the EU communities no 2160/2003] (Van Immerseel *et al.*, 2003). However, the cost of these have implications in terms of production costs and, with the increasing globalisation of poultry production, this is likely to affect economic production in individual countries. Moreover the consumption of meat and eggs from poultry reared in free-range systems or small back-yard flocks in developing countries make the application of hygiene to these flocks difficult and leads to increased environmental contamination with *Salmonella*. The effect of high temperature in tropical countries and open sided houses also increases environmental infection. Therefore, the cost and impracticability of some of the required improvements in hygiene and management to achieve *Salmonella*-free poultry flocks imply that biological means of control are likely to be increasingly sought.

These biological means include the use of antibiotics, competitive exclusion (CE) products and vaccines or combinations of these measures (Smith and Tucker, 1975a, Barrow *et al.*, 1987b, Zhang-Barber *et al.*, 1999, Methner *et al.*, 2001).

1.8 Antibiotics

Antibiotics have been used in Europe and the USA in earlier decades for growth promotion or, when chemotherapeutic antibiotics were banned from use in this way in Europe (Report, 1969, Report, 2005) were used solely to control infection. They have been used by in-feed administration, via water or as egg dips. However, these often do not produce the desired effect but, in addition, can increase susceptibility through their harmful effects on the gut flora. More importantly they lead to an inevitable increase in resistance both in pathogens but also in members of the normal flora (Smith and Tucker, 1975b, Smith and Tucker, 1975a, Smith and Tucker, 1980, Barrow *et al.*, 1998). Increasing globalisation is also leading to importation of strains which are increasingly resistant as a result of lack of regulation of antibiotic usage in many producing countries. The application of these antibiotics has been discussed very critically during the last years, because the widespread use of antibiotics in livestock production has been linked with the rise of multiple drug resistant bacteria (Threlfall *et al.*, 1998, Wray and Davies, 2000). Besides the appearance of unwanted antibiotic residuals in animal products, this essentially boosted public concerns regarding the use of antibiotics in feed. Consequently, the EU countries have banned the use of most of the antibiotics as growth promoters since the end of June-1999 (Van Immerseel, 2004).

1.9 Competitive Exclusion (CE)

Competitive exclusion (CE) describes the protective effect of the natural or native bacterial flora of the intestine in limiting the colonisation of some bacterial pathogens including food borne pathogens.

The normal flora of adult chickens is inhibitory to colonisation and cultures of such flora can be used to treat orally newly hatched chicks, which, within 24 hours acquire the full resistance of the parent.

In 1973, Nurmi and Rantala demonstrated that treatment of newly hatched chickens with intestinal contents (faeces) of adult chickens conferred resistance to infection by *S.* Infantis (Nurmi and Rantala, 1973). Since then many authors have contributed to this area of research. The advantages are that protection is rapid and effective against a wide range of bacterial pathogens. The exact mechanism by which CE products confer resistance to pathogens is still under investigation. Three mechanisms have been proposed to explain how CE products work namely: physical obstruction of attachment sites for *Salmonella* by the native flora lining the intestine, competition for essential nutrients by the native flora limits the ability of *Salmonella* to grow and the protective flora producing volatile fatty acids (especially in the caeca) that limit the growth of *Salmonellae*. This system is highly effective under experimental conditions but is less so in the field (Impey *et al.*, 1987). Nevertheless commercial preparations are available.

However, treatment with undefined flora is not permitted in many countries due to the potential risk of pathogens transmission, although this can be avoided by appropriate testing of the product. Because of some concerns associated with the use of undefined CE products, studies were commenced in the 1980s to search for bacterial strains that possess the colonisation characteristics of *Salmonella* but not their virulence determinants (Barrow and Tucker, 1986). Early studies revealed that this so-called colonisation inhibition effect existing between related bacteria was not the result of an immune response induced by bacterial antigens in the gastro-intestinal tract. It was specific to related bacterial taxonomy. Therefore strains of *E. coli, Citrobacter, Klebsiella* or any other related bacteria had no effect against *Salmonella*, but did inhibit colonisation by organisms from their own genera. Even among *Salmonella* not all strains are equally inhibitory (Berchieri and Barrow, 1990, Martin *et al.*, 1996).

The exact mechanism of colonisation-inhibition is poorly understood, although an early hypothesis that growth suppression operates because of the absence of an utilizable carbon source or electron acceptor emerged from the observations

that similar inhibition could be demonstrated in stationary-phase nutrient broth cultures (Zhang-Barber *et al.*, 1997) and interaction with the host, either by competition for sites of adhesion or through stimulation of the innate immune system (Van Immerseel *et al.*, 2005) may also be possible. Synergistic effects between these mechanisms are also likely.

The colonisation-inhibition mechanism has been studied using the *in vitro* system of stationary-phase broth cultures (Berchieri and Barrow, 1990, Berchieri and Barrow, 1991) and it appeared to relate to the use and depletion of carbon sources and other nutrients available under the relevant redox conditions under which the bacteria are growing (Maskell *et al.*, 1987, Zhang-Barber *et al.*, 1997).

The effect of genus-specific exclusion required by bacteria in young chickens' intestines is reduced by the development of the gut flora (Berchieri *et al.*, 1991, Martin *et al.*, 2002). The protective effect required high numbers of bacteria to confer long lasting effect in terms of reduction in faecal excretion. This effect is induced in a matter of 6 h or so but only becomes completely effective after 18-24 h (Iba *et al.*, 1995, Martin *et al.*, 2002). Unfortunately, so far, no strain was found to be fully effective against all *Salmonella* strains (Iba *et al.*, 1995, Martin *et al.*, 2002) and there appeared to be a serovar-specific effect.

As a consequence it was suggested it might be possible to administer live vaccine strains to newly hatched chickens in order to colonise the gut extensively and rapidly before the normal flora develops. This could induce a profound resistance to colonisation by strains that may be present in the poultry house or may also emerge in the hatchery. A search for a *Salmonella* strain with a wide-spectrum of inhibition and that would be capable of preventing colonisation by an extensive selection of strains was carried out. A strain of *S.* Infantis (Berchieri and Barrow, 1990) and strain of *S.* Hadar (Nogrady *et al.*, 2003b) were found to be more effective in inhibition than other serovars. These serovars are characteristically poorly invasive but highly colonising (Desmidt *et al.*, 1998b) and because they are good colonisers they inhibit other serovars.

1.10 Vaccines

Vaccines against host-specific *Salmonella* serotypes that cause severe systemic disease in particular host species (such as *S.* Gallinarum in poultry) induce a strong serotype-specific protective immunity against infection and disease (Smith, 1956, Barrow and Wallis, 2000). On the other hand, vaccination against non-host specific *Salmonella* serotypes have resulted in variable success rates. These two types of infections (specific & non-specific salmonellosis) display very different epidemiological patterns (Timms *et al.*, 1994). The efficacy of vaccine product is judged by the level of intestinal and systemic colonisation, morbidity and mortality rates after vaccination and experimental infection using the parenteral or oral routes of administration. The level of protection relies on the challenge strain, the route of administration, the infection dose, the age of birds and the species/line of birds. Therefore, it is difficult to compare strictly the efficacy of available current vaccines.

The vaccination of poultry has become one of the leading contributory measures to control *Salmonella* infections of poultry because of the costs, impracticability and disadvantages of other approaches mentioned above. Both live attenuated and killed vaccines are currently in use.

Killed vaccines have been used extensively to control host non-specific Salmonellosis in poultry with very varying success. Some authors' (Timms *et al.*, 1994, Liu *et al.*, 2001b) results support earlier observations that these vaccines may be used to reduce the mortality, although this is of little practical significance in the field. The relevance of this decline in mortality for colonisation of organs and shedding is also not clear as *Salmonella* infection in the field is asymptomatic.

Earlier experiments with killed vaccines report variable effects on faecal shedding and colonisation of the intestine and internal organs. For example, maternal vaccination with bacterins reduced mortality, but did not reduce

significantly the excretion of *Salmonella* in the progeny (McCapes *et al.*, 1967, Truscott and Friars, 1972).

A vaccine containing inactivated *S.* Enteritidis grown under iron-restricted conditions is available on the market in some European countries (Woodward *et al.*, 2002). Another related vaccine containing *S.* Enteritidis and *S.* Typhimurium, both grown under environments of iron restriction, is also commercially available (Clifton-Hadley *et al.*, 2002). Iron-restriction is recognized to up-regulate bacterial factors that stimulate virulence and therefore may induce important immunogens. Many other genes relevant to virulence and therefore immunogenicity have been shown to be up-regulated in the intracellular (macrophage) environment (Eriksson *et al.*, 2003), therefore it might be more appropriate to generate vaccines from bacteria cultured under the conditions experienced in this or other relevant environments (Woodward *et al.*, 2002).

The Fe-restricted inactivated *S.* Enteritidis vaccine, (Salenvac T vaccine; Intervet) was efficient at decreasing egg contamination after intravenous challenge with *S.* Enteritidis (Woodward *et al.*, 2002). But this work is difficult to evaluate since the field challenge is normally introduced through oral or respiratory routes and not intravenously. However, the combined *S.* Enteritidis and *S.* Typhimurium vaccine, when administrated intramuscularly at day 1 and week 4 was shown to decrease shedding after oral challenge with *S.* Typhimurium in a seeder-bird challenge model (Clifton-Hadley *et al.*, 2002) in which less than 30% of the vaccinated birds shed *Salmonella* bacteria, while at 10 days post-challenge more than 80% of the unvaccinated animals shed *Salmonella*.

Attention has been paid to the development of avirulent live vaccine strains of *Salmonella* because of the accumulation of evidence that such strains of *Salmonella* are more immunogenic in mice and poultry than killed vaccine (Collins, 1974, Zhang-Barber *et al.*, 1999). Live vaccines have been tested widely in mice and in poultry. Although many different live *Salmonella* strains have been tested for their efficacy for experimental and semi-field studies, only

a few of them were commercially registered for use in European poultry industry. The commercially available live *S.* Typhimurium and *S.* Enteritidis vaccine strains are either auxotrophic double marker mutants produced by chemical mutagenesis (Meyer *et al.,* 1993, Springer *et al.,* 2000) or developed on the basis of the principle of metabolic drift mutations (Linde *et al.,* 1997, Hahn, 2000). These are negative mutations in essential enzymes and metabolic regulatory centres, as a consequence of which the resulting metabolic processes resulted in prolonged generation times and corresponding reduction in virulence (Linde *et al.,* 1997).

S. Gallinarum 9R (Smith, 1956) is a rough live vaccine which in addition to its effectiveness against *S.* Gallinarum, is also registered for prophylactic use against *S.* Enteritidis. This vaccine strain has been tested more extensively in recent years since it has been indicated to confer cross-protection against *S.* Enteritidis (Barrow *et al.,* 1991), a member of the same serogroup (group D). In a large field experiment in the Netherlands, eighty commercial flocks were vaccinated with the 9R vaccine strain, the flock level occurrence of *S.* Enteritidis infection was 2.5% [2/80 flocks]. This was significantly less than the flock level occurrence in unvaccinated flocks [214 out of 1854, 11.5%] (Feberwee *et al.,* 2001a). Interestingly, in 4,500 eggs derived from five 9R-vaccinated flocks, no vaccine strain bacteria were detected and there was also no evidence implicating faecal shedding of the vaccine strain (Feberwee *et al.,* 2001b).

Many other live attenuated *Salmonella* vaccines have been developed by mutating virulence genes. Genetic modification of the vaccine strain is made to minimize the risk of spread or persistence in the environment while at the same time inducing an adaptive immune response. The completion of genome sequence of many bacteria including *S.* Typhimurium and *S.* Enteritidis (www.sanger.ac.uk/projects/*Salmonella*) has facilitated the construction of complete rational mutations. Genes coding for metabolic functions or virulence factors are the main targets for producing safe vaccine strains. There is a certain rationale for inactivation of housekeeping genes, which will reduce

bacterial growth and virulence without greatly affecting the expression of key virulence determinants, required for appropriate immunogenicity (Klose and Mekalanos, 1997). Double or even triple mutations of genes can be applied to increase the safety of the vaccine strain by reducing its risk to revert to a wild type by acquisition of these genes by horizontal transfer (Tacket *et al.,* 1997, Methner *et al.,* 2004). Whichever mutation is applied, it is important that the vaccine strain retains its capacity of invasiveness in order to stimulate sufficient immunity to be protective. At the same time the vaccine strain needs to be eliminated before the broiler slaughter age and before onset of lay in layer and breeder chickens.

Live attenuated vaccines produce better protection than killed inactivated vaccines. Live vaccines stimulate both cell-mediated and humoral immune arms of immunity. Killed vaccines stimulate mainly antibody production (humoral protection only) and represent only the antigens present at the time of *in vitro* harvesting (Collins, 1974, Barrow and Wallis, 2000). Killed vaccines may also be poorly immunogenic due to the destruction of relevant antigens during vaccine preparation, the fast destruction and elimination of the vaccine from the inoculated hosts and because they are unable to induce cytotoxic T cells (Barrow *et al.,* 1991, Rajashekara *et al.,* 1999). Live vaccines have been proven to be more effective in increasing lymphocyte proliferation in response to *S.* Enteritidis in laying hens (Babu *et al.,* 2003). Moreover killed vaccines do not stimulate secretory IgA responses, which play an important role in protecting mucosal surface (Barrow *et al.,* 1992, Desmidt *et al.,* 1998c).

Live vaccines have additional protective effects, particularly when administered orally, which can be exploited during their development and application. These effects include 1) genus-specific colonisation-inhibition and/or competitive exclusion (see section 1.9) demonstrated to be primarily an effect of microbial metabolism and 2) the stimulation of primed polymorphonuclear (PMN) cells in the gut. It is worth mentioning that the two German approved attenuated live *Salmonella* vaccines [Zoosaloral H "Impfstoffwerk Dessau – Tornau GbH, Germany" & *Salmonella* Vac T "TAD

Pharmazeutisches Werk GbH"] are immunogenic but generally not, or only briefly, able to inhibit intestinal colonisation of homologous or heterologous *Salmonella* challenge bacteria by the mentioned mechanism of CE (Methner *et al.*, 1997, Van Immerseel *et al.*, 2002). Killed vaccines are clearly unable to induce these effects. Their protective efficacy is additionally restricted by their low immunogenicity in unprimed hosts and in fact they do not induce cytotoxic T cells (Rajashekara *et al.*, 1999). However, there are concerns over public acceptability of live vaccines since those currently commercially available in Europe are genetically undefined and may be antibiotic resistant, whilst the better defined deletions, which may be antibiotic sensitive, are produced by genetic modification which is seen by the public as a cause for concern. Moreover live vaccines can pose a risk of residual virulence and reversion to pathogenic wild types as well as provide a potential source of environmental contamination (Meeusen *et al.*, 2007).

There is thus considerable scope for improvement in inactivated vaccines through a more rational approach and the work presented in this project could form the basis to such a vaccine. The rationale is that microarray technology (see section 1.11) can identify exactly the conditions to which the bacteria are subjected during infection. These conditions can be reproduced *in vitro* and bacteria cultured under these conditions, thus producing antigens which are normally presented *in vivo*.

1.11 Microarrays and their value

The mechanism of intestinal colonisation and virulence in *Salmonella* has been carried out largely through mutations studies (section 1.9). The increasing availability of gene expression technology applied at the level of the whole genome has opened up new approaches to the study of virulence.

Microarray technology can be used in gene expression analysis, gene discovery and gene mapping, diagnostics and drug discovery. In differential gene expression analysis, levels of specific transcripts in two or more RNA samples are compared to identify differences in the abundance and identity of the

transcripts they contain. This generates information about the physiological state of the cell studied through the activity of genes. Changes in mRNA levels are related to proteome changes as they are precursors of translated proteins. Differential gene expression analysis has been applied to all kinds of tissues, plants, yeast and bacteria (Baldwin *et al.*, 1999, Braxton and Bedilion, 1998, Mirnics *et al.*, 2001, Schulze *et al.*, 2001, van Berkum and Holstege, 2001, Higgins *et al.*, 2011). In gene discovery and gene mapping, microarrays have been used in the identification of new genes for the annotation of genomes and in the identification of functional regulatory elements leading to the understanding of gene regulation (Lieb *et al.*, 2001, Shoemaker *et al.*, 2001b, Shoemaker *et al.*, 2001a). Moreover, they have been applied to the analysis of genomic fragents derived from genomic analysis methods like genomic mismatch scanning and representational difference analysis, and for the prediction of splice variants, the analysis of single nucleotide polymorphisms (SNPs) and mutations, and for sequencing (Drobyshev *et al.*, 1997, Sapolsky *et al.*, 1999, Hu *et al.*, 2001, Larsen *et al.*, 2001, Meltzer, 2001). In the field of drug discovery, microarrays have been useful during different stages of the drug discovery process including the identification of potential drug targets and the analysis of their toxic properties and their function modes by examining the expression profiles they induce (Gray *et al.*, 1998, Jain, 2000, Lockhart and Winzeler, 2000, Meltzer, 2001, van Berkum and Holstege, 2001).

The microarray also allows the analysis of bacterial genomic content, and the identification of genes involved in host-pathogen interactions (Cummings and Relman, 2000). It can also be used to highlight cross-gene interactions between bacteria and their host during the course of infection (Cummings and Relman, 2000, Ehrenreich, 2006).

As a result of falling costs for equipment and oligonucleotides, DNA microarrays are becoming common tools in the microbiological laboratories although there is constant discussion about their replacement by deep-sequencing which currently, however, remains much more expensive. Microarrays allow a dynamic view on the physiology of the living cell and

have been compared with the advent of the microscope (Brown and Botstein, 1999). An inherent limitation of the microarray is that the resulting transcriptome does not account for post-translational events. Nevertheless, in most cases, there is a high correlation between transcriptome and proteome (Hecker and Engelmann, 2000). The transcriptome data are usually more comprehensive because of the limited number of proteins that can be resolved in two-dimensional gels. In addition, the relatively detailed knowledge of the genetics and biochemistry of bacteria allows direct interpretation of the transcriptome data in active pathways. Several studies have indicated that enzyme levels correlate directly with their respective gene expression profiles (Arfin *et al.*, 2000).

The advent of full genome sequencing of many bacteria, including *Salmonella* serotypes (www.sanger.ac.uk/projects/*Salmonella*) has enabled transcriptional analysis at the level of the genome to be carried out on bacteria harvested from a variety of environments. This is done either by using synthetic oligonucleotides representing each gene or by amplification of every ORF using synthetic primers. These are spotted onto prepared glass slides and hybridisation carried out using cDNA prepared from RNA taken from bacteria harvested from the environment investigated. Competitive hybridisation is performed using two cDNA preparations from different niches and labelled differentially enabling an estimate of the comparative strength of the signals from both environments for each gene.

This approach has been found to reflect well changes in transcription measured by more traditional means (Richmond *et al.*, 1999, Jansen and Yu, 2006, Niba *et al.*, 2007, Fardini *et al.*, 2007) and has been used to identify gene expression during host-pathogen interactions. This approach has identified the intracellular transcriptome of *Mycobacterium tuberculosis* within murine macrophages (Schnappinger *et al.*, 2003), the transcriptome of *Neisseria meningitidis* during infection (Dietrich *et al.*, 2003) and has been used to monitor changes *in vivo* such as during infection of macrophages by *S.* Typhimurium (Eriksson *et al.*, 2003).

Schnappinger and others (2003) captured *M. tuberculosis* transcriptomes from murine macrophages derived from bone-marrow, which are from either wild type or nitric oxide synthase 2-deficient mice. They concluded that the *M. tuberculosis* phagosomal environment is nitrosative, oxidative, functionally hypoxic, carbohydrate-poor and capable of troubling the pathogen's cell envelop indicating the value of array technology in studying microbial physiology in detail during infection (McKinney *et al.*, 2000).

Snyder and her colleagues (2004) analysed the transcriptome of uropathogenic *E. coli* during the murine urinary tract infection. This study showed the up-regulation of genes associated with the cells' translation process indicating that bacteria were in a rapid growth state despite specific nutrient limitations. Type 1 fimbriae, adherence virulence factor, capsular polysaccharide, lipopolysaccharide genes and five iron acquisition systems were expressed during urinary tract infection (Snyder *et al.*, 2004).

Bower and others (2009) showed that urinary pathogenic *E. coli* can be conditioned to grow at high rates in the presence of acidified sodium nitrate, suggesting that *E. coli* interactions with polyamines or stresses such as reactive nitrogen intermediates can in effect reprogram the bacteria enabling them to better colonise the host (Bower *et al.*, 2009).

More recently, Nielsen and others (2010), used microarray and *in situ* single cell expression methods to study *Vibrio cholerae* growth and virulence gene expression during infection of the rabbit ligated ileal loop model of cholera. Genes encoding for the toxin-coregulated pilus and cholera toxin were among the powerfully expressed genes early in the infection process (Nielsen *et al.*, 2010).

1.12 Aim of Project

The aim of the project was to define the transcriptome for *S.* Enteritidis during colonisation of the caeca of the chicken and to identify genes associated with colonisation using microarray technology.

1.13 Objectives

- Obtain *S.* Enteritidis RNA from the gut of infected chickens and look for the pattern of gene expression by microarray.
- Assess the importance of selected genes in colonisation by mutation.
- Assess the importance of tri carboxylic acids such as fumarate in colonisation.
- Assess the importance of osmoprotectant genes in colonisation.
- Assess the immunogenicity of bacteria harvested from chicken caeca as a candidate killed vaccine.

Chapter - 2**: Materials and Methods**

2.1 Materials

2.1.1 Growth media

Nutrient broth (CM0001, Oxoid) was used for general bacteriological culture. Nutrient agar (CM0003, Oxoid) was used with antibiotics where appropriate for bacterial enumeration or mutation.

2.1.2 Bacterial strains, plasmids and phages

Salmonella Enteritidis Phage Type 4 (antibiotic sensitive), strain number 125109 (Barrow, 1991); www.sanger.ac.uk/Projects/*Salmonella* PT4; EMBL (accession number: AM933172) was used. The genome structure and comparison with related serovar, *S.* Gallinarum has been determined recently (Thomson *et al.*, 2008). In the UK phage type 4 (PT4) strains are implicated in human food-poisoning cases more frequently than other phage types. This phage type is highly virulent for young broiler chickens with systemic disease and pericarditis observed in the field (Lister, 1988, O'Brien, 1988) and the virulence of this strain for young chickens has been demonstrated experimentally (Barrow, 1991, Barrow and Lovell, 1991). The strain was isolated from a laying flock which was associated epidemiologically with an outbreak of human food poisoning.

The strain was used either as a nalidixic acid resistant (Nal^R) or spectinomycin resistant (Spc^R) mutant.

2.1.3 Birds

Fertile one day-old commercial broiler eggs were purchased from P D Hook, Thirsk, UK. Their parents have not been treated with salmonella vaccines or reported to be infected with salmonella. They were incubated in animal house fumigated room incubators (Sutton Bonington, University of Nottingham). These birds were only provided with sterile water until they had been

inoculated with *Salmonella* and not given food at all for very short experiments (18 h). These birds were used for microarray and competitive exclusion experiments.

One day commercial layers (egg production breed) were used for vaccination experiments because they accumulate less weight during the course of experiment than would broilers. Their parents have not been treated with salmonella vaccines or reported to be infected with salmonella. They were purchased from Hy-line UK Ltd, Millennium Hatchery. Commercial starter feed with no added antibiotics or other additives and drinking water were both available *ad libitum*. Cleaning and feeding were organised which prevented cross contamination effectively throughout the course of experiments.

2.1.4 Agilent microarray slides

Agilent Microarray slides "8x15K" (Agilent Technologies) were used for microarray experiment. Each slide contains 8 identical array squares; each array square contains 15000 spots (probes). Each gene of *S.* Enteritidis is represented by 3 probes fixed randomly on the slide. Each probe is a 60 mer probe sequence. Therefore 15000 spots are enough for all *S.* Enteritidis genome (4380 open reading frames) plus control probes.

2.2 Methods

2.2.1 Bacterial enumeration

Routine bacterial enumeration of cultures was determined by spot plate counting method. Briefly, serial decimal dilutions were prepared in phosphate buffered saline (PBS). Dilutions from neat to 10^{-8} were made by sequentially transferring 20 µl of culture into 180 µl of PBS in a 96-well-plate, followed by plating 100 µl of each dilution (in triplicate) onto dried nutrient agar (NA). After overnight incubation at 37^{o}C, the viable count was expressed as colony forming units per ml (cfu/ml).

2.2.2 Assessment of bacterial growth rate

Volumes of 10 ml nutrient broth (NB) in universal bottles were pre-warmed and inoculated from NA slopes with *S.* Enteritidis mutants or with the wild-type. The broths were incubated at 37^{o}C for 18 h (overnight, o/n) in a shaking incubator (150 rpm). On the following day the optical density (OD_{600}) reading of each culture was recorded and then 1 ml of each was inoculated into 100 ml NB in a 250 ml flask pre-warmed at 37^{o}C. A second 1 ml aliquot of the same o/n culture was introduced into a 100 ml in 250 ml NB flasks pre-warmed at 42^{o}C; by which means that the strain was inoculated into 2 x 100 ml volumes of NB in 250 ml flasks. Then following brief shaking by hand an aliquot was removed from each culture for OD_{600} reading (time-point 0). The flasks were incubated in shaking incubator at the temperature selected. The OD_{600} reading for each was recorded every 20 min for 3 h. The bacterial growth rate was calculated as shown in the following equation in which the difference between two points OD_{600} reading on the Y axis logarithmic line is divided by the value between the corresponding time-points on X axis (Figure 2.1).

The bacterial growth rate = $(Y_2-Y_1) / (X_2-X_1)$; where Y_1 and Y_2 are any 2 points on the Y axis (OD_{600} logarithmic reading) and X_1 and X_2 are the two time-points corresponding for Y_1 and Y_2 points.

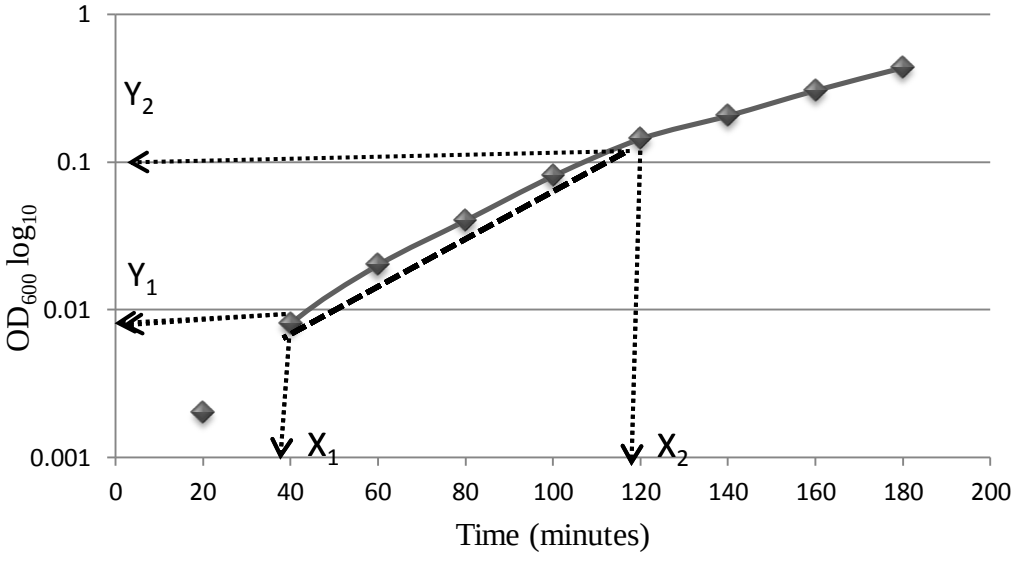

Figure 2.1: Bacterial growth curve. The dashed line represents the log phase, which started at min 40 and ended at min 120 (X1 & X2); while Y1 & Y2 represent the OD600 readings for those time-points respectively.

2.2.3 Microarray experiment

2.2.3.1 *In vivo* culture for RNA isolation

A total of one hundred and eighty chickens were orally inoculated within 10 h of their hatching to avoid the development of gut flora. Chickens were housed in fumigated cages and were handled with sterile gloves to avoid contamination. Chickens were orally infected with 0.1 ml of an antibiotic sensitive strain of *S.* Enteritidis PT4 P125109, grown for 18 h in NB at 37ºC and diluted in sterile NB to contain 10^7 cfu/ml. Sterile water only was provided during the infection period since the yolk sac is not fully absorbed for up to 3-4 days. At about 17 h post-inoculation the birds were killed one-by-one. Post-mortem bacterial harvesting was performed by recovering the caecal contents from both caeca of each bird. The 1st three randomly selected birds were killed and their caecal contents recovered separately into 3 sterile universal bottles on ice for bacterial viable count and bacterial purity estimation. Caecal contents from the remaining birds were pooled into RNA protect reagent (Qiagen, UK) in groups of between 5 and 7 birds per 10 ml RNAprotect, vigorously vortexed for 5s and centrifuged at 5000 x g for 12 min at 20^{o}C then tubes containing caecal content pellets were kept frozen at -70^{o}C until they were processed further within four weeks of their RNA harvesting as described in section 2.2.3.3.

2.2.3.2 *In vitro* culture for RNA isolation

A volume of 100 µl of an o/n statically incubated NB culture of *S.* Enteritidis was transferred into a pre-warmed 10 ml universal bottle of NB and incubated at 37^{o}C in a shaking incubator (150 rpm) for 2 h. One ml of this culture was transferred into 100 ml of pre-warmed NB in a 250 ml conical flask which was incubated at 37^{o}C with shaking (200 rpm) for 2 h. Ten ml of the culture were added into 20 ml of RNAprotect. Two ml of the culture was also used for bacterial viable count estimation using MacConkey agar plates (CM0007,

Oxoid, UK) as well as for spectrophotometer OD_{600} reading. The RNAprotect-bacterial culture mixture was mixed further by vortexing for 5s; incubated for 5 min at RT (15-25^{o}C) and centrifuged at 5000 x g for 12 min. After centrifugation, the supernatant of each tube was carefully discarded without disturbing the unseen pellet in the tube bottom. Tubes were kept at -70^{o}C until required for RNA isolation as described in the following section.

2.2.3.3 RNA Isolation and Purification

The pellet of each tube (from sections 2.2.3.1 or 2.2.3.2) was re-suspended in 700 µl TE buffer (30mM Tris and 1mM EDTA pH 8) containing lysozyme 1mg/ml (Sigma) and proteinase K 1:20 (Sigma). Tubes were incubated at RT (15-25^{o}C) for 10 min and vortexed for 10s every 2 min. RLT buffer (700 µl, Qiagen) containing 1/100 β-mercaptoethanol was added to the mixture of each tube and vigorously vortexed for 30 s. Avoiding the top layer of foam and bottom layer of precipitation, the middle layer of pure yellow solution was carefully recovered and transferred into new labelled sterile mini-tubes. Absolute ethanol (500 µl) was added into each tube and mixed by pipetting. A 700 µl aliquot from each tube were transferred into RNeasy mini-spin columns which were then centrifuged at 8000 x g for 15s.

2.2.3.4 DNA digestion

Commonly, DNase digestion is not required with RNeasy Kits (Qiagen, UK) since RNeasy silica membrane technology is efficient in eradicating most of the DNA without DNase treatment. Nevertheless, further DNA elimination may be necessary for certain RNA applications that are sensitive to the presence of very small amounts of bacterial genomic DNA. Following the centrifugation step in the last section (section 2.2.3.3); the samples were prepared and loaded onto RNeasy mini-spin columns as indicated above then the following steps were applied: a volume of 350 µl RW1 buffer (a mixture of guanidine thiocyanate salt and ethanol) from the kit was added into each mini-spin column and centrifuged at 8000 x g for 15s, to wash-spin column membrane; and the flow-through was discarded. A volume of 20 µl DNase I

stock solution (Qiagen) was added to 140 µl of RDD buffer, on-column digestion (10 µl of DNase I stock solution per 70 µl RDD buffer) for each column. Then a volume of 80 µl DNase I mixture was added directly on to the membrane of each column and incubated at RT for 15 min. Again, a volume of 350 µl of RW1 buffer was added to each column and incubated at RT for 5 min before they were centrifuged at 8000 x g for 15s, to wash the spin-column membrane; and then the collection tubes containing the flow-through were discarded. The mini-spin columns were placed on new lidless collection tubes, and then 500 µl of RPE buffer (wash ethanol buffer) was added to each mini-spin column and then centrifuged at 8000 x g for 15s to wash the spin-column membranes; and then the flow-through was discarded. The last step was applied twice. Then the mini-spin columns were placed this time on new 1.5 ml lidded collection tubes, and then 30 µl RNase-free-water were added directly onto the membrane of each column; and centrifuged at 8000 x g for 1 min. Another 30 µl RNase-free-water were added and centrifuged at the same speed and time. Then the spin-columns were discarded; while the collection tubes containing the RNA extracts were kept on ice until their RNA concentration and quality were measured using a NanoDrop 1000 spectrophotometer (Nano Drop Technologies Inc; USA).

2.2.3.5 RNA cleaning up

The extracted *in vivo* or the *in vitro* RNA using Qiagen RNeasy Kits were classified into one of the following three categories, pure, cleanable or bad RNA quality according to preliminary measurements with NanoDrop 1000 spectrophotometer (Nano Drop Technologies Inc; USA). This instrument can give good indication for RNA concentration and preliminary indication for RNA quality. So when high concentrated RNA samples were obtained with 260/230 absorbance reading at the range of 1.8-2.4 (using NanoDrop 1000 spectrophotometer) they were considered as pure RNA samples (requiring further quality evaluation using Agilent 2100 Bioanalyser "Agilent Technologies"), while those samples with a high concentration of RNA and their 260/230 absorbance reading in the range of 1.0-1.8 were considered as cleanable RNA samples. RNA samples, whatever their concentrations with

260/230 absorbance reading ≤ 1.0 were considered bad RNA samples and therefore discarded. RNA clean up using RNeasy mini kit reagents (Qiagen, UK) was only applied to the cleanable RNA samples. Starting with the most cleanable RNA samples, which were removed from -70^{o}C and thawed at RT for 10 min, 100 µl of this sample were collected and transferred into a new 1.5 ml sterile mini tube (Eppendorf, UK); while the rest of the mixture was kept frozen at -20^{o}C. Then the following reagents were applied: a volume of 350 µl RLT buffer was added to the mixture and mixed, then 250 µl of 96-100% ethanol were added and mixed by pipetting. A volume of 700 µl of this mixture was transferred into RNeasy spin column and centrifuged at 8000 x g for 15 s. The flow-through was discarded. A volume of 500 µl RPE buffer was added and centrifuged at 5000 x g for 15 s. A further 500 µl of RPE buffer was added and centrifuged at 5000 x g for 2 min and the flow-through was discarded. The column was placed on a new sterile collection tube and centrifuged at full speed (13000 x g) for 1 min. The column was then placed in a new 1.5 ml collection tube. A volume of 30 µl of RNase-free water were added into the mini-spin column and centrifuged at 8000 x g for 1 min. A further 30 µl of RNase-free water were added to the column and centrifuged at 8000 x g for 1 min. The spin-columns were discarded; while the collection tubes containing the purified RNA were kept on ice until their RNA concentration and the quality was measured using the NanoDrop 1000 spectrophotometer as above.

2.2.3.6 Bactericidal effect of RNAprotect reagent

RNAprotect (Qiagen, UK) is commonly used in harvesting of bacterial cells in order to stabilise the *in vivo* or *in vitro* grown *S.* Enteritidis RNA and prevent its degradation or induction of genes. Consequently representative gene expression of both target environments (RNA samples isolated from *S.* Enteritidis grown under *in vivo* and *in vitro* environments) can hopefully be obtained as they are during the moment of *Salmonella* collection. It was important to determine whether the RNAprotect reagent was efficient in killing bacterial cells completely which might otherwise result into misleading gene expression. The experiment was performed as following: pre-warmed 10 ml NB was inoculated with a culture of *S.* Enteritidis PT4 and incubated statically

at 37°C o/n. On the following day, 100 µl of the broth culture were transferred into pre-warmed 10 ml NB and incubated at 37°C in a shaking incubator for 2 h. After 2 h, 1 ml of the broth culture was transferred into a pre-warmed 100 ml broth and incubated at 37°C in a shaking incubator (150 rpm) for 2 h. A 12 ml volume of this culture was added to 24 ml of RNAprotect reagent in a 50 ml Falcon tube. An aliquot of 1 ml from the same flask was collected for bacterial viable counting. Once the bacterial culture was added to RNAprotect the mixture was mixed immediately by vortexing for 5s followed by incubation for 5 min at RT (15-25°C) and centrifugation at 5000 x g for 12 min. After centrifugation the supernatant of the tube was carefully discarded without disturbing the unseen pellet in the tube' bottom, which was re-suspended in pre-warmed 10 ml NB, vortexed for a few seconds and incubated at 37°C shaking incubator for 20 min. Then a 1 ml aliquot from this tube was collected for bacterial viable count by serial dilution on MacConkey and NA plates. This method was performed in triplicate.

2.2.3.7 Evaluation of the RNA concentration and quality using Agilent 2100 bioanalyzer

The collected *in vitro* and *in vivo S.* Enteritidis RNA, which was considered as pure samples by NanoDrop 1000 spectrophotometer, were further evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). A volume of 1 µl of each RNA sample was diluted in 4 µl of RNA-free water for analysis, and then the obtained concentration for each sample was multiplied by 5 to get the actual concentration per µl. These RNA concentrations then were compared to those obtained by NanoDrop using coefficient correlation of Excel software.

The quality of each sample RNA was also assessed from the electropherogram of each sample using Agilent 2100 Bioanalyzer. It allows a visual inspection of mRNA integrity, and generates ribosomal ratios. Each sample RNA shows two peaks of ribosomal RNA (rRNA), 16S and 23S rRNA peaks (bands). The quality of bacterial mRNA can easily be determined through visual inspection of the electropherogram of each sample. The rRNA ratios (16S/23S) shown by

this electropherogram is indicative for messenger RNA quality. The rRNA ratios (16S/23S) assess RNA quality in terms of degradation.

2.2.3.8 Bacterial RNA amplification

The selected *in vitro* and *in vivo* RNA samples for microarray were subjected to amplification using the Message Bacterial RNA Amplification kit (Ambion) by incorporating aminoallyl UTP (Cat No: 8437, Ambion) to enhance the binding between the amplified RNA and Cy dyes. A mass of 100 ng of each sample RNA diluted in 5µl RNase-free water is considered as starting material for RNA amplification (considering Agilent Bioanalyzer 2100 RNA Readings).

2.2.3.8.1 Polyadenylation of RNA template

RNA samples (100 ng / 5µl) were incubated at 70 ^{o}C for 10 min; then kept on ice for 3 min; during which time the polyadenylation polymerase master mix (PAP-MM) was prepared by mixing the following components with the right amounts in a non-stick Ambion mini-tube at room temperature: nuclease-free water 3µl, 10X poly (A) tailing buffer 2 µl, RNase inhibitor 2 µl, poly (A) tailing ATP 1 µl and PAP 2 µl. Then the 10 µl PAP MM was gently mixed and briefly centrifuged. Then 5µl of this mix were introduced into each RNA sample and then gently mixed and briefly spun; before they were incubated at 37^{o}C for 15 min; during which time the reverse transcriptase master mix (RT-MM) was prepared.

2.2.3.8.2 Reverse transcription to synthesis 1st strand cDNA

The RT MM was prepared in non-stick Ambion mini-tube at RT as following:

Nuclease-free water 6 µl, T7 oligo (dT) VN 2 µl, 10X first strand buffer 2 µl, dNTP mix 8µl and arrayscript 2 µl.

Then the 20 µl RT MM was gently mixed and briefly centrifuged. Then 10 µl of this mix were introduced into each 10 µl RNA-mix sample and then gently mixed and briefly spun; before they were incubated at 42^{o}C for 2 h. During last 30 mins of previous incubation second strand of cDNA synthesis master mix (SS cDNA MM) was prepared on ice as mentioned below.

2.2.3.8.3 Second strand cDNA synthesis

The SS cDNA MM was prepared in non-stick Ambion mini-tube on ice as following: nuclease-free water 126 µl, 10X second strand buffer 20 µl, dNTP mix 8 µl, DNA polymerase 4µl and RNase H 2 µl. Then the 160 µl SS cDNA was gently mixed and briefly centrifuged. Then 80 µl of this mix were introduced into each 20 µl RNA-mix sample and then gently mixed and briefly spun; before they were incubated at 16^{o}C thermal cycler for 2 h. Once the 2 h of incubation was over the double strands of cDNA samples were placed on ice; and immediately the following step was carried out.

2.2.3.8.4 cDNA purification

A volume of 250 µl of pre-warmed cDNA binding buffer was added to each cDNA sample; mixed gently by vortexing, then 350 µl of the cDNA sample plus cDNA binding buffer were loaded into the centre of the filter cartridge already placed on the wash tube and centrifuged at 10 000 x g for 1 min. The flow-through was discarded, the filter cartridge was replaced on the same tube and then 500 µl wash buffer was added to each sample and centrifuged at 10 000 x g for 1 min. The flow-through was discarded; the filter cartridge was replaced on the same tube. An extra-1 min centrifugation was applied to eliminate trace amounts of ethanol. Then the cDNA filter cartridges were transferred into cDNA elution tubes. A volume of 18 µl of pre-heated (50 ^{o}C) nuclease-free water were added to the centre of each filter cartridge and incubated at RT for 2 min, before being centrifuged at 10 000 x g for 1.5 min. The pure double-stranded "ds" cDNA ~15 µl accumulated in the bottom of each elution tube was kept on ice.

2.2.3.8.5 *In vitro* transcription (IVT) to synthesise amplified RNA using aminoAlyll-UTP-Labelled Reactions

The 5-(3-aminoallyl)-UTP (Ambion) was incorporated into the amplified RNA to provide an amine-reactive group for addition of label with any moiety bearing an N-hydroxySuccinimidyl (NHS) ester (as a pre-labelling stage). The 5-(3-aminoallyl)-UTP) is a 50 µM solution of amino allyl modified UTP, used with the MessageAmp II Bacteria kit to synthesis amino-reactive aRNA which

can then be post-labelled with any amino-reactive label moiety (e.g. Cy3 and Cy5). The volumes of the reagents involved in this step are as mentioned in Table 2.1.

Table 2.1: *In vitro* transcription master mix (IVT MM) reagents

Component	Quantities	Kept
T7 ATP solution (75 mM)	8 µl	on ice
T7 CTP solution (75 mM)	8 µl	on ice
T7 GTP solution (75 mM)	8 µl	on ice
T7 UTP solution (75 mM)	4 µl	on ice
AminoAlyll UTP (50mM)	6 µl	on ice
10X T7 Reaction Buffer	8 µl	on ice
T7 Enzyme Mix	8 µl	on ice
Total	50 µl	on ice

Then the *in vitro* transcription master mix including aa-UTP (aa-UTP-IVT) was gently mixed and briefly centrifuged. Then 25 µl of this mix were introduced into each 15 µl of cDNA sample and then gently mixed and briefly spun before they were incubated at 37 ^{o}C for 14-18 h (o/n).

2.2.3.8.6 aRNA purification

This step was performed at RT. Once the overnight 37^{o}C incubation period (14 h) was over, the sample (s) were placed on ice. A volume of 60 µl of nuclease-free water was added to each sample (15µL ds cDNA + 25 µl IVT MM); collectively the total volume of each tube was 100 µl. All samples were gently mixed up, then 350 µl aRNA binding buffer were added to each aRNA sample; which was then briefly vortexed and spun. Then a volume of 250 µl 100% absolute ethanol was added into each aRNA sample; and mixed by pipetting 3X. No vortexing was applied. Then 700 µl (100 + 350 + 250) of each sample were loaded into the centre of an aRNA filter cartridge and centrifuged at 10 000 x g for 1 min. The flow-through was discarded and the filter was replaced on the aRNA collection tube. A volume of 650 µl of wash buffer were loaded into each aRNA filter cartridge and centrifuged at 10 000 x g for 1 min. The flow-through was discarded and the filter was replaced on the aRNA collection

tube. The aRNA filter cartridge for each sample was spun for an additional 1 min to remove the trace amounts of ethanol. Then the filter cartridge of each sample was transferred into its corresponding fresh aRNA collection tube (A), into which 75 µl pre-heated (55 ^{o}C) nuclease-free water were added to the centre of the filter of each sample, incubated at RT for 2 min before being centrifuged at 10 000 x g for 1.5 min. Then the filter cartridge of each sample was transferred onto another fresh aRNA collection tube (B) into which 75 µl pre-heated (55 ^{o}C) nuclease-free water were added into the centre filter of each sample, incubated at RT for 2 min before being centrifuged at 10 000 x g for 1.5 min. Each RNA sample ended with two collection tubes (A and B). The total volume of each collection tube was 75 µl. The RNA samples for array work were mainly collected from the 1st tube, because they reflect better quality. All RNA samples concentration and optical quality at 230 and 280 nm was assessed after they have been diluted 10x in nuclease free water using the NanoDrop 1000 spectrophotometer and confirmed with the Agilent Bioanalyser 2100 (Agilent). Then they were all labelled and kept at -80 ^{o}C freezer until they were required for labelling with fluorescent dyes (e.g. Cy3 and Cy5).

2.2.3.8.7 RNA precipitation method

A 1/10 volume of sodium acetate and 2.5 volumes of 100% ethanol were added to each aRNA sample volume. Then they were gently mixed by inverting the tubes. Then all samples were incubated at -20^{o}C for 30 mins, and then they were centrifuged in a microcentrifuge at 13 300 rpm (17 000 x g) at 4^{o}C for 20 min. The supernatants of all RNA samples were removed without disturbing the RNA pellets, removed by aspirating off from the opposite side of the tube to RNA pellet. A volume of 250 µl of 70% ethanol was added to each RNA pellet, gently mixed, and then all tubes were centrifuged at 13 300 rpm at 4^{o}C for 10 min. The supernatant in each tube was removed, and then the tube lids were left open for 1-2 min to evaporate any remaining ethanol. The dried RNA pellet in each tube was dissolved with an appropriate amount of coupling buffer. Awareness was considered for not over drying the RNA pellets, which could result in them being very difficult to re-dissolve.

2.2.3.8.8 Amino allyl amplified RNA (Barnass *et al*.) coupling with Cy dyes

A mass of 10 µg of dry amino allyl aRNA of each sample, dried by sodium acetate and ethanol precipitation were re-dissolved in 9 µl coupling buffer (Invitrogen, UK) by gently vortexing. One aliquot of Cy dyes Cy_3 or Cy_5 (Amersham, UK) were prepared for each RNA sample. Cy_3 was used for *in vitro* RNA samples; Cy_5 was used for *in vivo* RNA samples. Each Cy dye frozen pellet was dissolved in 11 µl dimethyl sulfoxide (DMSO, Ambion) and mixed by pipetting as many as 20 times in the dark. Then 11 µl of Cy_3 or Cy_5 was added to its corresponding 9 µl RNA sample (Cy_3 into *in vitro* RNA samples; Cy_5 into *in vivo* RNA samples) and both well mixed by pipetting.

All samples then were incubated for 30 min at RT in the dark wrapped in foil. A volume of 4.5 µl hydroxylamine was added into each sample, mixed and incubated for 15 min at RT in the dark in order to quench the reaction and remove the active amine-residues on the unincorporated dye molecules. A volume of 5.5 µl nuclease-free water was added into each sample; so the volume of each sample became 30 µl. The coupled RNA tubes were covered with foil where possible to protect the dye molecules from the light, as too much exposure to light would cause dye bleaching.

2.2.3.8.9 Dye labelled aRNA purification

A volume of 105 µl of an aRNA binding buffer was added into each 30 µl dye coupled aRNA sample. Then 75 µl of 100% grade ethanol were added into each sample, mixed by pipetting. Then immediately 210 µl of each sample was loaded onto the centre of an aRNA filter cartridge (with a collection tube attached), centrifuged for 1 min at 10 000 x g, the flow-through of each sample was discarded and the aRNA filter cartridge for each sample was replaced into the collection tube. A volume of 500 µl of aRNA wash buffer was applied to each aRNA filter cartridge, centrifuged for 1 min at 10 000 x g, and the flow-through of each sample was discarded and the aRNA filter cartridge for each sample was replaced into the 2 ml wash tube. All sample tubes were spun for 1 min to remove any trace amounts of ethanol; the wash tube of each sample was

then discarded and the filter of each was placed on a fresh aRNA collection tube. A volume of 10 µl of pre-heated nuclease-free water (50 ^{o}C) was applied to the centre of the filter of each sample, left at RT for 2 min then centrifuged for 1.5 min at 10 000 x g. The previous step was repeated with an extra 10 µl of preheated nuclease-free water. Then all coupled RNA samples (20 µl each) were kept wrapped in foil on ice and the frequency of incorporation calculated (as described in section 2.2.3.9) before being stored at -80 ^{o}C.

2.2.3.9 Calculating the frequency of incorporation

Once the aRNA coupled with Cy dyes samples were purified, the incorporation of dye molecules with RNA was measured using the NanoDrop. The frequency of incorporation (FOI) calculated: using the information from the NanoDrop1000 spectrophotometer (Microarray setting). The FOI was calculated according to the following equations:

For Cy_3 FOI = 58.5 x Absorbance at 550 / Absorbance at 260.

For Cy_5 FOI = 35.1 x Absorbance at 650 / Absorbance at 260.

If the FOI resulted in reading > 20 the incorporation between the dye and RNA was regarded as good (http://www.nanodrop.com/Library/NanoDrop-1000-Microgenomics-Application-Notes).

2.2.3.10 Hybridization

2.2.3.10.1 Hybridization samples preparation

For each microarray, the following components were added into a 1.5 ml nuclease-free microfuge tube: 300 ng Cy_3-aRNA, 300 ng Cy_5-aRNA, 10x blocking agent (Agilent), nuclease free water and 25x fragentation buffer (Agilent), with volumes as indicated in Agilent microarray web site, two-colour microarray-based gene expression analysis.

http://www.chem.agilent.com/en-US/Pages/HomePage.aspx

The Fragentation mixture was incubated at 60°C water bath for 30 min to fragent RNA. Then 200 µl of 2x GE hybridization buffer were added into the tube of fragentation mix to stop the fragentation reaction. The components were well mixed by careful pipetting. The tube was centrifuged at 17 000 x g (Thermo, UK) for 1 min at RT to drive the sample off the walls and lid and to aid in bubble reduction. The hybridization mix was loaded onto the array slides immediately.

2.2.3.10.2 Hybridization assembly preparation

In a warm and dark area a clean gasket slide was loaded into the Agilent hybridization chamber base with the label facing up and aligned with the rectangular section of the chamber base. For each microarray slide (8 x 15k); 40 µl of the hybridization sample were slowly dispensed into each array portion onto the gasket bounded squares.

The array active side for each microarray slide was slowly and gently placed down onto the hybridization gasket slide, so that the "Agilent"-labelled barcode was facing down and the numeric barcode was facing up. The chamber cover was placed onto the sandwiched slides and clamped. The clamp was well tightened onto the chamber. Then the microarray slide was incubated (hybridised) in Agilent hybridisation oven at 65°C, rotating at 10 rpm for 17 h.

2.2.3.11 Microarray washes

2.2.3.11.1 Microarray slide washes preparation

A volume of 250 µl Triton X-102 was added to 500 ml of wash buffer "1 and 2" (Agilent). The wash buffer-2 was kept at 37^{o}C o/n. Staining dishes, racks and stir bars that were used in previous experiments with the Agilent stabilization and drying solution were washed with acetonitrile to remove any remaining residue. The slide rack and stir bar were put to the staining dish containing 100% acetonitrile. This was conducted in a vented fume hood. The staining dish was transferred with the slide rack and stir bar to a magnetic stir

plate. The magnetic stir plate, speed setting 4 (medium speed) was set. The staining dishes were air dried in the vented fume hood.

2.2.3.11.2 Microarray slides wash

Five plastic dishes were labelled for this experiment. Dish-1 was filled with 500 ml gene expression (GE) wash buffer 1 plus 250µl Triton and kept at RT for the disassembly. Dish-2 was filled with 500 ml GE wash buffer 1 plus 250µl Triton and kept at RT and designed for the 1st wash of 1 min (Table 2.2).

Table 2.2: Wash conditions and procedures with stabilization and drying solution.

Wash steps	Wash buffer	Container	Temp	Time
Disassembly	GE wash buffer 1	500ml Dish	RT	-
1st Wash	GE wash buffer 1	50ml Falcon Tube (1)	RT	1 min
2nd Wash	GE wash buffer 2	50ml Falcon Tube (2)	Elevated Temp	1 min
Acetonitrile wash	Acetonitrile	50ml Falcon Tube (3)	RT	10 sec
3rd Wash	Stabilization & drying solution	50ml Falcon Tube (4)	RT	30 sec

Dish-3 was filled with 500 ml GE wash buffer 2 plus 250µl Triton and kept at 37°C o/n for the 2nd wash of 1 minute. Dish-4 was filled with 500 ml acetontrile and kept at RT for the 3rd wash of 10 seconds. Dish-5 was filled with 500 ml stabilization and drying solution and kept at RT for the last wash of 30 seconds.

The hybridization chambers were removed from incubator. If there was any bubble formation this was recorded. Then the hybridization chambers were disassembled as following:

The hybridization chambers assembly were placed on a flat surface and the thumbscrew loosened. The chamber cover was removed. Then with gloved fingers, the array-gasket sandwich was removed from the chamber base by holding the slides by their ends. The microarray slide numeric barcode was kept facing up as the sandwich was quickly transferred to the Dish containing GE wash buffer 1, where the array-gasket sandwich was submerged. Once the sandwich was completely submerged in GE wash buffer 1, the sandwich was

open from the barcode end only by penetrating one of the blunt ends of the forceps between the slides.

Then gently the forceps was turned upwards or downwards to separate the slides. Then the gasket slide was placed in the bottom of the dish. The microarray slide was removed and placed into slide rack in dish-2 containing WB-1 on magnetic rotator plate for 1 min at RT. During the previous wash step, the dish-3 containing wash buffer 2 was brought out from the 37°C oven. Then the slide rack was transferred from dish-2 into dish-3 containing GE wash buffer 2 at elevated temperature on the magnetic plate for 1 min. Then the slides were removed from GE wash buffer 2 and were tilted slightly to minimize wash buffer carry-over. The slide-rack was then immediately transferred to dish-4 containing acetonitrile and rotated gently for less than 10 seconds. Afterwards the slide-rack was transferred into dish-5, which was filled with stabilization and drying solution and rotated gently for 30 seconds.

The slide rack was slowly removed to minimize droplets on the slides. It took 5 to 10 seconds to remove the slide rack. Then the slides were dried with an air pressure gun and they were wrapped in foil after being flushed with nitrogen gas. Then the slides were immediately scanned to minimize the impact of environmental oxidants on signal intensities. Until the scan was performed the slides were stored in the dark and flushed with nitrogen.

2.2.3.12. **Microarray data acquisition**

The microarray slides were scanned using an Axon 4000B scanner (Scan resolution 5µm) and the Genepix software was used to quantify the signal intensities. Quality control software features were routinely used. For each of the two fluorophores used, a separate scan was done and the images were then combined for analysis. A bounding box, fitted to the size of the DNA spots was placed over each array element. A scatter plot was visualized before normalization for the quick and easy comparison of slide replicates (1 forward and 1 reverse slide). Data from spots that were marred by dust particles or hybridization artefacts were excluded from further analysis. A gene array/annotation list (gal) file containing the feature name and any comment

related to its synthesis as well as its coordinates in the array was created by Dr Tristan Cogan, University *of* Bristol, UK and loaded into the software. For each hybridised slide, a set of two Tif files (one for each channel), a settings file (gps) and a results file (gpr) were created.

2.2.3.13. Microarray data analysis

The Genepix results file (gpr) for each slide was slightly modified. Those array features, for which the percentage of pixels greater than two standard deviations (2SD) were below 85 % in at least one of the channels were labelled as marginal "M" in the flags column.

The modified gpr files were imported into GeneSpring GX 10.0 software (Agilent Technologies Inc., USA), a software package designed to display and analyse microarray data. For normalization, for each array feature the median pixel intensity for the local background was subtracted from the median pixel intensity of the feature independently of their status as being flagged "A" for bad or "M" for marginal. The intensities of the test strain per feature or spot were divided by those of the control strain and finally normalised per slide to the median. For all values of the control reference below 0.001 the value of 0.001 was arbitrarily used. Three commonly applied methods were used for the analysis of the normalized ratio data to determine the up or down regulation of the respective genes (Cooke *et al.*,2007; Witney *et al.*,2005). These were as follows: (i) twofold cutoff, (ii) 3 standard deviation "3SD", and (iii) Gack software.

The twofold cutoff method is an arbitrary cutoff which was used to identify those genes that are specific to one of the strains. Therefore, for all strains, the upper cutoff was set at a ratio of 2 and the lower cutoff at a ratio of 0.5. Genes with a ratio greater than the upper cutoff were deemed to be specific to the test strain, genes with a ratio less than the lower cutoff were deemed to be specific to the reference strain, and genes with ratios between 0.5 and 2 were deemed to be present in both strains.

The 3SD, rather than using a fixed-value cutoff for all arrays as above, a cutoff based on the variation in the ratio data of the core genes was determined for each strain. For each strain, the standard deviation of ratios for genes within the subset of core genes was calculated to measure variation in the data, and then the ratio cutoffs for each strain were set at 3 standard deviations (3SD) on either side of the median value. The standard deviation was calculated for each test strain independently.

The GACK software uses the distribution of the ratio data for each strain to classify genes based on the probability that a gene is either present or absent/divergent (Kim *et al.*, 2002).

The following structure (Figure 2.2) illustrates the Microarray experiments steps sequence, starting with bacterial RNA extraction ending with features extraction and data analysis.

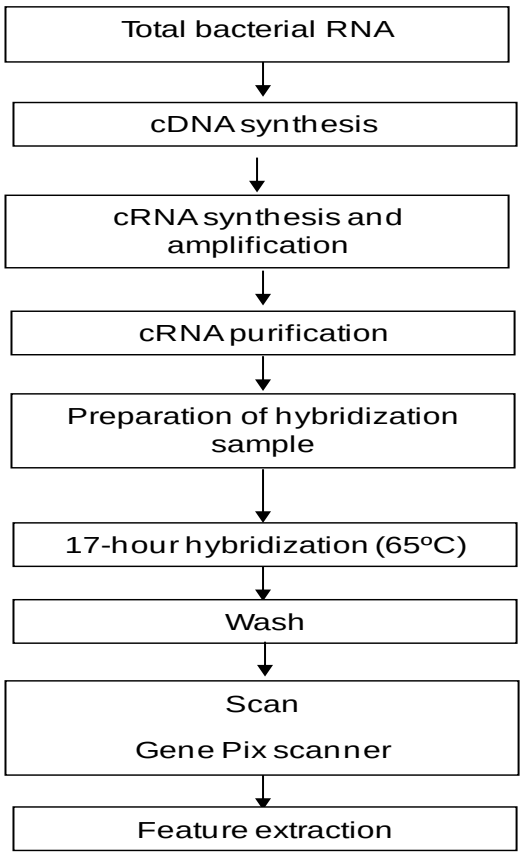

Figure 2.2 Workflow for sample preparation and array processing

2.2.4 Microarray evaluation by 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 few randomly selected genes were designed using the universal probe library assay design centre (Roche Applied Science, UK) available at: 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. A standard $\log_{10}$ dilution (10, 100 and 1000) series was 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.

2.2.5 Bacterial mutation

The *S.* Enteritidis genes of interest were subjected for mutation by λ red mutagenesis (Datsenko and Wanner, 2000). The pKD46 plasmid provides the function of lambda red system by producing some specific enzymes which mediate the incorporation of PCR-products (linear DNA) into the bacterial genome of interest. The pKD46 enables the antibiotic sequence (s) (e.g. chloramphenicol "Cm" or kanamycin "Km") to replace the target gene (s).

Briefly the sequences of the target genes for mutation were identified and approximately 50 bases of flanking regions were also selected on both ends of the open reading frames (ORF).

2.2.5.1 Primers design for the target genes

The genes of interest were identified and had their primers designed as mentioned in following schematic illustrations. Briefly, the Sanger Institute

web site (http://www.sanger.ac.uk/research/projects/pathogengenomics) and the Primer Design Tool (CLC) were utilised in designing these primers. Two antibiotic cassettes were used in designing these primers (Cm and Km). The 50 bases selected from the non-coding strand made up the reverse mutagenesis primer, together with the antibiotic-specific sequence on the 3' end. The 3' end sequences of the mutagenesis primers depended only on the antibiotic cassette that has been selected (Cm or Km). A pair of target specific test control primers was also designed for checking the replacement of the target gene to the antibiotic cassette. The binding site of the test primers had to be outside of the coding region of the target gene (Figures 2.3 and 2.4). The published Cm and Km cassette specific test primers (C1, C2 and K1, K2 respectively) were also used in combination with the target specific test primers for checking the incorporation of the antibiotic cassettes to the desired place.

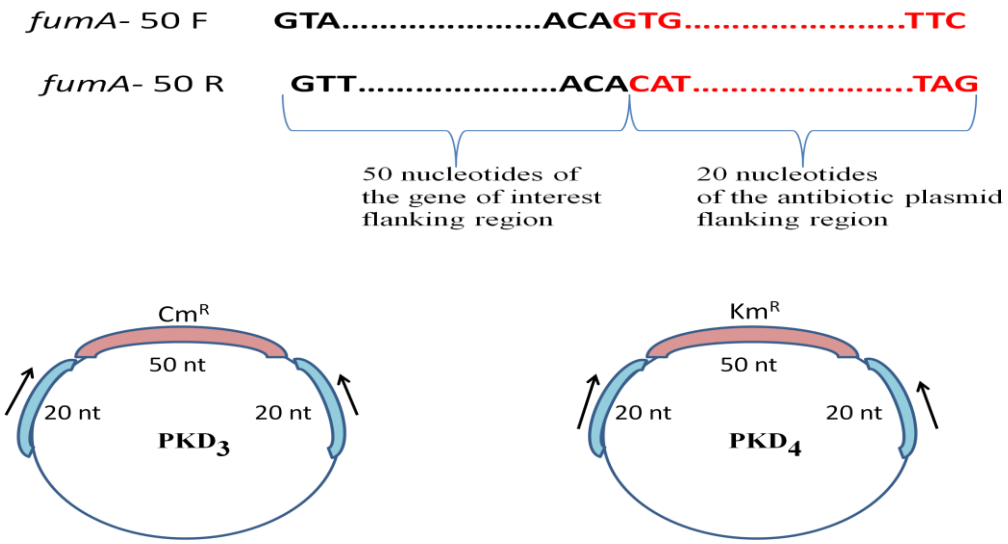

Figure 2.3: These schematic figures show the gene of interest is replaced by the antibiotic sequence by using plasmids (pKD3 "Cm resistant" or pKD4 "Km resistant")

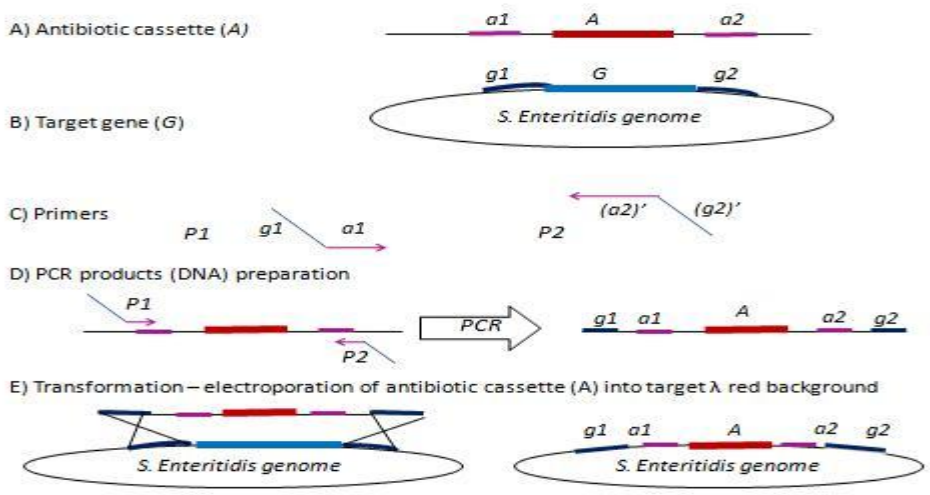

Figure 2.4: This schematic sequence shows the steps performed in order to mutate the gene of interest by replacing it by the antibiotic cassette after integrated forward and reverse primers for the gene of interest and the antibiotic have been designed and electroporated into *Salmonella* genome by the action of λ red recombinase system.

The mutagenesis control primers specific for the target gene were designed (20 nucleotides). For the complete deletion of an ORF from the first base to the last, the 50 bases right before the start codon on the coding strand, and the 50 bases right after the stop codon on the non-coding strand were selected. The 50

bases from the coding strand formed the 5' end of the forward mutagenesis primer, while the antibiotic cassette specific sequence formed the 3' end.

2.2.5.2 Plasmid purification

A colony of *E. coli* harbouring pKD3 or pKD4 plasmids (Datsenko and Wanner, 2000) was subcultured into 10 ml NB supplemented with 20 µl Cm or Km (20 µg / ml) as selective agents. Then the broth was incubated at 37^{o}C in a shaking incubator (150 rpm/min) o/n. On the following day, a volume of 1.5 ml of *E. coli* culture was loaded into 2 ml eppendorf tube, centrifuged at 9000 x g for 3 min, the supernatant was discarded, the rest of the culture was loaded, centrifuged and supernatant discarded; by which a clean pellet was obtained at the bottom of each tube. Then a volume of 250 µl buffer P1 (Qiagen Prep Spin Kit, UK) was added to the tube, vortexed vigorously until the pellet completely suspended. Then a volume 250 µl buffer P2 was added and mixed thoroughly by inverting the tube 4 – 6 times; a good result was indicated a by blue homogenous colour. Then 350 µl of N3 buffer were added and mixed immediately by inverting the tube 4 – 6 times when solution colour became cloudy. A homogenous colourless suspension indicated that the SDS had been effectively precipitated.

The tube was centrifuged at 10 000 rpm (high speed) for 10 min (Avant J-E Centrifuge, Beckman Coulter); after which speed a compact white pellet was formed in the tube bottom. The supernatant was carefully collected and transferred into its corresponding Qia Prep spin column by pipetting. Spin columns were centrifuged at high speed for 30 sec and the flow-through was discarded. A volume of 0.5 ml PB buffer was added into each column as wash step and centrifuged for 60 sec; then the flow-through was discarded. A volume of 0.75 ml PE buffer was added into each column as further wash step and centrifuged for 60 sec; then the flow-through was discarded. The columns were centrifuged for additional min to eliminate any trace amounts of ethanol or residual wash buffer. Then the filter column was transferred into their corresponding labelled 1.5 ml eppendorf tubes and then 50 µl of RNase free water was added into the centre of spin column. Then the tubes were

centrifuged at high speed for one min. A further 1 min centrifugation was applied using the same flow-through over the filter again for 1 min. Then the plasmid (pKD3 and pKD4) DNA quantity and quality were measured using the NanoDrop Spectrophotometer (Agilent Technologies, USA). Plasmids were kept frozen at -20^{o}C until needed.

2.2.5.3 Gene primers test by PCR

Commercially designed primers for the target genes were PCR-tested and then their PCR-products amplified. PCR products were generated by using several pairs of 70-nt-long primers that included 50-nt homology extensions and 20-nt priming sequences for pKD3 or pKD4. The purchased primers were dissolved in pure distilled water to form (0.1mM) as stock solution which was then kept at -20^{o}C. A volume of 50µl of stock solution of each primer was mixed with 50µl pure water to produce a working solution of it (0.05mM). Half of the target genes were replaced by the chloramphenicol (pKD3) cassette, and the other half were replaced with the kanamycin (pKD4) cassette.

Table 2.3: PCR reagents and their volumes required to test gene primers using ordinary Taq-polymerase

PCR-Reagents	**Volume 1x (µl)**	**Volume 5x (µl)**
H_2O	16	112
PCR-Buffer	2.5	17.5
$MgCl_2$	2	14
dNTP	1	7
Taq polymerase	0.5	3.5
Primer $_{Forward}$	1	-
Primer $_{Reverse}$	1	-
Template pKD_3 or pKD_4	1	-

2.2.5.4 Amplification of genes PCR-products

PCR products for the genes of interest were amplified using Klen Taq polymerase for more accuracy and amplification.

PCR setting (Alpha Laboratories) was performed to test the amplification of double strand DNA (PCR-products) for the gene (s) of interest. The setting was as following: 94 ^{o}C for 3 min, 94 ^{o}C for 20 sec (35 cycles), 60 ^{o}C for 30 sec, 72 ^{o}C for 90 sec; 72 ^{o}C for 5 min and then samples were kept automatically at 4 ^{o}C until needed.

The other PCR setting (Alpha Laboratories) was performed to test the location of mutated genes after been replaced by antibiotic cassette. The setting was as following: 94 ^{o}C for 1 min, 94 ^{o}C for 30 sec (35 cycles), 68 ^{o}C for 3 min (35 cycles) and 68 ^{o}C for 5 min then samples were automatically kept at 4 ^{o}C until needed. The setting applied for mutation of the fumarate reductase (*frd*ABCD) operon was as following: 94 ^{o}C for 3 min, 94 ^{o}C for 20 sec (2 cycles), 58 ^{o}C for 30 sec, 72^{o}C for 4 min, 72^{o}C for 5 min (35 cycles) and then samples were kept at 4 ^{o}C until needed.

2.2.5.5 Gel electrophoresis

The amplified PCR products were visualized by gel electrophoresis. Briefly, A 1.2 g aliquot of agarose was dissolved in 80 ml TAE buffer. Ethidium bromide (4 µl) was used for staining. Separation was achieved at 80 volts, 500 mA, 250 watt. The gel was carefully imaged by an Image Quant 300 (GE Healthcare Life Sciences).

2.2.6 DNA-precipitation by sodium acetate and ethanol

The method mentioned in section 2.2.3.8.7 of this Chapter was applied for these DNA samples. This method resulted in precipitated dry PCR-products which kept frozen (-20^{o}C) until needed for electropration.

2.2.7 Electroporation

The aim of this experiment was to incorporate the produced linear DNA, (concentrated and amplified PCR products of the genes of interest) into the *S.* Enteritidis PT4 harboring pKD46 plasmid by electroporation.

S. Enteritidis harboring the plasmid pKD46 grown on NA supplemented with ampicillin (100 µg/ml) was inoculated into 10 ml NB supplemented with

ampicillin (100 µg/ml), incubated at 29°C (pKD46 is temperature sensitive), in shaking incubator (150 rpm) for o/n. On the following day, 0.5 ml of o/n *S.* Enteritidis PT4 harboring pKD46 culture was inoculated into 20 ml NB containing 50 µl ampicillin (100 µg/ml), 5 ml arabinose (10mM). Arabinose is added to induce the enzyme activity system of pKD46 to express recombination activity between PCR-products and bacterial genome. Arabinose was added to a final concentration of 0.2% approximately 2 h before performing electrophoresis. However, the mix was incubated at 29°C, in shaking incubator (150 rpm) for 4 h until the culture reached mid-log phase [OD_{600} = 0.5] at which time-point an extra 5 ml arabinose were added and incubated under the same conditions for further 20 min. Then the flask content was divided into two 50 ml Falcon tubes, the tubes were centrifuged at 4 000 x g, 4°C for 10 min. The supernatant of each tube was decanted. The pellet of each tube was re-suspended by pipetting with 1 ml cold distilled water then filled with 27 ml cold distilled water, vortexed and centrifuged as before (4000 x g, 4°C for 10 min). This wash step was performed 3 times for both tubes. After the last wash, the supernatant was decanted leaving a drop (160 µl liquid) on each pellet. The drop was mixed with the pellet by pipetting, a homogenous suspension was produced. A volume of 80 µl of this suspension (*S.* Enteritidis pKD46) transferred into a frozen electroporator pulser cuvette (Gene Pulser Xcell™, BioRad). Then 10 µl PCR products for the gene of interest (section 2.2.5.6), were introduced into the cuvette containing the suspension of pKD46. The Electroporator (Gene Pulser Xcell™, BioRad) was set at the setting: voltage 2500 volt, capacitance 25 µF, resistance 200 ohms, and cuvett (2 mm). The bacterial suspension was pulsed for few seconds. The performance of electroporation on bacterial cells is indicated by the time constant (TC) which should be varied around 4-5. Once the pulse was performed and TC reading for each sample was taken, the cuvette contents were introduced into pre-warmed 1.5 ml fresh NB and incubated at 37 °C static incubator for 20 min, followed by incubation at 37°C in a shaking incubator 60 min. Then the culture was transferred into 2 ml sterile tubes, centrifuged at 12 000 x g for 4 min and the supernatant was discarded while ~ 100 µl were spread on NA supplemented with the relevant antibiotics, incubated at 37°C for 36 h. A small amount of the

culture, which remained in the Falcon tube before being electroporated, was spread on to another NA plate supplemented with the same antibiotic as control. Another control plate of NA supplemented with Cm or Km was plated with some drops of o/n culture and incubated under the same conditions. After 24-48 h a small number of isolated colonies were observed on the NA plates plated with *S.* Enteritidis pKD46 incorporated with PCR-products. They were streaked and inoculated onto NA plates and NB tubes respectively supplemented with Cm or Km and incubated at 37^{o}C for 24 h.

2.2.7.1 *S.* Enteritidis generated mutants' crude DNA extraction

A volume of 1.5 ml of 24 h of bacterial culture of every presumed mutant was centrifuged in an eppendorf tube at 13 000 x g for 2 min. The supernatant of every tube was decanted. The formed pellets were re-suspended in 200 µl pure water by vortexing until a homogenous suspension was formed in all tubes. Then the tubes were incubated at 100 ^{o}C heater block for 20 min. Then tubes were incubated at -20 ^{o}C for 20-30 min. After such time the sample were brought out from the freezer to defrost, then they were centrifuged at 13 000 x g for 2 min to precipitate the cell debris. Then all presumably *S.* Enteritidis generated mutants' crude DNA was tested by confirmatory PCR using specific control primers.

2.2.8 Competitive exclusion

Interaction between cultures was tested *in vitro* in three different ways as described below:

2.2.8.1 *In vitro* competitive exclusion of generated mutants against the parent strain

This was designed to test the ability of stationary phase cultures of mutants to inhibit multiplication of small numbers of the parent strain introduced into the culture in NB incubated aerobically.

Universal bottles of 10ml NB were inoculated with 100 µl of 24 h broth cultures of Nal^{R} derivatives of mutants of *S.* Enteritidis. Two control bottles

were included, the positive control inoculated with 10µl of the wild type Nal^R; while the negative control was uninoculated broth. The experiment was performed in triplicate. Then the cultures were incubated for 18 h at 42^{o}C in a shaking aerobic incubator (150 rpm). On the following day 10 µl from a culture of the wild type Spc^R (challenge) were diluted (x1000) in 10 ml fresh NB and mixed well. From this, 100 µl were introduced into the stationary phase cultures of the mutants. Each culture was then mixed briefly by hand before an aliquot of 100 µl of each was collected for bacterial viable count estimation (time-point 0). These bottles were incubated at 42^{o}C for 24 h in a shaking incubator at 150 rpm. A further 100 µl sample was taken at 24 h. Viable counts were done on these samples as described.

2.2.8.2 *In vitro* co-culturing experiment of generated mutants with the parent strain

The competitive ability of mutants and parent strain was tested by inoculating equal number of strain (either Nal^R or Spc^R) simultaneously into NB. Culture conditions and the numbers inoculated were identical to those mentioned in section 2.2.8.1 (e.g. 10 µl of a x 1000 dilution of an o/n culture).

2.2.8.3 *In vitro* competitive exclusion of the parent strain against the generated mutants

This was designed to test the ability of stationary phase cultures of the parent strain to inhibit multiplication of small numbers of mutant strains introduced into the culture in NB incubated aerobically. The method used is similar to that in section 2.2.8.1.

Chapter - 3: *S.* Enteritidis PT4 Gene Expression in 1-Day Chicken Caeca

3.1 Introduction

This chapter describes the expression of *S.* Enteritidis PT4 P125109 genes in the caeca of newly-hatched chickens compared with mid-log phase cultures in nutrient broth (NB).

3.2 Experimental Plan

A total of one hundred and eighty 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 P125109 culture grown for 18 h in nutrient broth (NB) at 37^{o}C in 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 for viable count estimations. The caecal contents from the remaining birds were removed and mixed with RNA protect (Qiagen). The RNA was produced (section 2.2.3.3) amplified (section 2.2.3.8) and gene transcription analysed (section 2.2.3.13).

For *in vitro* control cultures, 3 ml of an overnight NB culture *S.* Enteritidis was inoculated into 300 ml of pre-warmed NB and incubated with shaking (200 rpm) for 2 h at 37^{o}C. Cultures were pre-treated with RNA protect before being centrifuged and subjected for RNA isolation (section 2.2.3.3).

The *S.* Enteritidis extracted and purified RNA that grown *in vivo* 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.

The gene expression of *S.* Enteritidis grown *in vivo* was compared with *in vitro* grown *S.* Enteritidis (log-phase) gene expression in 3 groups of chickens; each

represents 30 birds pooling *S.* Enteritidis RNA. These were compared separately with 3 *in vitro* culture preparations of *S.* Enteritidis RNA.

The validity of using microarray was confirmed by qRT-PCR analysis (Chapter 2; section 2.2.4) of selected genes (Table 3.1) again comparing with an *in vitro* NB culture.

Table 3.1: Primers used in Quantitative real-time-PCR:

Gene ID	Gene Symbol	Sense / antisense (5'- 3')
SEN 1762	*gap*A	CGTATCGGTCGCATTGTT
		TTCGTCCCATTTCAGGTT
SEN 1304	*yci*E	CATTTCTCGTTCGGTGTT
		AATTGTTCCGTTGTCTGC
SEN 0215	*cda*R	CGGCGAACCAGAGCATCT
		ATCCCAGCGACCAAACGA
SEN 2725	*sip*C	GTCTTCCAGTGCCGTTGC
		GTGGCTTTCAGTGGTCAGTTTA
SEN 0073	*cai*B	GAAACGGGTAAAGGTGAAAG
		GCAACCAGCGTAGTAGGG
SEN 0221	*map*	CCGAAGTGCTGGAAATGA
		GAGGTATCGCCGTGGAAT
SEN 0375	*rdg*C	AGATTCCCTGAAGGATGAAGT
		AACCCTGAGCGACCGTAC
SEN 2278	*ais*	GTGCTGGCATTTACCCTA
		GGCGGAATAACACGACTA
SEN 2079	*udg*	TGGTGGCGTTAGACATTG
		CACGATACGGGAGGGATA

3.3 Results

3.3.1 *In vitro* bacterial growth

To determine the culture conditions required for NB culture to reach mid-log phase, a full growth curve was performed using the method described in Chapter 2, sections 2.2.1.1 and 2.2.1.2. The viable count "Log_{10} transformation" (Figure 3.1) and spectrophotometer optical density (OD_{600}) readings (Figure 3.2), show that time point 2 h was approximately the mid-log phase point, which considered for bacterial *in vitro* growth point.

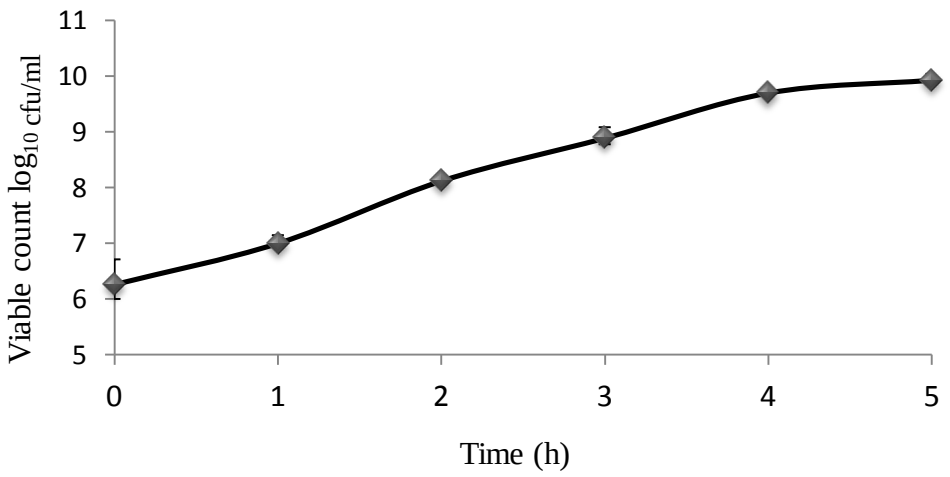

Figure 3.1: *S.* Enteritidis growth curve in 100 ml nutrient broth flask showing the bacterial logarithmic numbers at different time-points and 2 h as a mid-log phase. Mean ± standard errors are shown for triplicate samples.

The viable count at each hour time-point here was combined with optical-density (OD_{600}) readings by spectrophotometer as shown on Fig 3.2.

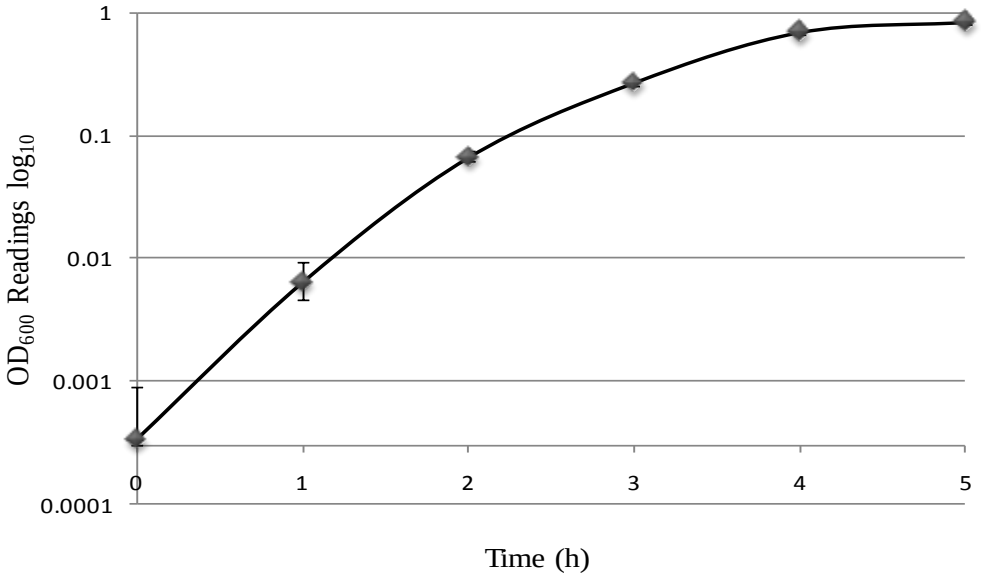

Figure 3.2: *S.* Enteritidis growth curve in 100 ml nutrient broth flask showing the $\log_{10}$ of spectrophotometer absorbance readings at OD_{600}. Mean ± standard errors are shown for triplicate samples.

3.3.2 *In vitro* culture RNA isolation

S. Enteritidis was thus grown under the same conditions described in the methods section in NB (300 ml) for 2 h (mid-log phase). These bacterial cells RNA harvested by using RNeasy mini kit (Qiagen). Initially the samples were measured by NanoDrop 1000 spectrophotometer (ND) then samples regarded initially as pure (when the absorbance rate of 260/280 is ~ 2 and 260/230 at 1.8~ 2) were evaluated using 2100 Bioanalyser "AG" (Agilent). The RNA concentrations and quality for each sample were assessed by NanoDrop and Agilent graphs respectively as shown in Figures 3.3 and 3.4.

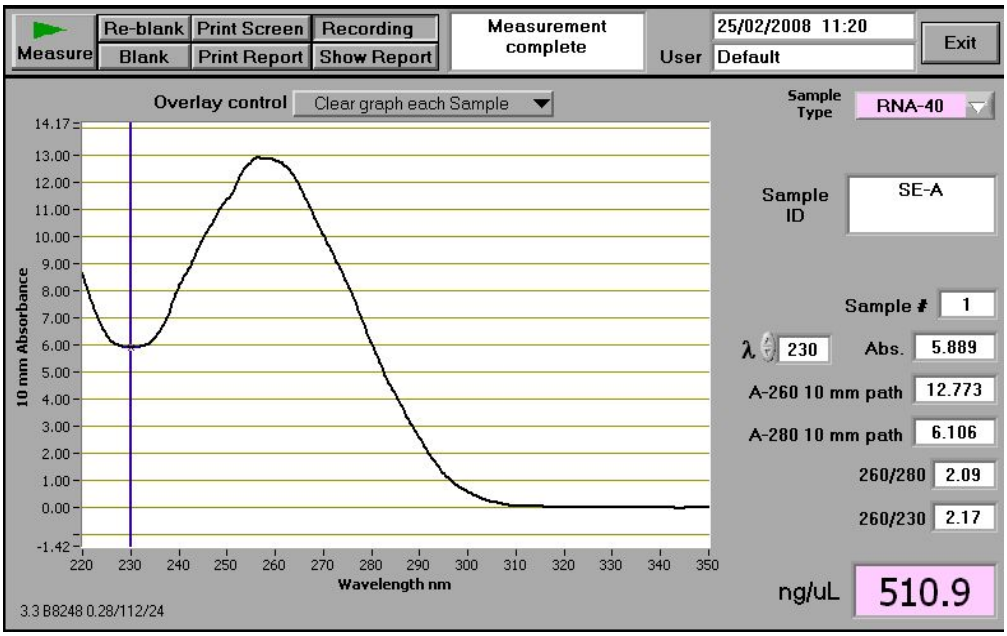

Figure 3.3: An example of ND Spectrophotometer graph for a pure sample of RNA extract from *S.* Enteritidis grown *in vitro* as it shows as the absorbance rate of 260/280 & 260/230 at ~ 2 and its concentration is 510.9 ng/µ L.

A ratio of ~1.8 is generally accepted as pure for DNA; while a ratio of ~2.0 is generally accepted as pure for RNA. The RNA purity also indicated by the peak location on 260 nm "represents the RNA highest absorbance" (Figure 3.3). The shift of this peak from this reading is an indication for protein or phenol contamination. The above ND spectrophotometer graph is just an example for one of the good RNA samples (Figure 3.3) indicating good concentration and quality of RNA.

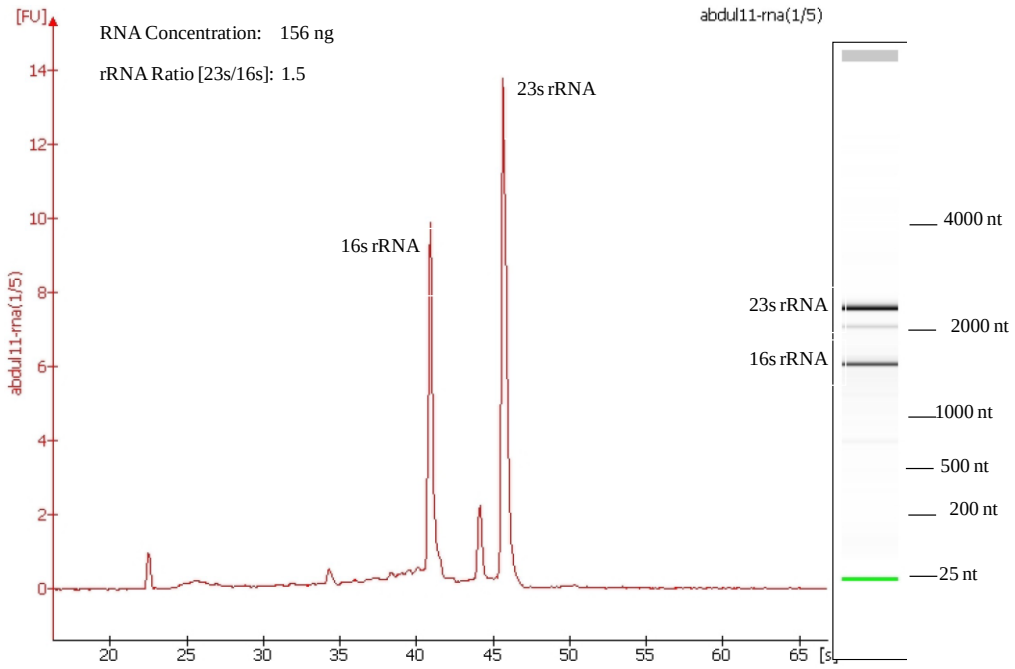

Figure 3.4: An example of an AG 2100 bioanalyser graph for a pure sample (SE-A) of RNA extract from *S.* Enteritidis grown *in vitro* with concentration readings (156 x 5 = 780 ng/µL).

The RNA concentration and quality for each sample were evaluated by AG 2100 bioanalyser (Figure 3.4). The ratio of rRNA [23S/16S]: 1.5 is indicative for the pure quality of RNA as well as the 2 clear bold bands of 16S and 23S rRNA on the gel track at the right side. In Figure 3.4, the RNA concentration was 156 ng multiplied by 5 to get the actual RNA concentration per µL (780); because the measurement was taken from the aliquot diluted 5 times in RNase free water. Therefore, RNA preparations which, when analysed on the Agilent Bioanalyser, gave a 23S/16S ratio ≥ 1.5 were regarded as pure and their machine read off produced concentrations by machine multiplied by 5 to give the actual RNA concentration.

3.3.3 *In vivo* bacterial viable counts

All bacterial cells counts on nutrient and MacConkey agar plates used for viable count estimations for all three batches of chickens indicated that they were found to be free of any other bacteria ($\leq 2 \times 10^2$ cfu/ml) other than the inoculated *Salmonella.* The average of *Salmonella* numbers from the randomly

selected three birds from each lot (1, 2 and 3) were Log_{10} 9.15-9.96, 9.56-9.99, and 9.38-9.95 cfu/ml respectively.

3.3.4 *In vivo* culture RNA isolation

The quantity and quality of *S*. Enteritidis RNA, which was harvested from the chickens' caeca, were assessed for three experimental infected chickens. Sufficient RNA was produced for hybridisation studies using microarray.

According to the Agilent 2100 analyser, some samples of *S.* Enteritidis RNA showed partial degradation, which indicated by rising of the baseline and the emergence of many ribosomal peaks (Figure 3.5) and was also indicated by the presence of many bands in the gel track (Figure 3.5). Additionally, the ratio of rRNA [23S/16S] of 0.7 is indicative of degraded RNA. Therefore, all samples showing evidence of degradation were avoided for further analysis. It was noticed that 23s rRNA is more susceptible to degradation than 16S rRNA.

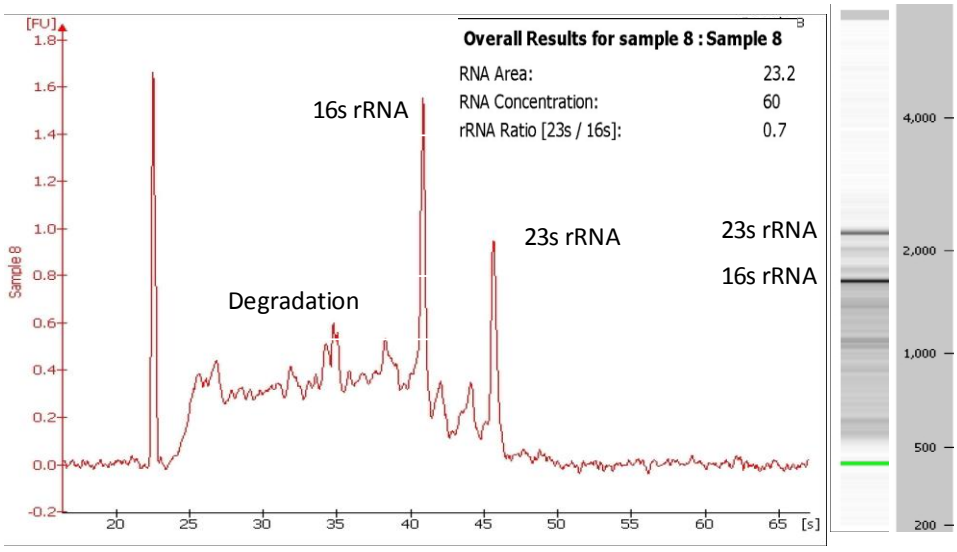

Figure 3.5: An example of an AG 2100 Bioanalyzer graph showing partial degradation of *S*. Enteritidis RNA with concentration readings (60 x 5 = 300ng/µL). The rRNA ratio [23S/16S] is 0.7, rising of the baseline and the emergence of many ribosomal peaks and the presence of many bands in the gel track are indicative of poor quality of RNA.

Despite the challenge of trying to obtain high quality RNA for *Salmonella* isolated from chicken caeca, it was possible to extract sufficient pure RNA representative for the three lots of chickens.

3.3.5 Evaluation of the collected RNA concentration and quality:

The *in vitro* and *in vivo* extracted *S.* Enteritidis RNA samples, assessed as high quality by the NanoDrop 1000 spectrophotometer were further evaluated for their purity and concentration using the Agilent 2100 Bioanalyzer. The concentration readings using NanoDrop 1000 spectrophotometer and Agilent 2100 Bioanalyser for both *in vivo* and *in vitro* samples were compared using coefficient correlation (Excel). The readings were found to be 93 % compatible with each other indicating that both instruments were reliable methods (Fig 3.6).

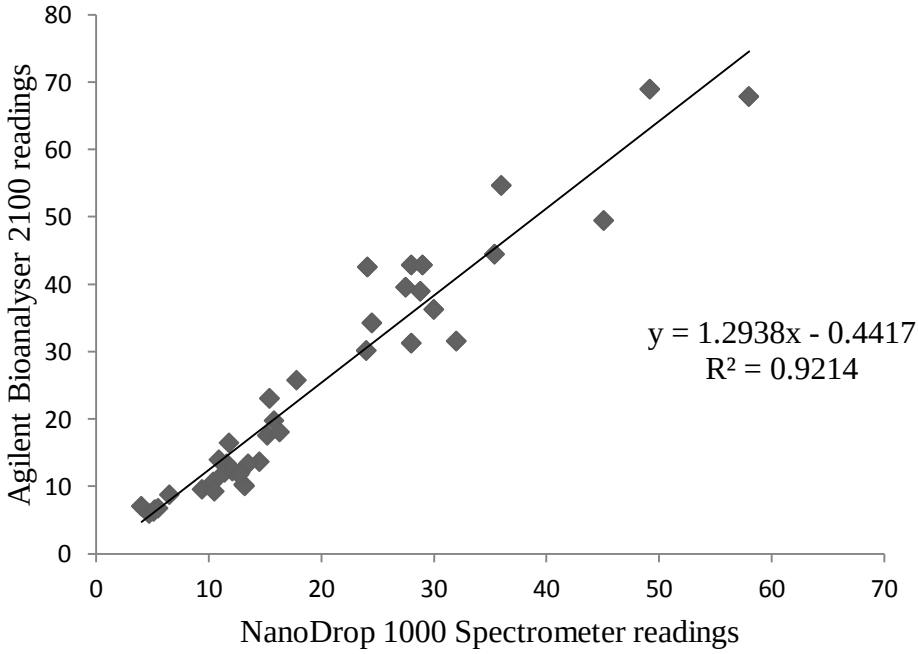

Figure 3.6: The correlation of *in vitro* and *in vivo S.* Enteritidis RNA concentration readings (93 % similarity) between NanoDrop 1000 spectrophotometer and Agilent 2100 analyzer

3.3.6 Bactericidal effect of RNAprotect reagent

RNAprotect reagent (Qiagen) was found to be very effective in killing all *Salmonella* cells added to it; and therefore preserving the picture of RNA expression at the time of harvesting. This was shown by the total bacterial viable number after mixing with this reagent which was $\leq 2 \times 10^2$ cfu/ml on both nutrient and MacConkey agar plates. The bacterial total number before adding the reagent varied from 1.1-1.5 x 10^8 cfu/ml.

3.3.7 Host RNA interference

Caecal contents from birds inoculated with nutrient broth only contained < 1 x 10^2 cfu/ml *Salmonella* and subjected to prokaryotic RNA extraction kit (Qiagen) and it showed no bacterial RNA signal at all (using Nanodrop1000 and Agilent bioanalyser 2100) also confirming that uninfected *in vivo* samples would not generate a signal if applied to the *Salmonella* microarray.

3.3.8 Bacterial RNA amplification

The in-put *Salmonella* RNA from the *in vitro* sample before amplification was 100 ng/µl. The end-product of this sample amplification using this protocol was 1154 ng/µl in a volume of 150 µl (173.1 µg a tube). However, the amplification rate using this protocol was > 1000 fold, 260/280 ratio = 1.86 and 260/230 ratio = 1.69 as been proved NanoDrop1000 spectrophotometer. According to the Agilent 2100 Bioanalyser graph (Figure 3.7), the end product of amplified RNA concentration for the same *in vitro* sample was (304 x 5) = 1520 ng/ µl; (~ 200 µg a tube), the amplification rate using this protocol was > 1000 fold according to this tool. The same rate of amplification was also shown for *in vivo* grown *S.* Enteritidis samples (Figure 3.8). The concentrations of the pooled *in vivo* grown *S.* Enteritidis RNA for the three lots of chickens were as follows 1165, 1790 and 1875 ng/µl. While the concentrations of the *in vitro* grown *S.* Enteritidis RNA for the three samples were as follows 1105, 1117 and 1105 ng/µl.

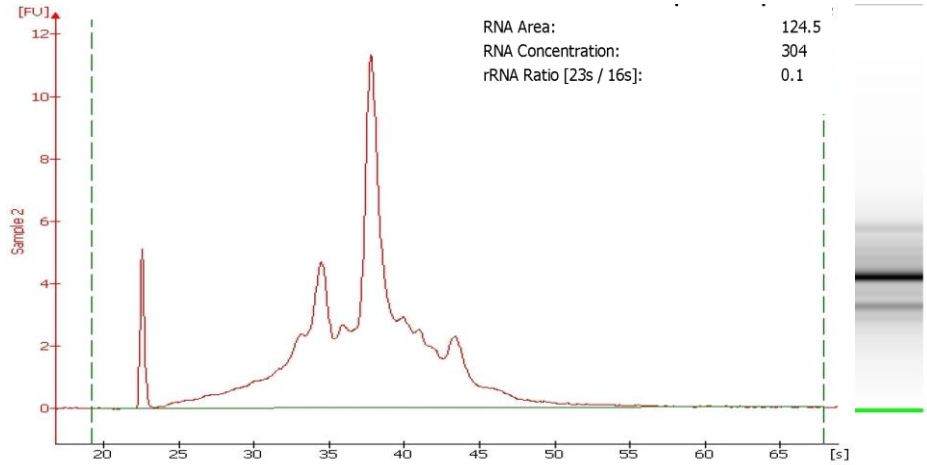

Figure 3.7: An example of Agilent 2100 Bioanalyzer graph showing *in vitro* grown *S.* Enteritidis RNA sample (control) subjected to amplification.

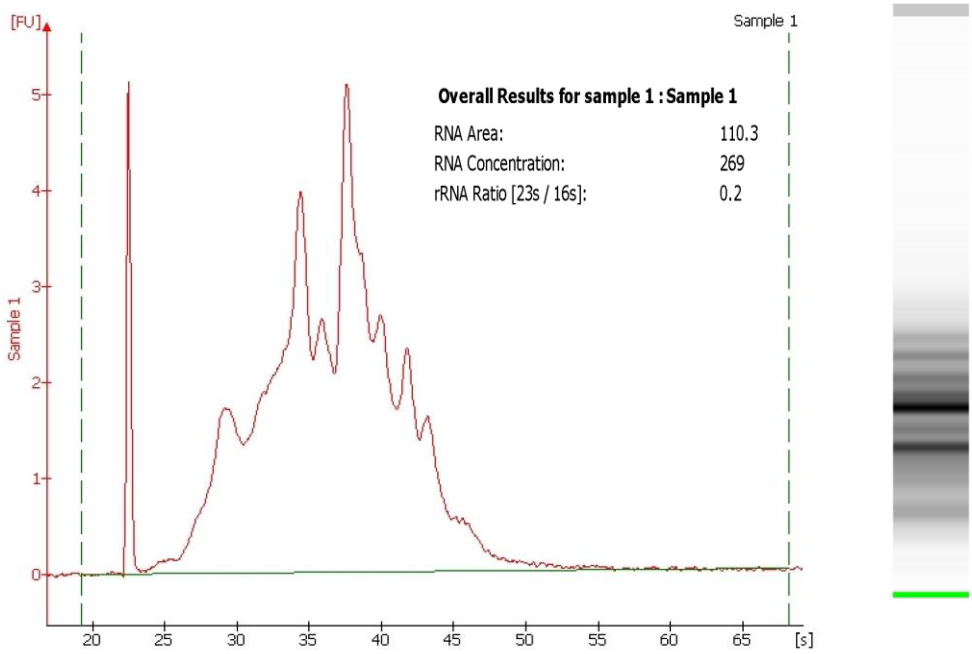

Figure 3.8: An example of Agilent 2100 Bioanalyzer graph showing one of the *in vivo* grown *S.* Enteritidis RNA sample subjected to amplification.

The binding rate or the frequency of incorporation (Sheppard *et al.*, 2004) between the fluorescent dye Cy_3 and *in vitro* grown *S.* Enteritidis amplified RNA samples was calculated according to their NanoDrop1000 spectrophotometer absorbance reading at 550 and 260 of Microarray setting; while the FOI between the fluorescent dye Cy_5 and *in vivo* grown *S.* Enteritidis

amplified RNA samples was calculated according to their absorbance reading at 650 and 260 of microarray setting (Chapter 2; section 2.2.3.9). The Cy_3 FOI with *in vitro* grown *S.* Enteritidis aRNA samples were 46.4, 31 and 44.3; while the Cy_5 FOI with *in vivo* grown *S.* Enteritidis aRNA samples were 37, 32 and 30.2. However, all scored readings were considered as a good binding between the Cy dye and the RNA (http://www.nanodrop.com/Library/NanoDrop-1000-Microgenomics-Application-Note.pdf).

3.3.9 *S. Enteritidis* gene expression (transcription) analysis

The general overview of the differences in global gene expression in *S.* Enteritidis during its colonisation of the caeca in newly-hatched chickens compared with the mid-log phase NB culture is shown in Figure 3.9. It is reflecting the results of the 3 biological comparisons.

The total number of *S.* Enteritidis genes, coding sequences (CDS) is 4380 (Thomson *et al.*, 2008); Nick Thompson's file and Tristan Cogan "Personal Communication"), of which 1870 genes (42%) changed in expression during caecal colonisation (>2 fold up or down regulated and statistically significant, $P < 0.05$) compared to the growth of same strain in log-phase NB. The remaining 2510 genes (58%) were equally expressed in both environments or not expressed at all. The total number of up-regulated genes was 937 (21.4%), while the total number of down-regulated genes was 933 (21.3%). These results are presented in Figure 3.9.

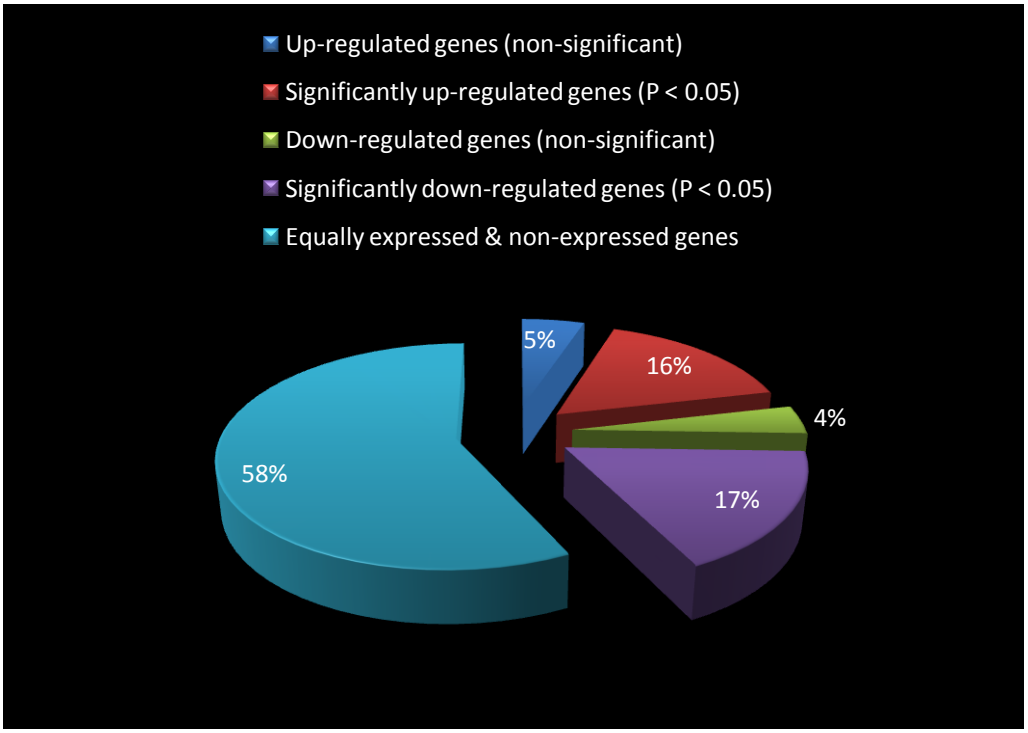

Figure 3.9: Pie chart illustrating the percentage *S.* Enteritidis genes showing changes or no change in expression during its colonisation in 1-day chicken caeca.

The significantly expressed genes of *S.* Enteritidis were grouped according to the Clusters of Orthologous Groups of proteins (COGs) classification [http://www.ncbi.nlm.nih.gov/COG/old] (Table 3.2 and Figure 3.10). The results show that the pattern of gene transcription was different in the intestine compared with broth culture with 714 genes (16%) significantly up-regulated and 753 (17%) significantly down-regulated in the intestine (Table 3.2).

Table 3.2: The number (n) and percentage (%) of the significant ($P < 0.05$) up-regulated genes (fold change > 2) of *S.* Enteritidis at *in vivo* and *in vitro* cultures according to Clusters of Orthologous Groups of genes / proteins (COGs) functional categories

COGs Category	***in vivo***		***in vitro***	
	n	%	n	%
Not found in COGs	349	48.9	362	48.1
Energy production C	59	8.3	13	1.7
Cell cycle D	1	0.1	7	0.9
Amino acid transport / metabolism E	40	5.6	67	8.9
Nucleotide transport / metabolism F	2	0.3	16	2.1
Carbohydrate transport / metabolism G	49	6.9	27	3.6
Co-enzyme transport / metabolism H	18	2.5	25	3.3
Lipid transport / metabolism I	7	1.0	13	1.7
Translation, ribosomal structure J	5	0.7	44	5.8
Transcription K	29	4.1	27	3.6
Replication, recombination & repair L	5	0.7	31	4.1
Cell envelop, outer membrane biog.M	10	1.4	29	3.9
Cell motility N	6	0.8	10	1.3
Posttranslational, protein turnover O	26	3.6	13	1.7
Inorganic ion transport / metabolism P	19	2.7	39	5.2
Secondary metabolites biosynthesis Q	8	1.1	9	1.2
General function prediction only R	44	6.2	65	8.6
Function unknown S	41	5.7	32	4.2
Signal transduction mechanisms T	26	3.6	17	2.3
Intracellular trafficking secretion U	1	0.1	-	-

The COGs 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, inorganic ion transport/metabolism, co-enzyme transport/metabolism, nucleotide transport/metabolism, cell motility, translation, replication and cell membrane and outer membrane biogenesis (Table 3.2 and Figure 3.10).

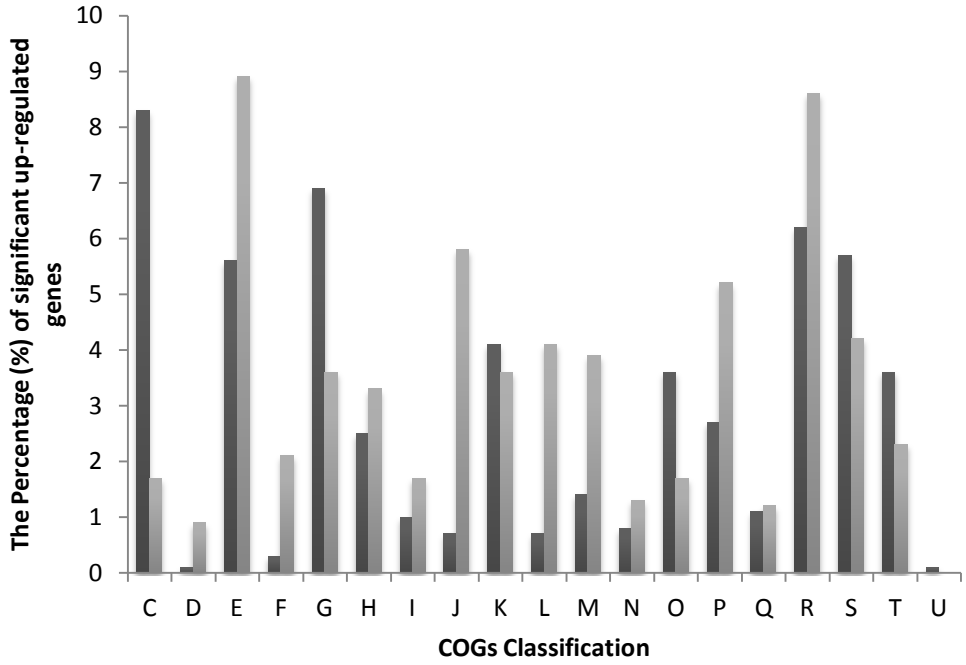

Figure 3.10: The percentage (%) of the significantly ($P < 0.05$) up-regulated genes (fold change > 2) of *S.* Enteritidis during caecal colonisation (black columns) and *in vitro* growth (grey columns) with genes clustered according to COGs functional categories. For category description refer to Table 3.2.

Genes which showed a statistically significant differential expression between *in vivo* and *in vitro* conditions (2-fold change; $P < 0.05$) were considered to be of interest. The genes with increased and decreased levels of expression, and which fulfilled this criterion, are listed in Table 3.3 and Table 3.4 respectively. However, supplementary tables for significantly up-and-down regulated genes of *S.* Enteritidis in chicken caeca are presented in the Appendix, to facilitate others to study or refer to individual genes of their own interest

Table 3.3: Genes of interest of *S.* Enteritidis which were significantly ($P < 0.05$) up-regulated (< 2 fold) during colonisation of the chicken caeca. The genes were classified according to COGs.

COGs Class	Gene Designation	Function	Fold (n)
Not in COGs	SEN0068	Transcriptional activator *cai*F	17.4
	SEN0634	ABC transporter periplasmic binding protein	3.5
	SEN1155	Type III secretion system protein (*sop*E)	19.6
	SEN1182	Invasion-associated protein (*sop*E2)	8.5
	SEN1184	Serine/threonine protein phosphatase 1 *prp*A	3.4
	SEN1287	Alcohol dehydrogenase *adh*E	4.5
	SEN2034	*pdu/cob* regulatory protein *poc*R	3.0
	SEN2041	Propanediol utilization protein *pdu*G	4.1
	SEN2438	Ethanolamine ammonia-lyase chain *eut*B	7.3
	SEN2444	Ethanolamine utilization protein *eut*M	11.9
	SEN2445	Ethanolamine utilization protein *eut*N	12.3
	SEN2446	Putative phosphate acyltransferase *eut*D	7.5
	SEN2447	Putative cobalamin adenosyltransferase *eut*T	4.0
	SEN2450	Putative ethanolamine utilization pr. *eut*S	23.5
Cell division	SEN0672	Deoxyribodipyrimidine photolyase *phr*B	12.8
	SEN0744	Excision nuclease ABC subunit B *uvr*B	2.2
	SEN0776	DNA protection during starvation protein *dps*	50.3
Energy production conversion And Respiration	SEN0059	Citrate (PRO-3S)-lyase ligase *cit*C2	5.1
	SEN0062	Citrate lyase alpha chain *cit*F2	6.5
	SEN0687	Succinyltransferase component (E2) *suc*B	3.4
	SEN0689	Succinyl-CoA synthetase alpha chain *suc*CD	3.0
	SEN0690	Cytochrome d ubiquinol oxidase subunit I *cyd*A	2.8
	SEN0869	Anaerobic dimethyl sulfoxide reductase chain A precursor *dms*A	30.6
	SEN0870	Anaerobic dimethyl sulfoxide reductase chain B *dms*B	20.5
	SEN1321	Aconitate hydratase 1 (citrate hydro-lyase 1) *acn*A	15.4
	SEN1277	Respiratory nitrate reductase 1 delta chain *nar*J	3.1
	SEN1278	Respiratory nitrate reductase 1 gamma chain *nar*I	3.9
	SEN2238	Cytochrome c-type *nap*C	5.5
	SEN2239	Cytochrome c-type *nap*B precursor	2.2
	SEN2240	Ferredoxin-type *nap*H	3.9
	SEN2241	Ferredoxin-type *nap*G	6.5
	SEN2244	Ferredoxin-type *nap*F	3.4
	SEN4049	Cytochrome c-type biogenesis *nrf*C	2.8

	SEN4056	Formate dehydrogenase H *fdh*F	7.8
	SEN4110	Fumarate reductase complex subunit D; *frd*D	13.9
	SEN4111	Fumarate reductase complex subunit C; *frd*C	13.3
	SEN4112	Fumarate reductase, iron-sulfur *frd*B	14.5
	SEN4113	Fumarate reductase, flavoprotein subunit *frd*A	16.9
Carbohydrate Transport and metabolism	SEN0192	Sugar fermentation stimulation protein *sfs*A	3.0
	SEN1045	Cytoplasmic alpha-amylase *amy*A	3.0
	SEN1075	Trehalose phosphatase *ots*B	16.9
	SEN1076	Trehalose-6-phosphate synthase *ots*A	17.0
	SEN1115	Pyruvate kinase A *pyk*A	3.5
	SEN1241	Periplasmic trehalase *tre*A	9.3
	SEN2265	Glycerol-3-phosphate transporter *glp*T	4.8
	SEN2819	Fuculose-1-phosphate aldolase *fuc*A	14.1
	SEN2821	L-fuculose kinase *fuc*K	14.9
	SEN2822	Fuscose operon fucU protein *fuc*U	17.1
	SEN3359	Glycogen synthase *glg*A	6.9
	SEN3360	Glucose-1-phosphate adenyltransferase *glg*C	7.5
	SEN3361	Glycogen operon protein *glg*X	13.6
	SEN3362	1,4-alpha-glucan branching enzyme *glg*B	6.5
Amino acid transport and metabolism	SEN0736	Histidine ammonia-lyase *hut*H	2.5
	SEN0793	Putative L-asparaginase *ybi*K	2.6
	SEN1737	Succinylarginine dihydrolase *ast*B	4.3
	SEN1739	Arginine N-succinyltransferase *ast*A	3.8
	SEN2539	Lysine decarboxylase *cad*A	2.7
	SEN2635	Succinate-semialdehyde dehydrogenase *gap*D	8.1
	SEN2636	4-aminobutyrate aminotransferase *gap*T	2.4
	SEN2896	Glycine dehydrogenase (decarboxylating) *gcv*P	3.3
	SEN2897	Glycine cleavage system H protein *gcv*H	3.3
	SEN2898	Glycine cleavage system T protein *gcv*T	4.6
	SEN2901	Proline aminopeptidase II *pep*P	4.3
Cell envelop and cell membrane	SEN0721	UDP-glucose 4-epimerase *gal*E	2.3
	SEN2159	Putative periplasmic protein *yeh*Z	10.4
	SEN4109	Putative lipoprotein *blc*	13.4
Cell motility	SEN0524	Type-1 fimbrial protein, a chain precursor *fim*A	7.6
	SEN0526	Fimbrial chaperone protein *fim*C	10.8
	SEN2145B	Putative fimbrial subunit protein *peg*A	3.3
	SEN2873	Probable fimbrial protein *std*A	11
	SEN3463	Long polar fimbriae *lpf*A	5.1
Co-enzyme transport /	SEN0470	Ferrochelatase *hem*H	3.2
	SEN0702	*pnu*C protein	7.7

metabolism	SEN0843	Pyruvate dehydrogenase *pox*B	6.4
	SEN2014	Nicotinate-nucl-dimethylbenzimidazole *cob*T	2.6
	SEN2021	Cobalamin biosynthesis protein *cbi*M	4.7
	SEN2023	Sirohydrochlorin cobaltochelatase *cbi*K	9.1
	SEN2026	Cobalamin biosynthesis protein *cbi*G	7.4
	SEN2030	Deacetylating cobalt-precorrin-6A synthase *cbi*D	9.2
	SEN2032	Cobalamin biosynthesis protein *cbi*B	3.7
General function	SEN0871	Anaerobic dimethyl sulfoxide reductase chain C *dms*C	12.3
	SEN4095	Anaerobic C4-dicarboxylate transporter *dcu*A	5.9
	SEN4323	Putative periplasmic protein *osm*Y	31.3
Inorganic ion transport	SEN0670	Potassium-transporting ATPase A chain *kdp*A	3.7
	SEN1073	Ferritin-like protein *ftn*B	11.9
	SEN1111	Zinc uptake system ATP-binding protein *znu*C	2.8
	SEN1276	Respiratory nitrate reductase 1 beta chain *nar*H	4.5
	SEN1607	Copper-zinc superoxide dismutase sodC	17.4
	SEN1725	Catalase HPII *kat*E	19.0
	SEN2018	Putative cobalt transport ATP-binding protein *cbi*O	3.2
	SEN4047	Cytochrome c552 precursor *nrf*A	19.7
	SEN4061	Proline/betaine transport system *pro*P	6.4
	SEN4172	CysQ protein	2.5
Lipid transport / metabolism	SEN0292	Possible acyl-CoA dehydrogenase *yaf*H	4.0
	SEN0758	Putative phospholipase *ybh*O	11.3
	SEN2371	Putative 3-ketoacyl-CoA thiolase *yfc*Y	4.3
	SEN4143	Probable acyl Co-A dehydrogenase *aid*B	17.2
Nucleotide transport / metabolism	SEN0067	Carbamoyl-phosphate synthase *car*B	2.6
	SEN0067	Carbamoyl-phosphate synthase large chain *deo*A	2.6
Posttranslational, protein turnover	SEN0577	Alkyl hydroperoxide reductase c22 protein *ahp*C	2.8
	SEN0853	ATP-dependent Clp protease ATP-binding subunit *clp*A	4.1
	SEN0976	Curved DNA-binding protein *cbp*A	3.7
	SEN1492	Osmotically inducible protein C *osm*C	10.0
	SEN1596	Glutathione S-transferase *gst*	4.4
	SEN1672	Putative amintransferase *suf*S	9.0
	SEN1703	Vitamin B12 transport protein *btu*E	3.2
	SEN2583	ClpB protein (heat shock protein f84.1)	5.5
	SEN3634	Heme exporter protein B1 *ccm*B	3.4
	SEN3635	Heme exporter protein A2 *ccm*A	3.0
Secondary metabolites	SEN0732	Imidazolonepropionase *hut*L	2.7
	SEN2011	Putative membrane transport protein *yee*O	2.9

biosynthesis	SEN2056	Putative propanediol utilization protein *pdu*X	2.8
	SEN2165	Putative oxidoreductase *yoh*F	3.4
	SEN3198	Possible exported protein *yhc*Q	4.9
Signal transduction mechanisms	SEN0090	Bis(5'-nucleosyl)-tetraphosphatase *apa*H	3.5
	SEN0428	BolA protein	4.3
	SEN0653	Citrate-proton symporter	2.5
	SEN0987B	PhoH protein (phosphate starvation-inducible protein PsiH)	3.7
	SEN2369	Phosphohistidine phosphatase *six*A	2.7
	SEN2459	Nitrate/nitrite sensor protein narQ	3.1
	SEN2565	Sigma-E factor regulatory protein *rse*B precursor	2.4
	SEN2566	Sigma-E factor negative regulatory *rse*A	7.2
	SEN3414	Universal stress protein A *usp*A	2.8
Transcription	SEN0603	lysR-family transcriptional regulator *ybe*F	2.6
	SEN1350	Transcriptional regulatory protein *tyr*R	2.1
	SEN2312	NADH dehydrogenase operon transcriptional regulator IrhA	3.5
	SEN2434	Ethanolamine operon transcriptional regulator *eut*R	2.9
	SEN2533	Stationary phase inducible protein *csi*E	12.2
	SEN3391	RNA polymerase sigma-32 factor *rpo*H	2.3
	SEN4068	Melibiose operon regulatory protein *mel*R	2.6
Translation, ribosomal structure	SEN0931	Ribosome modulation factor (protein E) *rmf*	43.5
	SEN1711	Threonyl-tRNA synthetase thrS	2.8
	SEN2587	Putative sigma(54) modulation protein YfiA	28.0
	SEN3504	Selenocysteine-specific elongation factor *sel*B	2.8

Table 3.4: Genes of interest of *S.* Enteritidis which were significantly ($P < 0.05$) down-regulated (< 2 fold) during colonisation of the chicken caeca. The genes were classified according to COGs.

COGs Class	Gene ID	Function	Fold (n)
Not in COGs	SEN0020	Fimbrial subunit *bcf*A	2.9
	SEN0027	Hypothetical protein *bcf*H	2.5
	SEN0201	Outer membrane usher protein *stf*c	2.6
	SEN0211	Cobalamin periplasmic binding protein *btu*F	2.8
	SEN0341	Type III restriction-modification system *res*	3.1
	SEN0490	Outer membrane protein	3.7
	SEN1015	Outer membrane protein S1 *omp*S	2.9
	SEN1174	Inner membrane protein *pag*O	9.2
	SEN1803	Outer membrane invasion protein *pag*C	11.0
	SEN1806	Putative lipoprotein *env*E	3.5
	SEN1807	Putative virulence *msg*A	4.0
	SEN2278	Ais protein	36.0
	SEN2281	Putative lipopolysaccharide *yfb*G	3.1
	SEN2794	Major fimbrial subunit	5.3
	SEN3459	Fimbrial gene (*lpf*E)	3.4
	SEN3537	LPS core biosynthesis *rfa*Z	4.7
Cell division	SEN0265	Ribonuclease H *mnh*A	4.6
	SEN0378	Exonuclease SbcC	2.7
	SEN0379	Exonuclease SbcD	2.6
	SEN1105	Crossover junction endodeoxyribonuclease *ruv*C	2.4
	SEN1594	Endonuclease III *nth*	4.3
	SEN1741	Exodeoxyribonuclease III *xth*A	2.3
	SEN2817	5'-3' exonuclease *exo*	2.8
	SEN2848	DNA mismatch repair protein *mut*H	2.6
	SEN3310	DNA adenine methylase *dam*	3.2
	SEN3548	Formamidopyrimidine-DNA glycosylase *mut*M	3.4
	SEN3551	Putative DNA repair protein *rad*C	2.6
	SEN3956	Histone like DNA-binding protein *hup*A	2.8
	SEN4285	Putative Type I restriction-modification system specificity subunit M *hsd*S	5.9
	SEN4286	Type I restriction-modification system methyltransferase *hsd*M	2.7
Energy production conversion	SEN0424	Cytochrome o ubiquinol oxidase subunit I	2.7
	SEN0425	Cytochrome o ubiquinol oxidase subunit II	2.9

And Respiration	SEN1691	Putative electron transfer flavoprotein subunit *ydi*R	3.8
	SEN2261	Putative Ferredoxin *yfa*E	4.6
	SEN2518	Ferredoxin *rdx*	2.9
	SEN3689	MioC protein	4.7
	SEN4183	Inorganic pyrophosphatase *pp*A	4.4
	SEN4188	Soluble cytochrome b562 *cyb*C	3.2
Carbohydrate transport /metabolism	SEN2200	Sugar efflux transporter *set*B	2.6
	SEN2894	6-phospho-beta-glucosidase *bgl*A	2.7
	SEN3338	High-affinity gluconate transporter	4.4
	SEN3598	Putative inner membrane transport protein *yic*M	3.8
	SEN3696	High affinity ribose transport protein *rbs*A	5.3
	SEN3698	D-ribose-binding periplasmic protein *rbs*B	2.2
	SEN3875	Putative glycerol metabolic protein *glp*X	2.3
	SEN4204	Trehalose-6-phosphate hydrolase *tre*C	6.9
Amino acid transport and metabolism	SEN0171	Spermidine synthase *spe*E	4.1
	SEN0773	Glutamine transport ATP-binding protein *gln*Q	2.3
	SEN0833	Arginine transport system *art*M	2.9
	SEN1068	Tyrosine-specific transport protein *tyr*B	5.6
	SEN1471	L-asparagine permease	3.7
	SEN2654	Glycine betaine/l-proline transport ATP-binding protein *pro*V	3.5
	SEN2655	Glycine betaine/L-proline transport system permease protein *pro*W	3.1
	SEN2656	Glycine betaine-binding periplasmic protein precursor *pro*X	5.0
	SEN3487	Valine--pyruvate aminotransferase *avt*A	4.8
	SEN3711	Threonine deaminase *ilv*A	2.3
	SEN4017	Aromatic-amino-acid aminotransferase *tyr*P	4.1
Cell envelop, outer membrane	SEN0303	Outer membrane pore protein E precursor *pho*E	2.3
	SEN0363	D-alanine:D-alanine ligase A *ddl*A	3.5
	SEN0635	Apolipoprotein N-acyltransferase	2.5
	SEN1716	Putative outer membrane protein	3.3
	SEN2162	Penicillin-binding protein *pbp*G	2.5
	SEN2279	Putative lipopolysaccharide biosynthesis protein *yfb*E	8.9
	SEN2511	Penicillin-binding protein 1C *pbp*C	2.6
	SEN2832	Membrane-bound lytic murein transglycosylase A precursor *mlt*A	2.3

	SEN2833	N-acetylmuramoyl-L-alanine amidase *ami*C	2.7
	SEN3140	UDP-N-acetylglucosamine 1-carboxyvinyltransferase *murA*	3.7
	SEN3535	O-antigen ligase *rfa*L	5.0
	SEN3536	Lipopolysaccharide 1,2-n-Acetylglucosaminetransferase *rfa*K	4.6
	SEN3539	Lipopolysaccharide 1,2-glucosyltransferase *rfaJ*	4.8
	SEN3541	Lipopolysaccharide 1,6-galactosyltransferase *rfaB*	3.8
	SEN3545	Lipopolysaccharide core biosynthesis *rfaQ*	2.6
	SEN3546	3-deoxy-D-manno-octulosonic-acid Transferase *kdt*A	2.9
	SEN3687	Glucose inhibited division protein *gid*B	3.2
	SEN4005	Diacylglycerol kinase *dgk*A	3.2
Cell motility	SEN0390	Protein-export membrane *sec*D	2.4
	SEN0889	Tetraacyldisaccharide 4'-kinase *lpx*K	2.8
	SEN1028	Flagellar biosynthetic *fli*R	12.1
	SEN1032	Flagellar motor switch protein *fli*N	2.2
	SEN1033	Flagellar motor switch protein *fli*M	2.5
	SEN4298	Methyl-accepting chemotaxis protein *tsr*	5.4
Co-enzyme transport / metabolism	SEN0149	Nicotinate-nucleotide pyrophosphorylase *nad*C	2.4
	SEN0185	Aspartate alpha-decarboxylase *pan*D	2.4
	SEN0186	Pantoate--beta-alanine ligase *pan*C	2.4
	SEN0187	3-methyl-2-oxobutanoate hydroxymethyltransferase *pan*B	2.4
	SEN0399	6,7-dimethyl-8-ribityllumazine synthase (riboflavin synthase beta chain) *rib*H	2.4
	SEN0559	Ferric enterobactin transport ATP-binding protein *fep*C	3.0
	SEN0561	Ferric enterobactin transport protein *fep*D	5.3
	SEN0604	Lipoate-protein ligase B (lipoate biosynthesis protein B) *lip*B	5.0
	SEN0791	Molybdopterin biosynthesis MoeB protein	2.9
	SEN3880	Menaquinone biosynthetic protein *men*A	4.2
	SEN3928	Pantothenate kinase *coa*A	8.7
General function prediction	SEN0008	Integral membrane protein *yaa*H	5.9
	SEN0254	Putative ABC transporter permease protein *yae*E	2.4
	SEN0255	Putative ABC transporter ATP-binding prot. *Abc*	2.8
	SEN1255	Putative ATP/GTP-binding protein *ych*F	3.1
	SEN1850	Putative secreted protein *yce*G	2.2

Lipid transport /metabolism	SEN0359	*sbm*A protein	2.8
	SEN0402	Phosphatidylglycerophosphatase A *pgp*A	3.2
	SEN0811	Putative permease protein *ybj*G	3.9
	SEN0932	D-3-hydroxydecanoyl-(acyl carrier-protein) *fab*A	5.7
	SEN1298	putative acyl-coA hydrolase *yci*A	3.6
	SEN1856	3-oxoacyl-[acyl-carrier-protein] synthase III *fab*H	2.9
	SEN1857	Fatty acid/phospholipid synthesis protein *pls*X	4.2
	SEN2373	Long-chain fatty acid transport protein *fad*L	6.6
Nucleotide transport /metabolism	SEN0145	GP reductase *gua*C	3.5
	SEN0175	Hypoxanthine phosphoribosyltransferase *hpt*	5.7
	SEN0212	MTA/SAH nucleosidase *pfs*	4.9
	SEN0225	Uridine monophosphate kinase *pyr*H	2.3
	SEN0464	adenine phosphoribosyltransferase *apt*	4.2
	SEN2260	Ribonucleoside-diphosphate reductase 1 beta chain *nrd*	2.6
	SEN2506	Nucleoside diphosphate kinase *ndk*	4.9
	SEN2843	Thymidylate synthetase thyA	2.4
	SEN4120	Oligoribonuclease *orn*	3.0
	SEN4132	Adenylosuccinate synthetase *pur*A	2.1
Posttranslational, protein turnover	SEN0096	DnaJ-like protein	2.3
	SEN0517	Peptidyl-prolyl cis-trans isomerase B *ppi*B	3.6
	SEN1230	Disulfide bond formation protein B	3.9
	SEN1452	Putative peptidase *ydc*P	2.6
	SEN3716	Peptidyl-prolyl cis-trans isomerase C *ppi*C	7.3
	SEN3823	FdhE protein	4.2
Secondary metabolites biosynthesis	SEN0564	Isochorismate synthase EntC	2.5
	SEN1750	Pyrazinamidase/nicotinamidase *pnc*A	3.2
	SEN1854	3-oxoacyl-[acyl-carrier protein] reductase *fab*G	4.1
	SEN2246	Putative ABC transporter ATP-binding protein yojI	3.5
	SEN2860	2-keto-3-deoxygluconate oxidoreductase *kdu*D	2.2
	SEN3224	Acriflavine resistance protein E (protein envc)	2.8
Signal transduction mechanisms	SEN1273	Nitrate/nitrite sensor protein *nar*X	2.3
	SEN1818	Transcriptional regulatory gene *pho*P, regulator of virulence determinants	3.8
	SEN2745	Possible serine/threonine protein phosphatase	2.8

	SEN3564	Guanosine-3',5'-bis(diphosphate) 3'-pyrophosphohydrolase; CG Site No. 156	2.7
	SEN3604	Two-component system sensor histidine kinase *uhp*B	2.8
Transcription	SEN0098	Probable ATP-dependent helicase *hep*A	2.6
	SEN0380	Phosphate regulon transcriptional regulatory protein PhoB	2.9
	SEN0585	Regulator of nucleoside diphosphate kinase *rnk*	2.6
	SEN1027	Colanic acid capsullar biosynthesis activation protein A *rcs*A	10.3
	SEN1057	Cell-division regulatory protein *sdi*A	2.4
	SEN1617	Purine nucleotide synthesis repressor *pur*R	3.4
	SEN1818	Transcriptional regulatory protein *pho*P, regulator of virulence determinants	3.8
	SEN2400	Xanthosine operon transcriptional regulator *xap*R	3.2
	SEN2907	Chromosome initiation inhibitor *ici*A	2.6
	SEN3139	Ner-like regulatory protein *npl*	8.9
	SEN3884	Transcriptional repressor *cyt*R	2.2
	SEN3932	Transcription antitermination protein *nus*G	2.4
	SEN3969	Acetate operon repressor *icl*R	6.4
	SEN4339	Probable trp operon repressor *trp*R	2.5
Translation, ribosomal structure	SEN0189	Poly(A) polymerase *pnc*B	2.8
	SEN0190	Glutamyl-tRNA synthetase-related protein *yad*B	3.2
	SEN0221	Methionine aminopeptidase *map*	3.0
	SEN0224	Elongation factor *tsf*	2.8
	SEN0248	Putative release factor yaeJ	3.1
	SEN0250	Prolyl-tRNA synthetase pros	4.4
	SEN0387	S-adenosylmethionine:tRNA ribosyltransferase-isomerase	4.5
	SEN0388	Queuine tRNA-ribosyltransferase; tRNA-guanine transglycosylase	3.0
	SEN0586	Ribonuclease I precursor	2.8
	SEN0639	MiaB protein	3.7
	SEN0904	Asparaginyl-tRNA synthetase *asn*S	2.8
	SEN1264	*hem*K protein	2.3
	SEN1862	Ribosomal large subunit pseudouridine synthase C *rlu*C	6.6
	SEN2399	Glutamyl-tRNA synthetase *glt*X	2.5
	SEN2594	50S ribosomal subunit protein L19 *rpl*s	2.5
	SEN2595	tRNA(guanine-N1)methyltransferase *trm*D	3.0

	SEN2596	16S rRNA processing protein *rim*M	2.7
	SEN2597	30S ribosomal subunit protein S16 *rps*P	2.5
	SEN2668	Alanyl-tRNA synthetase alaS	2.8
	SEN2883	Lysyl-tRNA synthetase lysS	3.3
	SEN3119	tRNA pseudouridine 55 synthase, *tru*B	2.5
	SEN3565	tRNA (guanosine-2'-O)-methyltransferase *spo*U	2.5
	SEN3657	RNase P, protein component *rnp*A	3.0
	SEN3923	tRNA (uracil-5)-methyltransferase *trm*A	2.7
	SEN4104	Elongation factor P efp	5.7
	SEN4157	30s ribosomal protein S6 *rps*F	2.3
	SEN4322	Peptide chain release factor 3 *prf*C	2.6

Tables 3.3 and 3.4 show the significant changes in genes expression (transcription) occurred in chicken caeca compared with log-phase of *in vitro* grown bacteria which were observed in genes associated with:

3.3.9.1 Cell division

Thirty one genes associated with chromosome replication (including *sbc*C, *sbc*D, *ruv*C, *dam*, *mut*M, *rad*C, *hsd*S, *hsd*M and *hup*A) were down-regulated *in vivo* compared with *in vitro* grown bacteria. On the other hand there were only five genes were significantly up-regulated *in vivo* (including *phr*B, *uvr*B and *dps*). There was a significant reduction in expression of 44 genes associated with translation, including *pnc*B, *yad*B, *map*, *pro*S, *mai*B, *asn*S, *rlu*C, *glt*X, *rpl*S, *trm*D, *rim*M, *rps*P, *rnp*A, *rps*F and *prf*C. Only five genes were significantly up-regulated *in vivo* (including *rmf*, *YfiA* and *sel*B).

3.3.9.2 Energy, carbohydrate and respiration

Fifty nine genes associated with energy production including citrate (*cit*C2, *cit*F2, *suc*B, *acn*A, *suc*CD and *fr*ABCD); the cytochrome (*cyd*A, *nap*BCFGH; *nrf*C and *fdh*F); anaerobic dimethyl sulfoxide reductase (*dms*A and *dms*B); respiratory nitrate reductase (*nar*JI) were significantly up-regulated *in vivo* compared to *in vitro* culture. Moreover a number of genes such as serine/threonine phosphatase (*prp*A), alcohol dehydrogenase (*adh*E),

propanediol regulatory and utilization proteins (*pdu/cob* and *pdu*G) and ethanolamine utilization proteins (*eut*BDMNST) were up-regulated in the caeca. On the other hand only 13 genes were significantly down-regulated *in vivo* including *ydi*R, *yfa*E, *rdx*, *pp*A and soluble cytochrome *cyb*C.

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 gene associated with C4 carbohydrates, fumarate, gluconate, fucose, sialic acid, ethanolamine and 1,2-propanediol and unusual carbon sources such as allantoin (*all*PBCD), in addition to peptidases (*pepT*, *ptrB*) although this may also be a sources of raw amino acids. 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 (*ttr*SABC) in addition to 9 other electron acceptors including nitrate (*nar*HIJKV, *nap*ABDFGH) thiosulphite (*phs*ABC), hydrogen (*hya*A-E, *hyb*A-G) and DMSO (*dms*ABC) although a number of oxidoreductases both putative and *cyd* genes were also up-regulated.

A number of different loci involved in the utilization of carbohydrates showed different levels of up-and down-regulation. Genes associated with carbohydrate utilization such as sugar fermentation *sfs*A, cytoplasmic amylase *amy*A, pyruvate kinase *pyk*A, trehalose utilization and synthesis *ots*AB, glycerol transporter *glp*T, fucose utilization *fuc*AKU and glycogen/glucose utilization *glg*ACXB were significantly up-regulated *in vivo* culture compared to the *in vitro* growth. On the other hand, fewer genes associated with carbohydrate utilization were significantly down-regulated *in vivo*. These include sugar efflux transporter *set*B, glucosidase *bgl*A and ribose transport/utilization proteins *rbs*AB.

3.3.9.3 Amino acid utilization

There was a significant level of up-regulation of expression of histidine ammonia-lyase *hut*H, putative L-asparaginase *ybi*K, arginine utilization *ast*BA, lysine decarboxylase *cad*A, glycine dehydrogenase and cleavage systems *gcv*PHT and proline amino peptidase *pep*P. On the other hand, there was a

significant level of down-regulation of expression of spermidine synthase *spe*E, glutamine transport system *gln*Q, arginine transport system *art*M, tyrosine-specific transport protein *tyr*B, L-asparaginase permease, glycine betaine / l-proline transport system *pro*VWX, valine-pyruvate aminotransferase *avt*A, threonine deaminase *ilv*A and aromatic amino acid aminotransferase *tyr*P *in vivo*.

3.3.9.4 Bacterial surface

The majority of genes involved in flagella production were not significantly down-regulated ($P > 0.05$) which including *fli*RQPNMLJFEDBA and were significantly down-regulated ($P < 0.05$) including flagellar basal body and hook formation protein encoded genes *flg*ACDFGHL. Few fimbrial-associated genes were up-regulated, which include type 1 fimbrial protein, chaperone protein and major pillin protein *fim*ACDI, long polar fimbriae *lpf*A, putative fimbrial subunit protein *peg*A (SEN 2145B) and fimbrial major subunit protein *std*A. More fimbrial-associated genes were down-regulated *in vivo*. These genes were as follows fimbriae W protein *fim*W, fimbrial subunit *bcf*ABH, long polar fimbriae *lpf*DE, lipoprotein *saf*A and fimbrial chaperone *stb*DEC (Figure 3.11). Few genes associated with membrane integrity were up-regulated *in vivo*. These included genes encoding phage shock proteins *psp*ABCDE, multiple antibiotic resistance *mar*AR ($P > 0.05$), UPD-glucose 4-epimerase *gal*E, putative periplasmic protein *yeh*Z and putative lipoprotein *blc* ($P < 0.05$). The significantly down-regulated genes associated with membrane integrity included outer membrane pore protein E precursor *pho*E, D-alanine ligase *ddl*A, penicillin-binding protein *pbp*C, membrane-bound lytic murein transglycosylase *mlt*A, N-acetylmuraamoyl-L-alanine amidase *ami*C, UDP-N-acetylglucosamine 1-carboxyvinyltransferase *mur*A and lipopolysaccharide (LPS) *rfa*IJKLJBQYZ) as shown in Tables 3.3, 3.4 and Figure 3.11.

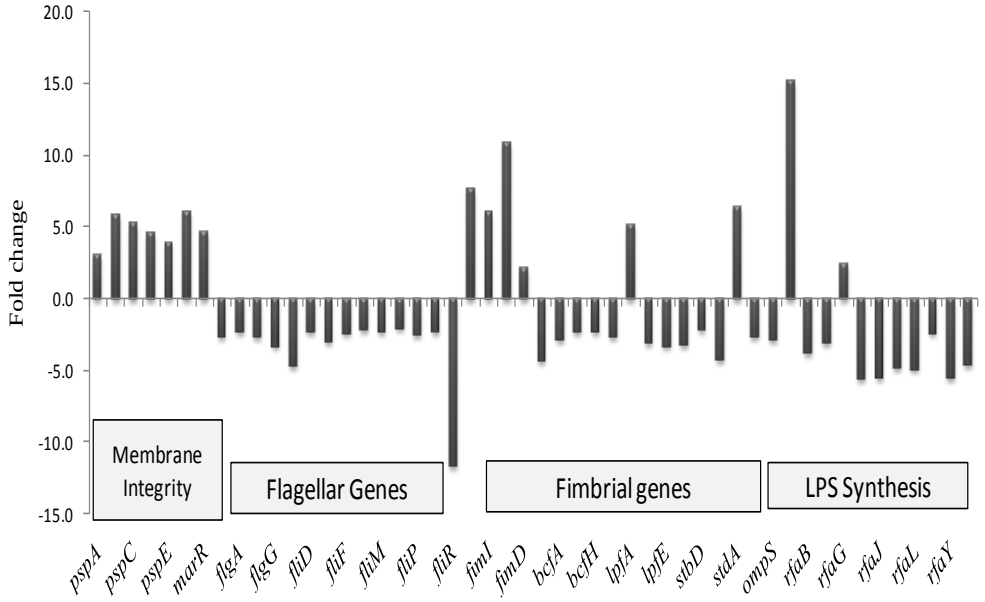

Figure 3.11: Significant changes ($P < 0.05$) in genes' expression of membrane, fimbrial, flagellar and LPS genes during colonisation of S. Enteritidis in 1-day chicks' caeca.

3.3.9.5 Ion utilization genes

Ion utilization and transportation genes were varied in their regulation in the chick' caeca, the potassium transport system *kdp*A was up-regulated by 3.7, while some iron utilization/transport genes, *ftn*B, *feo*A and *iro*N were up-regulated by 12, 3 and 3 fold respectively (Table 3.3). The iron transport operon *fhu*BD, which include, the ferrichrome-binding protein *fhu*D and *fhu*B were significantly down-regulated by 2.5, 9 fold respectively (Figure 3.12). The other iron binding/transport proteins were *fep*CDEG were also down-regulated by 3 fold. Calcium antiporter (*cha*A), magnesium transport (*mgt*A), magnesium and cobalt transport protein (*cor*A) and potassium uptake protein (*trk*H) were also significantly down-regulated by 5.5, 6.9, 3.3 and 2.4 fold respectively (Figure 3.12).

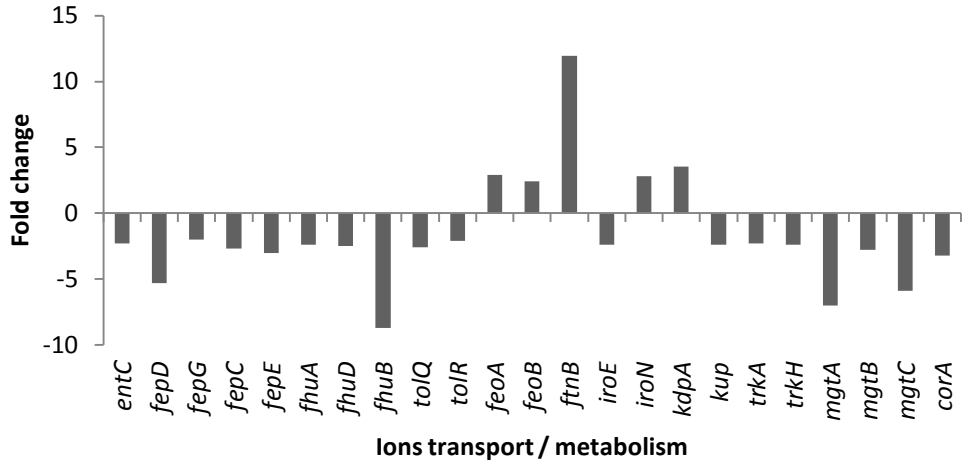

Figure 3.12: Significant changes in expression of ion transport systems genes during colonisation of *S.* Enteritidis in 1-day chicken' caeca.

3.3.9.6 Virulence and colonisation genes

Of the *Salmonella* Pathogenicity Islands (SPIs), SPI 1, SPI 2 and SPI 5 genes were up-regulated 3-17, 3-20 and 9-12 fold respectively in chicken caecal contents compared with *in vitro* levels of expression whilst SPI 3 genes were down-regulated (Figure 3.13) and SPI 4 was unchanged. The SPI 1 up-regulated genes in chicken caeca were as follows: iron transport protein *sit*B, pathogenicity 1 island effector proteins *prg*KJIH, tyrosine phosphatase *spt*P, pathogenicity island effector proteins *sip*ABCD and type III secretion virulence genes *inv*JICBAEGF. The only two SPI 1 genes which down-regulated in chicken caeca were cell invasion protein *org*A and cell adhesion/invasion protein *inv*H (Figure 3.13). But for the *inv*H as its position among the last location of *inv* operon at does not matter for genes expression and translation as the rest of operon is expressed (Barrow personal communication).

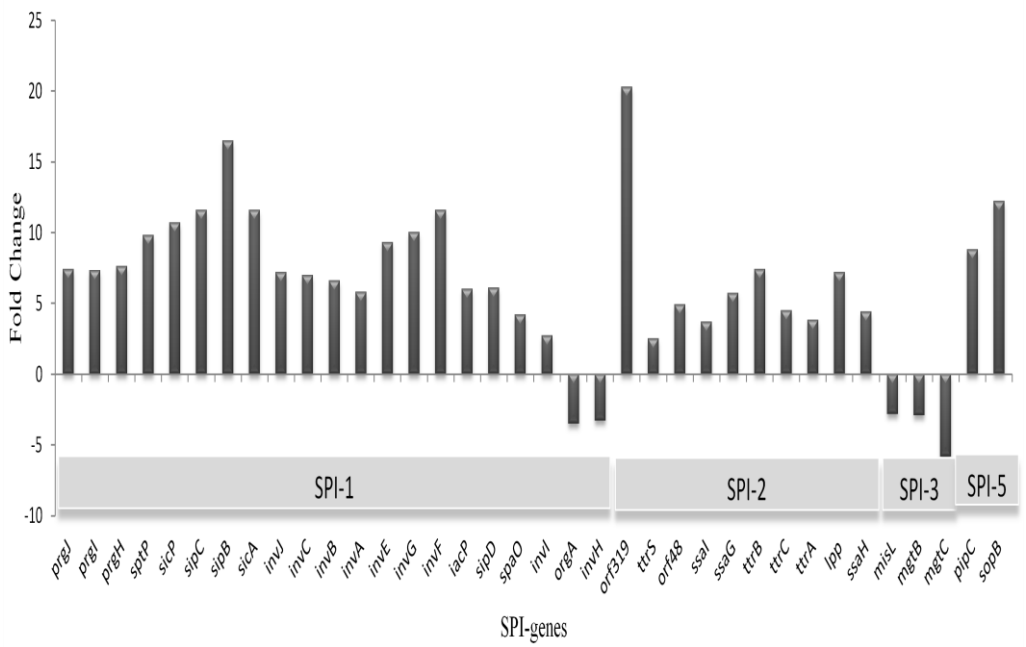

Figure 3.13: Significant changes ($P < 0.05$) in genes' expression of different SPI 1-5 genes showing during the colonisation of *S.* Enteritidis in 1-day chickens' caeca.

The SPI 2 up-regulated genes in chicken caeca were as follows: putative pathogenicity island protein *orf*319, putative histidine kinase *ttr*S, putative ribokinase / regulatory protein *orf*48, putative pathogenicity islands *ssa*AGHIJKLMQ, tetrathionate reductase subunits ABC, *ttr*ABC and major outer membrane lipoprotein *lpp* (Figure 3.13).

The SPI 3 down-regulated genes in chicken caeca were as follows: putative autotransported protein *mis*L, magnesium transport ATPase *mgt*B and magnesium transport protein C *mgt*C (Figure 3.13).

The SPI 5 up-regulated genes in chicken caeca were as follows: cell invasion proteins *pip*C and *sop*B (Figure 3.13).

A number of genes associated with stress were up-regulated including higher temperature *clp*ABCP, *hsc*C, *dna*K, *csi*E, *rpo*S, *rpo*H, *usp*AB, *ibp*AB and *mop*B in addition to cold shock genes *csp*CDE. A number of toxin efflux/inactivation systems were also operational including the multiple antibiotic resistance genes *mar*RAB, thioredoxin (*trx*C) and glutathione S-

transferases. Surprisingly, increased expression of oxidative stress response elements (*sox*RS, *kat*E and *sod*C) and and phage encoded genes was observed.

3.3.9.7 TCA cycle and osmotic associated genes

Two areas of particular interest were carbon source utilisation and respiration and the effect of osmotic pressure on colonisation. 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 colonise the intestinal niche. It was noticed that most operons and genes linked to bacterial fumarate respiration (TCA cycle) and osmotic stress-associated genes were significantly ($P < 0.05$) up-regulated during the colonisation of chicks' caeca compared to the *in vitro* growth (Figures 3.14 and 3.15 respectively).

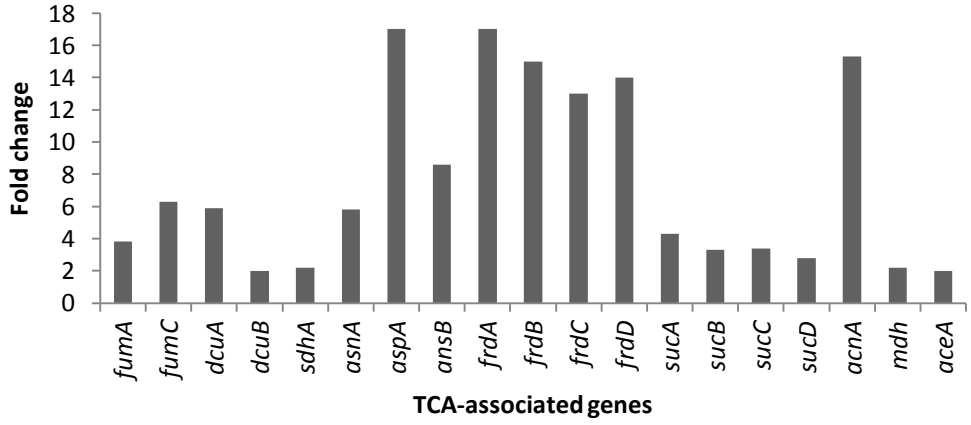

Figure 3.14: Significant up-regulation ($P < 0.05$) in genes associated with TCA-cycle and fumarate respiration during the colonisation of *S.* Enteritidis in 1-day chicken' caeca.

The TCA- associated genes that were up-regulated were as follows: fumarate hydratase class I aerobic *fum*A, fumarate hydratase class II anaerobic *fum*C, anaerobic C4-dicarboxylate transporters (*dcu*A and *dcu*B), succinate dehydrogenase *sdh*A, asparagine synthetase *asn*A, aspartate ammonia-lyase *asp*A, L-asparaginase II *ans*B, fumarate reductase flavoprotein *frd*A, fumarate reductase iron sulphur protein *frd*B, fumarate reductase, membrane anchor polypeptide subunits (*frd*C and *frd*D), 2-ketoglutarate dehydrogenase subunits E1 and E2 (*suc*A and *suc*B), succinyl-CoA synthetase α and β (*suc*C and

*suc*D), aconitate hydratase *acn*A, malate dehydrogenase *mdh* and isocitrate lyase *ace*A (Figure 3.14). 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 involvement of *sucABCD* suggests that the TCA cycle was showing anaerobic behaviour and acting in a non cyclic manner.

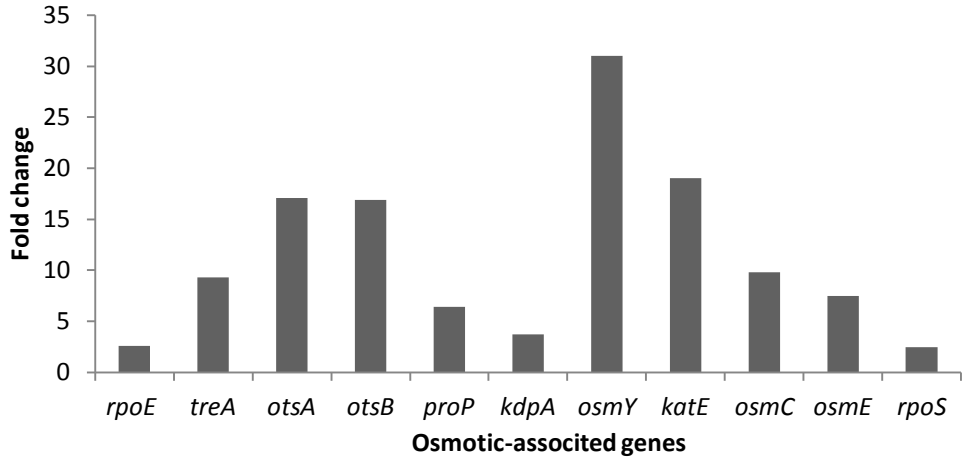

Figure 3.15: Significant changes ($P < 0.05$) in genes associated with stress (e.g. osmotic stress) during the colonisation of *S.* Enteritidis in 1-day chick caeca.

The osmotic- associated genes that were up-regulated were as follows: alternative sigma factor *rpo*E, periplasmic trehalase *tre*A, trehalose-6-phosphate synthase *ots*A, trehalose phosphatase *ots*B, proline/betaine transporter *pro*P, potassium-transporting ATPase A chain *kdp*A, osmotic stress protein-associated with anaerobic environment (*osm*Y and *kat*E), osmotic induced proteins (*osm*C and *osm*E) and sigma factor *rpo*S (Figure 3.15). The *rpo*S sigma factor is known as starvation/stationary phase sigma factor, while the alternative sigma factor *rpo*E is known as extracytoplasmic / extreme heat stress sigma factor.

3.3.10 RT-PCR

The changes in gene expression (n-fold) by microarray were validated by RT-PCR, in which 8 randomly selected up-and-down regulated genes were tested

by PCR (Chapter 2; section 2.2.4). The obtained data were converted to log2 values and plotted against the changes calculated from the array data which had also been log2 converted (Figure 3.16). The data for these 8 genes demonstrated a R^2 value of 0.96 indicating an excellent fit between the two methods, therefore validating the microarray generated results.

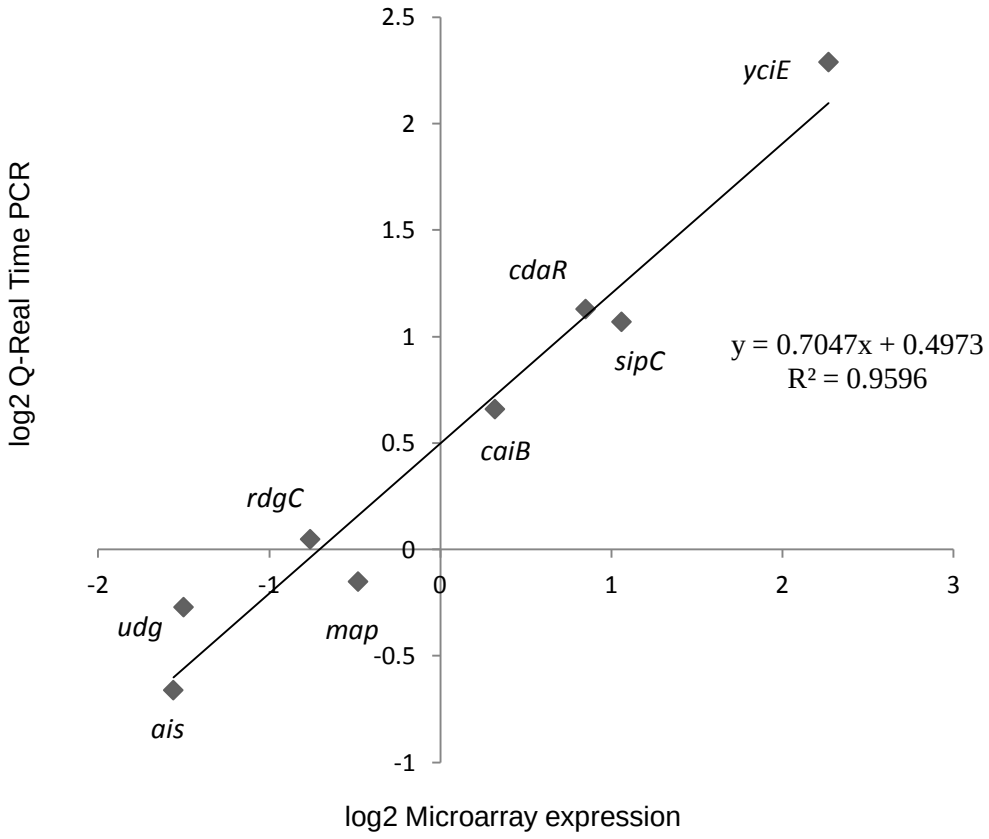

Figure 3.16: Correlation between microarray and quantitative Real-Time (RT-PCR) expression values. These are Log_2 transformed expression values for 8 genes from bacterial total RNA extracted from chick caecal contents in triplicate. The best-fit linear regression line is shown together with the r^2 value and calculated equation for the slope.

3.4. Discussion

The results illustrated above demonstrate the extensive transcriptional changes occurring in the caeca following infection of day-old chickens with *S.* Enteritidis P125109 with more genes being down-regulated in expression than the number that were up-regulated indicating decreased metabolic activity in comparison with the broth culture. Those genes which were up-regulated reflect a degree of adaptation to the caecal environment.

The *S.* Enteritidis-grown *in vitro* and *in vivo* RNA was subjected to amplification using the Message Amp^{TM} II Prokaryotic Amplification protocol with incorporating the pre labeling step aminoallyl UTP (aaUTP). According to Dr P. Tighe, Nottingham University Post Genomic Technologies Department (http://genomics.nottingham.ac.uk) and current publications recommendations (Naderi *et al.,* 2004, Scheler *et al.,* 2009), it was decided to include pre-labeling step prior to Cy dyes coupling with RNA by incorporating the aminoallyl-UTP (aa-UTP) (Chapter-2, section 2.2.3.8.5) in order to enhance the binding of Cy dyes to RNA molecules and to avoid dye swabbing. This is known as indirect labelling, in which both RNA preparations are reverse-transcribed to cDNA in the presence of aminoallyl-modified dUTP or dCTP, respectively. Since both preparations are labeled with the same molecule, there is no bias. While direct labeling which was more common in the past, it has the fundamental problem that Cy3 and Cy5 are incorporated with different yields. In practice this difference can be quite substantial because the Cy3 and Cy5 molecules have different sizes. In fact, there is plenty of evidence in the literature that amplification is useful for array work and does not bias results (Polacek *et al.,* 2003, Li *et al.,* 2004, Kaposi-Novak *et al.,* 2004, Ginsberg, 2005, Rachman *et al.,* 2006, Waddell *et al.,* 2008).

The 16S rRNA and 23S rRNA peaks shown by Agilent 2100 bioanalyser graphs (Figure 3.4) are ribosomal RNA, which represent the majority of RNA. The rRNA ratio is indicative of pure and good quality mRNA. The mRNA molecules are much smaller and would not be noticed on the gel. But since the rRNA was pure we can assume that the mRNA was also pure.

To study gene expression of *Salmonella* during colonisation of chicken the most appropriate model uses birds which are 2-6 weeks old (Barrow, personal communication) with an established gut flora which would be numerically more dominant than the colonising pathogen. The limitations imposed by studying gene expression by microarray meant that experiments had to be performed in newly hatched chickens to avoid false positive signals from the presence of numerically dominant floral components. This model reflects the situation that occurs during infection in newly-hatched chickens which does take place within hatcheries. However, patterns of global gene transcription in *Campylobacter jejuni* in a similar model were found to resemble those from older birds with existing gut flora (Woodall *et al.*, 2005) and other similar models, spectinomycin-treated mouse, which have been used with *E. coli* successfully (Jones *et al.*, 2007b, Jones *et al.*, 2008). Accordingly it was concluded that the pattern of transcription may have also been similar in older birds with a gut flora.

The requirement for large number of chickens to generate sufficient RNA also meant that bacteria present in the caecal content of different birds would also have been at different stages of the growth cycle depending on whether the caeca were full, had just emptied or freshly filled (Barrow, unpublished). This potential variation had implications for measuring the expressions of genes associated with logarithmic versus stationary-phase growth so differences associated with growth rate and cell division may have been smaller than were real.

Upon colonisation of the GI tract, the highest viable count of *Salmonella* are obtained from caecum, cloaca, and ileum (Fanelli *et al.*, 1971, Snoeyenbos *et al.*, 1982, Barrow *et al.*, 1988) and this could be due to the low flow rate of this part of the gut (Smith, 1965), which would allow greater bacterial multiplication and the re-absorption of fluids from the intestinal contents may also contribute to this.

It was aimed to rely on gene expression from *S.* Enteritidis RNA grown in pools of 30 birds rather than *S.* Enteritidis RNA collected from individual birds mainly to avoid gene expression variation between individual birds. Moreover,

less variation still existed between *S.* Enteritidis gene expression in the 3 lots of chickens, but again it was relied on the genes that were expressed where at least one of the three triplicate spots showed a signal.

In the array data, many genes show small changes in expression ratio (e.g. 1.6 fold) that can fall below commonly used, but arbitrary 2-fold cut off, yet may represent biologically meaningful and statistically significant changes. Small changes in mRNA may produce big changes in phenotype (Butcher, 2004). RNA polymerase sigma factor (*rpo*S), alternative sigma factor (*rpo*E) and RNA polymerase 32 sigma factor (*rpo*H) are good examples of such expression (Humphreys *et al.,* 1999). These three genes expression in chicken caeca array varied between 2-3 fold.

The microarray data obtained was validated by the similar changes in expression that observed in selected genes tested by RT-PCR as found by other authors (Eriksson *et al.,* 2003, Woodall *et al.,* 2005, Aldridge *et al.,* 2006).

Those groups of genes that might play a significant role in colonisation and virulence in the caeca of one-day old chicks were the focus for study. Because the time span between *Salmonella* inoculation and harvesting was less than 24 h and because the immune system at this age is still undeveloped, it was expected that the majority of harvested *Salmonella* RNA represented cells that are freely found in the lumen of the caeca; which known as intestinal phase of colonisation, prior to intra-cellular colonisation.

According to the COGs classification (Tables 3.2, 3.3, 3.4 and Figure 3.10) the pattern of gene transcription was different in the intestine compared with broth culture with 714 genes up-regulated and 753 down-regulated in the intestine.
This overarching classification indicated major changes occurring from adaptation to the caecal environment with up-regulation of genes required for energy generation, respiration, 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.

The exact mechanism whereby enteric pathogens colonise the gut of livestock is still relatively poorly 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).

Salmonella pathogenicity islands (SPI) are large sections of horizontally acquired DNA which carry genes required for virulence. Although there are up to 14 different SPI, the presence of which varies among different serovars of *S. enterica*. Five of these can be found in all *S. enterica* serovars (McClelland *et al.*, 2001, Parkhill *et al.*, 2001, Karasova *et al.*, 2010). SPI 1 and SPI 2 pathogenicity islands are considered the most important for *S. enterica* virulence.

SPI 1 encodes a type 3 secretion system (TTSS-1) and secreted translocator proteins which together mediate delivery of effector proteins into epithelial cells resulting in internalisation of *Salmonella* and induction of enteropathogenic responses (Wallis and Galyov, 2000, Zhang *et al.*, 2003). In our array data, most of SPI 1 genes including pathogenicity island effector proteins *prg*HIJ, *sic*P, *sip*CBD, *sic*A, tyrosine phosphatase *spt*P, chaperone, secretory and surface antigen presenting proteins *inv*ABCEFG, probable acyl carrier protein *iac*P and surface antigen presenting protein *spa*O were found to be up-regulated. This could be indicative that virulence and invasive genes are playing an important role in the intestinal phase of colonisation prior to intra-cellular infection.

Two studies that screened transposon mutant libraries of Typhimurium for reduced colonisation of the chicken GI tract either found mutations in SPI1 but not in SPI 2 (Turner *et al.*, 1998) or that SPI 1 mutations had greater impact (Morgan *et al.*, 2004). Despite the fact that caecal swabbing was used to recover strains in these two studies, which may fail to catch low level colonisation, both studies still identified SPI 1 as important in intestinal colonisation. Caecal colonisation was also reported to decrease substantially after the deletion of SPI 1 T3SS components in one day chickens (Porter and

Curtiss, 1997). *S.* Enteritidis SPI 1 genes have a minor effect in colonisation of chicken gut (Morgan *et al.,* 2004).

Moreover, Jones and others (2007a) analyzed the contribution of SPI 1 and SPI 2 to the colonisation of chickens by Typhimurium through the deletion of a single T3SS structural gene in each. They concluded that the SPI 2 T3SS was required for systemic infection and played a significant role in the colonisation of the GI tract, while the SPI 1 T3SS was involved in both compartments without being essential (Jones *et al.,* 2007a).

More recent study indicated that *S.* Typhimurium SPI 1 contributes more than SPI 2 genes in chicken gut colonisation (Dieye *et al.*, 2009). In the last study the whole SPI genes were mutated, the chickens with mixtures of the two strains being compared and determined the competitive index.

Other authors indicated that SPI 2 genes play an important role in the intestinal phase of *Salmonella* infection in mice (Coombes *et al.,* 2005, Coburn *et al.,* 2005). Moreover, SPI 2 genes encode a second type 3 secretion system (TTSS-2) that secrete effector proteins across the membrane of *Salmonella* containing vacuoles (SCV) and enables persistence of *Salmonella* inside host cells by modulating vesicular trafficking (Cirillo *et al.,* 1998, Hensel *et al.,* 1998, Waterman and Holden, 2003). Our array data indicated that few of SPI 2 genes including *orf*319, *orf*48, *ttr*S, *ttr*ABC, *ssa*GHL and *lpp* were expressed by *S.* Enteritidis during its colonisation of the caeca. Because SPI 2 encoded T3SS is required for the transport of *S. enterica* proteins across the phagosomal membrane and increases *S. enterica* survival inside phagocytic cells (Cirillo *et al.,* 1998, Hensel *et al.,* 1998).

On other hand, SPI 1 encoded T3SS is required for the transport of *S. enterica* proteins across the cytoplasmic membrane of a host cell into its cytosol where they induce cytoskeletal rearrangements resulting in the uptake of *S. enterica* even by non-phagocytic cells (Kaniga *et al.*, 1995).

The up-regulation of SPI 1 could be indicative of the presence of bacteria free in the lumen of the caeca or on the caecal epithelia; this was compatible with immunohistochemistry results obtained by Desmidt and others (1998a). In this study they indicated that at 6 h post-infection of infecting 1-day old chickens,

numerous *S.* Enteritidis PT4 were adhering to the apical surface of epithelium of the chicken caeca; while at 18 h post-infection a large number of *S.* Enteritidis PT4 were present in the lumen of caecal crypt (Desmidt *et al.*, 1998a). Besides, the down-regulation of SPI 3 genes (*mgt*A and *mgt*BC) and its regulators (PhoPQ) is indicative that the majority of *Salmonella* cells are free in the caecal lumen during harvesting (Eriksson *et al.*, 2003) and minority of bacterial cells are on epithelial caecal cells.

SPI 4 genes are required for the intestinal phase of disease in cattle (Morgan *et al.*, 2004) and in systemic phase in mice (Kiss *et al.*, 2007). SPI 4 genes in our data were not expressed in chicken caeca at all. In line with this, Morgan and others (2004) have indicated that SPI 4 genes have only a minor or no effect in chicken caeca colonisation. More recent study has also indicated that no effect for SPI 4 in chicken caeca colonisation (Rychlik *et al.*, 2009). They also indicated that the major role of *S.* Enteritidis SPI 1 and SPI 2 on chickens virulence and colonisation of caeca, liver and spleen, but SPI 3, SPI 4 and SPI 5 have no effect.

Other studies indicated that SPI 5 genes are co-regulated with either SPI 1 or SPI 2 genes and therefore represent a dually controlled system (Knodler *et al.*, 2002, Papezova *et al.*, 2007).

The array data showed the down regulation of the majority of *S.* Enteritidis fimbrial, flagellar, LPS and outer membrane genes in chicken' caeca; a few fimbrial genes were significantly up-regulated ($P < 0.05$), including type 1 fimbriae (*fim*A or SEF21) and others fimbrial subunit genes encoded by *peg*A, *std*A, *lpf*A (SEF14). Therefore it was thought that *fim*A, *peg*A, *std*A and *lpf*A genes have a major role in chicken caecal colonisation. These appendages thought to be involved in physical attachment of *Salmonella* and *E. coli* to host mucosal layer or even epithelial cells (Gophna *et al.*, 2001, Edelman *et al.*, 2003, Morgan *et al.*, 2004, Snyder *et al.*, 2004). *S.* Enteritidis type 1 fimbria (*fim* or SEF21) and curli fimbria (SEF17) have a significant role in hen reproductive tract colonisation and have therefore been implicated in egg contamination (Cogan *et al.*, 2004).

More recently Clayton and others (2008) indicated that *S.* Enteritidis PT4 possesses 13 major fimbrial subunits; the majority of which have no significant role in chicken caecal colonisation (Clayton *et al.,* 2008). The fimbrial subunit *peg*A have shown to influence significantly in chicken caecal colonisation. The *peg* fimbriae is a unique operon for *S.* Enteritidis, which displays 60-70% sequence conservation with the *stc* operon of *S.* Typhimurium and is located in the same relative position (Clayton *et al.,* 2008, Thomson *et al.,* 2008).

S. Enteritidis *in vivo* exhibited down-regulation of most motility-associated genes (*flg*AG and *fli*DFMNPR) compared to the growth in nutrient broth. This is compatible with other findings that bacteria in chicken intestinal lumen or the murine urinary tract displayed poor-motility compared to *in vitro* grown bacteria, as demonstrated by phase contrast microscopy (Harvey *et al.,*unpublished) and (Snyder *et al.,* 2004). This could be the fact that motility requires a more liquid environment than the semi-liquid nature of the caecal contents. Therefore it cannot be assumed that the flagella are not essential for colonisation. It is unclear whether down-regulated genes such as LPS synthesis genes (e.g. *rfa*) play a role in colonisation of chicken'caeca. A signature-tagged mutagenesis screening by Morgan and co-workers (2004) proved that mutations in genes for enzymes involved in the biosynthesis of O-antigen side chains attenuated bacteria in their ability to colonise chick and calf intestines (Turner *et al.,* 1998, Morgan *et al.,* 2004).

Within the chicken caecal contents degradation of 1, 2-propanediol appeared to be occurring, although, this generally requires endogenous adenosylcobalamin (coenzyme B12) biosynthesis. The *pdu* genes are co-regulated with cobalamin biosynthetic gene clusters *cob* or *cbi* (McClelland *et al.,* 2001, Rondon *et al.,* 1995, Klumpp *et al.,* 2009). However, in the current experiment there was no significant up-regulation of the *cob* or *cbi* operons within the lumen, possibly because some vitamin B_{12} is already present in egg yolk (Coates, 1963, Coates M. E., 1963) and therefore would be available in the gut of newly hatched chickens as the yolk sac complete desorption lasts 2-3 days.

Many intestinal pathogens including *Salmonella* are able to utilize ethanolamine as a sole source of carbon, nitrogen and energy (Blackwell *et al.,*

1976, Chang and Chang, 1975, Roof and Roth, 1992, Del Papa and Perego, 2008). The precursor molecule for the ethanolamine is phosphatidylethanolamine, an abundant phospholipid in bacterial and mammalian/avian cell membrane that is broken down to glycerol and ethanolamine by phosphodiesterases *glp*Q (Larson *et al.*, 1983, Roof and Roth, 1988, Sheppard *et al.*, 2004). The ethanolamine ammonia-lyase *eut*BC degrades ethanolamine to acetaldehyde and ammonia within a multi protein complex called a carboxysome (Roof and Roth, 1988). The ammonia serves as cellular supply of limited/reduced nitrogen; while acetaldehyde is converted to acetyl co-enzyme A (acetyl Co-A) by the aldehyde dehydrogenase *eut*E-encoded enzyme (Roof and Roth, 1988). Acetyl-CoA is subsequently utilized in many metabolic cycles such as tri carboxylic acid (TCA) cycle. All above ethanolamine linked enzymes were expressed in the chicken array data (Table 3.3 or Appendix Tables) which could indicate their importance in bacterial metabolism as a source for carbon, nitrogen and energy.

Iron utilization and a transport system (*ftn*B, *feo*AB and *iro*N) were up-regulated *in vivo* compared to NB growth culture. Iron represents an essential component for respiratory enzymes, which contain iron-sulphur clusters (Beinert *et al.*, 1997, Takahashi and Tokumoto, 2002, Tokumoto *et al.*, 2002) and regulatory proteins (Tsolis *et al.*, 1995). This result indicates that *S.* Enteritidis cells are starved for iron within chicken caeca in a similar way to *Vibrio cholerae* in the rabbit upper intestine (Xu *et al.*, 2002). Other ion utilization and transport systems for calcium (*cha*A), magnesium and cobalt (*mgt*ABC and *cor*A) were down-regulated, these ions as mentioned could be available as yolk sac remaining in the gut. A sulphur oxoanion, tetrathionate is reduced to thiosulfide and further to H_2S with the products of *ttr*ABC, *phs*ABC and *asr*ABC genes, which were up-regulated in chicken caeca of array study. It is likely that tetrathionate results in part from material from the yolk sac which is rich in sulphur. The role of sulphur-based electron acceptors in respiration in the gut has been shown recently by researchers (Winter *et al.*, 2010) who demonstrated that mice with acute intestinal infection, reactive oxygen is released which generates thiosulphate to be used as electron acceptors.

It seems that nitrogen source (glutamine, arginine and tyrosine) is abundant in the chicken caeca or maybe there are other sources for nitrogen which require less energy to utilize. This was indicated by the down-regulation of transport system of these amino acids *gln*Q, *art*M and *tyr*B respectively as is shown in Table 3.4. This is in contrast to the murine urinary tract environment, where nitrogen source is limited for *E. coli* to utilize and therefore transport systems for these compounds were up-regulated (Snyder *et al.,* 2004). These two models are not strictly comparable since the nutrients available and redox environment of chicken GI tract is totally different than murine urinary tract. Although nitrogen is abundant in urine "e.g. urea is present at ~ 0.5M" (Griffith *et al.,* 1976), it is limiting resource in the urinary tract for *E. coli*, due to its lack of the urease enzyme, which is required for catalyzing the hydrolysis of urea to ammonia and CO_2.

Stationary phase *csi*E, heat shock temperature (*clp*B, *hsc*C, *ibp*AB and SEN1800) and low oxygen (e.g. *frd*ABCD*)* are among the expressed genes in chicken caeca. This reflects the sort of environment which exists in chicken caeca. Some of these regulatory genes have been found to be required for *S.* Enteritidis to colonise chicken gut (Porter and Curtiss, 1997, Turner *et al.,* 1998, Morgan *et al.,* 2004).

Many peptides were also up-regulated in chicken caeca 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 colonisation and to virulence in *S.* Typhimurium for the chicken and mouse (Matiasovicova *et al.,* 2011).

However, it is difficult to interpret how the set of down-or-equivalent regulated genes in the array data might play a role in *S.* Enteritidis colonisation as many of them only have putative assignments. The down-regulation of thiamine biosynthesis (*apb*E and *thi*HGFE) would indicate that there is a ready source of thiamine in the intestine. The data as presented do not highlight important genes expressed at equivalent levels under both conditions which may nevertheless be important in colonisation. By focusing on genes showing

increased expression *in vivo*, a subset of genes that may be important in adaptation to the host environment was focused on.

The results indicate that *S.* Enteritidis adapts to conditions within the chicken caeca; by increasing the expression of specific genes so that the bacterium can efficiently make use of the limited or absence of oxygen and nutrient supplies. The presence of multiple respiratory mechanisms and their differential expression under various conditions may be an advantage for *S.* Enteritidis in coping with the changes in oxygen availability *in vivo*. There is evidence that both oxygen-dependent and oxygen-independent pathways are activated to ensure the survival of *S.* Enteritidis in the chicken' caeca, although which pathways are essential for colonisation and survival is unknown. Respiration-relevant genes for *S.* Enteritidis that were up-regulated in chicken caeca were as follows: nitrate (*nar*HIJKV, *nap*ABDFGH) thiosulphite (*phs*ABC), hydrogenases (*hya*A-E, *hyb*A-G), DMSO (*dmsABC*) and fumarate (*frd*ABCD) respiration.

It was speculated that in the caeca, *S.* Enteritidis, as a facultative anaerobe, can adapt to growth in the various redox environments present in the caeca. Based on the pattern of gene expression of *S.* Enteritidis, the chicken caeca is less oxygen rich than the NB shaking culture under the cultural conditions used for the incubation. Many genes indicative of anaerobic metabolism were up-regulated under *in vivo* conditions. Most interestingly, *frd*ABCD, encoding fumarate reductase, *adh*E, encoding alcohol dehydrogenase, *glp*QTABC, encoding glycerol phosphate, *asp*A, encoding Aspartase, *dms*C, encoding dimethyl sulfoxide reductase, *fdh*F, encoding formate dehydrogenase and Fnr-regulated genes *ans*B, encoding L-asparaginase II were up-regulated during growth in the caeca. By contrast *cyo*AB, encoding components of cytochrome *o* oxidase indicative of respiration under conditions of relatively high oxygen tension were down-regulated *in vivo*. However, the success of *S.* Enteritidis in the GI tract looks to require respiratory flexibility and use the best available electron acceptor (Jones *et al.*, 2007b).

The majority of *S.* Enteritidis PT4 unique genes, which only present either in *S.* Enteritidis or *S.* Typhimurium but not in *S.* Gallinarum genome (Thomson *et*

al., 2008), includes: type three secretion systems T3SS effectors (invasion-associated secreted effector protein *sopE*$_2$, pathogenicity island protein *pip*B and putative virulence effector protein *sif*B) and genes involved in common metabolic processes such as cobalamine biosynthesis and relevant genes *cbi*ABDEFGHJKLMT, propanediol utilization *pdu*GXW and tetrathionate respiration *ttr*ABCS, and fimbrial proteins (*peg*A and *lpf*A), hydrogenase activity *hya*DE were up-regulated in the array data

Many genes associated with carbohydrate transport and metabolism were up-regulated in chicken caeca. These carbohydrates are trehalose, pyruvate, glycerol, fuculose, fucose, glycogen and glucose, which may utilized mainly as a carbon and energy sources. Carbon and energy source mechanism is considered to be essential during the early stages of many bacterial infections (Conway and Schoolnik, 2003). The majority of intestinal bacteria require a fermentable carbohydrate for growth, and fermentation is assumed to be the mode of metabolism used by most species (Salyers, 1979, Salyers *et al.*, 1978). The importance of defined carbon sources, including gluconate, during colonisation has been shown by Conway and his colleagues (Chang *et al.*, 2004, Fabich *et al.*, 2008). Glycogen synthesis by *S.* Typhimurium in chicken gut has been shown to be important for bacterial survival (McMeechan *et al.*, 2005). The activities of GlgA, GlgB and GlgC increase in the presence of rich medium containing glucose and expression also increases as the bacteria enter stationary phase (Preiss, 1984). In the array experiment, the up-regulation of *S.* Enteritidis glycogen synthesis genes *glg*A, *glg*B, *glg*C (harvested from chicken caeca) compared to *S.* Enteritidis (grown mid-log NB) is indicative that the environment in the chicken caeca looks representing the stationary phase environment (Preiss, 1984). Moreover the up-regulation of stationary-phase inducible protein *csi*E and *rpo*S sigma factor (Marschall and Hengge-Aronis, 1995) is another indication of the assumption that the environment in the caeca representing stationary phase environment. The transition of *E. coli* growth from exponential-phase into stationary-phase in LB rich medium resulted in the elevation of *rpo*S-controlled genes expression (Wei *et al.*, 2001). As it explained earlier in the discussion above, bacteria present in the caecal content

of different birds would also have been at different stages of the growth cycle depending on whether the caeca were full, had just emptied or freshly filled (Barrow, unpublished). This potential variation had implications for measuring the expressions of genes associated with logarithmic versus stationary-phase growth. Because the *S.* Enteritidis genes expressed at log phase in chicken caeca were neutralized with those expressed *in vitro* (log phase) on array slides, so the only appeared induced genes are those induced in stationary phase.

However, two areas of particular interest were carbon source utilisation and respiration and the effect of osmotic pressure on *S.* Enteritidis colonisation in chicken gut. 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 colonise the intestinal niche. Because there is limited knowledge of the role of the TCA- linked genes (Figure 3.14) and osmotic-associated genes (Figure 3.15) in intestinal colonisation mechanisms of *S.* Enteritidis in the chicken; it was decided to determine the role of some of these genes in colonisation and competitive-exclusion mechanisms. This was achieved by mutating genes using lambda-Red mutagenesis (Datsenko and Wanner, 2000) and evaluating their effects on colonisation using *in vitro* and *in vivo* competitive-exclusion experiments (Barrow *et al.,* 1988, Berchieri and Barrow, 1990, Berchieri and Barrow, 1991) as explained and shown in the following chapters 4 and 5 respectively.

Chapter - 4: The role of tricarboxylic acid cycle (TCA) substrates in intestinal colonisation

4.1 Introduction

Analysis of the gene expression by microarray (Chapter-3) indicated that all *S.* Enteritidis fumarate respiratory-associated substrates such as aspartate ammonia-lyase (*asp*A) involved in the TCA cycle intermediates and transport systems were up-regulated (Table-4.1), presumably due in part to the lack or absence of oxygen in the chicken caeca. Therefore, it is very important to present a brief introduction about the TCA cycle and the linked fumarate respiratory mechanisms as well as why some TCA genes were selected for mutational studies (section 4.1.1).

Table 4.1: *S.* Enteritidis PT4 TCA associated genes/enzymes, which were significantly ($P < 0.05$) up-regulated more than 2 fold during the colonisation of 1 day chickens intestine compared to *in vitro* growth (* not significant change).

Gene accession number	Gene Function	Symbol	Reference	Fold change	*P*
SEN1581	Fumarate hydratase class I aerobic	*fumA*	Woods and Guest, 1987	3.8	0.005
SEN1579	Fumarate hydratase class II anaerobic	*fumC*	Woods and Guest, 1987	6.3	0.005
SEN4095	Anaerobic C4-dicarboxylate transporter	*dcuA*	Engel *et al.* 1992	5.9	0.006
SEN4073	Anaerobic C4-dicarboxylate transporter	*dcuB*	Engel *et al.* 1992	2	*
SEN0684	Succinate dehydrogenase (Flavoprotein subunit)	*sdhA*	Guest, 1992	2.2	0.01
SEN3691	Asparagine synthetase A	*asnA*	Cedar and Schwartz, 1967	5.8	0.005
SEN4096	Aspartate ammonia-lyase	*aspA*	Creaghan and Guest, 1977	17	0.003
SEN2949	L-asparaginase II	*ansB*	Cedar and Schwartz, 1967	8.6	0.005
SEN4113	Fumarate reductase, flavoprotein	*frdA*	(Van Hellemond and Tielens,	17	0.007

			1994)		
SEN4112	Fumarate reductase, iron-sulfur protein	*frdB*	(Van Hellemond and Tielens, 1994)	15	0.005
SEN4111	Fumarate reductase, membrane anchor polypeptide subunit C	*frdC*	(Van Hellemond and Tielens, 1994)	13	0.006
SEN4110	Fumarate reductase, membrane anchor polypeptide subunit D	*frdD*	(Van Hellemond and Tielens, 1994)	14	0.004
SEN0686	2-ketoglutarate dehydrogenase (E1 subunit)	*sucA*	(Veit *et al.*, 2007)	4.3	0.006
SEN0687	2-ketoglutarate dehydrogenase (E2 subunit	*sucB*	Veit *et al.*,2007	3.3	0.005
SEN0688	Succinyl-CoA synthetase (β)	*sucC*	Veit *et al.*,2007	3.4	0.007
SEN0689	Succinyl-CoA synthetase (α)	*sucD*	Veit *et al.*,2007	2.8	0.01
SEN1321	Aconitate hydratase	*acnA*	(Prodromou *et al.*, 1991)	15.3	0.003
SEN3192	Malate dehydrogenase	*mdh*	Creaghan and Guest, 1977	2.2	0.02
SEN3966	Isocitrate lyase	*aceA*	Creaghan and Guest, 1977	2	*

4.1.1 Bacterial TCA cycle

The tricarboxylic acid (TCA) cycle, also known as the citric acid cycle or Krebs cycle, is responsible for the total oxidation of acetyl co-enzyme A, which is derived mainly from the pyruvate produced by glycosis (Figure 4.2).

Tricarboxylic acids (TCA) are organic carboxylic acids containing three carboxyl functional groups (-COOH) as shown in Figure 4.1. The citric acid contains an extra OH molecule.

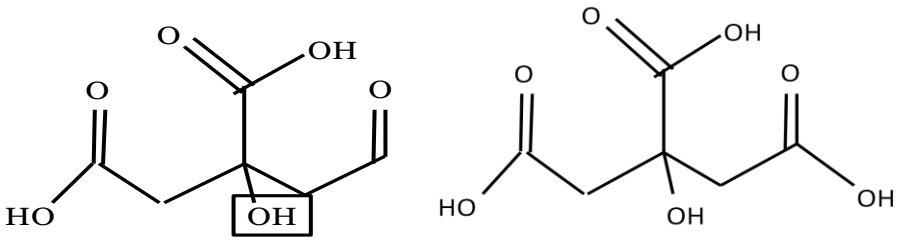

Figure 4.1: Tricarboxylic acid chemical structure (left) and citric acid chemical structure (right). TCA cycle intermediates are also required in the biosynthesis of several amino acids.

Until the 1990s, the TCA cycle was regarded as a constitutive "house-keeping" pathway, when confirmation was provided to indicate that in *E. coli* and *S.* Typhimurium the TCA pathway is inducible (Guest, 1992, Iuchi *et al.*, 1989, Iuchi and Lin, 1988). Thus, anaerobic repression of succinate dehydrogenase encoded by *sdh*ABCD was masked by the anaerobic induction of fumarate reductase encoded by *frd*ABCD (Guest, 1992). Also, the regulation of aerobic fumarase (*fum*A) was masked by the anaerobic *fum*B and the unregulated *fum*C (Woods and Guest, 1987). The expression levels of the TCA cycle enzymes respond mainly to the presence of oxygen and to the carbon source (Gray *et al.*, 1966b, Gray *et al.*, 1966a, Amarasingham and Davis, 1965).

Glycosis is the basis for both aerobic and anaerobic respiration and it is found nearly in all organisms including facultative anaerobic bacteria (Bowden *et al.*, 2009, Postma *et al.*, 1993). In facultative anaerobic bacteria (Smith and Neidhardt, 1983) such as *E. coli* and *Salmonella*, the full TCA cycle (Fig 4.2) is seen only during aerobic growth on glucose or acetate or fatty acids (Perrenoud and Sauer, 2005, Amarasingham and Davis, 1965, Ornston and Ornston, 1969). Such cultural conditions give the highest levels of TCA cycle enzymes, and the cycle provides all the energy and reducing potential needed to support growth.

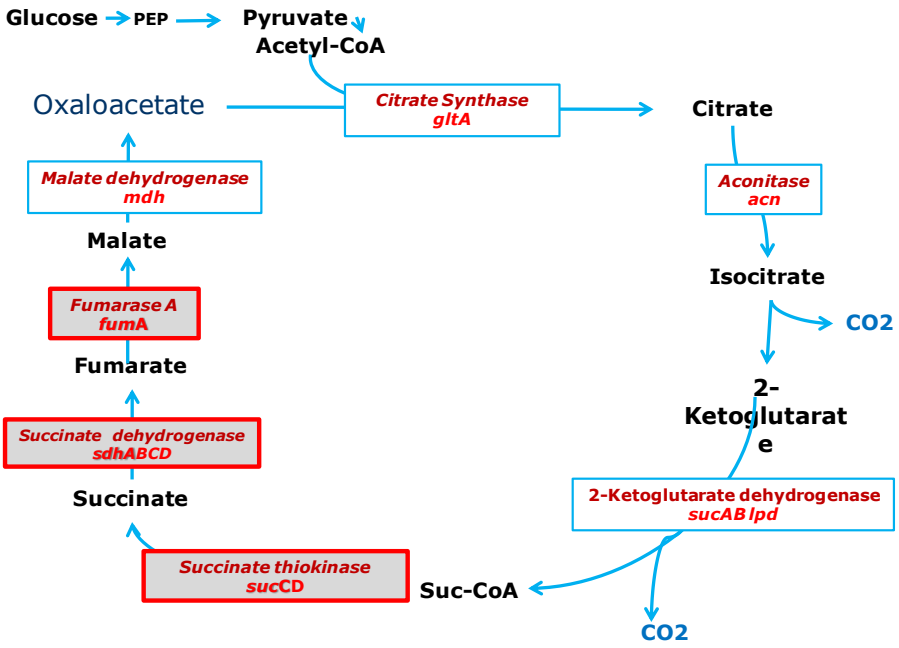

Figure 4.2 : TCA Cycle of facultative anaerobes during their aerobic growth on acetate or fatty acids. The grey highlighted genes are the ones subjected for mutagenesis individually (Chapter 2; section 2.2.5).

On the other hand under anaerobic conditions, the TCA cycle can no longer provide energy and instead operates as two biosynthetic pathways, a reductive pathway that produces succinyl-CoA and an oxidative pathway producing 2-ketoglutarate (Figure 4.3). These pathways are unable to form a cycle because 2-ketoglutarate dehydrogenase is almost absent during anaerobic growth (Amarasingham and Davis, 1965, Smith and Neidhardt, 1983). Synthesis of this enzyme is the most severely repressed of all the TCA cycle enzymes. Under these conditions, the level of other enzymes activities (and enzyme proteins) are much lower (10-20 fold) than those found during aerobic growth.

During anaerobic growth also, as shown in Figure 4.3, several other genes are expressed to supplement the biosynthetic pathways formed by the TCA cycle enzymes. For example, fumarate reductase (*frd*ABCD) substitutes for succinate dehydrogenase (*sdh*ABCD) to allow reductive production of succinyl-CoA (as well as providing an anaerobic respiratory pathway). Aspartase (*asp*A) gene is induced to assist together with the constitutive malate dehydrogenase (*mdh*) in the conversion of oxaloacetate to fumarate. Also, some carbon does flow

between the branches by the action of the glyoxylate cycle enzyme isocitrate lyase, which converts isocitrate to succinate and glyoxylate (Creaghan and Guest, 1977). The action of isocitrate lyase under these conditions is confirmed by the finding that anaerobically grown cells show a succinate requirement only when both isocitrate lyase and fumarate reductase are inactivated by mutation (Creaghan and Guest, 1977).

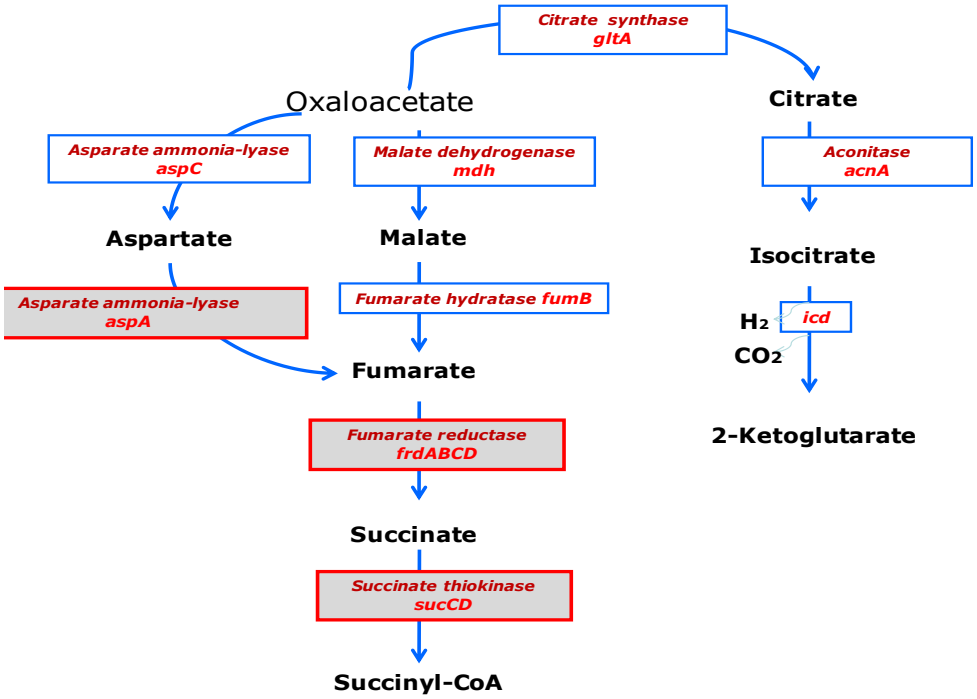

Figure 4.3: Function of the TCA cycle in anaerobic growth. The oxidative branch is to the right and the reductive branch is to the left. The grey highlighted genes are the ones subjected for mutagenesis individually (Chapter 2; section 2.2.5).

Escherichia coli synthesizes two asparaginases, asparaginase I (*asn*A) and L-asparaginase II (*ans*B); which are distinct in a number of ways. The asparaginase I (*asn*A) is located in the cytoplasm, whereas L-asparginase II (*ans*B) is located in the periplasmic space (Campbell *et al.*, 1967, Cedar and Schwartz, 1967, Willis and Woolfolk, 1974). Asparaginase I (*asn*A) is produced and synthesized constitutively; while L-asparaginase II (*asn*B), is produced primarily under anaerobic environments and only in medium containing high concentrations of amino acids and little or no sugars (Cedar

and Schwartz, 1967, Willis and Woolfolk, 1974). The L-asparaginase II (*asn*B) was found to have a higher affinity for asparagine than asparaginase I (Schwartz *et al.*, 1966). L-asparaginase II (*asn*B) has been studied extensively because of its widespread use in the treatment of childhood acute lymphocytic leukaemia (Durden and Distasio, 1980).

Under aerobic environments the uptake of C_4-dicarboxylates (fumarate, malate, and succinate) and L-aspartate is mediated by a secondary transporter known as the Dct system (Kay, 1971, Kay and Kornberg, 1971, Lo, 1977), as shown in Figure 4.4. Under anaerobic environment, the uptake and exchange of C_4-dicarboxylate compounds is mediated by the Dcu system, which is genetically distinct from the aerobic Dct system (Engel *et al.*, 1992, Engel *et al.*, 1994, Zientz *et al.*, 1996).

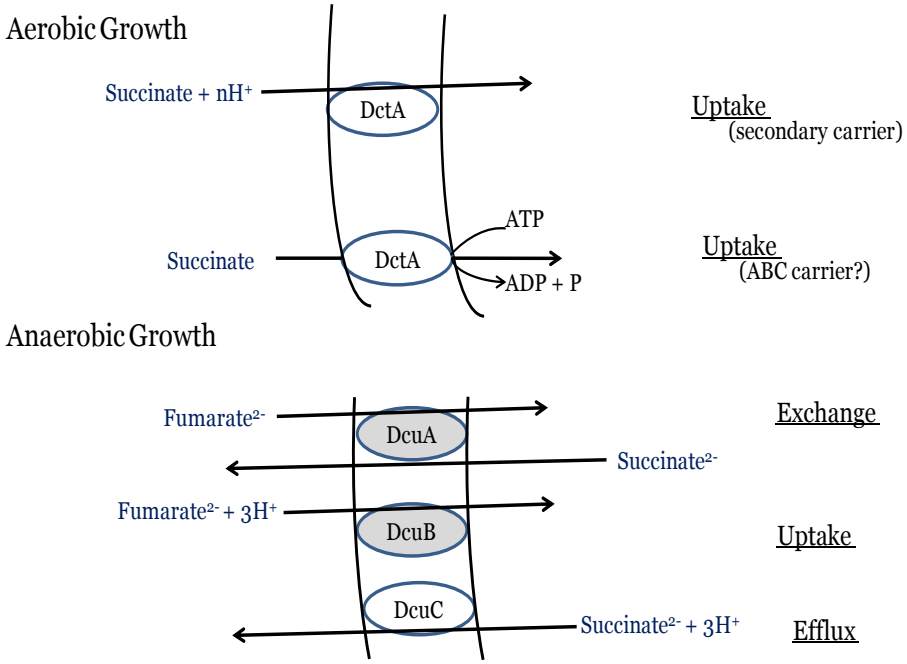

Figure 4.4: C_4-dicarboxylate carriers of aerobically or anaerobically grown *E. coli* and their mode of action (Unden and Bongaerts, 1997). The grey highlighted protein-encoded genes are the ones subjected for mutagenesis (Chapter 2; section 2.2.5).

Because there is limited knowledge of the role of the TCA cycle intermediates and transport system associated genes in intestinal colonisation mechanisms of *S.* Enteritidis in the chicken; therefore it was decided to determine the role of

some of these in colonisation. Initial studies on gene expression using newly-hatched chickens (Barrow *et al.*, 1988) showed that many bacterial strains, which are unable to colonise the chicken intestine when a mature gut flora is present, are able to do so when the flora is absent. It is therefore difficult to assess colonisation ability of mutants in newly-hatched chickens. Instead a competition exclusion experiment was used in which colonisation by a mutant is investigated for its ability to prevent colonisation by the parent strain (Barrow *et al.*, 1988, Berchieri and Barrow, 1990, Berchieri and Barrow, 1991, Turner *et al.*, 1998, Morgan *et al.*, 2004). Therefore to study *S.* Enteritidis PT4 TCA intermediates and TCA transport systems genes (8 genes and 1 operon in total) were subjected for individual mutagenesis using lambda-Red mutagenesis (Datsenko and Wanner, 2000); most of these are highlighted in grey in Figures 4.2, 4.3 and 4. 4. These include: fumarate hydratase *fum*A, succinate dehydrogenase *sdh*A, aspartate ammonia-lyase *asp*A, fumarate reductase *frd*ABCD (*frd*AD), asparagine synthetase *asn*A, L-asparaginase *ans*B, succinyl-CoA synthetase β *suc*C, anaerobic C4-dicarboxylate transporter *dcu*A and *dcu*B. This number of genes was selected, because they are the most common expressed ones which linked to the TCA cycle intermediates and transport systems (for relevant references see Table 4.1). To study the role of these genes in young birds' intestinal colonisation, these mutants' growth curve and growth rate compared to the wild type were assessed. Then the *in vitro* competitive-exclusion was carried out using *in vitro* model in which stationary nutrient broth cultures of the mutants were assessed for their ability to suppress growth of the parent strain inoculated 24 h later and vice versa (Zhang-Barber *et al.*, 1997, Berchieri and Barrow, 1991). The co-culture competitiveness of every mutant with the parent strain was also assessed. These assays were carried out aerobically and anaerobically. Finally competitive exclusion was transferred to the *in vivo* environment and the *in vivo* colonisation-inhibition of these mutants against the wild type in newly-hatched young chickens was also determined.

4.2 Materials and Methods

4.2.1 TCA mutants generation

TCA mutants were constructed by insertion of a Nal^R or Spc^R cassette into the open reading frame "ORF" of the gene of interest (Chapter-2, section 2.2.5). Briefly, a pair of primers and specific test control primers was designed for every TCA gene of interest (Table 4.2 and 4.3) for checking the replacement of the target gene by the antibiotic cassette. The published Cm and Km cassette specific test primers (C1, C2 and K1, K2 respectively) were used in combination with the target specific test primers for checking the incorporation of the antibiotic cassettes to the desired place. Once the mutants have been confirmed by PCR confirmatory tests they were further tested by growing them on selective culture, performing slide agglutination test, and acriflavin test to make sure that the bacterial cell wall still intact. Then they were subjected for recombination (transduction) test, in which Bacteriophage P22 [kindly provided by Dr G. Dougan, Welcome Biotechnology, Beckenham] was used to transfer *S.* Enteritidis mutants DNA (donor) to *S.* Enteritidis wild type (Nal^R) strain (recipient), to reduce the likelihood that the phenotypes are result of second site defect. All mutants passed this test successfully, after which they were streaked on NA plates supplemented with their respective antibiotics and incubated at 37^{o}C for overnight, then on the following day an isolated typical colony from each was streaked on NA plate contained no antibiotics and incubated at 37^{o}C for overnight. On the following day one loop-full of bacterial culture was collected and inoculated into 20% glycerol nutrient broth tubes (2 ml glycerol + 8 ml NB); well mixed and then split over sterile 1-ml mini-glass tubes and kept frozen at -80^{o}C freezer.

Table 4.2: Primer used to construct TCA gene mutation individually. Grey shading indicates the primer sequence homologous to the chloramphenicol (Cm) or kanamycin (Km) antibiotic cassettes (Datsenko and Wanner, 2000).

Name	Sequence (5'- 3')
*fum*A_50F	TGGAACACCTGCCCAGAGAATAACCATACCGAGCGGTAAGTGAGAGC ACAGTGTAGGCTGGAGCTGCTTC
*fum*A_50R	GTTCTCCGGCAAAATAGCGAATCATGTTACCGCCCCGCAGGGCGGCG ACACATATGAATATCCTCCTTAG
*dcu*A_50F	GTATTGGATTCGCAGGTGGGCTGGGTGTTCTGGTACTTGCCGCCATC GGCGTGTAGGCTGGAGCTGCTTC
*dcu*A_50R	GTCAAAGGGCGGGAGAATGCGGGGCGTGATACGCCCCGCGGGTGAAA CACCATATGAATATCCTCCTTAG
*dcu*B_50F	TGTGATCCATTTATCAAAATTGACTGGGTTTATCGCGAGGATAAATA AAAGTGTAGGCTGGAGCTGCTTC
*dcu*B_50R	GCCAGGCTCTAAGTCGCGCGCCGCGCATAACCGGGCGGCGCGCGCGG CCACATATGAATATCCTCCTTAG
*sdh*A_50F	GCGCTGGTGGTTTACGTCATCTATGGATTTGTTGTGGTGTGGGGTGT GTAGTGTAGGCTGGAGCTGCTTC
*sdh*A_50R	GGTTATAACGATAAATCGAAAATTCGAGTTTCATCATCCTGTCTCCG CAACATATGAATATCCTCCTTAG
*asn*A_50F	GCTCTCGTTTTGTTGCTTAATCATAGGCAACAGGACGCAGGAGTAAA AAAGTGTAGGCTGGAGCTGCTTC
*asn*A_50R	TGACACCGGGATGCGAAGCCGCCTGCTGAGACGCTGGCGGCGCTAAC GGCCATATGAATATCCTCCTTAG
*asp*A_50F	CTGTGTGTTTTAAAGTAAAAATCATTGGCAGCTTGAAAAAGAAGGTT CACGTGTAGGCTGGAGCTGCTTC
*asp*A_50R	GAAAAAAGGCACGTCATCGTGACGTGCCTCTTTGGTACTACCCTGTA CGACATATGAATATCCTCCTTAG
*ans*B_50F	GTCTGCAATATAGAGATAATGCGACCAGTTGACATAACTGGAGATAT AACGTGTAGGCTGGAGCTGCTTC
*ans*B_50R	GCGAGAGGTCTTCCAAAAATAGCCCCGGCCTTCCGACCGGGGCATTA TCACATATGAATATCCTCCTTAG
*frd*A_50F	GCTTTATCTGGCTGCGCGAGGGTGAAATTACAATAATCTGGAGGAAT GTCGTGTAGGCTGGAGCTGCTTC
*frd*D_50R	AAAAGAAAAAACGCCCTCTTATCGGGTAGATAAGAGGGCGTCGTGGC AACCATATGAATATCCTCCTTAG
*suc*C_50F	CAGGCCTACAGGTCTAAAGATAACGATTACCTGAAGGATGGACAGAA CACGTGTAGGCTGGAGCTGCTTC
*suc*C_50R	GGTGAAGCCCTGGCAGATAACCTTGGTATCTTTATTAATTAAAACGG ACACATATGAATATCCTCCTTAG

Table 4.3: Primer combinations used to validate each TCA gene mutation. Primers are specific to the flanking regions of the specific TCA gene (ctrF = control forward; ctrR = control reverse) or the antibiotic resistance cassette (Cm1 and Km1 = reverse; Cm2 and Km2 = forward).

Name	Sequence (5'- 3')	Predicted size (bp)
*fum*A_ctrF	ATTCTTTTCGATGGCGTCAC	2350 nt
*fum*A_ctrR	GAAATCCGAAAATGCTCCAG	
*dcu*A_ctrF	TCATTGTTTTGCTGGCAATC	1420 nt
*dcu*A_ctrR	CGAAAGAACAAAAAGACCCG	
*dcu*B_ctrF	TACTGCTCTCGCTGACTCCA	1770 nt
*dcu*B_ctrR	TGACGCTGACATAATCGGAG	
*sdh*A_ctrF	CTGACCTTTGAAGCCTGGAC	2200 nt
*sdh*A_ctrR	ATTCAAACCATCAGAACCGC	
*asn*A_ctrF	GCATTTTCCATTAAGGCGTC	1400 nt
*asn*A_ctrR	GAGCAAAGTGGGAGAGTTGC	
*asp*A_ctrF	TTCGATATGGTGGTGCGTAG	1980 nt
*asp*A_ctrR	ATCGAATGGAATTGTCCCTG	
*asn*B_ctrF	AAAGATGTCTGTAGCCGCGT	1450 nt
*asn*B_ctrR	GTCGAACCACTTGTGGACCT	
*frd*A_ctrF	TTCCCTCACATCCCTGAGAC	3700 nt
*frd*D_ctrR	GCGGAGTAGGCGAACTACAG	
*suc*C_ctrF	GTACCTGGCGCTCTCTTACG	1670 nt
*suc*C_ctrR	ATGGAGTCTTTGCAGAACGG	
Cm1	TTATACGCAAGGCGACAAGG	-
Cm2	GATCTTCCGTCACAGGTAGG	-
Km1	CAGTCATAGCCGAATAGCCT	-
Km2	CGGTGCCCTGAATGAACTGC	-

4.2.2 Assessment of growth rate of mutants of *S.* Enteritidis defective in TCA cycle and linked genes.

This was designed to assess whether the particular TCA gene mutation affected its growth rate, this could contribute to the poor growth of *S.* Enteritidis TCA-generated mutant using *in vitro* culture model. The methodology of this experiment was explained in (Chapter-2; section 2.2.2). The bacterial growth rate for *S.* Enteritidis wild type and its TCA-defective mutants was calculated for both temperatures (37^{o}C and 42 ^{o}C) as shown in result section of this chapter. Moreover the growth cultural characteristics of these mutants on NA and MacConkey agar plates aerobically and anaerobically was assessed compared to *S.* Enteritidis wild type

4.2.3 *In vitro* competitive exclusion and co-culturing experiments for mutants of *S.* Enteritidis defective in TCA genes and wild type

Eight different experimental formats were used for *in vitro* colonisation-inhibition and co-culturing of the mutants of *S.* Enteritidis defective in one of TCA genes and wild type. These experiments were explained in detail in Chapter-2; section 2.2.6. Briefly; these 8 experiments were numbered from 1-to-8 to facilitate clarity. The different conditions for these 8 experiments are listed in Table 4.4 below.

Table 4.4: *In vitro* colonisation inhibition and co-culturing experiments for mutants of *S.* Enteritidis defective in TCA genes and wild type incubated at 42°C or 37°C.

Experiment No	Method	Environment	1st Strain (Stationary Phase)	2nd Strain (Challenge strain)	Incubation Time
1	Ability of stationary-phase cultures of mutants of *S.* Enteritidis to suppress growth of the wild type	Aerobic 42°C	TCA mutants 3-7 x 10^8 cfu/ml	Parent strain 2-7 x 10^3 cfu/ml	24 h
2	Ability of stationary-phase cultures of mutants of *S.* Enteritidis to suppress growth of the wild type	Anaerobic 42°C	TCA mutants 1-5 x 10^8 cfu/ml	Parent strain 2-6 x 10^3 cfu/ml	24 h
3	Ability of stationary-phase cultures of mutants of *S.* Enteritidis to suppress growth of the wild type	Aerobic 42°C	TCA mutants 6-9 x 10^8 cfu/ml	Parent strain 6-9 x 10^3 cfu/ml	72 h
4	Ability of stationary-phase cultures of mutants of *S.* Enteritidis to suppress growth of the wild type	Anaerobic 37°C	TCA mutants 2-5 x 10^8 cfu/ml	Parent strain 2-5 x 10^3 cfu/ml	72 h
5	Co-culturing of *S.* Enteritidis Mutants with the wild type	Aerobic 42°C	-	-	24 h
6	Co-culturing of *S.* Enteritidis Mutants with the wild type	Anaerobic 42°C	-	-	24 h
7	Ability of stationary-phase cultures of *S.* Enteritidis wild type to suppress growth of the *S.* Enteritidis mutants	Aerobic 42°C	Parent strain 3-7 x 10^8 cfu/ml	All TCA mutants 3-8 x 10^3 cfu/ml	24 h
8	Ability of stationary-phase cultures of *S.* Enteritidis wild type to suppress growth of the *S.* Enteritidis mutants	Anaerobic 42°C	Parent strain 1-9 x 10^7 cfu/ml	All TCA mutants 4 x 10^2 – 3 x 10^3 cfu/ml	24 h

4.2.3.1 Experiment 1

This was designed to test the ability of stationary phase cultures of mutants defective in TCA genes (*fum*A, *dcu*A, *dcu*B, *sdh*A, *asn*A, *asp*A, *ans*B, *frd*ABCD and *suc*C) to inhibit multiplication of small numbers of the parent strain introduced into the culture in nutrient broth aerobically 24 h. The method is explained in detail in Chapter-2; section 2.2.6.1.

4.2.3.2 Experiment 2

This is designed to test the ability of stationary phase cultures of mutants defective in TCA genes to inhibit multiplication of small numbers of the parent strain introduced into the culture in nutrient broth anaerobically for 24 h.

This method as described in Experiment 1 method above; but the nutrient broth media in this experiment were incubated in an anaerobic chamber (Bactron Anaerobic / Environmental Chamber, Anaerobe Systems). Two control tubes were included: one universal tube containing 10 ml fresh nutrient broth (NB) was inoculated with 2 - 6 x 10^3 cfu/ml of wild type Spc^R (challenge); and the other universal tube contained the stationary phase of *S.* Enteritidis wild type (NaL^R) 4-9 x 10^8 cfu/ml was inoculated with the challenge inoculum (2-3 x 10^3 cfu/ml) of wild type $Spc^{R.}$

NB: The incubation in anaerobic chamber was static at all times.

4.2.3.3 Experiment 3

This is designed to test the ability of stationary phase cultures of mutants defective in TCA genes (*dcu*A, *dcu*B, *sdh*A, *asp*A and *frd*ABCD) to inhibit multiplication of small numbers of the parent strain introduced into the culture in nutrient broth aerobically at 42^{o}C for three days with time-points at 24, 48 and 72 h. This Experiment' method is as Experiment-1.above; but the test was performed for 3 days with time-points at 24, 48 and 72 h.

4.2.3.4 Experiment 4

This is designed to test the ability of stationary phase cultures of mutants defective in TCA genes (*dcu*A and *dcu*B) to inhibit multiplication of small numbers of the parent strain introduced into the culture in nutrient broth incubated anaerobically at 37^{o}C for three days with time-points at 24, 48 and 72 h. This Experiment' method is as described in Experiment-2; but the test was performed for 3 days with time-points 24, 48 and 72 h.

4.2.3.5 Experiment 5:

Using the co-culture method to test the ability of each of the mutants to out-compete the parent strain in nutrient broth aerobically was determined. The methodology of this experiment was explained in Chapter-2; section 2.2.8.2.

4.2.3.6 Experiment 6

This co-culture method was to test the ability of each of the mutants to out-compete the parent strain in nutrient broth under anaerobic conditions.

This Experiment is as described in Chapter-2; section 2.2.8.2.; but all nutrient broth cultures in this method were incubated statically in an anaerobic chamber for 24 h.

4.2.3.7 Experiment 7:

This was designed to test the ability of a small number of mutants defective in one of TCA genes (*fum*A, *dcu*A, *dcu*B, *sdh*A, *asn*A, *asp*A, *ans*B, *frd*ABCD and *suc*C) to overgrow the stationary phase cultures of parent wild strain in nutrient broth at aerobic environment for 24 h. The methodology of this experiment was explained in Chapter-2; section 2.2.8.3.

4.2.3.8 Experiment 8

This was designed to test the ability of small number of mutants defective in one of TCA genes to overgrow the stationary phase cultures of parent wild strain in nutrient broth statically in anaerobic chamber for 24 h. The method of

this experiment is as mentioned in Chapter-2; section 2.2.8.3.; but the nutrient broth media in this experiment was incubated statically in an anaerobic chamber. One positive control tube was included; which contained the stationary phase of *S.* Enteritidis wild type Spc^R inoculated with small numbers of *S.* Enteritidis wild type Nal^R and incubated for 24 h.

4.2.4 *In vivo* competitive exclusion experiments for mutants of *S.* Enteritidis defective in TCA

This experiment was designed first to test the ability of mutants defective in TCA genes (*fum*A, *dcu*A, *dcu*B, *sdh*A, *asn*A, *asp*A, *ans*B, *frd*ABCD and *suc*C) to grow in the one-day-old chicken caeca; and then evaluate these mutants' colonisation inhibition (competitive exclusion) against the wild type of *S.* Enteritidis spectinomycin resistant strain (Spc^R). The birds used were 1-day old broilers (P D Hook., Thirsk, UK). Briefly, eleven groups of ten birds were designed for this experiment; nine groups of them were inoculated with 0.1 ml 24 h broth culture of the *S.* Enteritidis mutants (4×10^7 cfu) defective in one of TCA mutants within the first 6 h of hatching. The birds in the positive control group were inoculated orally with a blunt needle with *S.* Enteritidis wild type Nal^R (4×10^7 cfu) while the negative control received nothing. On the following day 3 chicks of every group were randomly selected, humanely killed and their caecal contents collected to enumerate the inoculated strain on BGA plates supplemented with nalidixic acid and novobiocin. The remaining birds for every group (7 birds) were inoculated with 0.1 ml of 24 h shaken cultures of *S.* Enteritidis wild type Spc^R (challenge) after been diluted 1/1000 in PBS - the challenge dose was ~1.8×10^5 bacterial cells. Then the groups of birds were kept in separated rooms on straw bedded solid ground for further 48 h. When chicks reached 4 days of age (48 h after challenge inoculation) they were humanely killed and their caecal contents were collected for viable counts of all mutants and the challenge strain, which were done on these samples as described in the general methods section.

4.3 Results

4.3.1 Mutants PCR confirmation

In Chapter 2, section 2.2.5.1, Figures 2.3 and 2.4 illustrated clearly the steps performed in order to mutate the gene of interest by replacining it with the antibiotic cassette [chloramphenicol (1.3 kb) or kanamycin (1.6 kb)] after integrating forward and reverse primers for the gene of interest and the antibiotic cassette that has been designed and electroporated into *S.* Enteritidis genome by the action of λ red system (section 2.2.5).

According to Table 4.5, the Figures 4.5 and 4.6 indicate clearly that these are real mutants, as the expected size of gene or operon of interest is shown on the wild type (wt) DNA of *S.* Enteritidis; while the mutated genes or operon were replaced by antibiotic cassettes, Cm^R (1.3 kb) or Km^R (1.6 kb) after the mutants subjected to recombination (transduction) test., in which bacteriophage P22 was used to transfer mutants DNA (donor) to *S.* Enteritidis wild type (Nal^R) strain (recipient), to reduce the likelihood that the phenotypes are result of second site defect. All mutants passed this test successfully.

All mutants were tested by growing them on nutrient agar supplemented with chloramphenicol or kanamycin, on which they exhibited resistance to the antibiotic they possess and sensitivity to the other one.

Table 4.5: The test control primers and templates used to detect the chloramphenicol cassette (1.3 kb) replacing *sdh*A gene (2.2 kb) in *S.* Enteritidis genome by PCR experiment.

	Contents/lanes (Fig 4.5)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Primers	*sdh*A gene primer forward	+	+	+	+	+	+	+	+	-	-	-	-
	*sdh*A gene primer reverse	+	+	+	+	-	-	-	-	+	+	+	+
	Chloramphenicol control primer forward	+	+	-	-	-	-	-	-	+	+	+	+
	Chloramphenicol control primer reverse	+	+	-	-	+	+	+	+	-	-	-	-
Templates	*sdh*A mutant colony 1 DNA	+	-	-	-	+	-	-	-	+	-	-	-
	*sdh*A mutant colony 2 DNA	-	+	-	-	-	+	-	-	-	+	-	-
	S. Enteritidis wild type DNA	-	-	+	-	-	-	+	-	-	-	+	-

The + means the primer and/or the template was added to the PCR reaction, while – means the primer and/or the template was not added to the PCR reaction.

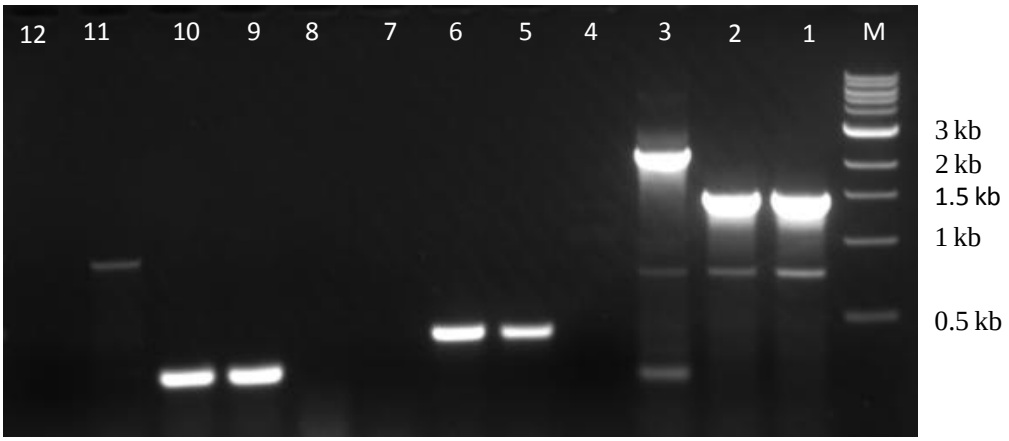

Figure 4.5: Agarose gel electrophoresis of the confirmatory PCR for *S.* Enteritidis *sdh*A mutant, in which the chloramphenicol cassette (~1.3 kb) [lanes 1 and 2] replaced the *sdh*A gene, which is present in the wild type (2.2 kb) [lane 3]. Lanes 4, 8 and 12 are non-template controls. Lanes 7 and 11 represent wild type amplification with incompatible forward and reverse primers. The rest of the lanes represent products resulting from amplification using the combination of *sdh*A forward primer with chloramphenicol reverse primer or vice versa. M represents 1kb molecular weight ladder.

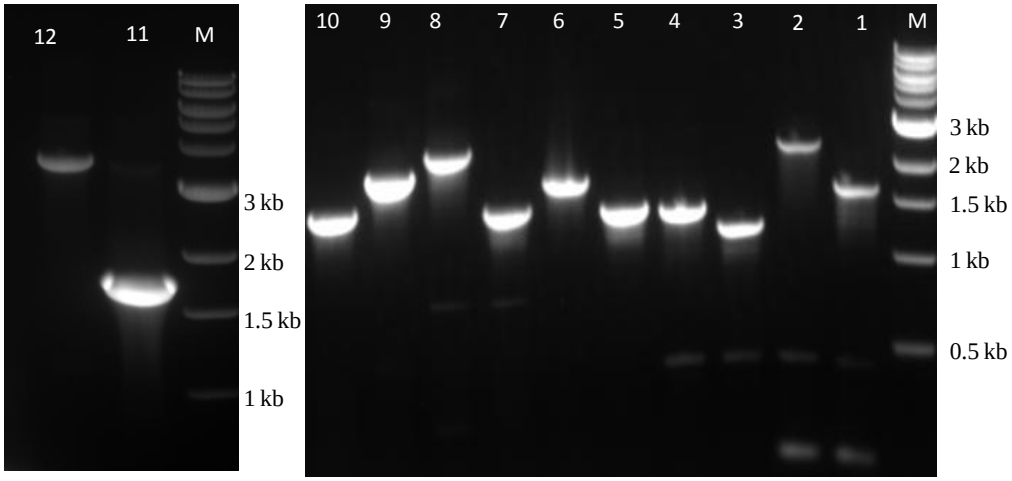

Figure 4.6: Agarose gel electrophoresis of the confirmatory PCR for some of *S.* Enteritidis TCA generated mutant; lanes 2, 4, 6, 8, 10 and 12 were the wild type with the primers for *fum*A (2.35 kb), *dcu*A (1.42 kb), *dcu*B (1.77), *sdh*A (2.2 kb), *asn*B (1.45 kb) and *frd*AD (3.7 kb) respectively. Respective mutants for each gene are in lanes 1, 3, 5, 7, 9 and 11 with kanamycin (1.6 kb) replacing the genes in 1, 9 and 11 and with chloramphenicol (1.3 kb) replacing the genes in 3, 5 and 7. Primers for each gene are shown in Table 4.3. M represents 1kb molecular weight ladder.

4.3.2 Assessment of growth rate of mutants of *Salmonella* Enteritidis defective in one of selected TCA genes

The growth rates for all mutants in comparison with the parent strain of *S.* Enteritidis are shown in Table 4.6. All mutants exhibited a similar pattern of growth curve to the parent strain at 37^{o}C (Fig 4.7). At 42^{o}C all mutants displayed slightly higher growth rates than the parent strain (Fig 4.8).

Table 4.6: The lag phase time and growth rate of *S.* Enteritidis wild type and its TCA generated mutants at temperature 37°C and 42°C.

Pathway	Strain/mutants	Symbol	Lag phase (min)		Growth Rate (OD_{600} h^{-1})	
			37°C	**42°C**	**37°C**	**42°C**
	S. Enteritidis *Wild type*	*wt*	40	40	0.1	0.11
Fumarate Respiratory Mutants	Fumarate hydratase (fumarase A), aerobic class I	*fumA*	40	20	0.13	0.1
	Anaerobic C4-dicarboxylate transporter *(dcuA)*	*dcuA*	20	00	0.09	0.08
	Anaerobic C4-dicarboxylate transporter *(dcuB)*	*dcuB*	40	20	0.1	0.1
	Succinate dehydrogenase complex subunit A	*sdhA*	40	40	0.09	0.12
	Asparagine synthetase A	*asnA*	40	40	0.11	0.1
	Aspartate ammonia-lyase	*aspA*	40	40	0.1	0.12
	L-asparaginase	*ansB*	20	40	0.1	0.1
	Fumarate reductase Complex operon	*frdAD*	40	40	0.1	0.11
	Succinyl-CoA synthetase beta chain	*sucC*	40	40	0.08	0.07

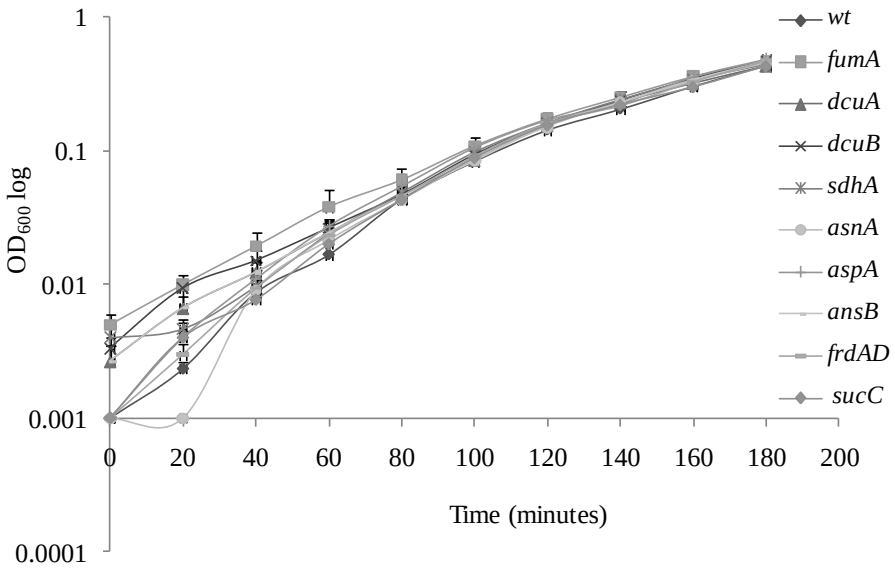

Figure 4.7: Growth curves of *S.* Enteritidis wild type and TCA-generated mutants in 100 ml nutrient broth flask incubator for 3 hours at 37°C. SE bars are shown.

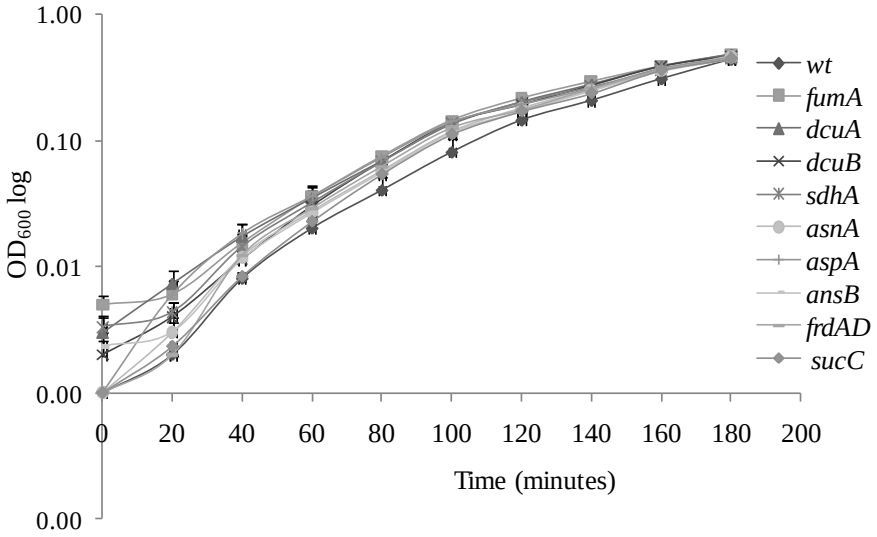

Figure 4.8: Growth curves of *S.* Enteritidis wild type and TCA-generated mutants in 100 ml nutrient broth flask incubator for 3 hours at 42°C. SE bars are shown.

4.3.3 Assessment of growth cultural characteristics of mutants of *S.* Enteritidis defective in selected TCA genes

All generated mutants exhibited similar cultural characteristic colonies to the parent strain when they were streaked on brilliant green agar (BGA) plates and incubated aerobically at 37°C for 18 h, except for the mutant defective in succinate dehydrogenase complex subunit A (*sdh*A), which showed a pale red background while the wild type showed pink background (Figure 4.9). But when the incubation for *sdh*A mutant was extended to 30 h the background gradually converted to pink background as observed with the rest of the mutants.

Figure 4.9: Cultural characteristics of *S.* Enteritidis wild type (right) and *S.* Enteritidis *sdh*A (left) on BGA plates and incubated at 37°C for 18 h.

4.3.4 *In vitro* competitive-exclusion and co-culturing experiments for mutants of *S.* Enteritidis defective in TCA genes and wild type

4.3.4.1 Experiment-1

All mutants reached stationary phase with a density of 8.3-8.9 $\log_{10}$ cfu/ml within 18 h of aerobic incubation at 42^{o}C. The results of inoculating the parent strain into the 18 h cultures of the individual mutants and continuing incubation for a further 24 h are shown in Table 4.7 and Figure 4.10. All the *S.* Enteritidis mutants inhibited/suppressed the growth of challenge strain (not more than 0.6 $\log_{10}$ increase). The exceptions were the *asn*A (asparagines synthetase) and the *sdhA* (succinate dehydrogenase) mutants where the parental challenge strain was able to increase by 2.4 and 1.4 $\log_{10}$s cfu/ml respectively. Under such conditions when the parent strain inoculated on its own increased its viable count by 5.3 $\log_{10}$ cfu/ml over the 0 h count (negative control). The increase in viable counts of the challenge strain in a stationary phase culture of the wild type strain (positive control) was 0.2 $\log_{10}$ cfu/ml only.

Table 4.7: Increase in viable counts of the parental *S.* Enteritidis Spc^{R} (challenge) in stationary phase broth cultures of the TCA-defective mutants when the conditions were 42^{o}C and under aerobic incubation for 24 h compared with wild type.

Strain	Log_{10} increase in viable numbers of challenge (Mean of 3)	SD	SE	*P* value compared to the wt
No background (Negative)	5.31	0.137	0.079	0.0023
*fum*A	0.26	0.289	0.167	0.45
*dcu*A	0.12	0.120	0.069	0.64
*dcu*B	0.06	0.140	0.081	0.42
*sdh*A	1.4	0.14	0.078	0.008
*asn*A	2.4	0.155	0.090	0.004
*asp*A	0.07	0.100	0.058	0.40
*ans*B	0.26	0.176	0.102	0.80
*frd*AD	0.05	0.075	0.043	0.56
*suc*C	0.61	0.328	0.189	0.33
Wild type (Positive)	0.2	0.314	0.181	-

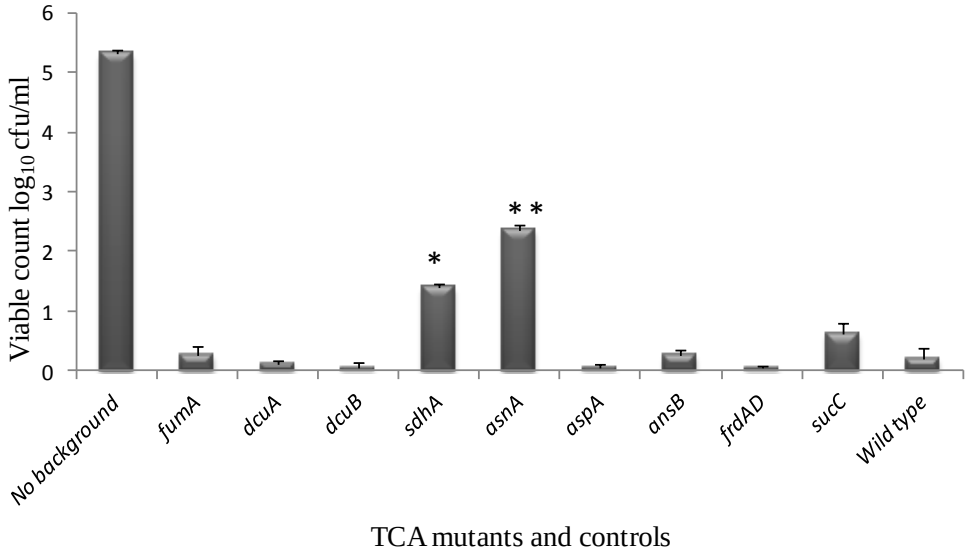

Figure 4.10: Increase in viable counts of *S.* Enteritidis wild type Spc^R in stationary phase broth cultures of TCA-defective mutants of the same *S.* Enteritidis Nal^R strain when the conditions were 42°C, under aerobic incubation conditions for 24 h. (SE bars are shown). One asterisk (*) indicates a significant difference between mutants ($P < 0.01$) and two asterisks (**) indicate highly significant difference between mutants ($P < 0.005$) according to the student t test.

4.3.4.2 Experiment-2

All *S.* Enteritidis TCA-defective mutants grew well in NB and reached the stationary phase with densities of 8-8.7 $\log_{10}$ cfu/ml within 24 h of anaerobic static incubation at 42°C. The results of inoculating the parent strain into the 24 h cultures of the individual mutants and continuing incubation for a further 24 h are shown in Table 4.8 and Figure 4.11. All TCA mutants inhibited the growth of the challenge completely, with the exception of anaerobic C4-dicarboxylate transporter (*dcu*A), aspartate ammonia-lyase (*asp*A) and fumarate reductase complex (*frd*ABCD), in which the challenge increased significantly ($P < 0.05$) by 1.6, 3.7 and 4 $\log_{10}$ cfu/ml in their stationary phase cultures respectively compared to the positive control. The viable numbers of the challenge strain in a stationary phase of the parent strain showed complete inhibition as it decreased by 0.01 $\log_{10}$ cfu/ml. The counts of the parental challenge only increased by 4.98 $\log_{10}$ in NB (negative control) within 24 h.

Table 4.8: Increase in viable counts of the parental *S.* Enteritidis Spc^R (challenge) in stationary phase broth cultures of the TCA-defective mutants when the conditions were 42^{o}C and under anaerobic incubation for 24 h compared with wild type.

Strain	Log_{10} increase in viable numbers of challenge (Mean of 3)	SD	SE	*P* value compared to the wt
No background	4.98	0.11	0.06	0.0007
*fum*A	-0.18	0.25	0.15	0.16
*dcu*A	1.55	0.47	0.27	0.03
*dcu*B	0.06	0.04	0.03	0.51
*sdh*A	-0.07	0.19	0.11	0.39
*asn*A	-0.07	0.17	0.1	0.72
*asp*A	3.72	0.12	0.07	7.3×10^{-5}
*ans*B	-0.06	0.22	0.13	0.47
*frd*AD	3.95	0.07	0.04	7.9×10^{-5}
*suc*C	-0.05	0.09	0.05	0.63
Wild type	-0.01	0.12	0.07	-

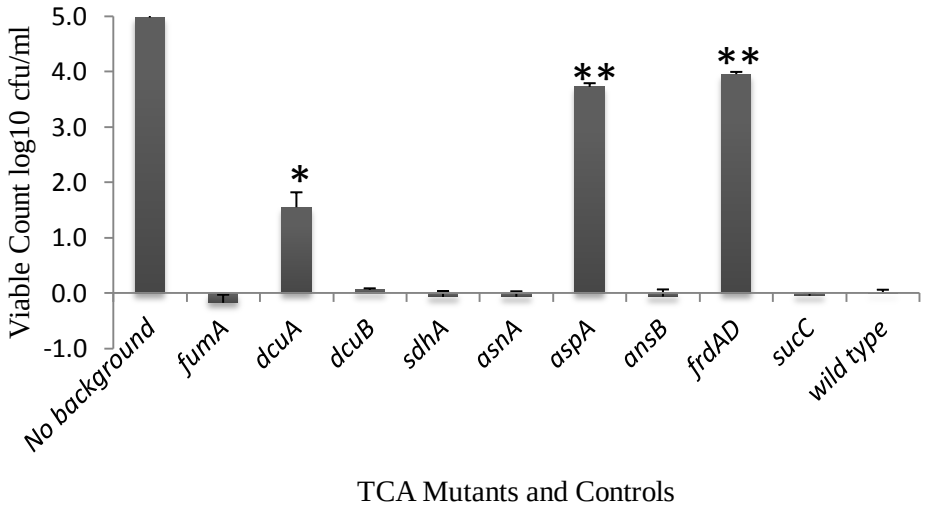

Figure 4.11: Increase in viable counts of *S.* Enteritidis wild type Spc^R in stationary phase broth cultures of TCA-defective mutants when the conditions were 42^{o}C, under anaerobic incubation for 24 h. (SE bars are shown). One asterisk (*) indicates a significant difference between mutants ($P < 0.05$) while (**) indicates a highly significant difference between mutants ($P < 0.005$) according to the student t test.

In Figure 4.12 below the *dcu*A, *sdh*A, *asn*A, *asp*A and *frd*ABCD mutants exhibited variable extent of inhibition to the parent strain under aerobic and anaerobic environments.

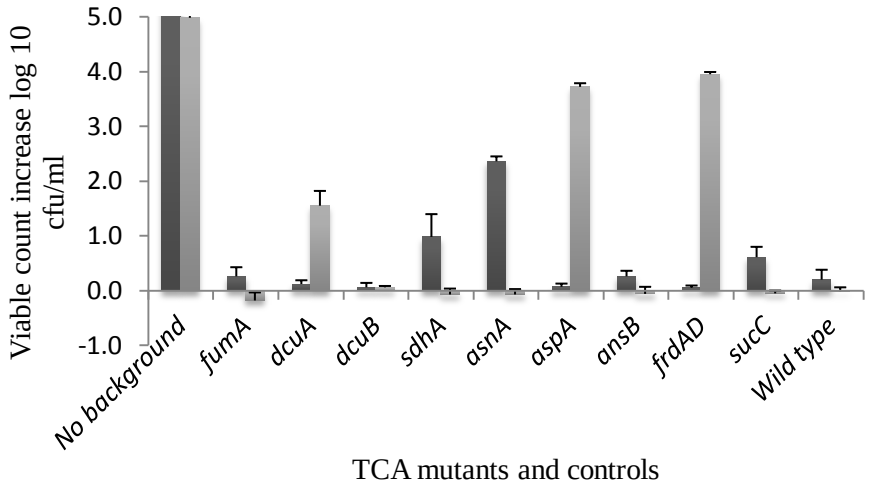

Figure 4.12: Increase in viable counts of *S.* Enteritidis wild type Spc^R in stationary phase broth cultures of TCA-defective mutants when the conditions were 42°C,aerobically (black columns) or anaerobically (grey columns). SE bars are shown.

4.3.4.3 Experiment-3

Because *dcu*A, *dcu*B, *sdh*A, *asp*A and *frd*ABCD mutants exhibited variable extent of inhibition to the parent strain (Figure 4.12) under aerobic and anaerobic environments; therefore experiment-1 methodology was applied for these mutants for further period, incubated aerobically for 72 h and the challenge counted every 24 h. Apart from *sdh*A mutant all tested mutants completely inhibited the growth of the challenge strain (Table 4.9, Figure 4.13 and Figure 4.14).

S. Enteritidis *sdhA*, defective in succinate dehydrogenase, exhibited a very significant difference of inhibition ($P < 0.005$) for the three time points tested when compared with the positive control. The parent challenge managed to increase growth over the *sdh*A mutant by 5.28, 6.48 and 7.73 $\log_{10}$ cfu/ml over 24, 48 and 72 h respectively. The rest of the TCA mutants exhibited a similar pattern of inhibition to the wild type at the three time-points tested.

Table 4.9: Increase in Log_{10} viable numbers of *S.* Enteritidis wild type Spc^R in stationary phase broth cultures of TCA-defective mutants of the same *S.* Enteritidis strain when the conditions were 42°C, aerobic incubation for time-points 24, 48 and 72 h.

Time-Point	Strain	Log_{10} cfu/ml increase in viable numbers of challenge (Mean of 3)	SD	SE	*P* value compared to the wt
24 h	Negative	5.24	0.06	0.04	0.0002
	*dcu*A	-0.38	0.68	0.39	0.48
	*dcu*B	0.06	0.07	0.04	0.37
	*sdh*A	1.44	0.05	0.03	0.003
	*asp*A	0.20	0.02	0.01	0.26
	*frd*ABCD	0.04	0.1	0.06	0.82
	Wild type (Positive)	-0.02	0.08	0.04	-
48 h	No background (Negative)	5.46	0.03	0.02	0.0003
	*dcu*A	-0.57	0.58	0.34	0.69
	*dcu*B	-0.25	0.19	0.11	0.52
	*sdh*A	2.65	0.18	0.1	0.002
	*asp*A	0.16	0.21	0.12	0.15
	*frd*ABCD	0.18	0.11	0.06	0.002
	Wild type (Positive)	-0.39	0.13	0.08	-
72 h	No background (Negative)	5.28	0.22	0.13	0.0001
	*dcu*A	-0.67	1.11	0.64	0.82
	*dcu*B	-0.07	0.35	0.2	0.16
	*sdh*A	3.90	0.07	0.04	0.002
	*asp*A	0.50	0.49	0.28	0.04
	*frd*ABCD	0.37	0.35	0.2	0.1
	Wild type (Positive)	-0.81	0.33	0.19	-

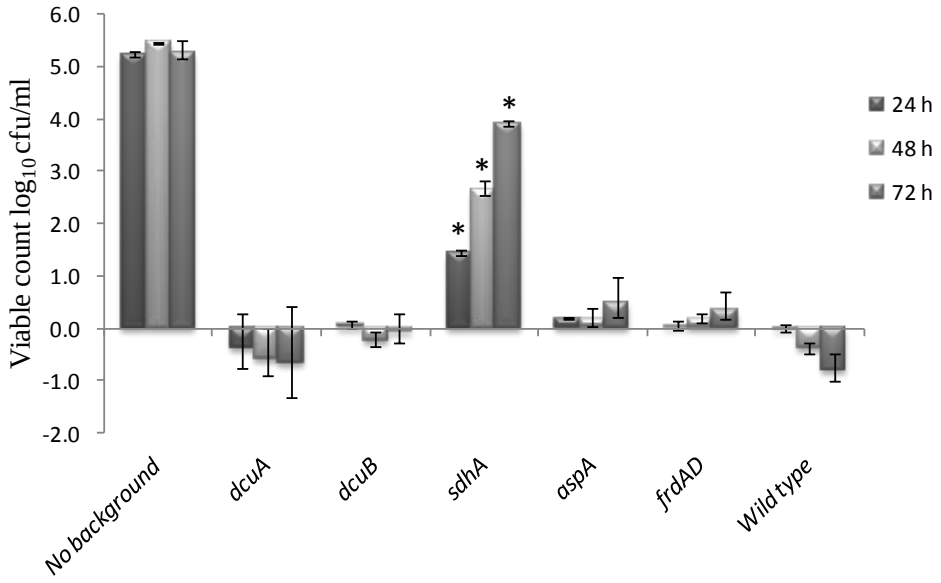

Figure 4.13: Increase in viable counts of *S.* Enteritidis wild type Spc^R in stationary phase broth cultures of TCA-defective mutants of the same *S.* Enteritidis strain when the conditions were 42^{o}C, under aerobic incubation. (SD and SE bars are shown). Asterisk (*) indicates a highly significant difference between mutants ($P < 0.005$) according to the student t test.

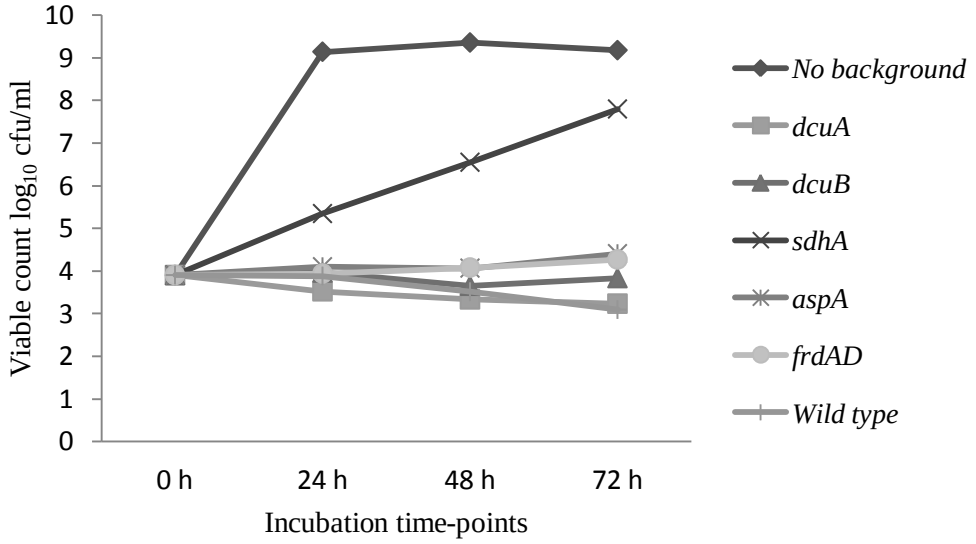

Figure 4.14: Increase in viable counts of *S.* Enteritidis wild type Spc^R in stationary phase broth cultures of TCA-defective mutants of the same *S.* Enteritidis strain when the conditions were 42^{o}C under aerobic incubation.

4.3.4.4 Experiment-4

In Experiment 2 (Fig 4.11), *S.* Enteritidis *dcu*A and *dcu*B mutants showed variable results in their *in vitro* competitive-exclusion against the wild type challenge growth during 24 h incubation anaerobically at 42^{o}C, despite possessing the same Dcu transporter system. Therefore the challenge strain was incubated and counted over a longer 72 h period. Throughout this period the parental *S.* Enteritidis wild type completely inhibited the growth of the challenge strain in comparison of the growth of the challenge strain in nutrient broth when inoculated on its own (Table 4.10, Figure 4.15 and Figure 4.16). The *S.* Enteritidis *dcu*B completely inhibited the challenge over the three time-points tested, while the *S.* Enteritidis *dcu*A was less competitive than the dcuB mutant ($P < 0.005$) for the three time points when compared with the positive control. The *dcu*B mutant exhibited a similar pattern of inhibition to the wild type; which is similar to the previous results shown in Fig 4.11.

Table 4.10: Increase in Log_{10} viable numbers of *S.* Enteritidis wild type Spc^{R} in stationary phase broth cultures of TCA-defective mutants of the same *S.* Enteritidis strain when the conditions were 37^{o}C, under anaerobic incubation for time-points 24, 48 and 72 h.

Time-Point	Strain	Log_{10} increase in viable numbers of challenge (Mean of 3)	SD	SE	*P* value compared to the wt
24 h	No background (Negative)	5.36	0.06	0.03	0.0004
	dcuA	2.82	0.21	0.12	0.0049
	dcuB	0.06	0.11	0.06	0.08
	Wild type (Positive)	-0.08	0.13	0.08	-
48 h	No background (Negative)	5.29	0.09	0.05	0.0002
	dcuA	2.87	0.08	0.05	0.0003
	dcuB	0.07	0.07	0.04	0.38
	Wild type (Positive)	-0.15	0.03	0.01	-
72 h	No background (Negative)	5.39	0.08	0.04	0.0004
	dcuA	2.93	0.04	0.02	0.001
	dcuB	0.13	0.05	0.03	0.71
	Wild type (Positive)	-0.08	0.13	0.07	-

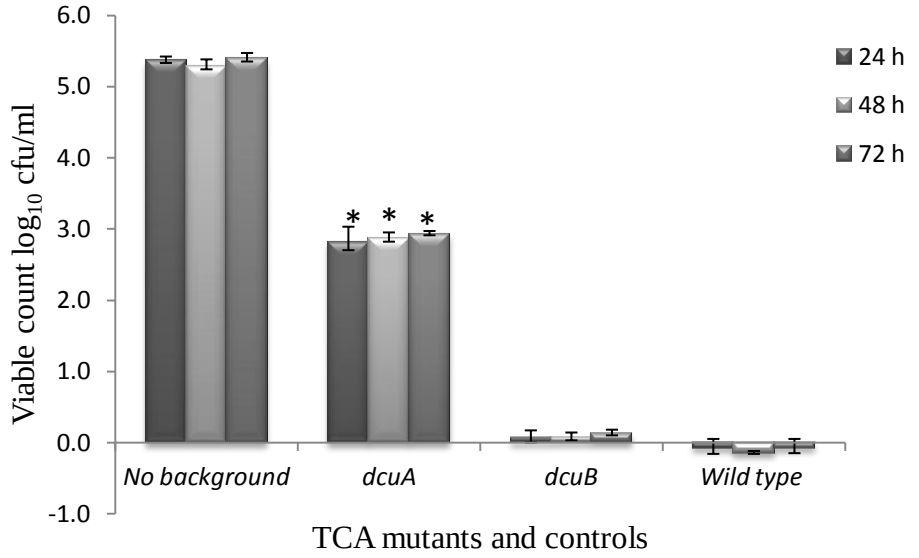

Figure 4.15: Increase in viable counts of *S.* Enteritidis wild type Spc^R in stationary phase broth cultures of TCA-defective mutants of the same *S.* Enteritidis strain when the conditions were 37^{o}C, under anaerobic incubation. (SD and SE bars are shown). One asterisk (*) indicates very significant difference between mutants ($P < 0.005$) according to the student test.

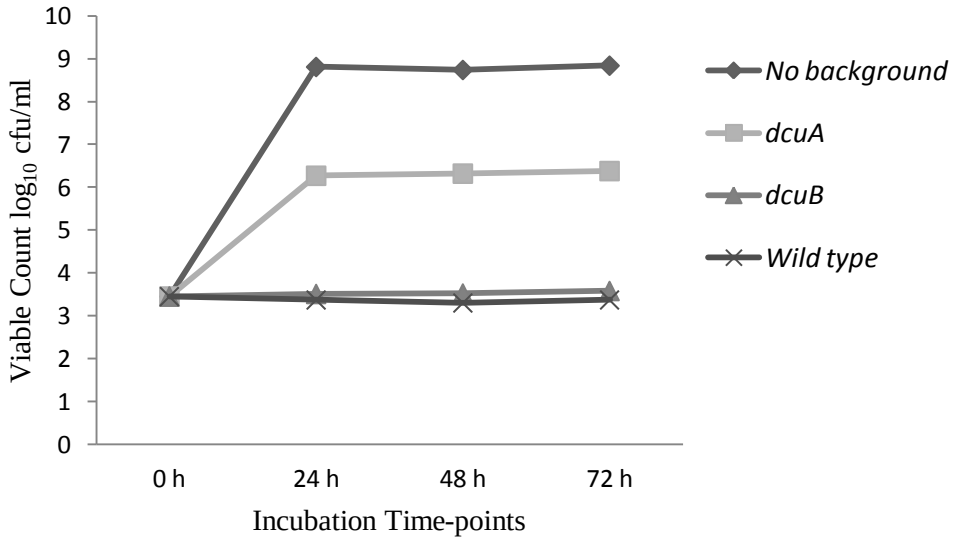

Figure 4.16: Increase in viable counts of *S.* Enteritidis wild type Spc^R in stationary phase broth cultures of TCA-defective mutants of the same *S.* Enteritidis strain when the conditions were 37^{o}C, under anaerobic incubation.

4.3.4.5 Experiment-5

The results of inoculating individual mutants simultaneously with the parent strain into NB aerobically are shown in Table 4.11 and Figure 4.17. The mutants showed slightly similar increases in comparison with the parent strain; the increase of growth varied from 4-4.4 $\log_{10}$ cfu/ml. The *S.* Enteritidis *dcu*B, defective in anaerobic C4-dicarboxylate transporter, showed the highest increase of growth in comparison with the parent wild type strain while the mutant defective in fumarase hydratase (*fum*A) showed the lowest increase.

Table 4.5: Increase in viable count of *S.* Enteritidis TCA-defective mutants and the parental *S.* Enteritidis wild type when they were cultured simultaneously in nutrient broth at 42°C and under aerobic incubation conditions for 24 h

Strain	Log_{10} cfu/ml increase in viable numbers of mutants (Mean of 3)	Mutants *P* value	Log_{10} increase in viable numbers of Parents (Mean of 3)	Parents *P* value
No background	0.00	0.001	4.41	0.12
*fum*A	4.21	0.42	4.02	0.04
*dcu*A	4.22	0.97	4.21	0.36
*dcu*B	4.41	0.002	4.10	0.11
*sdh*A	4.09	0.50	4.33	0.007
*asn*A	4.04	0.10	4.20	0.14
*asp*A	4.22	0.82	4.14	0.008
*ans*B	4.26	0.56	4.26	0.04
*frd*ABCD	4.25	0.63	4.15	0.18
*suc*C	4.04	0.39	4.13	0.21
Wild type	4.23	-	3.87	-

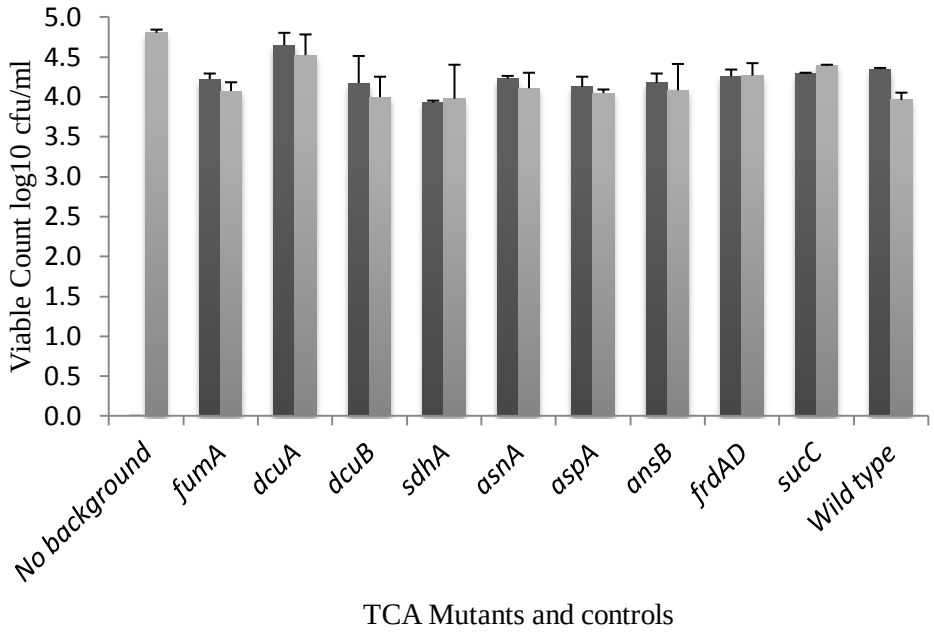

Figure 4.17: Increase in viable counts of *S.* Enteritidis wild type (grey columns) and mutants of *S.* Enteritidis defective in TCA genes (black columns) when they co-cultured simultaneously into nutrient broth cultures at 42°C and under aerobic incubation conditions for 24 h. (SE bars are shown).

4.3.4.6 Experiment-6

The results of inoculating individual mutants simultaneously with the parent strain into nutrient broth anaerobically are shown in Table 4.12 and Figure 4.18. The increase of mutants and the parent strains was approximately 2 $\log_{10}$ less than it was in experiment-5 (aerobic incubation). The increase of mutants and parents was variable. The mutants and the parent strains showed variable increases in growth varied from 1.7–3 and 1.9-2.7 $\log_{10}$ cfu/ ml respectively. *S.* Enteritidis *fum*A, defective in fumarase A, showed the highest increase in growth in comparison with the parent wild type strain while the mutant defective in aspartate ammonia-lyase (*asp*A) showed the lowest increase (Figure 4.14).

Table 4.6: Increase in viable count of *S.* Enteritidis TCA-defective mutants and the parental *S.* Enteritidis wild type when they were cultured simultaneously in nutrient broth at 42°C and under anaerobic incubation conditions for 24 h.

Strain	Log_{10} increase in viable numbers of mutants (Mean of 3)	Mutants *P* value	Log_{10} increase in viable numbers of Parents (Mean of 3)	Parents *P* value
No background	0.0	0.03	2.9	0.16
*fum*A	3.0	0.22	2.7	0.28
*dcu*A	2.7	0.69	2.7	0.30
*dcu*B	2.8	0.45	2.6	0.65
*sdh*A	2.1	0.48	2.2	0.32
*asn*A	1.7	0.23	1.9	0.11
*asp*A	1.7	0.05	2.0	0.19
*ans*B	2.3	0.48	2.2	0.17
*frd*ABCD	2.2	0.10	2.7	0.57
*suc*C	1.9	0.13	2.0	0.03
Wild type	2.5	-	2.5	-

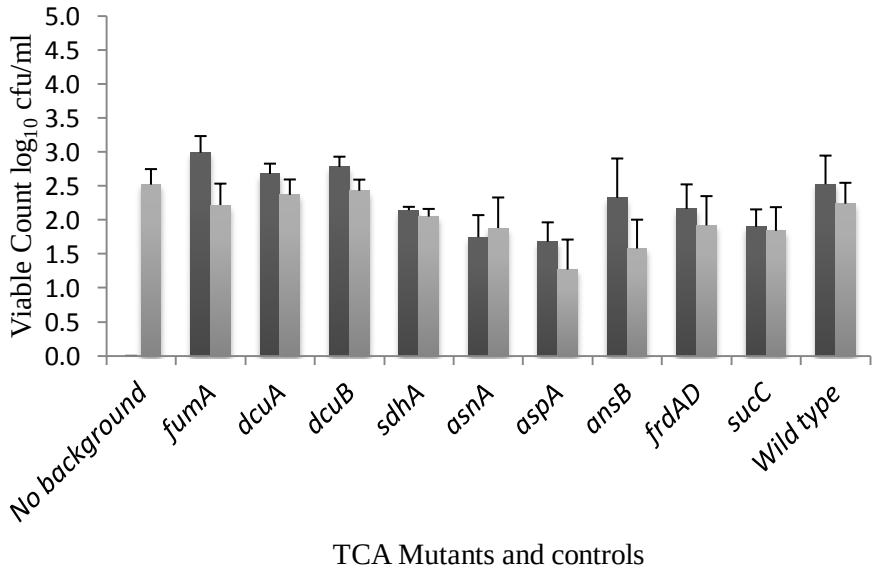

Figure 4.18: Increase in viable counts of *S.* Enteritidis wild type (grey columns) and mutants of *S.* Enteritidis defective in TCA genes (black columns) when co-cultured in nutrient broth cultures at 42°C and under anaerobic incubation conditions for 24 h. (SE bars are shown).

4.3.4.7 Experiment-7

The results of inoculating the mutants individually into 24 h cultures of the parental wild-type and continuing aerobic incubation for a further 24 h are shown in Table 4.13 and Figure 4.19. All mutants' growth was inhibited by the stationary phase of the wild type in nutrient broth at 42°C under aerobic incubation.

Table 4.7: Change in viable counts of *S.* Enteritidis TCA-defective mutants in stationary phase broth cultures of the parental *S.* Enteritidis wild type when the conditions were 42°C under aerobic incubation for 24 h.

Strain	Log_{10} cfu/ml increase in viable numbers of wild type (Mean of 3)	SD	SE	*P* value compared to the wt
fumA	0.16	0.11	0.06	0.96
dcuA	0.24	0.07	0.04	0.59
dcuB	0.10	0.05	0.03	0.65
sdhA	0.10	0.21	0.12	0.66
asnA	0.09	0.13	0.07	0.57
aspA	-0.03	0.06	0.04	0.23
ansB	0.09	0.07	0.04	0.30
frdAD	0.09	0.10	0.06	0.33
sucC	-0.01	0.03	0.01	0.22
Wild type	0.16	0.15	0.09	-

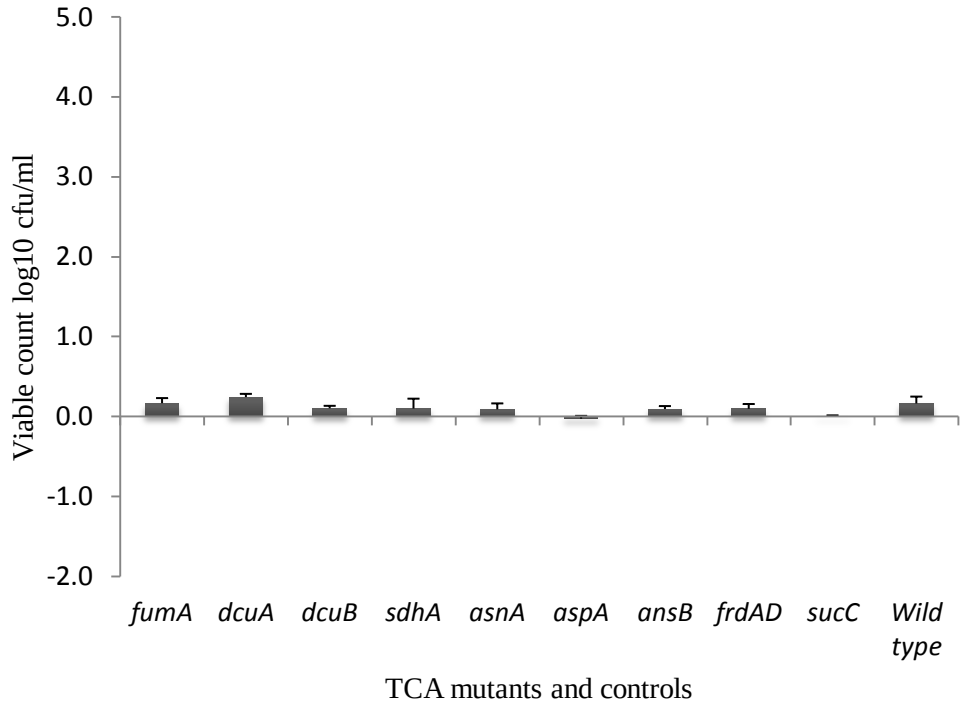

Figure 4.19: Increase in viable counts of *S.* Enteritidis TCA-defective mutants in stationary phase broth cultures of the parental *S.* Enteritidis wild type when the conditions were 42°C, under aerobic incubation conditions for 24 h. (SE bars are shown).

4.3.4.8 Experiment-8

The results of inoculating the mutants individually into 24 h cultures of the parental wild-type and continuing anaerobic incubation for a further 24 h are shown in Table 4.14 and Figure 4.20. In an anaerobic environment the TCA mutants showed a greater ability to overgrow the wild type than in an aerobic environment (Figure 4.20 and 4.21), with an increase varying from 1-2.65 $\log_{10}$ cfu/ml. The *S.* Enteritidis defective in anaerobic C4-dicarboxylate transporters (*dcu*B) and (*dcu*A) mutants showed the greatest increase over the wild type stationary phase of 2.65 and 2.54 $\log_{10}$ cfu/ml respectively. In contrast *S.* Enteritidis defective in L-asparaginase (*asn*B) showed the lowest increase of 1 $\log_{10}$ only.

Table 4.8: Increase in viable counts of *S.* Enteritidis TCA-defective mutants in stationary phase broth cultures of the parental *S.* Enteritidis wild type when the conditions were 42°C under anaerobic incubation for 24 h.

Strain	Log_{10} increase cfu/ml in viable numbers of wild type (Mean of 3)	SD	SE	*P* value compared to the wt
fumA	1.75	1.38	0.80	0.85
dcuA	2.54	1.27	0.73	0.51
dcuB	2.65	0.97	0.56	0.12
sdhA	1.65	1.46	0.84	0.89
asnA	1.79	1.07	0.62	0.63
aspA	1.37	1.12	0.65	0.99
ansB	1.01	1.32	0.76	0.47
frdAD	1.85	1.63	0.94	0.59
sucC	1.58	1.55	0.90	0.41
Wild type	1.36	1.83	1.06	

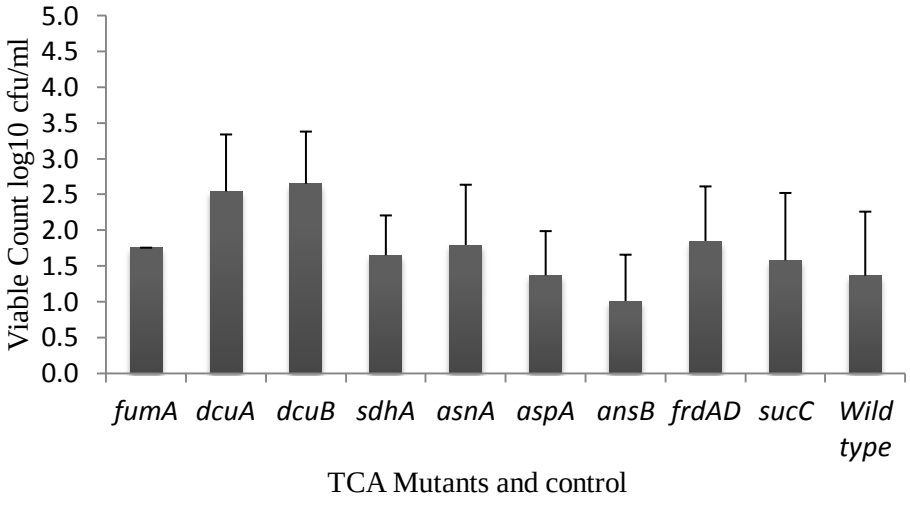

Figure 4.20: Increase in viable counts of *S.* Enteritidis TCA-defective mutants in stationary phase broth cultures of the parental *S.* Enteritidis wild type when the conditions were 42°C under anaerobic incubation conditions for 24 h. (SE bars are shown).

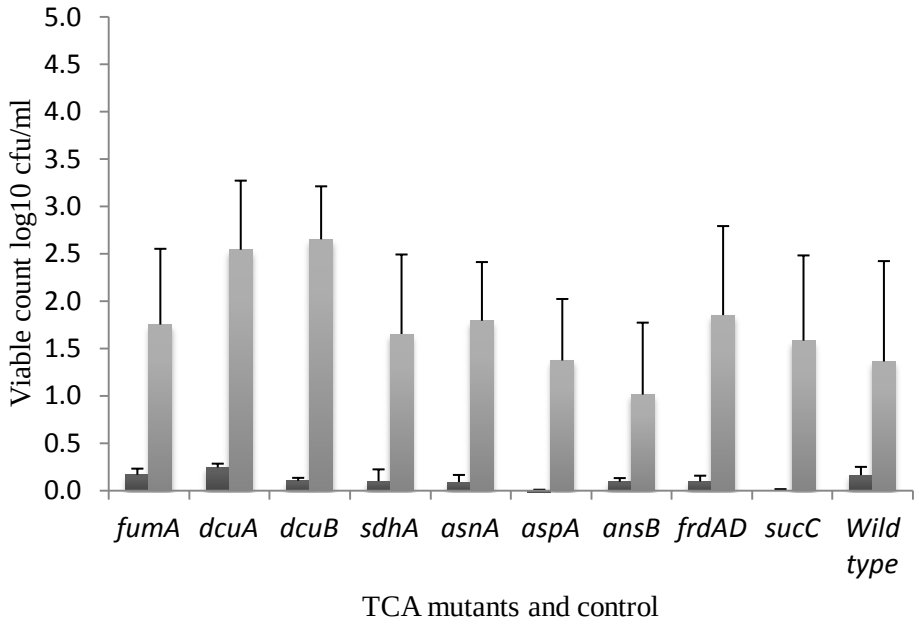

Figure 4.21: Increase in viable counts of *S.* Enteritidis TCA-defective mutants in stationary phase broth cultures of the parental *S.* Enteritidis wild type when the conditions were 42°C, aerobic incubation (black columns) or anaerobic incubation (grey columns). SE bars are shown.

4.3.5 *In vivo* competitive-exclusion experiments for mutants of S. Enteritidis defective in TCA and wild type

This assay was performed to assess the ability of each individual mutant to exclude a parent strain (Spc^R) inoculated 24 h later (Zhang-Barber *et al.*,1997). At the time of challenge inoculation, all mutants tested (*fum*A, *dcu*A, *dcu*B, *sdh*A, *asn*A, *asp*A, *ans*B, *frd*ABCD and *suc*C) colonised the gut well according to the viable count in the caeca of three birds killed from each group at the time of challenge (log_{10} 9.3-10.9 cfu/ml). When the birds were killed 48 h after challenge all mutants were still colonising well with the mean caecal count ranging from log_{10} 8.3-9.1 cfu/ml (Table 4.15). Compared to the wild type growth (positive control), significant growth of the challenge ($P < 0.05$) over the chicks' caeca pre-colonised with *dcu*A and *dcu*B mutants was exhibited, where the count reached log_{10} 3 and 3.1 cfu/ml respectively (Figure 4.22). Compared to the wild type inhibition for the challenge growth no significant difference ($P > 0.05$) was shown for the rest of mutants in inhibiting the growth of the challenge (Table 4.14 and Figure 4.22).

Table 4.9: The effect of intestinal colonisation of newly hatched chicks with *S.* Enteritidis (Nal^R) or one of its generated mutants on the caecal colonisation by the parental challenge (Spc^R) given orally 24 h later. Mean of 7 birds. These readings were taken 48 h post-challenge inoculation.

TCA mutant	Log_{10} cfu/ml of caecal contents of pre-colonising and challenge strain in 4-days chicks (48 h post challenge)				
	Pre-colonising strain (Nal^R)	Challenge strain (Spc^R)	SD	SE	*P* value compared to the wt
fumA	8.5	3.0	0.58	0.22	0.172
dcuA	8.8	3.0	0.00	0.001	0.030
dcuB	9.0	3.1	0.38	0.14	0.008
sdhA	9.1	2.1	0.38	0.14	0.356
asnA	8.7	2.7	0.49	0.18	0.356
aspA	9.0	2.0	0.00	0.001	0.078
ansB	8.3	2.1	0.38	0.14	0.356
frdABCD	8.5	2.3	0.49	0.18	0.604
sucC	8.7	2.9	0.38	0.14	0.078
wild type	8.7	2.4	0.53	0.20	-
Negative	< 2	7.9	0.28	0.11	6.6×10^{-6}

Day-1 birds were inoculated orally with 4×10^7 cfu in 0.1 ml of Nal^R strain (mutants); 24 h later challenged with 1.8×10^5 cfu in 0.1 ml of Spc^R strain.

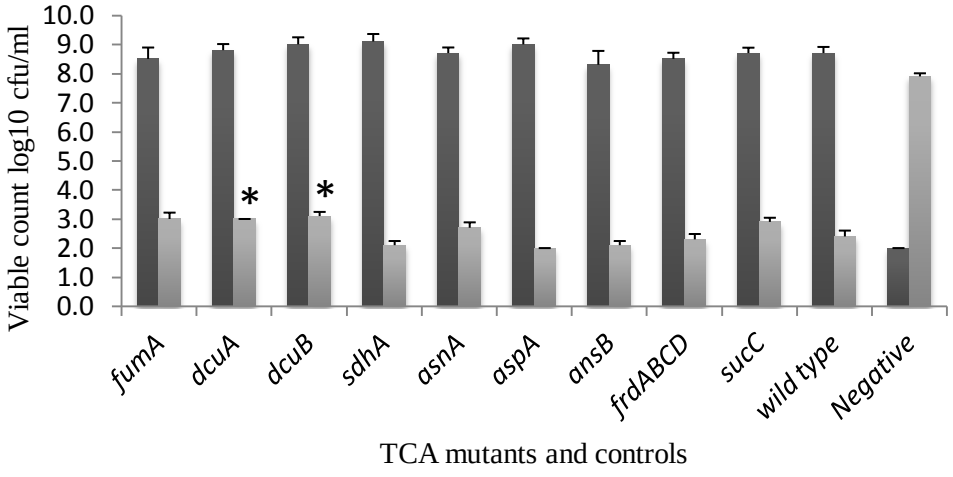

Figure 4.22: The effect of intestinal colonisation of newly hatched chicks with wild type *S.* Enteritidis (Nal^R) or one of its TCA-generated mutants on the caecal colonisation by the Spectinomycin resistant of the parent (challenge) given orally (1.8×10^5 cells) 24 h later. These readings were taken 48 h post-challenge inoculations; pre-colonised strain (black columns); challenge (grey columns). One asterisk (*) indicates significant difference between mutants ($P < 0.05$) according to the student test.

4.4 Discussion

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 4.1. There was consistent up-regulation of expression in the caeca with the greatest changes observed with genes associated with respiration using fumarate as terminal electron acceptor (*frd*ABCD) with increase of expression varied between 13-17 fold. The involvement of *suc*ABCD suggest that the TCA cycle was showing anaerobic behaviour and acting in a non-cyclic manner (Fig 4.3) supporting the contention that the caeca is a low redox environment even in newly hatched chickens. However, to study the role of individual genes in cellular physiology, deletion mutants lacking the gene of interest were constructed using lambda-Red mutagenesis (Datsenko and Wanner, 2000). The TCA associated genes of interest in *S.* Enteritidis PT4 were successfully disrupted and successfully transduced into *S.* Enteritidis PT4 (Nal^R) using Bacteriophage P22. All the mutants produced were verified by PCR.

Assessment of colonisation ability for all these mutants was difficult since many microorganisms that are normally unable to colonise the gut of the adult birds possessing a full and complex gut flora are nevertheless able to colonise 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, microbial competition between related bacterial strains in the intestine of the newly-hatched chicken was used as a model to determine colonisation 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 colonise the caeca of chick nor their ability to inhibit the establishment of the parental challenge strain. This can be referred to the alternative available TCA substrates or amino acids available in the gut (array data), which allow other bypass enzymes takes place, for example, during fumarate respiration (Fig 4.3) knocking out aspartate lyase (*asp*A) gene stopped the conversion of aspartate into fumarate,

so the alternative option for the bacteria to use is converting oxaloacetate to malate (malate dehydrogenase "*mdh*"), and then converting malate to fumarate (fumarate hydratase "*fum*B"); which very likely to be the case as *mdh* gene is expressed in the array data.

TCA generated mutants' growth rates were assessed in complex media because the gut is a complex nutritional environment. Because of this competition studies were performed in nutrient broth (Barrow *et al.*, 1987b, Barrow *et al.*, 1996b, Berchieri and Barrow, 1991, Zhang-Barber *et al.*, 1997) before being tested in the chicken gut (Amy *et al.*, 2004, Woodall *et al.*, 2005, Clayton *et al.*, 2008, Rychlik *et al.*, 2009). Therefore, the growth rate for every mutant generated was assessed in nutrient broth at 42^{o}C, which reflects the chicken temperature. The mutants' growth behaviour in rich media was tested in order to check if they still possess similar growth characteristics to their parent strain and test whether the nature of these mutations may have affected their growth and therefore it may affect competitive-exclusion activity (Nurmi and Rantala, 1973, Rantala and Nurmi, 1973). Collectively all mutants were able to grow well in NB aerobically and anaerobically at both temperatures 37^{o}C and 42^{o}C. During aerobic incubation all *S.* Enteritidis TCA mutants showed similar growth curve pattern to the parent strain when they were grown in nutrient broth at 37^{o}C; on the other hand they exhibited slightly higher growth curve than the parent strain when grown at 42^{o}C. Interpretation to this slight increase in the growth curve for TCA mutants compared to the wild type at 42^{o}C is unknown.

It is not known why *S.* Enteritidis defective in *sdh*A exhibited pale colonies on BGA plates during aerobic incubation for 18 h in contrast with the wild type and the rest of the mutants. However, this could be associated to the slow growth or alteration in metabolic activity to convert the amino acids (e.g. peptone) to ammonia (which converts plates to go red) and therefore the pH remained low during the first 18 h of *S.* Enteritidis defective in *sdh*A growth on solid media. Moreover, because the *sdh*A is knocked out its function was partially replaced by *frd* during aerobic growth (Hirsch *et al.*, 1963, Adsan *et*

al., 2002, Steinsiek *et al.*, 2011), this could be the reason behind the delay of its slow growth if it is the case.

Analysing *in vitro* competitive-exclusion experiments in an aerobic environment for the mutants of *S.* Enteritidis defective in one of TCA genes against the challenge (parent strain) showed that all mutants were as competitive as the wild type (positive control) against the challenge when they were grown in nutrient broth, except the asparagine synthetase *asn*A and succinate dehydrogenase *sdh*A mutants, which showed significantly less inhibition compared to the parent strain. This is a good indication that these two genes are required for *in vitro* competitive exclusion model aerobically. On the other hand, *in vitro* competitive-exclusion experiments in an anaerobic environment indicated that all mutants and wild type fully inhibited the challenge, except C4-dicarboxylate transport system *dcu*A, aspartate lyase *asp*A and fumarate reductase complex *frd*ABCD, which were significantly less competitive compared to the parent strain. This is a good indication that these three tested genes are required for *in vitro* competitive exclusion model but under anaerobic environment.

In experiment 3, the succinate dehydrogenase (*sdh*A) has shown to be important, and it was significantly less competitive than the wild type in inhibiting the growth of the challenge up to 72 h and if the incubation was extended for more days the challenge growth over *sdh*A stationary phase may reach the level of negative control. This again indicates that the *sdh*A gene is the most important among the tested genes at *in vitro* aerobic metabolism in rich media.

The above results indicate that asparagine synthetase (*asn*A) and succinate dehydrogenase (*sdh*A) are important at *in vitro* growth-inhibition mechanism aerobically. The amino acid asparagine can enter the TCA cycle and thereby improves function during the oxidation of acetoacetate (Taegtmeyer, 1983). It was suggested that utilization of substrates that can enrich TCA cycle (e.g. lactate and asparagine) may play a critical role in maintaining physiological function (Taegtmeyer, 1983). On the other hand, C4-dicarboxylate transport

system *dcu*A, aspartate lyase *asp*A and fumarate reductase complex (*frd*ABCD) were shown to be important components in TCA cycle at *in vitro* competitive exclusion mechanism at anaerobic environment. The results are in line with other work (Wood *et al.*, 1984, Guest, 1992, Golby *et al.*, 1998b, Nogrady *et al.*, 2003a, Woods and Guest, 1987) in that different situation exists for the interconversion of succinate and fumarate, in which the succinate dehydrogenase *sdh* is required aerobically (full TCA cycle, Figure 4.2) and fumarate reductase *frd* is required anaerobically (branched reductive TCA, Figure 4.3). Therefore, the results clearly demonstrate that C4-dicarboxylic acid metabolism with fumarate and succinate as the key metabolites is central for anaerobic growth exclusion expressed, and therefore also colonisation of the caeca in newly hatched chickens, by *S.* Enteritidis in nutrient rich media. It is very likely that the main reason for this is the utilization of fumarate as an electron acceptor during anaerobic respiration. Moreover, inactivation of aspartate-ammonia-lyase *asp*A, which generates fumarate from aspartate anaerobically, resulted in it being non-competitive for aspartate utilization. These results are compatible with study by Nogrady and others (2003a).

As for C4-dicarboxylic acid transport system the results showed that *dcu*A but not *dcu*B is needed for *in vitro* competitive exclusion anaerobically. This reflects the importance of *dcu*A gene in rich media during *S.* Enteritidis growth in anaerobic environment. This conclusion reflects the gene expression of the two genes (*dcu*A and *dcu*B) of *S.* Enteritidis in the array data, in which *dcu*A exhibited significant up-regulation by 5 fold, while *dcu*B exhibited insignificant up-regulation with only 2 fold in chicken caeca.

In contrast Nogrady and others (2003a) showed that both independent mutation of *S.* Typhimurium *dcu*A and *dcu*B resulted in being non-competitive against the wild type under strict anaerobic environment at 37^{o}C but in Luria broth, which is slightly different in nutrient ingredients from nutrient broth used in our experiments.

In the experiment-2, the *S.* Enteritidis *dcu*A and *dcu*B mutants showed variable results in the *in vitro* competitive-exclusion model against the wild type

challenge growth during 24 h incubation anaerobically at 42^{o}C, despite possessing the same Dcu transporter system. Therefore they were tested for a longer time period (24, 48 and 72 h) anaerobically at 37^{o}C. The *S.* Enteritidis *dcu*A was less competitive than *dcu*B mutants for the three time points when compared with the positive control, as the anaerobic C4-dicarboxylate transporter (*dcu*B) mutant exhibited a similar pattern of inhibition to the wild type at the three time-points tested. This again confirms the importance of *dcu*A gene in Dcu system in transporting di carboxylic compounds under anaerobic condition using *in vitro* growth model

Non growth-inhibition for *dcu*A, *asp*A and *frd*ABCD anaerobically *in vitro* model indicates clearly their importance in TCA cycle under anaerobic environment. On other hand *sdh*A is important in TCA cycle under aerobic environment.

The *S.* Enteritidis *asp*A and *frd*ABCD mutant showed different effects anaerobically in the *in vitro* competitive exclusion model to the effect generated *in vivo*, this is a clear indication that the *in vitro* model of competitive exclusion in rich medium did not reflect the environment existing in lower part of chicken gut. This could be because there are limited nutritional compounds *in vitro* compared to the *in vivo* environments and that is why apart of transport systems all tested genes seems somehow compensated in chick caeca plus the possible physical attachments to gut epithelial cells. Another interpretation for the challenge strain being able to outgrow the stationary phase cultures of *dcu*A, *asp*A and *frd*ABCD mutants *in vitro* anaerobically is that the end-metabolic products are consumable by the challenge strain or other alternative nutritional compounds still exist in the medium.

The *in vitro* co-culturing of the TCA mutants with the parent strain (Experiment 5) simultaneously in nutrient broth aerobically showed a similar level of competitiveness to the parent strain, but when the co-culturing was performed anaerobically (Experiment 6) TCA mutants exhibited variable competitiveness against the parent strain. This again indicates that these genes are important in anaerobic environment.

In Experiment 7, the parent strain of *S.* Enteritidis Spc^R stationary phase in the *in vitro* competitive-exclusion model inhibited all TCA mutants completely aerobically. But when the same model was performed anaerobically (Experiment 8) the growth pattern of these mutants over wild type stationary phases was totally different, as the parent strain was much less inhibitor/competitive to all TCA tested mutants anaerobically. This could indicate that these mutants can grow in adult birds' gut which already possess natural gut flora and where the environment is mostly anaerobic. The surprising and unexpected result is that the *S.* Enteritidis wild type Spc^R was unable to suppress the positive control, *S.* Enteritidis wild type Nal^R completely; when the positive control exhibited growth increase of 1.07 $\log_{10}$ cfu/ml. This could reflect the nature of spectinomycin mode of action, in interrupting protein synthesis in ribosomes, while nalidixic acid mode of action is blocking DNA replication, therefore using these two *S.* Enteritidis antibiotic markers as controls need to be reconsidered.

Fumarase A (fumarate hydratase class I, encoded by *fum*A gene, SEN 1581) is the enzyme that catalyzes the reversible hydration/dehydration of fumarate to malate under aerobic environment (Figure 4.2), while fumarase B (encoded by *fum*B gene; SEN 1580) converts malate to fumarate under anaerobic conditions (Figure 4.3) and because the assay of *in vitro* competitive exclusion at aerobic environment indicated that the action of *fum*A inhibition is similar to the wild type positive control, it was assumed that the *fum*A function may be compensated by *fum*C (fumarate hydratase class II; SEN 1579) as indicated by others (Weaver *et al.,* 1997) and shown to be up regulated by 6.3 fold in the array data. Moreover, asparagine synthetase A (encoded by *asn*A, SEN 3691) and L-asparaginase (encoded by *ans*B, SEN 294) also have similar effect of inhibition to the positive control during competitive exclusion *in vitro* under anaerobic conditions. This also applies for succinyl-CoA synthetase β (encoded by *suc*C, SEN 0688) in which a similar effect to the positive wild type, this also could interpreted that SucC function is compensated by CoA synthetase α aerobically (encoded by *suc*D, SEN 0689) as shown in Figure 4.2.

However, the significant up-regulation of both malate dehydrogenase, *mdh* expression (2.2 fold), combined with elevated fumarate reductase, *frd*ABCD expression (13-17 fold) in the experiment suggested a central role for fumarate respiration in the metabolism of *S.* Enteritidis during growth in 1-day chickens caeca.

Recent work, showed that anaerobic fumarate respiration is the pathway used by *S.* Typhimurium during its growth under *in vivo* mimicking conditions (Sonck *et al.,* 2009). Moreover, fumarate is an intermediate in the TCA cycle. Under anaerobic conditions, this pathway is altered to favour reactions in the direction of succinate, from oxaloacetate through malate and fumarate, refered to as the reductive branch of the TCA cycle (Figure 4.3). In rich media, like nutrient broth or Luria broth, this pathway is fed by C_4-dicarboxylates and related compounds such as malate and aspartate (www.ecosal.org). Both of these substrates can be converted to fumarate in a single reaction step (Golby *et al.,* 1998a, Woods and Guest, 1987). Recent experimental infection studies indicated that a *S.* Typhimurium *frd*ABCD and *sdhCDAB* double mutant is avirulent in mice (Mercado-Lubo *et al.,* 2008). They also showed that *S.* Typhimurium malate dehydrogenase (*mdh*) mutants are unable to convert malate to pyruvate and oxaloacetate and therefore were avirulent (Mercado-Lubo *et al.,* 2008). How far this result is applicable for *S.* Enteritidis in chicken requires further investigation.

It has been reported that there is a requirement for C4-dicarboxylate transporter gene transcription (*dcu*A and *dcu*B) for *Campylobacter jejuni* to adapt to living in chicken caeca (Woodall *et al.,* 2005) due to low oxygen tension in this part of the gut.

This indicates that C4-dicarboxylate compounds transportation into the bacterial cells is needed for fumarate respiration (reductive-cycle TCA cycle) in the absence of oxygen atoms during *in vitro* competition. The question raised now why these two genes behaved differently despite they belong to the same Dcu system, this could be because in *S.* Enteritidis the aspartate ammonia-lyase *asp*A (SEN 4096), is downstream of the anaerobic C4-

dicarboxylate transport gene *dcu*A (SEN 4095). However, in chicken caeca these two genes may be essential for C4-dicarboxylate compounds transportation, fumarate respiration and therefore for colonisation in this part of the gut, where they have shown significant difference to the wild type.

In *E. coli* and *Campylobacter jejuni* it is likely that these genes are contrascribed and regulated by similar mechanisms (Golby *et al.*, 1998b, Woodall *et al.*, 2005). Therefore, *S.* Enteritidis aspartate utilization under anaerobic growth may be linked to C4-dicarboxylate compounds utilization. Aspartate is deaminated by AspA, which can then be metabolised through the TCA cycle.

Engel and others (1992) demonstrated that *E. coli* can utilize C_4-dicarboxylates for energy production under aerobic and anaerobic environments. Under aerobic environments the uptake of C_4-dicarboxylates (fumarate, malate, and succinate) and L-aspartate is mediated by a secondary transporter known as the Dct system (Kay and Kornberg, 1971, Lo, 1977), as shown in Figure 4.4. The *dct*A gene has been sequenced, and the role of its generated protein (DctA) in the utilization of C_4-dicarboxylates is supported by complementation studies of *S.* Typhimurium *dct*A mutants (Baker *et al.*, 1996). Under anaerobic environment, the uptake and exchange of C_4-dicarboxylate compounds is mediated by the Dcu system, which is genetically distinct from the aerobic Dct system (Engel *et al.*, 1992, Engel *et al.*, 1994, Zientz *et al.*, 1996). The anaerobic transport activities were detected only in bacteria grown under anaerobic environments, and their synthesis requires intact FNR (fumarate nitrate reductase regulator), the transcriptional regulatory protein for anaerobic metabolism (Engel *et al.*, 1992, Engel *et al.*, 1994, Unden and Bongaerts, 1997). Three genes for the Dcu system (*dcu*A, *dcu*B and *dcu*C) were identified, which encode for C_4-dicarboxylate carriers, DcuA, DcuB and DcuC anaerobically (Figure 4.4). Bacterial growth tests and transport studies on *dcu*A, *dcu*B and *dcu*C single, double and triple mutants have shown that DcuA, DcuB and DcuC each mediate exchange as well as uptake of C_4-dicarboxylate

compounds such as succinate and fumarate anaerobically (Six *et al.*, 1994, Zientz *et al.*, 1996).

The role of L-asparaginase II (*asn*B) may be simply to provide a source of nitrogen and carbon from exogenous asparagines. The environmental inducer that regulates L-asparaginase II synthesis may reflect surrounding media rich in asparagine (Jennings *et al.*, 1993). Asparaginase II catalyses the hydrolysis of L-asparagine to aspartate and ammonia (Bonthron, 1990). A more attractive role of L-asparaginase II is in the provision of fumarate as a terminal electron acceptor. Under anaerobic environments bacterial growth on a non-fermentable carbon source (e.g. glycerol), metabolic energy can be generated by coupling of glycerol-3-phosphate oxidation and fumarate reduction (Smith *et al.*, 1983). All of the enzymes of this pathway, L-asparaginase II, aspartase, fumarate reductase and glycerol-3-phosphate are expressed by anaerobiosis via the FNR protein (Spiro and Guest, 1990, Fink *et al.*, 2007, Jennings and Beacham, 1993). The anaerobic regulation of the *S. enterica ans*B gene is not facilitated by the anaerobic transcriptional activator FNR. This is a different situation to the *ansB* gene of *Escherichia coli*, which is dependent on both CRP and FNR (Jennings and Beacham, 1993).

Collectively, from these experiments, it was clear that the genes affecting dicarboxylate metabolism did not compromise the ability of *Salmonella* to colonise 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 colonisation 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 colonisation and it is difficult to know how much colonisation would be affected by mutation affecting any one carbon source.

Chapter - 5: **The role of osmotic protection in intestinal colonisation**

5.1 Introduction

The work carried out in Chapter-3 indicated up-regulation of a number of genes in *S.* Enteritidis in the caeca of the newly-hatched chicken in comparison with broth cultures; including genes, channels and transport systems associated with osmotic stress (Table-5.1). It is thought that the osmotic environment in host gastrointestinal tract (e.g. chicken) has a big impact on enteric bacteria colonisation (Csonka, 1989, Weber *et al.,* 2006). Because there is limited knowledge of the role of the osmotic-associated genes in intestinal colonisation mechanisms of *S.* Enteritidis in the chicken, it was decided to determine the role of some of these in colonisation and competitive exclusion mechanisms. This was achieved by mutating genes using lambda-Red mutagenesis (Datsenko and Wanner, 2000) and evaluating their effects on colonisation using *in vitro* and *in vivo* competitive exclusion experiments (Barrow *et al.,* 1988, Berchieri and Barrow, 1990, Berchieri and Barrow, 1991). The *S.* Enteritidis PT4 defective in osmoregulation mutants produced in the lab were as following: RNA polymerase sigma-E factor "alternative sigma factor" (*rpo*E); periplasmic trehalase (*tre*A); trehalose-6-phosphate synthase (*ots*A); trehalose phosphatase (*ots*B); proline/betaine transporter (*pro*P) and potassium-transporting ATPase A chain (*kdp*A). Because the time for research the lab was limited, these six genes were selected for mutation as they are the most common ones involved in osmoprotectant transportation and regulation in *Escherichia coli* at different environments (Styrvold and Strom, 1991, Rod *et al.,* 1988, Csonka *et al.,* 1988, Altendorf *et al.,* 1992). The genes selected for mutation and competitive exclusion experiments are associated with the following osmoprotectants for bacteria: potassium (*kdpA*), proline (*pro*P), betaine (*pro*P) and trehalose (*tre*A, *ots*A and *ots*B). For example, *ots*A and *ots*B are genes responsible for trehalose synthesis and *tre*A for trehalose degradation. The gene *pro*P is responsible for transporting the amino acids

proline or betaine into the bacterial cells during osmotic stress, while *kdp*A transport system is responsible for transporting potassium ions. For further information for these genes see Chapter 1 section 1.6.

Table 5.1: *S.* Enteritidis PT4 genes and transport systems associated with osmotic-stress, which were significantly ($P < 0.05$) up-regulated more than 2 fold during the colonisation of 1 day chickens intestine compared to *in vitro* growth.

Osmotic stress-associated Genes					
Gene accession number	Gene Function	Symbol	Reference	Fold change	*P*
SEN2567	RNA polymerase sigma-E factor "sigma-24"	*rpo*E	McMeechan *et al.*,2007	2.6	0.004
SEN1241	Periplasmic trehalase	*tre*A	Styrvold and Ström, 1991	9.3	0.007
SEN1076	Trehalose-6-phosphate synthase	*ots*A	Rod *et al.*,1988	17.1	0.006
SEN1075	Trehalose phosphatase	*ots*B	Rod *et al.*,1988	16.9	0.005
SEN4061	Proline/betaine Transporter	*pro*P	Csonka, 1988	6.4	0.005
SEN0670	Potassium-transporting ATPase A chain	*kdp*A	Altendorf *et al.*,1992	3.7	0.009
SEN1306	Putative cytoplasmic protein	*yci*G	(Beraud *et al.*, 2010)	203	0.005
SEN1304	Osmotic stress protein	*yci*E	Weber, *et al.*,2006	188	0.005
SEN1305	Osmotic stress protein	*yci*F	Weber, *et al.*	161	0.005
SEN0776	Osmotic stress protein	*dps*	Weber, *et al.*,2006	50	0.004
SEN4323	Osmotic stress protein-associated with anaerobic environment	*osm*Y	Weber, *et al.*,2006	31	0.003
SEN1725	Oxidative stress protein-anaerobic environment. Hydroperoxidase II	*kat*E	Weber, *et al.*,2006	19	0.004
SEN1492	Osmotically induced protein C	*osm*C	Weber, *et al.*,2006	9.8	0.005
SEN1732	Osmotically induced protein E precursor	*osm*E	(Lacour and Landini, 2004)	7.5	0.004
SEN3380	Glycerol-3-phosphate-binding periplasmic protein	*ugp*B	(Taschner *et al.*, 2004)	3.6	0.006

To study the association between these genes in *S.* Enteritidis and intestinal colonisation in young birds (no gut flora) these genes were mutated individually and the growth curve and growth rate of the mutants were compared to the wild type in nutrient broth containing no added salt in an

aerobic environment. Because the *rpo*E mutant was found to be the poorest growing mutant compared to the rest of the tested mutants, it was re-tested for its growth in nutrient broth containing 4% added sodium chloride under the same conditions of aerobic incubation. It was found that by 24 h incubation the *rpo*E reached the same level of growth as the wild type. Then the association between gene function and colonisation ability was assessed. For this, competition experiments were used as described in Chapter 2; section 2.2.8. This was done *in vivo* in which colonisation of the gut by the mutant was assessed for its ability to prevent colonisation by the parent inoculated 24 h later (Barrow *et al.,* 1990). Competitive-exclusion was also carried out using an *in vitro* model in which stationary-phase nutrient broth cultures of the mutants were assessed for their ability to suppress growth of the parent strain inoculated 24 h later (Zhang-Barber *et al.,* 1997, Berchieri and Barrow, 1991).

5.2 Materials and Methods

5.2.1 Osmotic mutants generation:

Osmotic-associated mutants were also constructed by insertion of a Nal^R or Spc^R cassette into the open reading frame "ORF" of the gene of interest (Chapter-2, section 2.2.5). Briefly, a pair of primers and specific test control primers was designed for every gene of interest (Tables 5.2 and 5.3) for checking the replacement of the target gene by the antibiotic cassette. The published Cm and Km cassette specific test primers (C1, C2 and K1, K2 respectively) were used in combination with the target specific test primers for checking the incorporation of the antibiotic cassettes to the desired place. The mutants were confirmed by PCR tests, tested on selective culture, slide agglutination test, and acriflavin test to make sure that the bacterial cell wall was still intact. Moreover they subjected for recombination (transduction) test to reduce the likelihood that the phenotypes are result of second site defect. All mutants passed this test successfully, after which they were streaked on NA plates supplemented with their respective antibiotics and incubated at 37^oC for overnight, then on the following day an isolated typical colony from each was

streaked on NA plate contained no antibiotics and incubated at 37°C for overnight. Then on the following day one loop-full of bacterial culture was collected and inoculated into 20% glycerol nutrient broth tubes (2 ml glycerol + 8 ml NB); well mixed and then split over sterile 1-ml mini-glass tubes and kept frozen at -80°C freezer.

Table 5.2: Primer used to construct osmotic gene mutation individually. Grey shading indicates the primer sequence homologous to the chloramphenicol (Cm) or kanamycin (Km) antibiotic cassettes (Datsenko and Wanner, 2000).

Name	Sequence (5'- 3')
*rpo*E_50F	TGGCGTTTCGAAAGCGCGTGGAAATTTGGTTTGGGGAGACATTA CCTCGGGTGTAGGCTGGAGCTGCTTC
*rpo*E_50R	AAAGTTTTTCTTTCTGCATGCCTAATACCTTTTCCAGTATCCCG CTATCGCATATGAATATCCTCCTTAG
*tre*A_50F	TGTCATGGTAAATGCCGTTGGCTTTGGCTCACCGCTAAGGAGAT AACTTGGTGTAGGCTGGAGCTGCTTC
*tre*A_50R	GTGTAAGCGTTGACCCGGTCAGCGCCGGGTCAACCTACTATAAA CACGCGCATATGAATATCCTCCTTAG
*ots*A_50F	CAATTATCCACAACAAGAACAACAAGTAATGAATAACAGGAGAG ATGGCTGTGTAGGCTGGAGCTGCTTC
*ots*A_50R	GATGTGTTGCTGGTACCGTTAGCGGGCGACTAGTCGCCGCTCGC GATATTCATATGAATATCCTCCTTAG
*ots*B_50F	CTAATGAGACCGTTTGTGAGTCTCAATATGATGATAAGGAGGAG ACCAGGGTGTAGGCTGGAGCTGCTTC
*ots*B_50R	CAACGGCGAGGCCGCCGGCGCCGCCTTTATTATCCGGGGGGGCA ATTCGACATATGAATATCCTCCTTAG
*pro*P_50F	CCAGTGCCCGCCGTATATAGCGCTACAGGGCTTAGCCTATGAGG ACAGCTGTGTAGGCTGGAGCTGCTTC
*pro*P_50R	ATGGAGGAGAGTATGCCCGCGAGAGATTAAGCGAACCTTAAGCG CGAAATCATATGAATATCCTCCTTAG
*kdp*A_50F	TTACTTTTAGGTTATCTGGTCTATGCCCTGATTAATGCGGAGGC GTTCTGGTGTAGGCTGGAGCTGCTTC
*kdp*A_50R	TTCAAACAGCGCCAGTTGCTTGCGACTCATATCAATGTACTCCG CATCGCCATATGAATATCCTCCTTAG

Table 5.3: Primer combinations used to validate each osmotic gene mutation. Primers are specific to the flanking regions of the specific osmotic gene (ctrF = control forward; ctrR = control reverse) or the antibiotic resistance cassette (Cm1 and Km1 = reverse; Cm2 and Km2 = forward).

Name	Sequence (5'- 3')	Predicted size (bp)
*rpo*E_ctrF	ACTCCAACCTGTTGCTTGCT	920 nt
*rpo*E_ctrR	TAATGGCGACACCTGACGTA	
*tre*A_ctrF	GCGCCGATAAATTCTGTCTC	2070 nt
*tre*A_ctrR	TATTCTCGGTATTGTCGGGG	
*ots*A_ctrF	ATTTCCGTAAAAGTGGGCGT	1800 nt
*ots*A_ctrR	CTTTCATCGCATCAGGTGAG	
*ots*B_ctrF	TCTGGCAGCAGTTATCTTCG	1300 nt
*ots*B_ctrR	TTCACCCGCATATAGCCTTC	
*pro*P_ctrF	ATTCAGGCGTCAACAGGTTC	1840 nt
*pro*P_ctrR	AATACGTCGTGACCCACACA	
*kdp*A_ctrF	CTGGAGGTGCTCTGTGAGTG	2000 nt
*kdp*A_ctrR	GGTGAACCATAACCACAGGC	
Cm1	TTATACGCAAGGCGACAAGG	-
Cm2	GATCTTCCGTCACAGGTAGG	-
Km1	CAGTCATAGCCGAATAGCCT	-
Km2	CGGTGCCCTGAATGAACTGC	-

5.2.2 Assessment of growth rate of mutants of *S.* Enteritidis defective in osmotic-associated genes.

This was designed to assess whether the particular TCA gene mutation affected its growth rate, this could contribute to the poor growth of *S.* Enteritidis osmotic-defective mutant using *in vitro* culture model. The methodology of this experiment was explained in (Chapter-2; section 2.2.2). The bacterial growth rate for *S.* Enteritidis wild type and its osmotic-defective mutants was calculated for both temperatures (37^{o}C and 42 ^{o}C) as shown in result section of this chapter. Moreover the growth cultural characteristics of these mutants on NA and MacConkey agar plates aerobically and anaerobically was assessed compared to *S.* Enteritidis wild type.

5.2.3 *In vitro* competitive exclusion and co-culturing experiments for mutants of *S.* Enteritidis defective in osmoregulation and wild type

Competitive growth experiments were carried out in seven different ways (Chapter-2; section 2.2.8). Briefly; these 7 experiments were numbered from 1-to-7 to facilitate tracking and understanding. The different conditions for these 7 experiments are listed in Table 5-4 below.

Table 5.4: *In vitro* competitive exclusion and co-culturing experiments for mutants of *S.* Enteritidis defective in osmoregulation and wild type at 42°C, 150 rpm shaking aerobic incubator.

Experiment No	Method	Added NaCl (%)	1st Strain (Stationary Phase)	2nd Strain (Challenge strain)	Incubation Time
1	Ability of stationary-phase cultures of mutants of *S.* Enteritidis to suppress growth of the wild type in a non-osmotic aerobic environment	0	Osmotic mutants 5×10^8-1.2×10^9 cfu/ml	Parent strain $4\text{-}9 \times 10^3$ cfu/ml	24 h
2	Ability of stationary-phase cultures of mutants of *S.* Enteritidis to suppress growth of the wild type in an osmotic aerobic environment	4	Osmotic mutants $1\text{-}8 \times 10^7$ cfu/ml	Parent strain $1\text{-}4 \times 10^3$ cfu/ml	24 h
3	Ability of stationary-phase cultures of mutants of *S.* Enteritidis (*rpo*E & *ots*B) to suppress growth of the wild type in an osmotic aerobic environment	4	*rpo*E and *ots*B $1\text{-}8 \times 10^7$ cfu/ml	Parent strain $1\text{-}4 \times 10^3$ cfu/ml	72 h
4	Simultaneous co-culturing of *S.* Enteritidis Mutants with the wild type in a non-osmotic aerobic environment	0	$1\text{-}9 \times 10^4$ cfu/ml	$1\text{-}9 \times 10^4$ cfu/ml	24 h
5	Simultaneous co-culturing of *S.* Enteritidis Mutants with the wild type in an osmotic aerobic environment	4	$1\text{-}3 \times 10^3$ cfu/ml	$1\text{-}3 \times 10^3$ cfu/ml	24 h
6	Ability of stationary-phase cultures of *S.* Enteritidis wild type to suppress growth of the *S.* Enteritidis mutants in a non-osmotic aerobic environment	0	Parent strain $2\text{-}7 \times 10^8$ cfu/ml	Osmotic mutants $1\text{-}9 \times 10^3$ cfu/ml	24 h
7	Ability of stationary-phase cultures of *S.* Enteritidis wild type to suppress growth of the *S.* Enteritidis mutants in an osmotic aerobic environment	4	Parent strain $1\text{-}8 \times 10^7$ cfu/ml	Osmotic mutants 1×10^2 – 4×10^4 cfu/ml	24 h

5.2.3.1 Experiment 1

This is designed to test the ability of stationary phase cultures of mutants defective in osmoprotection (*rpo*E, *tre*A, *ots*A, *ots*B, *pro*P and *kdp*A) to inhibit multiplication of small numbers of the parent strain introduced into the culture in nutrient broth containing no added salt (4% sodium chloride) over 24 h. The method is explained in details in Chapter-2, section 2.2.6.1.

5.2.3.2 Experiment 2

This is designed to test the ability of stationary phase cultures of mutants defective in osmoprotection to inhibit multiplication of small numbers of the parent strain introduced into the culture in nutrient broth containing 4% sodium chloride over 24 h.

This method is explained in details in Chapter-2, section 2.2.6.1.; but the NB media in this experiment contained 4% sodium chloride to induce osmotic stress. Two control tubes were included: one universal tube (negative control) containing 10 ml fresh nutrient broth (NB) with 4% sodium chloride was inoculated with 1-4 x 10^3 cfu/ml of wild type Spc^R (challenge); and the other universal tube contained the stationary phase growth of *S*. Enteritidis wild type (Nal^R) 1-8 x 10^7 cfu/ml was inoculated with the challenge inoculum (1-4 x 10^3 cfu/ml) of wild type Spc^R (positive control).

5.2.3.3 Experiment 3

This is designed to test the ability of stationary phase cultures of mutants defective in osmoprotection (*rpo*E and *ots*B) to inhibit multiplication of small numbers of the parent strain introduced into the culture in NB containing 4% sodium chloride over three days with time-points at 24, 48 and 72 h. We selected these two mutants, because they exhibited the most comparable results in experiment-2 results when they tested for 24 h only. The method for this Experiment is as Experiment 2 method above; but the test was performed for 3 days with time-points at 24, 48 and 72 h.

5.2.3.4 Experiment 4

The co-culture method (Chapter-2, section 2.2.6.2.) was used to test the ability of each of the mutants to out-compete the parent strain in nutrient broth containing no added salt (non-osmotic environment), when each mutant was added simultaneously with the parent strain.

5.2.3.5 Experiment 5

This co-culture method to test the ability of each of the mutants to out-compete the parent strain in nutrient broth containing 4% added sodium chloride salt (osmotic environment) was also determined, when each mutant was added simultaneously with the parent strain.

The method of this experiment is as described in Chapter-2, section 2.2.6.2.; but all NB cultures in this method were supplemented with 4% sodium chloride, to induce osmotic stress and incubated for 24 h.

5.2.3.6 Experiment 6

This was designed to test the ability of small number of mutants defective in osmoprotection to overgrow the stationary phase cultures of parent wild strain in NB contain no added salt for 24 h. The methodology of this experiment is explained in Chapter-2, section 2.2.6.3.

5.2.3.7 Experiment 7

This was designed to test the ability of small number of mutants defective in osmoprotection to overgrow the stationary phase cultures of parent wild strain in nutrient broth containing added salt (4% sodium chloride) for 24 h.

The method of this experiment is as described in Chapter-2, section 2.2.6.1.; but the NB media in this experiment containing 4% added sodium chloride to induce osmotic stress. One positive control tube was included; which contained the stationary phase of *S.* Enteritidis wild type Spc^R inoculated with small numbers of *S.* Enteritidis wild type Nal^R and incubated for 24 h.

5.2.4 *In vivo* competitive exclusion experiments for mutants of *S.* Enteritidis defective in osmoregulation and wild type

This experiment was designed first to test the ability of mutants defective in osmoprotection (*rpo*E, *tre*A, *ots*A, *ots*B, *pro*P and *kdp*A) to grow and colonise in the one-day-old chicken caeca; and test their ability to compete against the colonisation of *S.* Enteritidis wild type spectinomycin resistant strain (Spc^R). Briefly, eight groups of ten birds were inoculated with 0.1 ml (4 x 10^7 cells) of the *S.* Enteritidis mutants defective in osmoprotection within the first 6 h of hatching. The birds in the positive control group were inoculated with *S.* Enteritidis wild type (Nal^R); while the negative control received nothing. On the following day 3 chicks of every group were randomly selected, humanely killed and their caecal contents collected for bacterial viable count on BGA plates supplemented with nalidixic acid (20 µg/ml) and novobiocin (1µg/ml). The remaining birds for every group (7 birds) were inoculated with 0.1 ml of 24 h incubated *S.* Enteritidis wild type Spc^R (challenge) after been diluted 1/1000 in PBS - the challenge dose was ~1.8 x 10^5 bacterial cells. Then the birds were kept in separated rooms for further 48 h. When chicks reached 4 days of age (48 h after challenge inoculation) they were humanely killed and their caecal contents were collected for bacterial viable counts, which were done on these samples as described in Chapter-2, section 2.2.1.1.

5.3 Results

5.3.1 Mutants PCR confirmation

Figure 5.1 indicates clearly that these are real mutants, as the expected size of gene of interest is shown on the wild type (wt) DNA of *S.* Enteritidis; while the mutated genes were replaced by antibiotic cassettes, Cm^R (1.3 kb) or Km^R (1.6 kb). All osmotic generated mutants were tested for growth on nutrient agar supplemented with Cm^R or Km^R, in which they exhibited resistant to the antibiotic they possess and sensitivity to the other one. Besides they did show bacterial cultural characteristics on MacConkey agar.

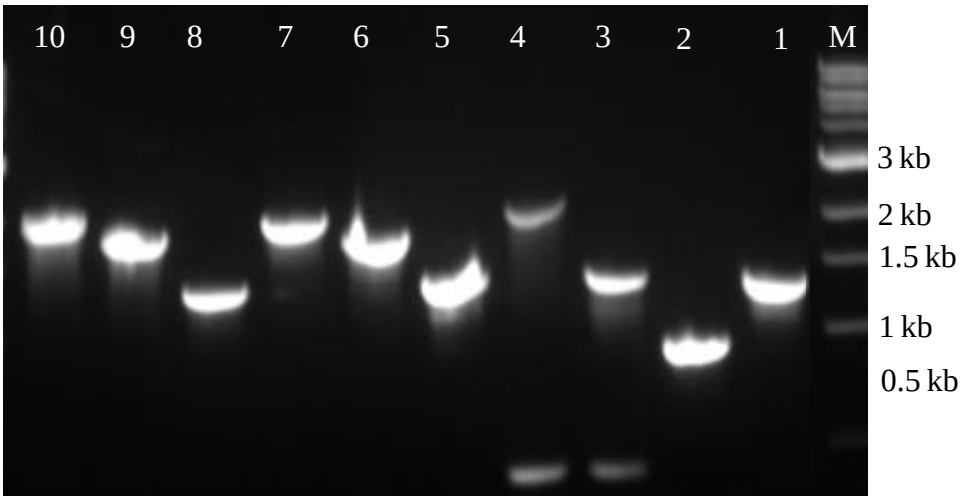

Figure 5.1: Agarose gel electrophoresis of the confirmatory PCR for *S.* Enteritidis osmotic generated mutant, lanes 2, 4, 6, 8 and 10 were the wild type with the primers for *rpo*E (0.92 kb), *tre*A (2.07 kb), *ots*A (1.8 kb), *ots*B (1.3 kb) and *kdp*A (2.0 kb) respectively. Respective mutants for each gene are in lanes 1, 3, 5, 7 and 9 with kanamycin (1.6 kb) replacing the genes in 7 and 9 and with chloramphenicol (1.3 kb) replacing the genes in 1, 3 and 5. Primers for each gene are shown in Table 5.3. M represents 1kb molecular weight ladder.

5.3.2 Assessment of growth rate of mutants of *S.* Enteritidis defective in osmoregulation

The growth rates for all mutants in comparison with the parent strain are shown in Table 5.5. All osmoprotection-defective mutants exhibited a similar pattern of growth curve to the wild type, with the exception of the *rpo*E mutant, which

exhibited a longer lag phase at temperature 37°C (Fig 5-2). At 42°C, apart from *rpo*E, all mutants of *S.* Enteritidis displayed slightly higher growth rates (Fig 5-3).

Table 5.5: The duration of the lag phase and growth rate of the parental and mutant strains of *S.* Enteritidis 125109 at 37°C and 42°C.

Strain/mutants	Symbol	Lag phase (min)		Growth Rate (OD_{600} /h)	
		37°C	42°C	37°C	42°C
S. Enteritidis wild type	wt	20	20	0.13	0.12
RNA polymerase sigma-E factor	*rpo*E	120	120	0.1	0.11
Periplasmic trehalase	*tre*A	20	40	0.16	0.17
Trehalose-6-phosphate synthase	*ots*A	40	40	0.13	0.15
Trehalose phosphatase	*ots*B	40	20	0.13	0.14
Proline/Betaine Transporter	*pro*P	20	40	0.13	0.15
Potassium-Transporting ATPase A chain	*kdp*A	40	40	0.12	0.13

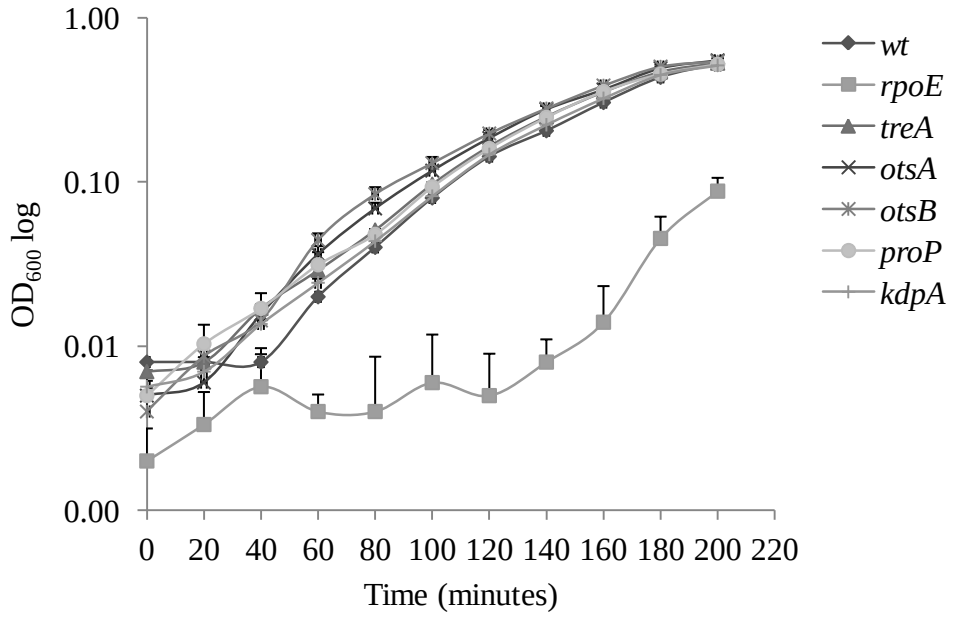

Figure 5.2: Growth curves of *S.* Enteritidis wild type and mutants at 37°C aerobic environment. SE bars are shown.

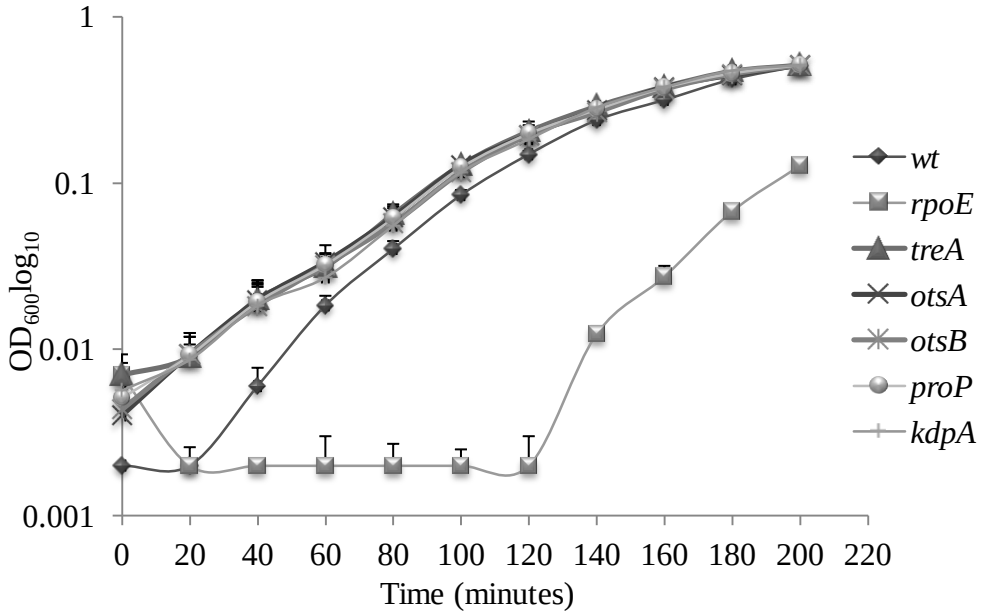

Figure 5.3: Growth curves of *S.* Enteritidis wild type and mutants at 42°C aerobic environment. SE bars are shown.

Because *S.* Enteritidis defective in the alternative sigma factor (*rpo*E) showed the longest lag phase at both temperatures (37 °C or 42 °C) when grown in nutrient broth with no added salt the time to reach stationary-phase for *S.* Enteritidis wild type and the *rpo*E mutant were compared in nutrient broth with 4% added salt. The result was 24 h is the time required for osmotic-generated mutant *rpo*E to reach the stationary phase stage (Fig 5-4), which is required for the following *in vitro* competitive-exclusion experiments. The rest of mutants were also tested and proven to reach the stationary phase (log_{10} 7-8.5) within 24 h.

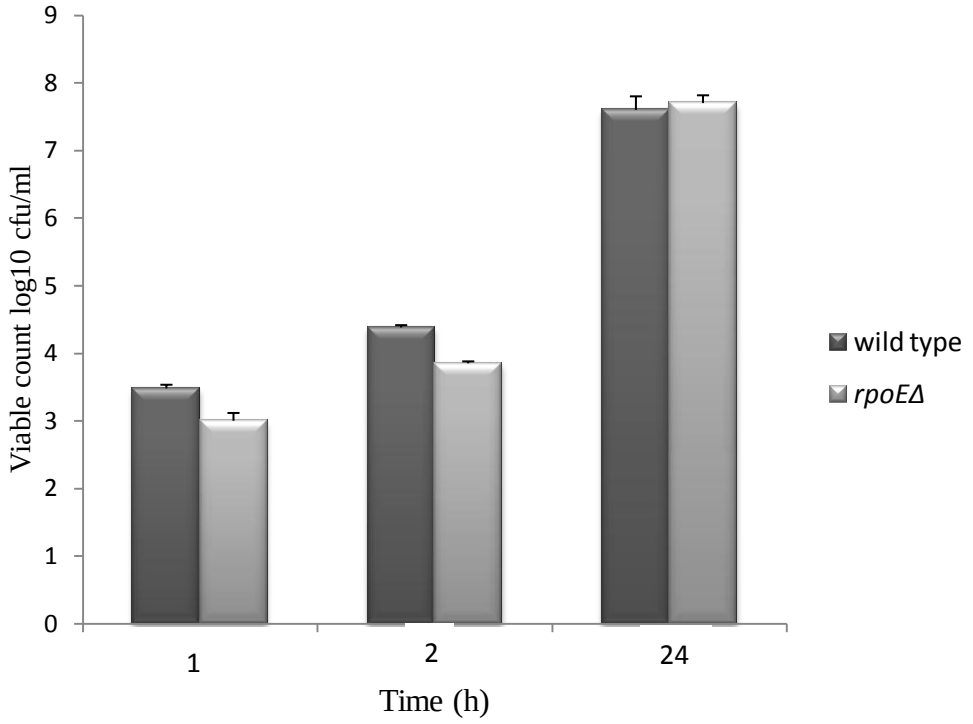

Figure 5.4: The effect of addition of 4% sodium chloride (w/v) to nutrient broth on bacterial growth of *S.* Enteritidis *rpo*E compared to the parental wild type. (SE bars are shown).

5.3.3 *In vitro* competitive-exclusion and co-culturing experiments for mutants of *S.* Enteritidis defective in osmoregulation and wild type

5.3.3.1 Experiment-1

All mutants reached the stationary phase at 2-5 x 10^8 cfu/ml within 18 h of aerobic incubation in NB contains no added salt at 42^{o}C. The results of inoculating the parent strain into the 24 h cultures of the individual mutants and continuing incubation for a further 24 h are shown in Table 5.6 and Figure 5.5. All the *S.* Enteritidis mutants inhibited/suppressed the growth of the challenge strain. With two exceptions the increase in viable counts of the challenge strain in the stationary phase cultures of the mutants did not exceed log 0.1 over 24 h. The exceptions were mutations in the periplasmic trehalase (*tre*A) and trehalose-6-phosphate synthase (*ots*A) where the challenge was able to increase slightly by 0.16 and 0.27 logs respectively. There was no significant difference of all these mutants in exclusion effect to the challenge compared to the parent strain effect ($P > 0.05$) as shown in Table 5.5. The increase in viable counts of

the challenge strain in a stationary phase culture of the wild type strain (positive control) was 0.2 logs only.

Table 5.6: Increase in viable counts of the parental *S.* Enteritidis Spc^R (challenge) in stationary phase broth cultures of the osmoprotection-defective mutants when the conditions were 42°C without 4% sodium chloride and under aerobic incubation for 24 h

Strain	Log_{10} cfu/ml increase in viable numbers of challenge (Mean of 3)	SD	SE	*P* value compared to the wt
No background (Negative)	5.31	0.14	0.08	0.002
*rpo*E	0.09	0.18	0.10	0.73
*tre*A	0.16	0.06	0.03	0.86
*ots*A	0.27	0.14	0.08	0.65
*ots*B	0.10	0.08	0.05	0.62
*pro*P	0.08	0.05	0.03	0.62
*kdp*A	0.09	0.12	0.07	0.65
Wild type (Positive)	0.20	0.31	0.18	-

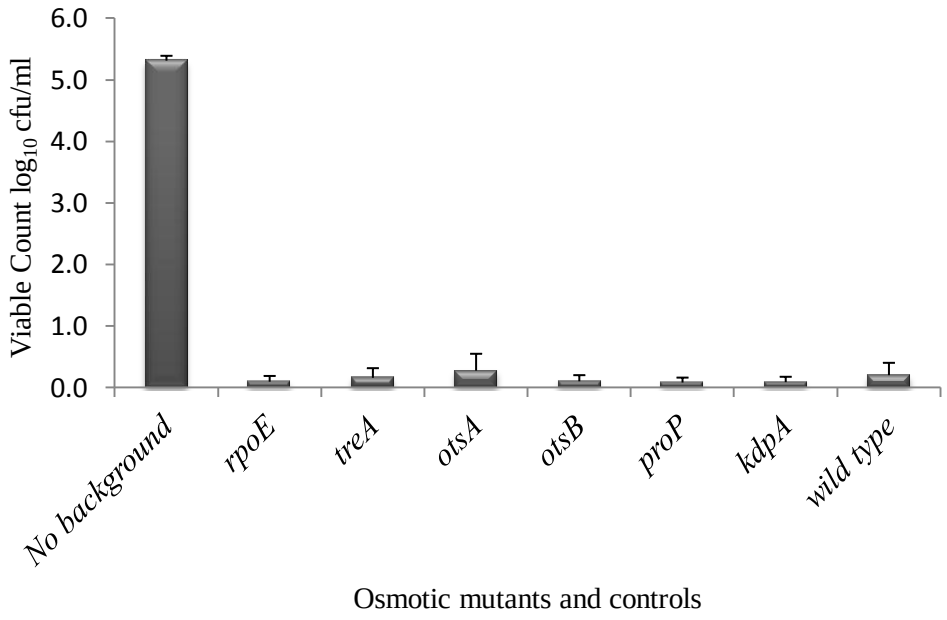

Figure 5.5: Increase in viable counts of *S.* Enteritidis wild type Spc^R in stationary phase broth cultures of osmoprotection-defective mutants of the same *S.* Enteritidis strain when the conditions were 42°C, without 4% added sodium chloride and under aerobic incubation conditions for 24 h. (SE bars are shown).

5.3.3.2 Experiment-2

All *S.* Enteritidis mutants grew well in nutrient broth containing 4% added sodium chloride reaching stationary phase of $\log_{10}$ 7.0-8.5 cfu/ml within 24 h of aerobic shaking incubation at 42^{o}C. The results of inoculating the parent strain into the 24 h cultures of the individual mutants and continuing incubation for a further 24 h are shown in Table 5.7 and Figure 5.6.

This time, the viable bacterial counts of the parental challenge strain only increased by 4.7 logs over the 0 h count within 24 h.

With the exception of mutants with the defective alternative sigma factor (*rpo*E) and potassium-transporting ATPase A chain (*kdp*A) all *S.* Enteritidis osmoprotection-defective mutants showed good inhibition / suppression of the challenge strain with less than 1 $\log_{10}$ of growth. By contrast the viable counts of the challenge stain increased by 1.5 and 1.0 logs in stationary phase cultures of the *rpo*E and *kdp*A mutants respectively. The increase in viable numbers of the challenge in a stationary phase of the parent strain was 0.8 logs only. There was a significant difference of *tre*A, *ots*A and *ots*B mutants in their exclusion effect to the challenge compared to the parent strain effect ($P < 0.05$) as shown in Table 5.7; while the rest of mutants did not show such difference. *S.* Enteritidis defective in trehalose phosphatase (*ots*B) was the best inhibitor of osmotic mutants in suppressing the growth of the wild type at *in vitro* rich medium in presence or absence of 4% sodium chloride (Figure 5.6).

Table 5.7: Increase in viable counts of the parental *S.* Enteritidis Spc^R (challenge) in stationary phase broth cultures of the osmoprotection-defective mutants when the conditions were 42°C with 4% added sodium chloride and under aerobic incubation for 24 h

Strain	Log_{10} cfu/ml increase in viable numbers of challenge (Mean of 3)	SD	SE	*P* value compared to the wt
No background	4.68	0.79	0.46	0.018
*rpo*E	1.50	0.49	0.29	0.203
*tre*A	0.32	0.23	0.13	0.015
*ots*A	0.38	0.21	0.12	0.018
*ots*B	-0.03	0.49	0.28	0.045
*pro*P	0.47	0.13	0.08	0.265
*kdp*A	1.01	0.19	0.11	0.140
Wild type	0.81	0.31	0.18	

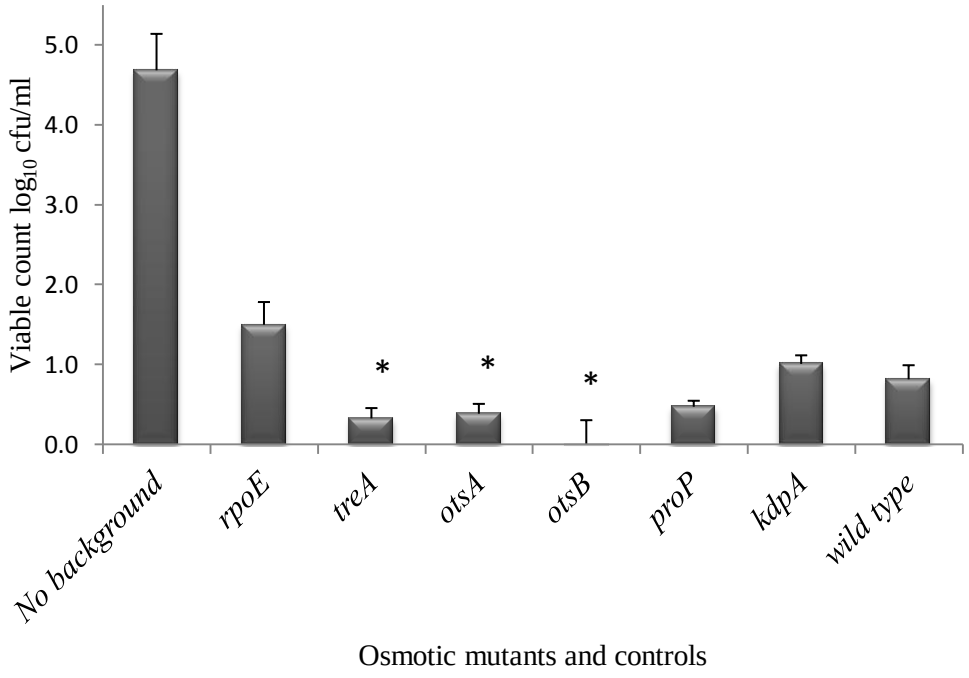

Figure 5.6: Increase in viable counts of *S.* Enteritidis wild type Spc^R in stationary phase broth cultures of osmoprotection-defective mutants of the same *S.* Enteritidis strain when the conditions were 42°C, with 4% added sodium chloride and under aerobic incubation conditions for 24 h. (SE bars are shown). Asterisk (*) indicates a significant difference between mutants ($P < 0.05$) according to the student t test.

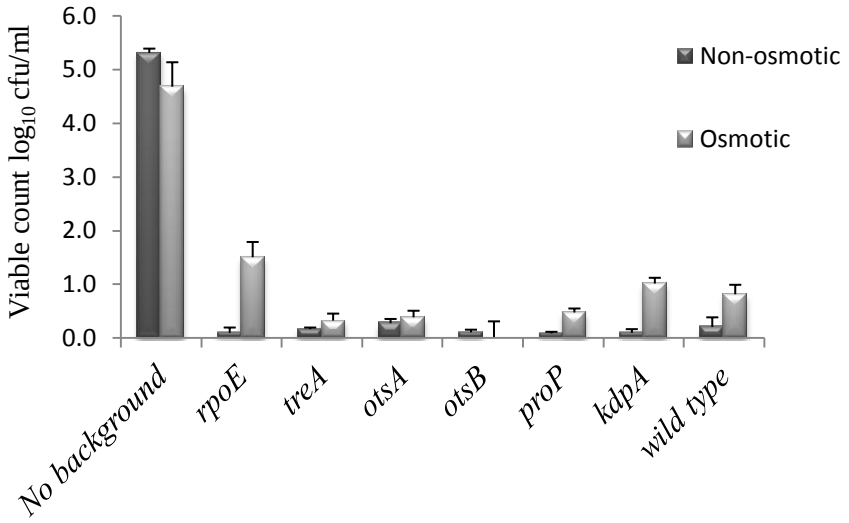

Figure 5.7: Increase in viable counts of *S.* Enteritidis wild type Spc^R in stationary phase broth cultures of osmoprotection-defective mutants of the same *S.* Enteritidis strain when the conditions were 42°C, without (black columns) or with 4% added sodium chloride (grey columns) and under aerobic incubation conditions. (SE bars are shown).

5.3.3.3 Experiment-3

In previous experiment (Figure 5.6) we found that the *S.* Enteritidis alternative sigma factor (*rpo*E) and trehalose phosphatase (*ots*B) mutants were the least and most (respectively) inhibitory mutants against the parent. Therefore the challenge strain was incubated and counted over a longer 72 h period. Throughout this period the parental *S.* Enteritidis wild type completely inhibited the growth of the challenge strain in comparison with growth of the challenge strain in nutrient broth when inoculated on its own (Table 5.8, Figure 5.8 and Figure 5.9). The *rpo*E and *ots*B mutants exhibited a significant difference of inhibition ($P < 0.05$) for time point 72 h when compared with the parental positive control.

Table 5.8: Increase in viable numbers of *S.* Enteritidis wild type Spc^R in stationary phase broth cultures of osmoprotection-defective mutants of the same *S.* Enteritidis strain when the conditions were 42°C, with 4% added sodium chloride and under aerobic incubation for time-points 24, 48 and 72 h.

Time-Point	Strain	Log_{10} cfu/ml increase in viable numbers of challenge strain (Mean of 3)	SD	SE	*P* value compared to the wt
24 h	No background (Negative)	6.42	0.09	0.055	0.001
	rpoE	2.38	0.13	0.07	0.02
	*ots*B	1.04	0.6	0.36	0.8
	Wild type (Positive)	1.18	0.4	0.21	-
48 h	No background (Negative)	6.27	0.1	0.06	0.002
	rpoE	3.54	0.2	0.096	0.005
	*ots*B	2.56	0.4	0.25	0.08
	Wild type (Positive)	1.21	0.34	0.195	-
72 h	No background (Negative)	6.01	0.24	0.14	0.004
	rpoE	4	0.15	0.09	0.004
	*ots*B	3.44	0.41	0.24	0.03
	Wild type (Positive)	1.09	0.47	0.27	-

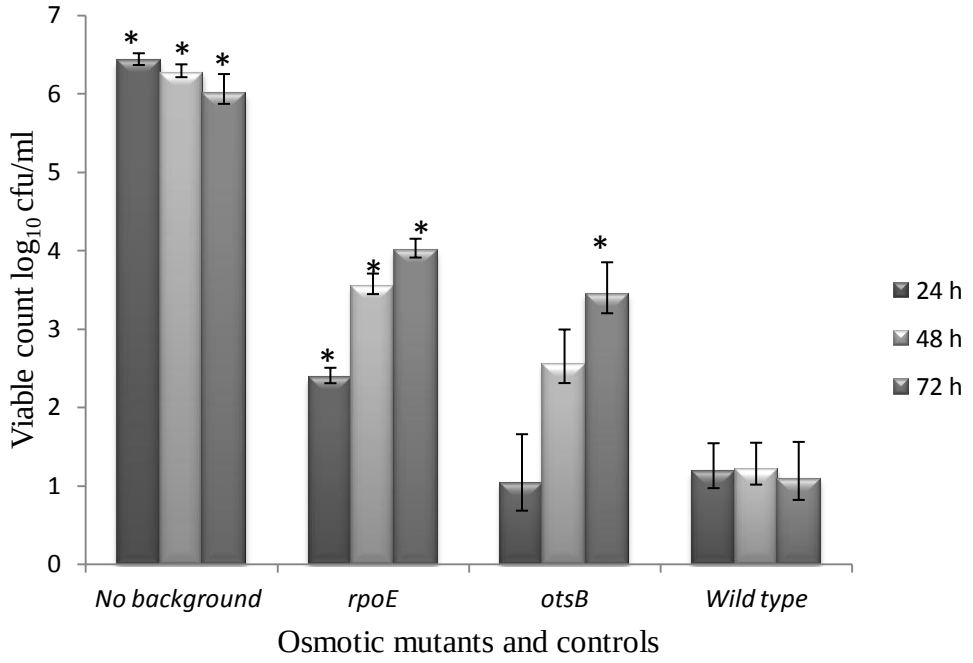

Figure 5.8: Increase in viable counts of *S.* Enteritidis wild type Spc^R in stationary phase broth cultures of osmoprotection-defective mutants of the same *S.* Enteritidis strain when the conditions were 42°C, with 4% added sodium chloride and under aerobic incubation conditions. (SD and SE bars are shown). Asterisk (*) indicates a significant difference between mutants ($P < 0.05$) according to the student t test.

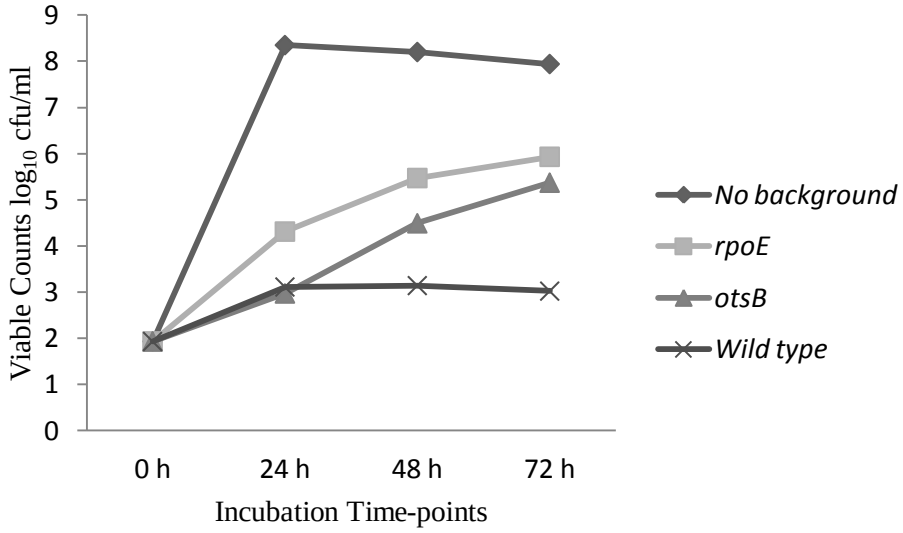

Figure 5.9: Increase in viable counts of *S.* Enteritidis wild type Spc^R in stationary phase broth cultures of osmoprotection-defective mutants of the same *S.* Enteritidis strain when the conditions were 42°C, with 4% added sodium chloride and under aerobic incubation conditions.

5.3.3.4 Experiment-4

The results of inoculating individual mutants simultaneously with the parent strain into nutrient broth containing no added salt and incubated for 24 h are shown in Table 5.9 and Figure 5.10. All mutants grew well in nutrient broth. The mutants showed increases in growth from 3.6 - 4.5 logs, while the *S.* Enteritidis wild type showed an increase of growth between 3.7-4.1 logs. The *S.* Enteritidis defective in periplasmic trehalase (*tre*A) mutant showed the highest increase of growth in comparison with the parent wild type strain; while the mutant defective in alternative sigma factor (*rpo*E) showed the lowest significant ($P < 0.05$) increase (Table 5.9 and Figure 5.10). But the rest of the mutant differences in growth were not significant ($P > 0.05$).

Table 5.9: Increase in viable count of *S.* Enteritidis osmoprotection-defective mutants and the parental *S.* Enteritidis wild type when they were cultured simultaneously in nutrient broth without 4% added sodium chloride at 42°C and under aerobic incubation conditions for 24 h

Strain	Log_{10} cfu/ml increase in viable numbers of mutants (Mean of 3)	Mutants *P* value	Log_{10} increase in viable numbers of Parents (Mean of 3)	Parents *P* value
No background	0.00	7.7×10^{-6}	4.80	0.002
*rpo*E	3.64	0.04	3.67	0.006
*tre*A	4.51	0.09	4.09	0.244
*ots*A	4.25	0.32	4.11	0.603
*ots*B	3.78	0.10	3.81	0.160
*pro*P	4.23	0.73	3.87	0.784
*kdp*A	4.32	0.93	3.89	0.832
Wild type	4.34	-	3.97	-

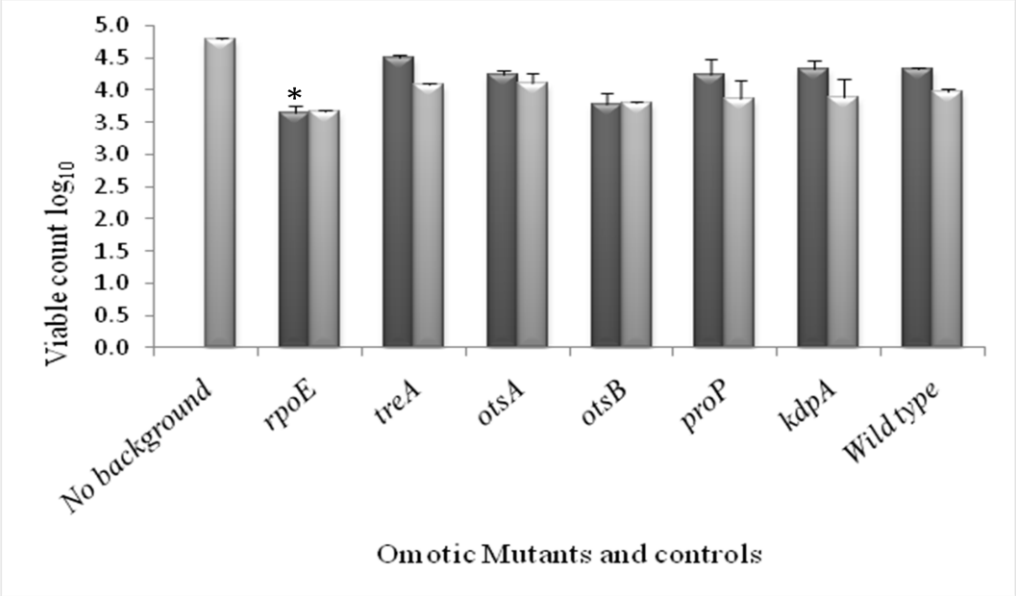

Figure 5.10: Increase in viable counts (cfu/ml) of *S.* Enteritidis wild type (grey columns) and mutants of *S.* Enteritidis defective in osmoregulation (black columns) when they co-cultured simultaneously in nutrient broth cultures without 4% added sodium chloride, at 42°C and under aerobic incubation conditions for 24 h (SE bars are shown). Asterisk (*) indicates a significant difference between mutants ($P < 0.05$) according to the student t test.

5.3.3.5 Experiment-5

The results of inoculating individual mutants simultaneously with the parent strain into nutrient broth contain 4% added salt and incubated for 24 h are shown in Table 5.10 and Figure 5.11. Mutants' growth varied from one to one. In general it was less than the parental strain. The mutants showed increases in growth from 3.1 - 4.4 logs, while the parental *S.* Enteritidis wild type showed increases which varied from 4.2 - 4.6 logs. The *S.* Enteritidis defective in potassium-transporting chain (*kdp*A) mutant showed the highest increase in growth in comparison with the parent wild type strain; while the mutant defective in proline/betaine transporter (*pro*P) showed the lowest increase but this difference was not significant. The *rpo*E, *ots*A and *ots*B showed significant difference ($P < 0.05$) in growth compared to the parent strain.

Table 5.10: Increase in viable count logs of *S.* Enteritidis osmoprotection-defective mutants and *S.* Enteritidis wild type when co-cultured in nutrient broth with 4% added sodium chloride at 42°C and under aerobic incubation conditions for 24 h for the three experiments performed

Strain	Log_{10} cfu/ml increase in viable numbers of mutants (Mean of 3)	Mutants *P* value	Log_{10} increase in viable numbers of Parents (Mean of 3)	Parents *P* value
No background	0.00	0.0004	4.36	0.3
*rpo*E	4.34	0.04	4.21	0.14
*tre*A	4.03	0.08	4.29	0.28
*ots*A	4.32	0.002	4.25	0.009
*ots*B	3.96	0.008	4.40	0.2
*pro*P	3.12	0.21	3.99	0.2
*kdp*A	4.44	0.16	4.59	0.8
Wild type	5.05	-	4.56	-

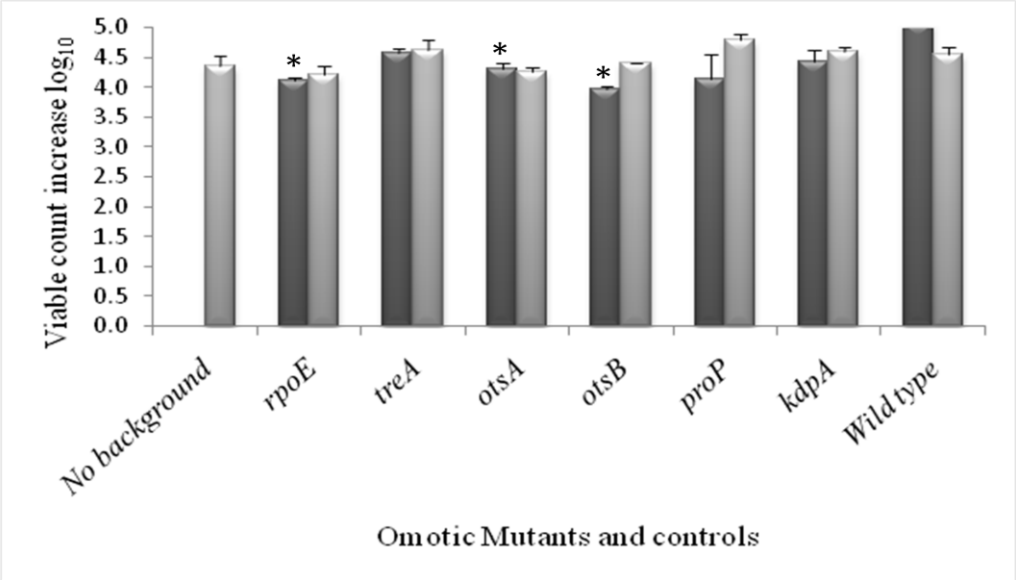

Figure 5.11: Increase in viable counts (cfu/ml) of *S.* Enteritidis wild type (grey columns) and mutants of *S.* Enteritidis defective in osmoregulation (black columns) when they co-cultured to nutrient broth cultures with 4% added sodium chloride, at 42°C and under aerobic incubation conditions for 24 h (SE bars are shown). Asterisk (*) indicates a significant difference between mutants ($P < 0.05$) according to the student t test.

5.3.3.6 Experiment-6

The results of inoculating the mutants individually into 24 h cultures (with no added salt) of the parental wild-type and continuing incubation for a further 24 h are shown in Table 5.11 and Figure 5.12. Apart from the *rpo*E mutant all mutants showed a variable increase over the stationary phase of the wild type in nutrient broth containing no added salt. This increase is small and varied from 0.07-0.26 logs. The trehalose-phosphate phosphatase (*ots*B) and trehalose-6-phosphate synthase (*ots*A) mutants showed the greatest increase over the wild type stationary phase of 0.26 and 0.12 logs respectively which was nevertheless small. In contrast the *S.* Enteritidis *rpoE* mutant was not able to grow over the wild type stationary phase; as the number declined with a change of -0.32 log.

Table 5.11: Change in viable counts logs of *S.* Enteritidis osmoprotection-defective mutants in stationary phase broth cultures of the parental *S.* Enteritidis wild type when the conditions were 42^{o}C without 4% sodium chloride and under aerobic incubation for 24 h.

Strain	Log_{10} cfu/ml increase in viable numbers of mutants (Mean of 3)	SD	SE	*P* value compared to the wt
*rpo*E	-0.32	0.17	0.10	0.05
*tre*A	0.07	0.12	0.07	0.5
*ots*A	0.12	0.06	0.04	0.6
*ots*B	0.26	0.31	0.18	0.6
*pro*P	0.08	0.11	0.06	0.6
*kdp*A	0.08	0.12	0.07	0.7
Wild type	0.16	0.15	0.09	-

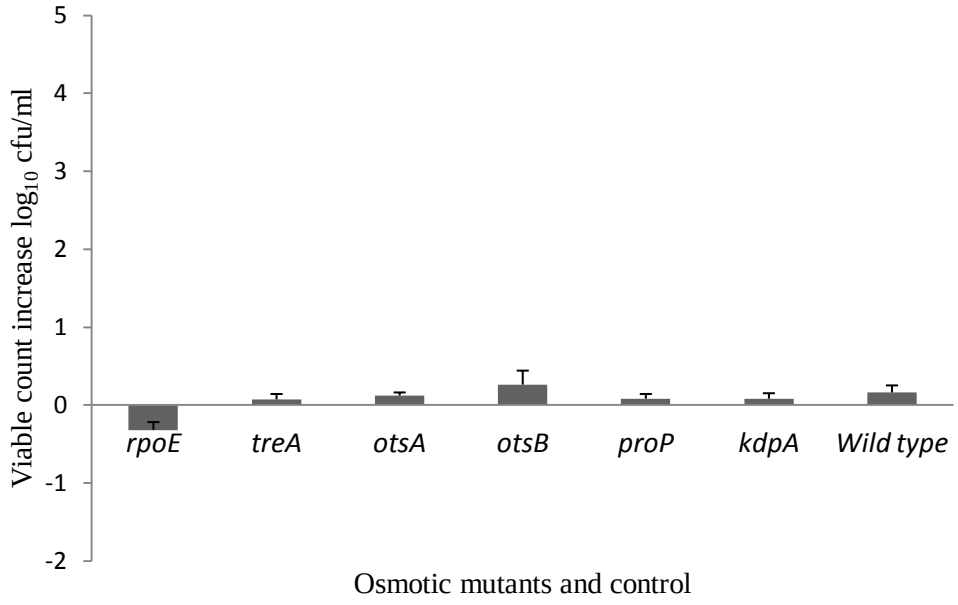

Figure 5.12: Change in viable counts of *S.* Enteritidis osmoprotection-defective mutants in stationary phase broth cultures of the parental *S.* Enteritidis wild type when the conditions were 42°C, without 4% added sodium chloride and under aerobic incubation conditions for 24 h. (SE bars are shown).

5.3.3.7 Experiment-7

The results of inoculating the mutants individually into 24 h cultures (containing 4% added salt) of the parental wild-type and continuing incubation for a further 24 h are shown in Table 5.12 and Figure 5.13. All mutants were suppressed by the stationary-phase culture of the parental strain in presence of 4% sodium chloride, the *S.* Enteritidis wild type Spc^R was not able to suppress the positive control, *S.* Enteritidis wild type Nal^R completely. It is comparable result to what produced in broth containing no salt (Figure 5.12).

The *S.* Enteritidis *ots*A and *pro*P mutants were able to grow very slightly over the parent strain stationary phase, with an increase of 0.15 and 0.09 logs respectively; which was nevertheless very small; while *rpo*E and *kdp*A mutants declined in viable counts decreasing by 1.14 and 0.52 logs cfu/ml respectively. All tested mutants showed significant difference ($P < 0.05$) compared to the wild type, except *kdp*A in growing over the stationary phase of the parent strain.

Table 5.12: Change in viable counts of *S.* Enteritidis osmoprotection-defective mutants in stationary phase broth cultures of the parental *S.* Enteritidis wild type when the conditions were 42°C, with 4% added sodium chloride and under aerobic incubation conditions for 24 h.

Strain	Log_{10} cfu/ml increase in viable numbers of mutants (Mean of 3)	SD	SE	*P* value compared to the wt
*rpo*E	-1.14	0.04	0.02	0.002
*tre*A	0.00	0.15	0.09	0.02
*ots*A	0.15	0.35	0.20	0.03
*ots*B	0.01	0.18	0.10	0.03
*pro*P	0.09	0.26	0.15	0.04
*kdp*A	-0.52	0.60	0.35	0.07
Wild type	1.07	0.17	0.10	-

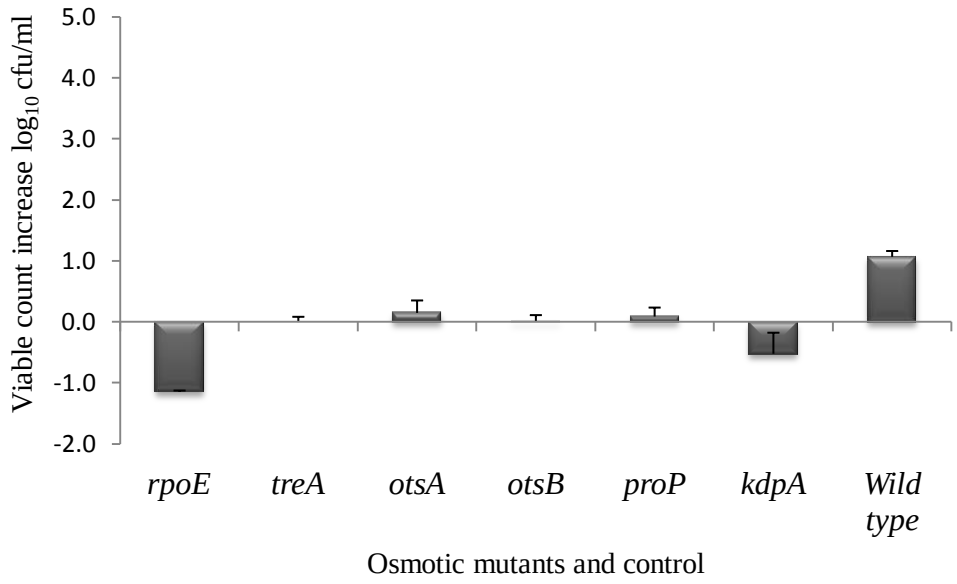

Figure 5.13: Increase in viable counts of *S.* Enteritidis osmoprotection-defective mutants in stationary phase broth cultures of the parental *S.* Enteritidis wild type when the conditions were 42°C, with 4% added sodium chloride and under aerobic incubation conditions for 24 h. (SE bars are shown).

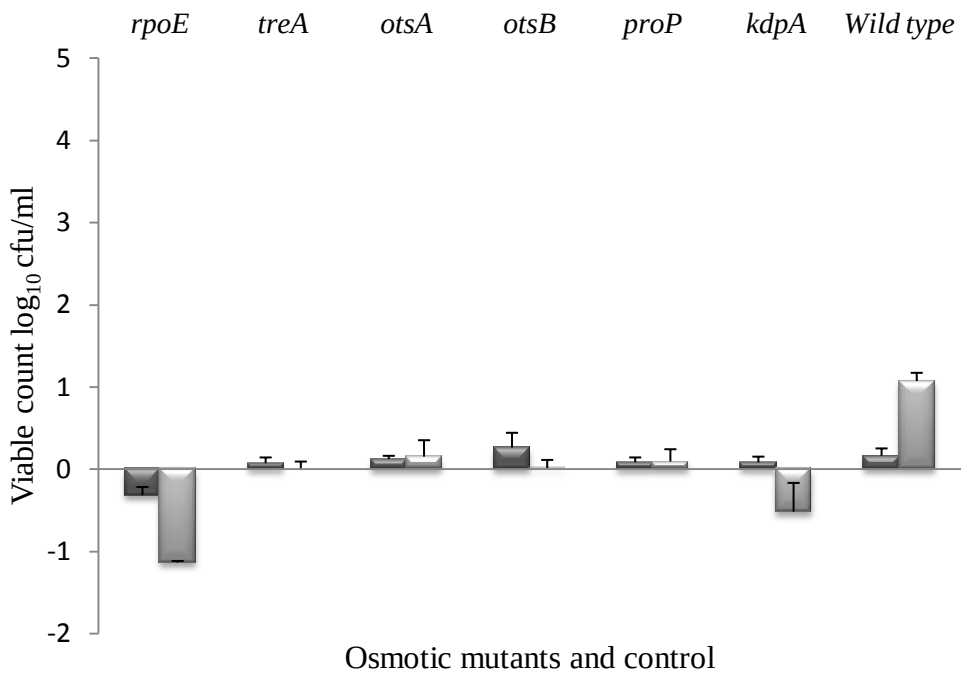

Figure 5.14: Increase in viable counts of *S.* Enteritidis osmoprotection-defective mutants in stationary phase broth cultures of the parental *S.* Enteritidis wild type when the conditions were 42°C, without 4% added sodium chloride (black columns) or with 4% added sodium chloride (grey columns) and under aerobic incubation conditions. (SE bars are shown).

5.3.4 *In vivo* competitive exclusion shown by mutants of *S.* Enteritidis defective in osmoregulation against the wild type

This assay was performed to assess the ability of each individual mutant to exclude a parent strain (Spc^R) inoculated 24 h later (Zhang-Barber *et al.*,1997). At the time of challenge all mutants tested (*rpo*E, *tre*A, *ots*A, *ots*B, *pro*P and *kdp*A) colonised the gut well according to the viable counts in the caeca of three birds killed at the time of challenge (log_{10} 7.2-10.7 cfu/ml). When the birds were killed 48 h after challenge inoculation all pre-colonised mutants were still colonising well with the mean caecal count ranging from log_{10} 7.8 – 8.8 cfu/ml (Table 5.13). Compared to the wild type growth (positive control), significant growth of the challenge ($P \leq 0.05$) over the chicks' caeca pre-colonised with *rpo*E, *tre*A, *ots*A and *pro*P mutants, in which the count reached log_{10} 5.8, 6.3, 6.2 and 6 respectively as shown in Table 5.13 and Figure 5.15. On the other hand, the challenge growth in birds pre-colonised with *ots*B and *kdp*A exhibited less growth count, log_{10} 3.6 and 5.2 respectively (Table 5.13

and Figure 5.15) with no significant difference compared to the wild type growth ($P > 0.05$). The challenge showed a growth of $\log_{10}$ 7.6 in control birds group, which was not pre-colonised with any bacteria.

Table 5.13: The effect of intestinal colonisation of newly hatched chicks with *S.* Enteritidis (Nal^R) or one of its generated mutants on the caecal colonisation by the parental challenge (Spc^R) given orally 24 h later. Mean of 7 birds. These readings were taken 48 h post challenge inoculation.

Mutation (Nal^R)	Log_{10} cfu/ml of caecal content of pre-colonising and challenge strain in 4 days chicks (48 h post-challenge)				
	Pre-colonised strain (Nal^R)	Challenge strain (Spc^R)	SD	SE	*P* value compared to the wt
*rpo*E	7.8	5.8	1.4	0.52	0.04
*tre*A	8.8	6.3	2.1	0.79	0.05
*ots*A	8.3	6.2	2.0	0.76	0.003
*ots*B	8.7	3.6	1.9	0.70	0.9
*pro*P	8.5	6.0	1.6	0.61	0.04
*kdp*A	8.7	5.2	2.3	0.87	0.14
Wild type (Positive)	8.5	3.5	1.8	0.66	-
No background (negative)	0	7.6	1.5	0.55	0.003

Day-1 birds were inoculated orally with 4 x 10^7 cfu ($\log_{10}$ 7.6) in 0.1 ml of Nal^R strain (mutants); 24 h later challenged with 1.8 x 10^5 cfu ($\log_{10}$ 5.3) in 0.1 ml of Spc^R strain.

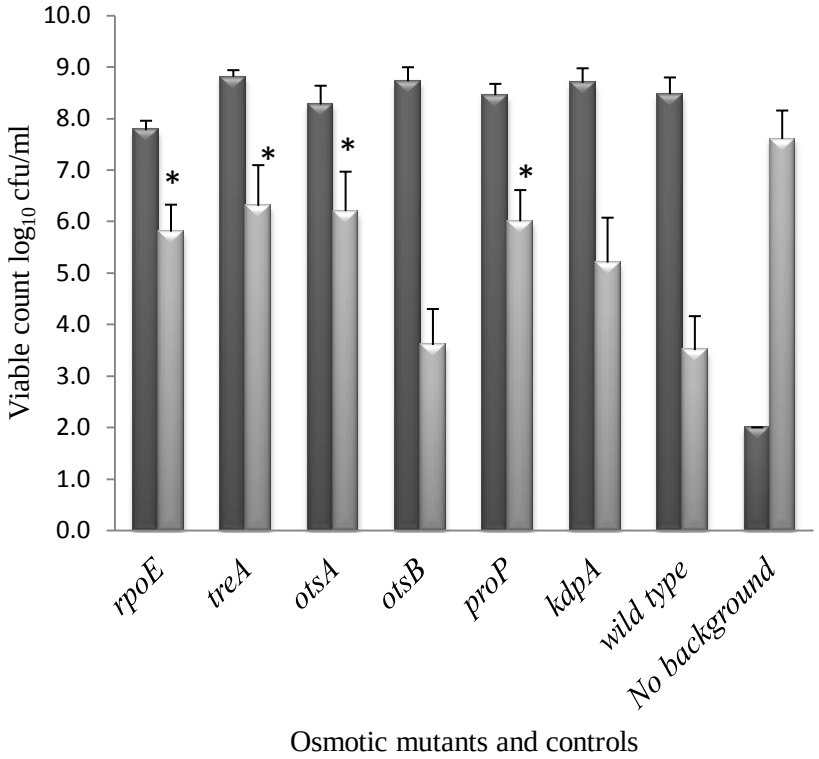

Figure 5.15: The effect of intestinal colonisation of newly hatched chicks with wild type *S.* Enteritidis (Nal^R) or one of its osmotic-generated mutants on the caecal colonisation by the parent strain (challenge) given orally (1.8×10^5 cells) 24 h later. These readings were taken 48 h post-challenge inoculations; pre-colonised strain (black columns); challenge (grey columns). Mean and SE of 7 birds. One asterisk (*) indicate a significant difference between mutants ($P \leq 0.05$) and the wild type according to the student t-test.

5.4 Discussion

To study the role of individual genes associated with osmoprotection in intestinal colonisation, deletion mutants lacking selected genes of interest were constructed using lambda-red mutagenesis (Datsenko and Wanner, 2000).

These genes were successfully disrupted and transduced into *S.* Enteritidis PT4 (Nal^R) using bacteriophage P22. All mutants produced were verified by PCR.

Mutant growth was tested in a complex media because the gut is a complex environment of many nutrients that originated from the egg yolk that is why the competition was performed in nutrient broth before being tested in chicken gut. Therefore the growth rate for every mutant generated in nutrient broth was

assessed *in vitro* at 42°C, which reflects chicken temperature. The initial checks on growth characteristics were to ensure that (i) they grew well in NB and whether (ii) there was a phenotype when tested in 4% NaCl (700mM). With the exception of the *S.* Enteritidis *rpoE* mutant all osmotic mutants showed similar growth patterns to the parent strain when they were grown in nutrient broth at 37°C; on other hand they exhibited slightly higher growth curve compared with the parent strain when grown at 42°C. The reason for this is not clear.

A percentage of 4% sodium chloride was added to the nutrient broth to induce osmotic stress, which is in line with other studies (Akman and Park, 1974, McKay and Peters, 1995). The growth rate for every mutant in nutrient broth at 37°C and 42°C was assessed. The *S.* Enteritidis defective in *rpo*E growth behaviour was an exception to the rest of tested mutants. Its exhibition of longer lag-phase could be that its generation-time become longer than the wild type and the rest of mutants. This required inoculating it into NB plus 4% NaCl and incubating it 3 h in advance to the remaining mutants to reach the stationary-phase within 24 h which required for *in vitro* competitive-exclusion environment.

However, despite extended lag phase and reduced growth rate, the *S.* Enteritidis defective in *rpo*E strain grew to a final density comparable to the wild type. This result indicated that *rpo*E is important for adaptation of *S.* Enteritidis to hyperosmotic environments. This finding is in line with other studies (McMeechan *et al.*, 2007, Du *et al.*, 2011b) that *S.* Typhimurium *rpo*E mutant exhibited long lag phase (~ 9 h) when inoculated in rich medium (LB) with or without added 6% salt. These studies demonstrated that *rpo*S- and *rpo*E-regulated genes are required for *Salmonella* optimal growth in media with high osmolarity (McMeechan *et al.*, 2007, Du *et al.*, 2011b). Previous genomic microarray analysis (Huang *et al.*, 2007) indicated that potential complementarity between the expression of *rpo*S and *rpo*E raised the possibility that some genes might be co-regulated by RpoE and RpoS in *Salmonella* under hyperosmotic stress. They also indicated that in the lumen of

human intestine *S.* Typhi is exposed to a significant increase in osmolarity equivalent to 300mM NaCl in small intestine (Huang *et al.*, 2007).

The *in vitro* competitive exclusion experiments performed indicate that all osmotic genes tested are not essential for *Salmonella* growth in nutrient broth containing no added salt, as they all showed no significant difference compared to the wild type positive control inhibition. But in presence of 4% sodium chloride it appeared that *rpo*E gene and *kdp*A transport system for potassium was needed despite non-significant difference with the wild type. The surprise in this assay is that trehalose mutants (*tre*A, *ots*A and *ots*B) were significantly more inhibitory than the wild type itself. To check whether this sort of inhibition is limited only for 24 h or can last for further period of time, the most inhibitory (*ots*B) and the least inhibitory (*rpo*E) were selected to perform the same experiment in nutrient broth containing 4% sodium chloride (~700mM) for 72 h. This confirmed the requirement for the alternative sigma factor *rpo*E under hyperosmotic conditions. On other hand trehalose phosphatase *ots*B showed similar inhibition to the wild type against the challenge at 24 h. Then the challenge managed to increase by 2.4 $\log_{10}$ at time-point 72 h. This means that this gene *ots*B in combination with *ots*A is much more needed for intracellular trehalose synthesis to function as osmoprotectant in late stages of growth (e.g. stationary or decline phases). However, it was aimed to assess colonisation ability of individual mutants of the parental *S.* Enteritidis. For comparison these mutants should have been assessed for colonisation ability also in newly-hatched chickens.

However, it was important to ensure comparability between the work described in this section of the thesis and the array results. Therefore it was decided to adapt the use of competitive colonisation experiments in which mutants were inoculated orally into newly hatched chickens followed 24 h by oral inoculation with the isogenic parent strain. There is a long history of such experiments both between isogenic mutants of *S.* Typhimurium but also between strains of *E. coli* in pigs (Impey *et al.*, 1982, Barrow and Tucker, 1986, Barrow *et al.*, 1987b, Berchieri and Barrow, 1990, Methner *et al.*, 1997,

Genovese *et al.*, 2000, Nisbet, 2002). However, these studies have proven that the vaccine strain colonises the gut extensively and prevents re-infection by other *Salmonella* strains by a genus-specific mechanism similar to that occuring during down-regulation of bacterial growth in stationary-phase nutrient broth cultures. The mechanism of this phenomenon has been studied (Berchieri and Barrow, 1991, Iba *et al.*, 1995, Barrow *et al.*, 1996b, Rychlik *et al.*, 2002, Nogrady *et al.*, 2003b). This work has been extrapolated to chickens with a similar degree of success. It is considered that during colonisation of the two caeca of chickens bacteria fluctuate between periods of logarithmic growth and stationary phase with the latter predominating (Barrow unpublished). The behaviour of *Salmonella* therefore resembles stationary-phase physiology with the additional stressful effects associated with growth in the gut, including high osmolarity. Before starting *in vitro* competitive-exclusion experiments under non-osmotic and osmotic stress it was shown that all osmotic mutants generated grew well in rich media reaching stationary phase at both temperatures (37^{o}C and 42^{o}C) after overnight (18 h) incubation in nutrient broth containing no added salt (4% sodium chloride) and within 24 h in nutrient broth containing 4% added sodium chloride. It is an indication that *Salmonella* grow slower in the presence of osmotic stress compared to its absence. This is expected to be the case for *Salmonella* growth in the intestine where a lot of salts exist (e.g. NaCl and bile salts). However, it was an attempt to mimic the conditions in the intestine and when done in 4% NaCl and at 42 ^{o}C.

According to the literature and the results gained in our research, some interpretation for the behaviour/mechanisms of osmotic-associated genes can be presented. It seems that for *Salmonella* or any other enteric bacteria to grow optimally *in vitro* or *in vivo* both general stress regulators, sigma factor (*rpo*S) and alternative sigma factor (*rpo*E) were required to be expressed (McMeechan *et al.*, 2007, Du *et al.*, 2011b) especially when *Salmonella* is exposed to one or multiple extracytoplasmic stress factors (e.g. high temperature, pH, osmotic stress). The environment of host lower intestinal tract is typical habitat for these factors. Therefore for bacteria to survive in such environment the

bacterial chemistry/physiology reacts/responds by transcription/expression of relevant genes/enzymes to resist these stresses.

The results were compatible with recent work (Du *et al.,* 2011b), which indicated the importance of osmoregulation of the alternative sigma factor *rpoE* or sigma factor *rpo*S in coregulating of many other osmoprotectants genes in hyperosmotic environments. The genes that are co-regulated by one of these factors were as follows: osmotic induced protein *osm*C, osmotic induced protein associated with anaerobic environment *osm*Y, trehalose synthase and phosphatase genes *ots*AB and glycerol-3-phosphate binding periplasmic protein *ugp*B. All these listed genes were significantly up-regulated in chicken caeca environment (Table 6.1).

It was expected that for *Salmonella* to withstand the osmotic stress inside chicken caeca potassium and/or proline/betaine are transported from caecal contents into the bacterial cytoplasm by the action of *kdp*A and/or *pro*P/*pro*U. When these electrolytes (K^+, proline and betaine) become limiting in the surrounding environment energy is required for trehalose to be synthesized intracellularly by the action of *ots*AB (Rod *et al.,* 1988); and because the caeca are filling and emptying around the time the osmotic stress fluctuates and at some points the synthesized trehalose could be in excess of that needed for bacterial turgor balance, so bacteria excrete it into outer cytoplasmic membrane where it is broken down to two molecules of glucose by the action of *tre*A (Styrvold and Strom, 1991, Giaever *et al.*, 1988). Giaever and others (1988) reported that osmotic stress on bacteria induces the *ots*AB operon 5-to-10 fold; this extent of change in gene expression is compatible with the microarray data for *S.* Enteritidis experimental infection recovered from chick caeca. On other hand, Hengge-Aronis and colleagues (1991) reported that the *ots*AB operon is also induced upon entry of cells into stationary phase, but the levels of trehalose in stationary phase cells are much lower than in cells under osmotic stress; and this again reflects the environment exposed the *Salmonella* during the experimental infection (Chapter 3).

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.,* 1987b, Berchieri and Barrow, 1991) and although the use of stationary-phase cultures has been used to study the contribution of respiration genes to colonisation it has been found to be inadequate (Zhang-Barber *et al.,* 1997).

The question is raised here whether these mechanisms of osmoprotectants transportation and synthesis occur simultaneously in the same bacterial cell or in a hierarchical manner. Further investigation is required to test double and multiple mutation effect of some of these genes in colonisation.

Collectively, a number of genes associated or thought to be associated with responses to high osmolarity were both highly up-regulated *in vivo* and showed reduced competitiveness *in vivo* but not *in vitro*. Osmolarity has been found previously to be important in gut colonisation (Dorman *et al.,* 1989, Bower *et al.,* 2009), although this has not previously reported for the chicken. The RpoE sigma factor regulates microbial characteristics associated with normal physiology including flagellation (Du *et al.,* 2011a, Du *et al.,* 2011b), 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 which would affect both *kdp*A and *pro*P (Balaji *et al.,* 2005).

The *tre*A mutants are unable to catabolise trehalose under 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, Strom and Kaasen, 1993). The phenotypes of trehalose synthase *ots*A and phosphatase *ots*B, 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 complex conditions in the intestine would be worth further study.

Chapter - 6: **Vaccination**

6.1 Introduction:

Experimental infection of chickens with invasive serotypes such as *S.* Typhimurium and *S.* Enteritidis results in their elimination from the gut and a high degree of resistance to subsequent challenge (Van Laere, 1989). This has been exploited in the development of both live (Anon, 1995, Anon, 1991, Report, 1969, Methner *et al.,* 1997, Methner *et al.,* 2004) and inactivated vaccines (Liu *et al.,* 2001b, Woodward *et al.,* 2002, Clifton-Hadley *et al.,* 2002). Both have advantages and disadvantages. Inactivated vaccines, as currently constituted, are generally less immunogenic since no microbial multiplication occurs. They may stimulate high levels of circulating antibody but may be poor at stimulating cell mediated immunity. Live vaccines are more immunogenic and since microbial activity occurs *in vivo* appropriate antigens are presented to the host. They have additional beneficial protective effects depending on route of administration (Report, 2005). However, there are concerns over public acceptability since those currently commercially available in Europe are genetically undefined and are antibiotic resistant, whilst the better defined deletions, which may be antibiotic sensitive, are produced by genetic modification which is seen by the public as a cause for concern.

Bacteria are killed for use as inactivated vaccines, and it is important that they remain as antigenically similar to the living bacteria as possible. Therefore crude methods of killing bacteria that causes extensive protein denaturation or lipid oxidation are usually unsatisfactory. If chemicals are used, they should not alter the antigens responsible for stimulating protective immunity. Formaldehyde is one of the chemicals used, which acts on proteins and nucleic acids to form cross-links and thus confer structural rigidity.

The current commercial inactivated killed vaccines for food-poisoning *Salmonella* infection are not comprehensively effective (e.g. Salenvac, Intervet) because the antigens presented to the host reflect those produced

during *in vitro* cultivation. Therefore, it was thought that vaccines prepared or generated from strains grown under *in vivo* environment would be more effective than that produced under *in vitro* environment.

There were two types of vaccination experiments performed in the animal facility on layer birds of 1 day chicks. The vaccine considered was an inactivated killed vaccine for *S.* Enteritidis PT4, which was grown in either *in vitro* or *in vivo* environments. Both were inactivated by formaldehyde (Section 6.2.3). The difference between the two experiments performed was the challenge inoculation site and the samples collected for bacterial counts. In both experiments the birds received the same regime of vaccination and treatment (Table 6.1); but in the 1st experiment birds were challenged orally to assess protection against intestinal colonisation, while in the 2nd experiment birds were challenged intra-venously to assess protection against systemic infection. Therefore the samples collected for the 1st experiment were cloacal and caecal samples; while the samples collected for the 2nd experiment were tissue portions of liver and spleen plus caecal contents. The intra-venous route of the challenge was mainly to ensure some degree of internal organ tissue invasion is taking place (Woodward *et al.,* 2002, Timms *et al.,* 1990, Timms *et al.,* 1994).

6.2 Methods:

6.2.1 The *in vitro* grown *S.* Enteritidis PT4 culture preparation:

S. Enteritidis PT4, antibiotic sensitive parent strain, P125109 was cultured in 2 flasks of 250 ml, each containing100 ml nutrient broth at 37^{o}C in a shaking incubator at 200 rpm for 2 h. In which time, bacteria reached the logarithmic phase (Chapter 2; Section 2.2.1). The total number of bacteria per ml was approximately 1 x 10^{8} cfu/ml. Therefore, each 100 ml grown culture was divided into 3 Falcon tubes, each contained approximately equal volumes centrifuged at 5000 x g; 20^{o}C for 30 min. The supernatant of each tube was carefully decanted without disturbing the pellets and the pellet of each tube was resuspended in 3.33 ml nutrient broth. The contents of the three Falcon tubes,

which belong to the same flask, were combined in one Falcon tube. This resulted in the total number of ~ 1 x 10^9 cfu/ml, which was nearly equivalent to the total number of *in vivo* grown bacteria (section 6.2.2) to ensure similar concentration of antigens. The last mix was subjected to formalization as explained in section 6.2.3.

6.2.2 The *in vivo* grown *S.* Enteritidis PT4 culture preparation:

Group of 60 birds of 1-day old chicks were dosed orally with 0.1 ml of *S.* Enteritidis PT4, antibiotic sensitive, parent strain nutrient broth culture diluted to 1 x 10^6 cells in nutrient broth. On the following day 3 caecal contents from three chickens were used to assess bacterial purity and bacterial viable count separately, while the remaining caecal contents were collected together into two Falcon tubes on dry ice, because they were collected over two days, according to the chicken's hatching days. The net weight of caeca collected was 5.5 g in approximately 5.5 ml; and the total number of bacteria in both tubes was approximately 1.25 x 10^{11} cells. Both tubes were diluted in 12.5 ml PBS, mixed well quickly by vortexing and then only 3 ml were collected from this dilution and added into 27 ml of nutrient broth at RT; this resulted in each ml containing 1 x 10^9 cfu. The last mix was subjected to formalization as explained in section 6.2.3.

6.2.3 Vaccine preparation by Formalization:

The *in vitro* and *in vivo* cultured *S.* Enteritidis preparation, as described above, were subjected to formalization to produce inactivated killed vaccines. Each 10 ml of bacterial growth received 0.215 ml of 37 % formaldehyde (Sigma – Aldrich) and then vortex-mixed and divided between 1 ml sterile tubes and kept at 4^{o}C until they were required for vaccination. An aliquot from the formalized *in vitro* and *in vivo* cultures of *S.* Enteritidis were streaked on nutrient and MacConkey agar plates and incubated at 37^{o}C for 24 h, and shown to be sterile.

6.2.4 Experiment-1 Plan:

Sixty 1-day layers (egg production breed) were put in 3 separate rooms, 20 birds per room. Group 1 birds were to be vaccinated with *S.* Enteritidis cultured *in vivo*, group 2 birds with *S.* Enteritidis cultured *in vitro* and group 3 birds would act as unvaccinated control vaccinated with nutrient broth. Birds in all rooms were kept on the floor bedded with wood straw and drinking water and food were provided *ad libitum*. On the day of arrival they all were dosed orally with 0.1 ml of neat Avigard gut flora (Microbial Developments Limited, UK) as it shown in Table 6.1. At 5 days of age they all were injected intra-muscularly (via both sides of the breast muscle) with 0.05 ml containing inactivated bacterial cells of *S.* Enteritidis equivalent to 1 x 10^8 cells, plus 0.1 ml dosed orally (containing the equivalent of 1 x 10^8 cells) of formalin killed *S.* Enteritidis. The vaccination regimen was repeated when the birds were 3 weeks old, but this time they were inoculated with 0.3 ml (3 x 10^8 cells) orally and 0.1 ml (1 x 10^8) intra-muscularly. Prior to challenge, a cloacal swab was taken from each bird and cultured for *Salmonella*. At week 5 of birds' age all birds were challenged with 0.5 ml (3 x 10^8 cells) of a nalidixic acid resistant (Nal^R) derivative of *S.* Enteritidis P125109 to facilitate enumeration. At days 1, 2, 3, 4, 7, 14, 21 and 28 post challenge inoculation cloacal swabs were collected from all birds for semi quantitative assessment (spot plate counting) of the challenge *S.* Enteritidis Nal^R by plating on BG agar supplemented with nalidixic acid (20 µg/ml) and novobiocin (1 µg/ml). At 9 weeks birds' age (28 day post challenge inoculation) all birds were humanely killed and their caecal contents were collected for semi quantitative assessment of the challenge in birds' caeca.

Table 6.1: Experiment-1of vaccination and challenge regimen

Birds Age	*In vivo* inactivated vaccine group	*In vitro* inactivated vaccine group	Control group
Day 1	0.1ml Aviguard orally	0.1ml Aviguard orally	0.1ml Aviguard orally
Day 5	0.1 ml *in-vivo* inactivated vaccine; i.m	0.1 ml *in-vitro* inactivated vaccine; i.m	0.1 ml nutrient broth i.m
	0.1 ml *in-vivo* inactivated vaccine; orally	0.1 ml *in-vitro* inactivated vaccine; orally	0.1 ml nutrient broth orally
Week 3 (Day 21)	0.1 ml *in-vivo* inactivated vaccine; i.m	0.1 ml *in-vtro* inactivated vaccine; i.m	0.1 ml nutrient broth i.m
	0.3 ml *in-vivo* inactivated vaccine; orally	0.3 ml *in-vtro* inactivated vaccine; orally	0.3 ml nutrient broth orally
Week 5 (Day 35)	Challenge with 0.3ml ($5x10^8$) live S.E orally	Challenge with 0.3ml ($5x10^8$) live S.E orally	Challenge with 0.3ml ($5x10^8$) live S.E orally
Cloacal swabs' collection	Day 1, 4; 7, 14, 21 and 28 post challenge inoculation swabs were collected for *Salmonella* count.		
Caecal contents collection	At week 9 of age; birds were humanely killed for *Salmonella* count in caeca.		

6.2.5 Experiment-2:

The vaccination regime of this experiment was the same as experiment-1, except the challenge dose and challenge administration was 0.1 ml (1×10^6) bacterial cells; which was injected intravenously in to the wing vein (Timms *et al.*, 1994, Woodward *et al.*, 2002). The collected samples were liver and spleen tissues at particular days post-challenge inoculation and caecal contents on the last day as mentioned in Table 6.2. On the day of arrival, all 60 birds were vaccinated as experiment-1. Prior to challenge administration, a cloacal swab was taken from each bird and cultured for *Salmonella.* At five weeks of age all birds were challenged with 0.1 ml (1×10^6 cells) of live *S.* Enteritidis Nal^R intra-venously in to the wing vein. Then at day 1, 4, 6 and 8 post-challenge, 5 birds from each group were randomly caught, for post-mortem examination and removal of samples of liver and spleen (section 6.2.7) as well as caecal content collection. All samples' tubes were kept on ice until subjected to homogenisation and viable count (section 6.2.8).

Table 6.2: Experiment-2 of vaccination and challenge regimen

Birds Age	Inactivated vaccine *in vivo* group	Inactivated vaccine *in vitro* group	Control group
Day 1	0.1ml Avigard orally	0.1ml Avigard orally	0.1ml Avigard orally
Day 5	0.1 ml *in-vivo* inactivated vaccine; i.m	0.1 ml *in-vitro* inactivated vaccine; i.m	0.1 ml nutrient broth i.m
	0.1 ml *in-vivo* inactivated vaccine; orally	0.1 ml *in-vitro* inactivated vaccine; orally	0.1 ml nutrient broth orally
Week 3 (Day 21)	0.1 ml *in-vivo* inactivated vaccine; i.m	0.1 ml *in-vitro* inactivated vaccine; i.m	0.1 ml nutrient broth i.m
	0.3 ml *in-vivo* inactivated vaccine; orally	0.3 ml *in-vitro* inactivated vaccine; orally	0.3 ml nutrient broth orally
Week 5 (Day 35)	Challenge with 0.1ml ($1x10^6$) Live S.E intra-venously		
Post-challenge Inoculation	Semi-quantitative bacterial count		
Day 1	5 birds from each group randomly selected, humanely killed and their caecal content, spleen and liver portions were collected for bacterial count		
Day 4	Same as Day 1		
Day 6	Same as Day 1		
Day 8	Same as Day 1		

6.2.6 Cloacal swabs processing:

Cloacal swabs were immersed in 2 ml selenite broth (Oxoid, CM0395), and on arrival in the laboratory they were mixed by vortex briefly then immediately streaked on BGA plates (Oxoid, CM0262) supplemented with nalidixic acid (20 µg/ml) and novobiocin (1µg/ml) using bacterial culture standard manner (spot plate counting). Then the swabs were left in selenite broth tubes for overnight incubation at 37^{o}C prior to plating on BGA, to encourage the growth of *Salmonella* and inhibit the growth of other flora.

6.2.7 Tissue processing:

All tissue samples were kept on ice until weighed and then proportional amounts (10 x weight expressed as volume) of PBS (pH 7.2) were added into

each tube. Liver and spleen portions were homogenised in a Griffiths tubes in PBS (pH 7.2) to obtain homogenous suspension (Barrow *et al.*, 1988) prior to dilution for counting.

6.2.8 Bacteriological Analysis:

The suspected *Salmonella* colonies on direct plating of BGA were subcultured into xylose lysine deoxycholate (XLD) plates (Oxoid, CM0469) and incubated at 37^{o}C overnight. Isolated colonies with black spots plus slide agglutination with O antiserum were indicative of *Salmonella*.

For experiment-2, the viable numbers of *S.* Enteritidis organisms in tissue samples (spleen and liver) and caecal contents were estimated using the method of spot plate counting as mentioned in Chapter-2.

6.2.9 Statistical Analysis:

For experiment-1, cloacal swabs were taken for culture from each bird 2 days prior to challenge inoculation to assure that birds were *Salmonella*-free (this also applied to experiment-2). The grown numbers of the *S.* Enteritidis (challenge) of the cloacal swabs on direct and enriched media at particular time-points were reported. The number and the percentage of positive birds for all groups were calculated. Excel software of Microsoft Office 2007 was used to make a linear chart for the challenge growth over the tested time-points and chi-square tests and Fisher's exact *P* tests were considered to assess the different group positivity to *Salmonella* culture.

As for experiment-2, again the grown numbers of the *S.* Enteritidis (challenge) of the tissue (spleen and liver) samples as well as caecal content on BGA plates at particular time-points were reported. The average (Mean), standard deviation (SD), standard of errors (SE) and the *P* value of each group compared to the control were determined for all groups. Statistical significance was assessed by using student's paired *t* test, and a *P* value of < 0.05 was considered significant (Excel, Microsoft Office 2007).

6.3 Results

6.3.1 Experiment-1

The effect of both killed inactivated vaccinations (grown at *in vivo* or *in vitro* environments) on the faecal excretion of *S.* Enteritidis (orally challenged) in treated birds compared to the control group are presented in Table 6.3. Moreover the positivity percentage, which is the percentage of positive birds' swabs according to enriched selenite broth, XLD media and slide agglutination tests, was also presented in the same Table.

The percentage of chickens excreting *Salmonella* in their faeces was 40-45 % on day 1 post-challenge inoculations for all groups. On day 4 post-challenge inoculation the percentage of positive birds were 45%, 55% and 50% for *in vivo*, *in vitro* and control vaccine groups respectively. On day 7 post-challenge inoculations the percentage of positive birds' faecal excretion was 60% for the *in vitro* vaccine group and 50% for the *in vivo* and control vaccine groups (Figure 6.1). On day 14 post-challenge inoculation the percentage of positive birds' were 30%, 20% and 15% for *in vivo*, *in vitro* and control vaccine groups respectively. On day 21 post-challenge inoculations the percentage of positive birds was 15 % for the *in vitro* vaccine group and 20% for the *in vivo* and control vaccine groups (Figure 6.1). On the last day of cloacal swabbing (day 28 post challenge inoculation) the percentage of positive birds was 15%, 10% and 20% for *in vivo*, *in vitro* and control vaccine groups respectively.

Collectively, the cloacal swabs results indicate that the comparison of *in vivo* preparation versus control (X^2 = 7.06; P = 0.2) or *in vitro* preparation versus control (X^2 = 5.68; P = 0.3) using chi square analysis showed no significant difference.

No *Salmonella* were cultured from the caecal swabs of the vaccinated groups on day 28 post-challenge inoculation (< 1 x10^2 cfu/ml) as shown in Table 6.3; while the control group exhibited 35% positivity to *Salmonella* growth.

Table 6.3: Protective effect of inactivated *S.* Enteritidis vaccines against faecal excretion by chickens of a virulent *S.* Enteritidis (Nal^R) strain (challenge), inoculated orally. The ≥50 and ≥1= colonies of the challenge on direct culture plates and E = *S.* Enteritidis (Nal^R) isolated by selenite enrichment broth, XLD plates or identified serological agglutination test.

Percentage of chickens (20 birds per group) excreting *S.* Enteritidis (orally-challenged) after:										
Samples	Age (days)	Inactivated *in vivo* vaccine (%)			Inactivated *in vitro* vaccine (%)			Control group		
		≥50	≥1	E	≥50	≥1	E	≥50	≥1	E
Cloacal swabs	1	0	0	40	5	35	45	0	20	45
	4	0	25	45	0	10	55	0	25	50
	7	0	30	50	0	5	60	5	5	50
	14	0	0	30	5	5	20	5	10	15
	21	0	0	20	0	0	15	5	15	20
	28	0	0	15	0	0	10	0	15	20
Caecal contents	28	0	0	0	0	0	0	5	20	35

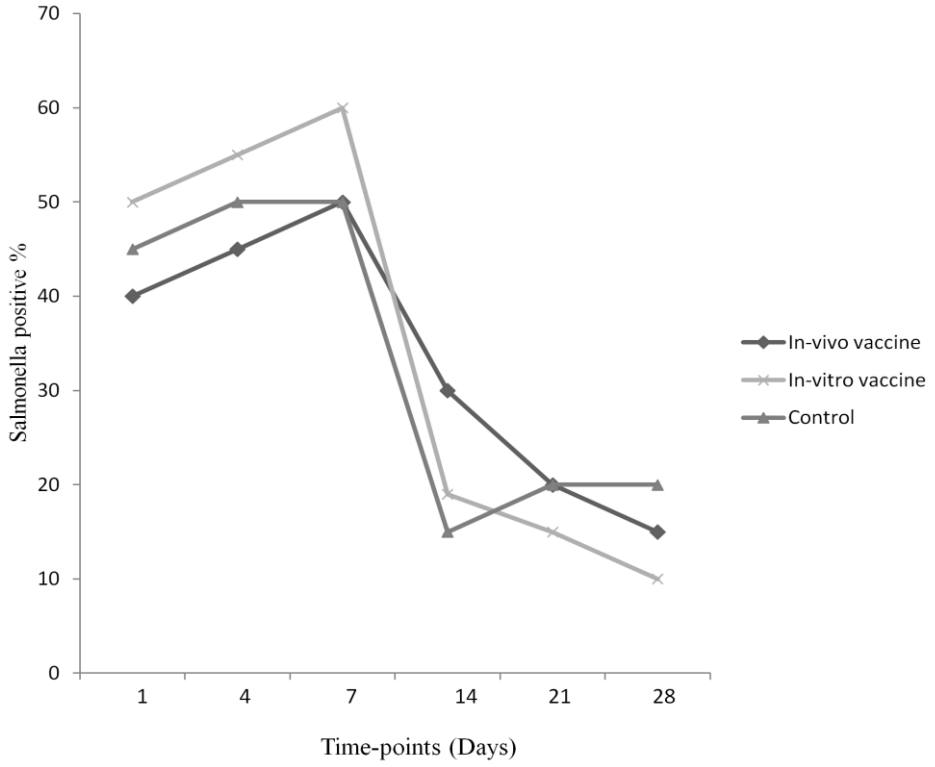

Figure 6.1: The percentage of chickens excreting the challenge *Salmonella* in the faeces according to cloacal swabs in the three groups of birds at particular days of post-challenge inoculation.

6.3.2 Experiment-2:

The effect of both killed inactivated vaccines (grown under *in vivo* or *in vitro* environments) on the internal organs tissues invasion/colonisation (spleen and liver) of intravenously challenged *S.* Enteritidis in birds compared to the control group was shown in Tables 6.4 and 6.5 respectively.

The viable bacterial numbers in the spleen at 1-day post intravenous challenge were 5, 4.7 and 4.8 $\log_{10}$ cfu/ml in the *in vivo, in vitro* and control vaccinated groups respectively (Figure 6.2). On days 4 and 6 post-challenge the two vaccinated groups showed similar *Salmonella* colonisation pattern in the spleen but unexpectedly significantly higher than the control group (P = 0.02 and 0.04) respectively. On day 8 post-challenge inoculation, *Salmonella* colonisation in the spleens of the *in vivo* vaccinated group declined by 0.4

log_{10}, while *Salmonella* colonisation in the *in vitro* vaccinated group showed no change compared to day-6. In contrast the control group showed *Salmonella* growth increase of 0.5 log_{10} in spleen on day 8 post-challenge.

Table 6.4: The protective effect of inactivated vaccines of *S.* Enteritidis against infection of the spleen in chicks challenged intravenously by the parent strain. Log_{10} mean viable counts / ml of homogenised spleen tissue.

Days post-challenge inoculation	Inactivated *in vivo* vaccine			Inactivated *in vitro* vaccine			Control		
	Aver (log_{10})	SE	*P*	Aver (log_{10})	SE	*P*	Aver (log_{10})	SE	P
1	5.0	0.04	0.23	4.7	0.2	0.7	4.8	0.15	-
4	5.3	0.15	0.12	5.2	0.1	0.2	4.8	0.19	-
6	4.9	0.13	0.02	4.9	0.2	0.04	3.6	0.48	-
8	4.5	0.6	0.6	4.9	0.3	0.05	4.1	0.13	-

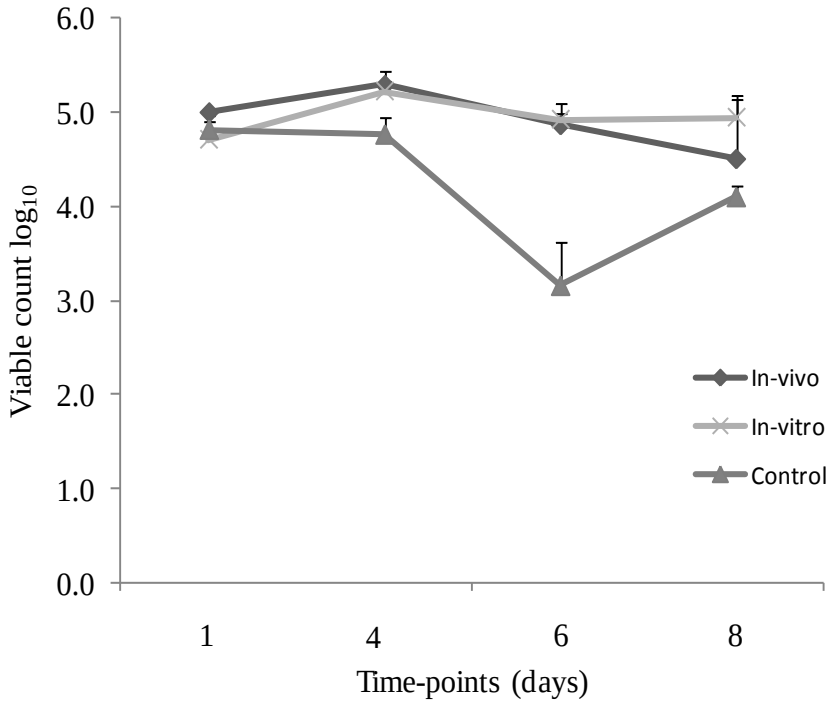

Figure 6.2: The number of *Salmonella* log_{10} (cfu/ml) in spleen tissue in the three groups of birds (*in vivo*, *in vitro* vaccinated and control groups) at particular days of post-challenge intra-venous inoculation.

In the liver, *Salmonella* counts were 3.8, 4.0 and 3.5 $\log_{10}$ (cfu/ml) in *in vivo, in vitro* and control vaccinated groups respectively at 1-day post-challenge (Figure 6.3). On day-1, *in vitro* vaccinated birds exhibited significantly higher ($P < 0.05$) *Salmonella* colonisation in their liver tissue compared to the control group birds. In general all groups tested showed variable gradual decline of *Salmonella* colonisation in liver from day 1-to-day 8 (Table 6.5; Fig 6.3). On day-4, *in vivo* vaccinated birds exhibited significantly higher ($P < 0.05$) *Salmonella* colonisation of the livers compared to the control group birds. By day 8 post inoculation, *Salmonella* colonisation in liver scored 2.1, 2.2 and 2.4 $\log_{10}$ (cfu/ml) at *in vivo, in vitro* and control vaccinated groups respectively as shown in Figure 6.3. No *Salmonella* were cultured from the caecal swabs of the vaccinated and control groups (< 1 $x10^2$ cfu/ml) at four time-points post-challenge inoculation.

Table 6.5: The protective effect of inactivated vaccines of *S.* Enteritidis against infection of the liver in chicks challenged intravenously by the parent strain. Log_{10} mean viable counts / ml of homogenised liver tissue.

Days post-challenge inoculation	Inactivated *in vivo* vaccine			Inactivated *in vitro* vaccine			Control		
	Aver (log_{10})	SE	*P*	Aver (log_{10})	SE	*P*	Aver (log_{10})	SE	*P*
1	3.8	0.17	0.07	4.0	0.12	0.03	3.5	0.07	-
4	3.6	0.13	0.02	3.1	0.16	0.29	2.7	0.24	-
6	2.6	0.16	0.12	3.0	0.09	0.002	2.2	0.12	-
8	2.1	0.06	0.3	2.2	0.15	0.61	2.4	0.25	-

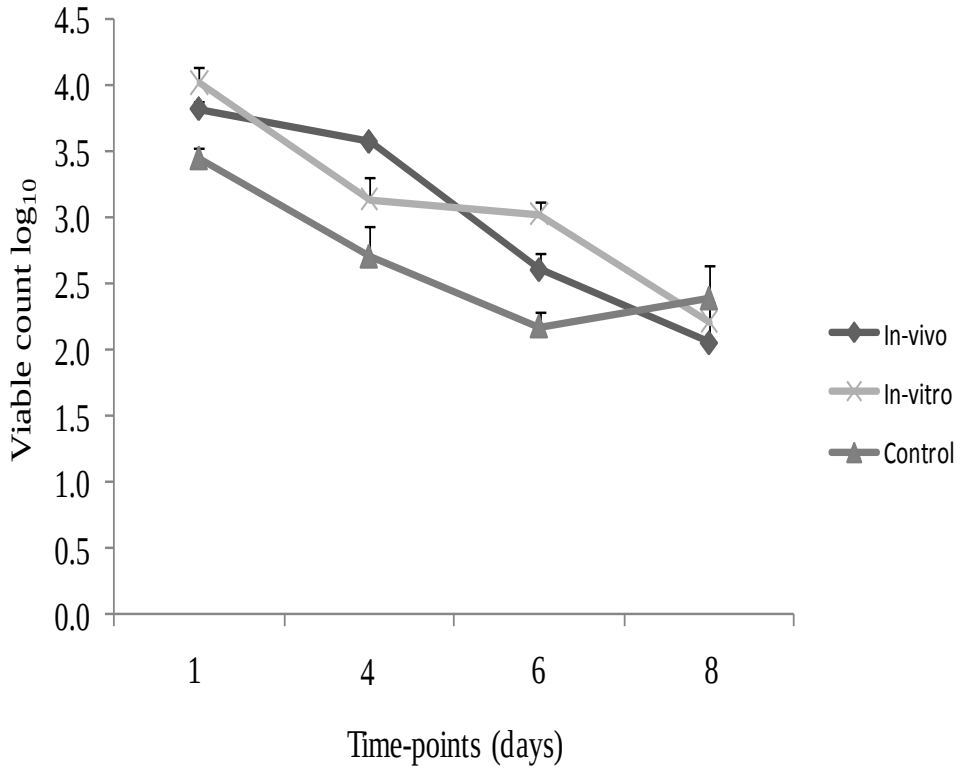

Figure 6.3: The number of *Salmonella* $\log_{10}$ (cfu/ml) in liver tissue in the three groups of birds (*in vivo*, *in vitro* vaccinated and control groups) at particular days of post-challenge intra-venous inoculation.

6.4 Discussion:

Control of *Salmonella* in poultry has been achieved largely through a combination of hygiene and management of extensive vaccination of laying flocks, as advocated in EU legislation [Council Directive 92/117, Commission of the EU Communities, 1992]. Live, attenuated and inactivated vaccines are also currently used in several countries. However, the live vaccines registered for use in EU have been produced by chemical mutagenesis, contain undefined mutations and antibiotic resistance. As mentioned in sections 1.10 and 6.1 the most extensively used inactivated vaccine is produced by culturing *Salmonella* bacteria under conditions of iron restriction on the basis that this will generate surface bacterial antigens required for iron uptake (Clifton-Hadley *et al.*, 2002).

It was hypothesised that *Salmonella* harvested directly from chicken caeca and which thus express antigens involved in colonisation (section 6.2.3) would be more immune-protective than bacteria cultured in nutrient broth *in vitro*.

The aim of this study was to assess the protective effect of a killed (inactivated) vaccine prepared in this way on point-of-lay pullets when challenged with the parent wild type strain of *S.* Enteritidis PT4. The challenge was given orally to assess *Salmonella* caecal colonisation by cloacal swabbing (experiment-1) and was injected intravenously to assess *Salmonella* systemic invasion to internal organs (e.g. spleen and liver) by tissue sampling (experiment-2).

The Aviguard gut flora product provided on day-1 is a mechanism to provide a mature gut flora that is naturally present in adult birds, because complete establishment of normal intestinal gut flora occurs only at an age between 2- 6 weeks (Barnes *et al.*, 1972). Then at day-5 and day-21 of age birds were vaccinated with relevant inactivated vaccination of *S.* Enteritidis in order to induce the humoral immune response of the birds in sufficient time before the challenge was administered orally or intravenously Cloacal swabbing has proven a useful semi-quantitative method for the estimation of caecal colonisation and faecal shedding of *Salmonella* in experimentally-infected

chickens (Smith, 1956, Smith and Tucker, 1975a, Cooper *et al.*, 1992, Allen-Vercoe and Woodward, 1999a). This was not completely the case for experiment-1 results, as direct bacterial counts made from caecal contents were compared at the end with bacteria recovered from cloacal swabs, there appeared little correlation with the semi quantitative measures determined by cloacal swabbing. This phenomenon is well known, and probably associated with intermittent caecal evacuation

The results of Experiment 1 showed that the vaccinatation with inactivated vaccines, whether prepared from *in vitro* or *in vivo* grown bacteria, had no protective effect against *Salmonella* faecal shedding compared to the control group ($P > 0.05$) when measured by cloacal swabs. Despite that the caecal swabs cultured on the last day of this experiment showed 35% of control group birds were positive for the infection while the vaccinated groups were negative ($< 1 \times 10^2$ cfu/ml). However, these inactivated vaccines were found ineffective in protecting the birds from faecal shedding. This may be due to destruction of *Salmonella* antigens during treatment with formaldehyde, or as a result of the fact that *Salmonella* proteins prepared from bacteria harvested from the chicken gut conditions showed evidence of enzymatic degradation from *in vivo* proteases (proteomic work performed by Barrow group).

In experiment-2, the results were unexpected in that the pattern of invasion of the challenge strain in the control group was slightly better with lower bacterial count than the two vaccinated groups. The obtained results for experiment-2 were somehow disappointing, as both vaccines did not exhibit a protective effect. This could be the result of loss of some antigens during the preparation of both vaccinations or due to a power cut that caused the temperature to decrease below 10^oC during the overnight prior to challenge inoculation day for few hours which could have had a variable critical effect on birds' immune status.

From the literature it was assume that the orally inoculated *S.* Enteritidis, as challenge, passed through the crop, with an acidic environment of pH 4-5, proventriculus and gizzard where the contents are much more acidic. Then

Salmonella pass through the small intestine before they reside and colonise in the large blind caeca that branch off from the distal ileum (Smith and Beal, 2008, Barrow *et al.,* 1988). Infection of the GI tract with wild-type *S.* Typhimurium results in an influx of heterophils and pro-inflammatory cytokines, chemokines IL-1β, IL-6 and chemokines "e.g. CXCLi" (Kaiser *et al.,* 2000). Therefore, it was thought that in experiment-1, the changes observed in the above immunological parameters were not the result of exposure to the vaccine strain but to the challenge infection.

Intra-venously inoculated *S.* Enteritidis challenge bacteria are removed rapidly from the blood by being ingested by macrophage or dendritic cells. The interaction between *Salmonella* and macrophages is the key to progression of systemic infection in both mammals and birds (Barrow *et al.,* 1994). *Salmonella* Pathogenicity Island (SPI 2) type three secretion system plays an essential role in *Salmonella* survival intra-cellularly (Hensel, 2000). The SPI 2 system injects its effectors into the host cells within the phagocytic vacuole of epithelial cells (SCV). The main effect of these effectors is to interfere with intracellular trafficking preventing fusion of the phagosome with lysosomes, though it also has effects on cytokine secretion and MHC (Cheminay *et al.,* 2005). Following the establishment of systemic infection the birds may clear or control the replication of bacteria. If replication is not controlled by innate immunity *Salmonella* replicates in liver and spleen resulting in pronounced hepatosplenomegaly, forming lesions in these organs.

In the work in experiment-2, no lesions were noticed in both organs apart from spleen slight enlargement in most birds; this could be indicative that the infection is under control for both the vaccinated and control groups. As the control group birds showed a similar pattern of challenge strain reduction up to 8 days post-infection, this is a clear indication that the host immune response is mediated by the challenge itself and the in-activated vaccines have no significant role in the immunity produced systemically.

In experiment-2, it is worthy to mention that no bacteria were detected in the caecal swabs of all groups. These findings are not in line with other findings in

which *S.* Enteritidis was shed in faeces by birds even though the challenge was given intravenously (Timms *et al.,* 1990, Timms *et al.,* 1994, Gast *et al.,* 1992, Woodward *et al.,* 2002). They suggested that the route of *Salmonella* colonisation clearance from deep tissues such as liver and spleen was via the gall bladder into the gastro-intestinal tract (Timms *et al.,* 1990, Woodward *et al.,* 2002). Woodward and others (2002) showed that the number of *Salmonella* detected in gall bladder samples were less in vaccinated groups than in unvaccinated control group. This surely means that after i.v inoculation most of the bacteria are removed from the blood probably by spleen and liver and do not reach the GI tract. This may correlate with the observation that biliary antibodies play a role in the clearance of *S.* Typhimurium from chickens (Lee *et al.,* 1981). Another reason for not detecting *Salmonella* in the caeca of experiment-2 birds challenged intravenously is that 8 days post-challenge inoculation might be still early for bacteria to reach the GI tract as the highest level of bacteria shed in the faeces of vaccinated and unvaccinated birds using similar method of challenge route was scored between 7-14 days (Woodward *et al.,* 2002). Therefore it is strongly recommend that future work is needed to detect bile antibodies in vaccinated and unvaccinated birds using our design of experiment. It is also recommend expanding cloacal samples analysis up to 21days post-challenge. Moreover, rethinking should be made about using an alternative way of inactivating *Salmonella* obtained from *in vitro* and *in vivo* conditions. Thinking is also needed about characterizing the humoral and cell mediated immunity of vaccinated and unvaccinated birds. This will enable us to assess the seroconversion of the birds after been vaccinated by measuring immunoglobulin titres using ELISA over the course of experiment (Cooper *et al.,* 1989).

The best immunogenic antigens for all bacteria are flagella and LPS (Hackett *et al.,* 1988, Van Amersfoort *et al.,* 2003). However, genes encoding these cellular components were down-regulated in chicken caeca (transcriptome results) and therefore a vaccine prepared in this way would contain low levels of these antigens while they were up-regulated in nutrient broth culture. LPS

would presumably be present as a major structural component. A serological examination is needed to prove this before performing next experiments.

As explained earlier in Chapter-1 several studies have suggested that live attenuated vaccies are more effective than bacterins in protecting birds from *Salmonella* infection (Babu *et al.,* 2003, Babu *et al.*, 2004, Curtiss and Hassan, 1996). However, because of the potential risks associated with the use of live attenuated vaccines, such as reversion to virulence (Hone *et al.,* 1988, Nnalue and Stocker, 1986), this option is not acceptable for many countries. Therefore, work on improving the efficacy of current available inactivated vaccines is needed. Many researches and field experiments were performed in the past in order to produce an effective inactivated vaccine against non-specific *Salmonella* serotypes in poultry flocks. Truscott (1981) immunised chickens with heated sonicates prepared from 6 different combinations of *Salmonella* serotypes and mixed with the feed and the extent of protection against the orally inoculated challenge was measured by the isolation of the challenge strains from cloacal swabs but protection was variable. For a vaccine to be of potential use in the field experimental results must show consistent, good protection. Salenvac and Salenvac®T vaccines (Intervet) are inactivated vaccines for *S.* Enteritidis and *S.* Typhimurium which have been introduced to the UK and other EU countries. Salenvac is a *S.* Enteritidis PT4 bacterin vaccine while Salenvac®T comprises inactivated *S.* Enteritidis and *S.* Typhimurium (bivalent inactivated vaccine). These organisms were grown under iron restriction, which reflects the host environment by iron limitation ((Wooldridge and Williams, 1993) and these factors may enhance the efficacy of the vaccine. However Clifton-Hadley and others (2002) assessed the efficacy of Salenvac ®T vaccine against orally challenge of *S.* Typhimurium in commercial broilers. The chicks were vaccinated at 1 day and 4 weeks of age. Birds were challenged at 8 weeks of age with a high (5×10^8) or low (2×10^6) doses. Following oral challenge with the low dose the counts of *S.* Typhimurium in the caecal contents were lower in vaccinated birds than unvaccinated controls. On the other hand oral challenge with the high dose of *S.* Typhimurium exhibited similar numbers of challenge organisms in faeces

and deep tissues such as liver and spleen. This could apply to the experiment-1 results in which the *S.* Enteritidis challenge dose was 3 x 10^8 cells. It is likely that the challenge doses of highly invasive and virulent *S.* Typhimurium or Enteritidis were sufficiently high to be overcome by immune system components associated with systemic or enteric phase of infection. Woodward and others (2002) assessed the efficacy of Salenvac vaccine against intravenous *S.* Enteritidis challenge in laying hens. They provided convincing evidence that the vaccine is efficacious and may contribute to the reduction of layer infection and egg contamination (Woodward *et al.,* 2002). They used the same strain of *S.* Enteritidis PT4 which used for the vaccination experiments [strain number 125109 (Barrow, 1991)], but the main differences between the vaccination experimental regimes and theirs are the method of growing and generating inactivated vaccines; they used 5800 commercial layer chicks, the Aviguard natural gut flora on day one was not provided, the birds were vaccinated on day one and week four with intramascular route only, their challenge dose size was 5-7.5 x 10^7cfu/ml, the birds were challenged at 8, 17, 23, 30 and 59 weeks of age. Birds' weight was assessed after challenge inoculation at different time-points of age. Cloacal swabs were collected from the infected birds at 1, 3, 7, 10, 14 and 21 day post challenge inoculation for *Salmonella* viable count detection. Egg production rates and bacteriological analysis of eggs were considered. However the intravenous challenge model used bypassed all mucosal barriers and did not represent whatever the probable field challenge. It may be argued that this model of challenge reflect the systemic phase of natural infection (Muir *et al.,* 1998).

Chapter - 7**: General Discussion**

7.1 Discussion

The last two decades of the 20th century witnessed an epidemic of human infections mediated by *S.* Enteritidis. Poultry and poultry products were considered as the primary source of human infection and it was recognised that measures to control infection in poultry flocks worldwide were required urgently (Anon, 1988). Improved biosecurity, hygiene measures, competitive-exclusion, antibiotics, vaccines, serological and bacteriological monitoring of poultry and its products were used to control the infection (Cooper *et al.*, 1989, Mead and Barrow, 1990). Therefore, during the last decade of the 20th century the incidence of human *S.* Enteritidis infection in the UK declined (Anon, 2001). Despite this, *S.* Enteritidis and S. Typhimurium remain a significant threat to human health and *S.* Enteritidis remains the dominant *Salmonella* serovar in flocks of laying hens in many European countries (EFSA., 2007). Intestinal colonisation is a major component of entry into the human food chain, either through carcass contamination or preceding systemic infection and subsequent egg contamination. The mechanism whereby the *S.* Enteritidis serovar colonises and interacts with the host in early stages of infection is still poorly understood.

The aim of the project was to define the transcriptome for *S.* Enteritidis during colonisation of the caeca of the chicken and to identify genes associated with colonisation using microarray technology (Chapter-3). Accordingly, the role of some metabolic pathways and respiratory genes that were up-regulated in colonisation of chicken caeca were assessed. Genes belonging to carbon source utilisation and TCA respiration pathways (Chapter-4) and osmoprotection (Chapter-5) were selected, mutated and their role in colonisation in chicken gut was assessed. Finally, in Chapter-6, an assessment of the immunogenicity of *S.* Enteritidis harvested from chicken caeca as a candidate killed vaccine in comparison to one generated from bacteria harvested from *in vitro* culture was also assessed.

Understanding the pattern of bacterial gene expression during the pathogen-host interaction is an important goal for many researchers who are interested in bacterial pathogenesis, physiology and host immune responses to infectious agents. An appreciation of the limitations of studying substitute *in vitro* signals as the cues for controlling gene expression has led researchers to explore genetic approaches which could define "*in vivo* induced genes" for pathogens (Mahan *et al.*, 1993a, Mahan *et al.*, 1993b, Camilli *et al.*, 1994, Valdivia and Falkow, 1997). However, reproduction *in vitro* of the *in vivo* environment is difficult because the parameters selected may be incomplete. In this study, the gene expression (transcriptome) *in vivo* and *in vitro* during comparable growth phases was measured and assessed whereas this is a dynamic situation *in vivo*.

The *S.* Enteritidis PT4 transcriptome in one-day chicken caeca using whole genome DNA microarrays was generated and analysed. The availability of the complete and fully annotated sequence of *S.* Enteritidis P125109 (Thomson *et al.*, 2008) plus the advent of microarray technology formed the basis of a custom microarray design (Agilent).

For microarray-generated data to be fully quantitative, a control containing a known amount of all mRNA species is required, which is logically not possible/practical, but a substitute correction factor, generated by labelling and hybridization with genomic DNA, has proved useful for obtaining measures of relative transcript abundance (Wei *et al.*, 2001, Li *et al.*, 2002). In practice, most researchers used control RNA from cells in a well-defined physiological state (e.g. mid-logarithmic phase of growth curve), so that conditions appropriately reflect the 'ground state' for their relative experimental variables (Conway and Schoolnik, 2003). However, the difficulty with using mid-log phase RNA as a reference is the absence of expression of genes associated with the remaining growth phases (lag, stationary and decline growth phases).

RNA amplification was used as a method to expand very small RNA samples so that there would be enough material for array hybridization. The Message Amp^{TM} II Prokaryotic Amplification kit was found to be a powerful tool in

amplifying the tiny amounts (as little as 50 ng) of *Salmonella* RNA to more than 100x amplification for both *in vitro* & *in vivo* extracted RNA.

It was obvious that *S.* Enteritidis behaved differently in the caeca of the newly hatched chicken in comparison with a mid-log phase nutrient broth culture grown aerobically. This is an expected conclusion given the differences in temperature, osmolarity, proximity of host tissue with its many innate immune factors, beside the availability of very different panoply of carbon and nitrogen sources and other nutrients. This has been found previously for the gut and other host environment during experimental infection with *S.* Typhimurium, *E. coli, V. cholerae* (Eriksson *et al.*, 2003, Bower *et al.*, 2009, Nielsen *et al.*, 2010, Liu *et al.*, 2011, Liu *et al.*, 2001a).

The array data indicated the possibility that type 1 fimbriae (*fim*A or SEF21) and other fimbrial subunit genes encoded by *peg*A, *std*A, *lpf*A (SEF14) could play a major role in chicken caecal colonisation. These appendages are thought to be involved in physical attachment of *Salmonella* and *E. coli* to host mucosal layer or even epithelial cells (Gophna *et al.*, 2001, Edelman *et al.*, 2003, Morgan *et al.*, 2004, Snyder *et al.*, 2004). Moreover, the involvement of SPI genes confirms previous mutational studies (Turner *et al.*, 1998, Morgan *et al.*, 2004) which suggest an intimate relationship with the mucosa during colonisation.

One of the main conclusions also is that the majority of *S.* Enteritidis PT4 genes (Thomson *et al.*, 2008) were found up-regulated in chicken caeca. These include: type three secretion system effectors (invasion-associated secreted effector protein *sopE*$_2$, and putative virulence effector protein *sif*B) and fimbrial proteins *peg*A. This is a good indication that these genes might play an important role in caecal colonisation.

Two pathways of particular interest were subjected to mutational experiments. These were carbon source utilisation and respiration and the responses to osmotic pressure. 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 colonise the intestinal niche.

The mutation of genes associated with carbon source utilization had a limited effect on colonisation probably reflecting the redundancy associated with the modular system used by enteric bacteria in respiration whether utilizing oxygen or other alternative electron acceptors as indicated by the up-regulation of such genes (Table 3.3 and Appendix Table).

In contrast, a number of genes and transport systems associated or thought to be associated with responses to high osmolarity were highly up-regulated *in vivo* and showed reduced competitiveness *in vivo* (but not *in vitro*). Osmolarity has been found previously (Dorman *et al.,* 1989, Bower *et al.,* 2009, Ni Bhriain *et al.,* 1989) to be important in gut colonisation although this has not previously been reported for the chicken.

Bacteria in the one-day chicken gut have a rich medium for nutrition whether from the remains of the yolk sac, which is rich in sulphur-rich proteins, or from the gut mucous membrane which is rich in mucin and polysaccharide. This was reflected by the gene expression pattern (Chapter 3), in which *Salmonella* was able to utilize a wide range of amino acids (such as tryptophan, threonine, isoleucine, leucine, lysine, methionine, allantoin, asparagines, aspartic acid, proline and glutamine) and carbohydrates as energy carbon, nitrogen and trace element source in chicken gut compared to the *in vitro* model (Table 3.3).

Facultatively anaerobic bacteria respond to the absence of oxygen as occurs in the caecal contents by replacing aerobic respiratory pathways with anaerobic respiratory or fermentative pathways, depending on the availability of different electron acceptors. Anaerobic growth of bacteria on non-fermentable carbon sources requires substances that can function as terminal electron acceptors for respiration (e.g. fumarate, nitrate, nitrite, trimethylamine *N*-oxide "TMAO", dimethylsulphoxide "DMSO" (Iuchi *et al.*, 1986, Stewart, 1988, Price-Carter *et al.,* 2001, Srikumar and Fuchs, 2011). The mechanisms regulating the expression of these pathways are organised in a hierarchical manner such that in any specific environment the most energetically-favourable process is used (Spiro and Guest, 1990). The rapidly changing or heterogeneous environment in the caeca for *S.* Enteritidis is indictaed by the expression of many genes in these pathways.

In chapter 4, it was concluded that fumarate is an essential component in *S.* Enteritidis respiration in 1-day chickens' gut. The Dcu system is important for *S.* Enteritidis to transport di-carboxylic acid compounds during anaerobic respiration in the chicken gut. It was also assumed that some *S.* Enteritidis TCA generated mutants are able to adapt to different environmental conditions and redox tension by compensating with substitution of the function with alternative genes. For example anaerobic repression of *sdh*A was masked and partially compensated by the induction of *frd* (Guest and Russell, 1992); and the regulation of aerobic *fumA* was masked by anaerobic *fum*B (Woods and Guest, 1987, Hirsch *et al.,* 1963, Adsan *et al.,* 2002, Steinsiek *et al.,* 2011).

In chapter 5 the results of investigating osmoregulation genes were compatible with recent work (Du *et al.,* 2011b), which indicated the importance of the alternative sigma factor RpoE or sigma factor RpoS in co-regulating of many other osmoprotectant producing genes in hyper-osmotic environments. This could explain why the *rpo*E mutant behaved differently compared to the rest of osmoprotectant mutants. The results indicated that the *rpo*E is required for *S.* Enteritidis optimal growth when exposed to high osmolarity as mentioned by others (McMeechan *et al.,* 2007).

There is no doubt that trehalose, proline, betaine and potassium compounds are important as osmoprotectants for *S.* Enteritidis in the chicken gut, as a number of genes associated with response to high osmolarity and relevant to synthesis or transportation of these osmoprotectants were both highly up-regulated *in vivo* and showed reduced competitiveness *in vivo* (but not *in vitro*). This was reported previously for the mouse model (Dorman *et al.,* 1989, Bower *et al.,* 2009); but it is the first time it is reported for chickens.

Collectively, the results in Chapter 4 and 5 indicated that the pattern of competitive exclusion *in vivo* was very different to that observed *in vitro* (NB culture). This is hardly surprising given the differences in osmolarity, proximity of host tissue with its many innate immune factors, plus availability of very different nutrients as carbon and nitrogen sources. 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.*, 1987a, Berchieri and Barrow, 1991) and although the use of stationary phase cultures has been used to study the contribution of respiration genes to colonisation, it has been found to be inadequate (Zhang-Barber *et al.*, 1997, Berchieri and Barrow, 1990). Bacteria growing in chicken gut are exposed to fluctuation in environmental stresses such as oxygen tension (oxidative stress), bile salts, fluctuating pH, antibacterial peptides and others, while bacteria growing in the *in vitro* model are under controlled and known environmental and nutritional parameters.

Therefore, the *in vitro* model of competitive-exclusion is not representative of *in vivo* colonisation inhibition. This was reflected by the different pattern of inhibition for all generated individual independent mutants (TCA and osmotic) in both conditions.

Vaccination has already proven to be efficient in laying hens, reducing faecal shedding and internal egg contamination of *Salmonella* leading to a reduction in the number of cases of human salmonellosis (Gantois *et al.*, 2006, Collard *et al.*, 2008).

In Chapter 6, two vaccination experiments were performed mainly to compare inactivated *S.* Enteritidis vaccines (grown in *in vivo* & *in vitro* environments) for their efficacy in preventing the parent challenge strain from colonising the gut (experiment-1) or invading internal organs (experiment-2). The vaccination of poultry has become one of the most important measures to control *Salmonella* infections of the birds because of the costs, impracticability and disadvantages of the other approaches mentioned in Chapter-1. It is well documented that live vaccines produce better protection than killed vaccines. Killed vaccines have been examined with varying results and mainly stimulate antibody production (Barrow *et al.*, 1996a, Chatfield *et al.*, 1993). Killed vaccines may also lead to poor immune protection due to the destruction of relevant antigens during vaccine preparation and therefore rapid destruction and elimination of the vaccine from the inoculated animals is very likely (Barrow, 1991). Commercial killed vaccines available on the market have

limited success in protecting laying hens against food-poisoning *Salmonella* infection. This is because these vaccines present only those antigens that were induced under the conditions of the *in vitro* fermentation process (Barrow and Wallis, 2000). Therefore, killed vaccine for *S.* Enteritidis PT4 was produced by formalising bacteria that had been harvested directly from chicken caeca. We thought that bacteria grown in the *in vivo* environment might present antigens representative of such environmental stress conditions and which might therefore stimulate antibodies to these relevant antigens which would therefore be more protective.

Unfortunately both vaccine regimes used were ineffective in protecting the birds against oral or intravenous administrated challenge and this might be due to the destruction of antigens with formaldehyde or by protease enzymes for the *in vivo* produced vaccine. To overcome this problem, the chemical agent used to produce these vaccines could be replaced by phenol or acetone (Gast *et al.,* 1992) after growing these bacteria under *in vivo* mimic conditions instead of chicken gut in order to avoid protease destructive enzymes.

In experiment-2, it is worth mentioning that no bacteria were detected on the caecal swabs of all groups that were challenged intravenously. These findings are not in line with other author's findings in that *S.* Enteritidis was shed in the faeces by vaccinated and unvaccinated control birds (Timms *et al.,* 1990, Timms *et al.,* 1994, Gast *et al.,* 1992, Woodward *et al.,* 2002). Because they suggested that the route of *Salmonella* clearance from deep tissues was via the gall bladder into the gastro-intestinal tract but other routs may also exist. Woodward and others (2002) reported lower number of *S.* Typhimurium in vaccinated chicken gall bladder than the unvaccinated control birds. This finding was correlated with the observation that biliary antibodies play a role in the clearance of *S.* Typhimrium from chickens (Lee *et al.,* 1981), therefore it was suggested that it is worthy to measure the immune response in the gall bladder using ELISA.

Characterising the humoral immunity of vaccinated and unvaccinated birds is very important. This should be applied to blood, liver, spleen and gall bladder

before and after vaccination. This will enable us to assess the seroconversion at different stages of the birds' life because humoral immunity plays a significant role in *Salmonella* clearance in systemic infections (Beal *et al.*, 2006).

Salmonella flagellin represents one of the most relevant antigens for the generation of protective immunity in mice (Hackett *et al.*, 1988) and strong inducer of inflammatory cytokines *in vitro* cultured human mononuclear cells (Wyant *et al.*, 1999) while the lipopolysaccharides, LPS represents the main surface antigens of Gram-negative bacteria (O-antigen) which possesses the binding sites for the antibodies (Van Amersfoort *et al.*, 2003). Therefore LPS is important in the recognition and elimination of bacteria by the host immune system (Morrison and Ryan, 1992). Because the majority of flagella and LPS-encoded genes were down-regulated in chicken caeca this may explain the poor immunogenicity of the bacteria harvested directly from the gut. By contrast the majority of flagella and LPS-encoded genes were up-regulated in the *in vitro* environment. However serology is required to study this further.

As mentioned above it is advisable to generate inactivated *S.* Enteritidis vaccine after being grown in conditions which mimic the *in vivo* environment. These conditions should reflect the environment of chickens' caecal environment. This is mainly to avoid the destruction of representing proteins by host proteases. These factors or parameters can be applied according to the transcriptome obtained for *S.* Enteritidis, presented in Chapter-3, mainly the significantly up-regulated genes during its growth in chicken caeca (Table 3.3 & Appendix Table). The parameters should include NaCl, bile salts, caeca equivalent-pH, right temperature, microaerophilic or anaerobic environments simultaneously, plus providing carbon and nitrogen sources (propnediol, tetrathionate and trehalose) for bacteria to grow.

7.2 Future Work:

1. Mutate other TCA and osmotic associated genes that were up-regulated *in vivo* and not been tested in the research. The TCA genes include: citrate synthase *glt*A, aconitate hydratase *acn*A, malate dehydrogenase *mdh*, aspartate ammonia-lyase *asp*C; while the osmotic genes include: putative cytoplamic protein *yci*G, osmotic stress proteins *yci*EF, *dps*, *osm*Y, *osm*C, *osm*E and glycerol-3-phosphate-binding periplasmic protein *ugp*B. Then subject these generated mutants to competitive-exclusion experiments at both environments *in vitro* and *in vivo* models.
2. Consider also testing these mutants in conditions which mimic the *in vivo* environment before they are tested in chicks would reduce animal usage. Such environmental parameters should include NaCl, bile salts, caeca equivalent-pH, microaerophilic or anaerobic environments simultaneously.
3. Performing further mutational studies for a wider range of *S.* Enteritidis PT4 unique genes, (Thomson *et al.*, 2008), which are found up-regulated in chicken caeca. These genes include: effector protein *sopE*$_2$, putative virulence effector protein *sif*B and fimbrial proteins *peg*A.
4. The phenotypes of trehalose synthase *ots*A and trehalose phosphatase *ots*B which exist in a single operon were identical in their osmotic sensitivity in glucose minimal medium (Giaever *et al.*, 1988). This was the case for *S.* Enteritidis grown *in vitro* (but not the *in vivo* model). Further investigation is therefore needed to understand this difference.
5. Consider making double mutants of *sdh* and *frd* or including *mdh* to see how multiple mutations could affect the colonisation process.
6. Consider testing the attenuation of single and double mutants affecting *S.* Enteritidis TCA and osmotic responses in the chick model. It was performed previously for *S.* Typhimurium in mice (Mercado-Lubo *et al.*, 2008).
7. Design vaccination experiments as explained in Chapter 6 to test inactivated *Salmonella* vaccines generated by the use of other

inactivating chemicals such as phenol. Once the vaccines are generated they should be evaluated for the presence of O and H antigens.

8. Strongly recommend future work on vaccination to consider using ELISA as an approach to detect/monitor and measure circulating IgG in blood, liver, spleen and bile; and secretory IgA in gastro-intestinal tract for vaccinated and unvaccinated birds.

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