Investigation of the molecular basis of variation in food chain related stress response of *Salmonella enterica*

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Abstract

Non-Typhoidal *Salmonella* is an important foodborne pathogen annually causing over 120,000 deaths worldwide. Cases and outbreaks of *salmonellosis* are typically associated with the consumption of contaminated animal products and more recently with a variety of other food types including fruits, vegetables, and confectionaries. Food manufacturers implement hurdle technology by combining more than one approach to prevent foodborne contamination, such as the use of preservatives, refrigeration, and high temperatures. *Salmonella* has the potential to evade these methods by invoking a stress response and can adapt to ever-changing environmental pressures. Multiple *Salmonella* strains are used simultaneously in a strain cocktail to assess survival during food challenge tests, but certain strains possess an increased resistance to stress, thus posing a greater risk to food safety. The variability of *Salmonella* survival in stresses relevant to food production was assessed to identify strains of importance and to determine the molecular mechanisms of stress tolerance. Sub-lethal heat treatment in a vegetarian food matrix, survival in desiccated conditions and growth in the presence of NaCl and organic acids were all investigated in a variety of *Salmonella* strains. *S.* Gallinarum strain 287/91 and *S.* Typhimurium strain SO1960-05 exhibited an increased sensitivity to stress. Variation in the survival of strains in stress conditions, indicated that response to stress is strain specific. A whole genome functional screen using transposon directed insertion site sequencing (TraDIS) contained 610,000 unique insertions in *S*. Typhimurium ST4/74 and was used to identify conditionally essential genes. Single gene knockouts of conditionally essential genes were constructed in ST4/74 to validate TraDIS results. An understanding of the diverse metabolic capacities of *Salmonella* strains, genes with central roles in food chain stress response, and phenotypic variation will result in a verified selection of target strains for process validations, improved processing, and more reliable risk assessments.

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Abbreviations

ANI Average Nucleotide Identity

ANOVA Analysis of Variance

ARIBA Antimicrobial Resistance Identification by Assembly

ATP Adenosine Triphosphate

BLAST Basic Local Alignment Search Tool

BR Broad Range

CCM Cold-chain management

CDS Coding Sequences

CEAC Central/Eastern African Clade

CFU Colony Forming Unit

DAP Diamidophosphate

DNA Deoxyribonucleic Acid

dNTPs Deoxynucleotide Triphosphates

dsDNA Double Stranded Deoxyribonucleic Acid

GEC Global Epidemic Clade

HF High Fidelity

HPC High Performance Cluster

HS High Sensitivity

HSPs Heat Shock Proteins

IDM Insertion Duplication mutagenesis

IPTG Isopropyl β- d-1-thiogalactopyranoside

KEGG Kyto Encyclopaedia of Genes and Gnomes

KO KEGG (Kyto Encyclopaedia of Genes and Genomes) Orthology

LB Luria-Bertani

LPS Lipopolysaccharide

LR Log Ratio

LSD Least Significant Difference

NEB New England Biolabs

NTS Non-Typhoidal *Salmonella*

OD Optical Density

PBS Phosphate Buffered Saline

PCR Polymerase Chain Reaction

RNA Ribonucleic Acid

SGSC *Salmonella* Genetic Stock Centre

SNP Single Nucleotide Polymorphism

T3SS Type 3 Secretion System

TraDIS Transposon Directed Insertion Site Sequencing

UKHSA United Kingdom Health Security Agency

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Chapter One

Introduction

1.1 *Salmonella* Microbiology

Organisms belonging to the *Salmonella* genus are Gram-negative, rod-shaped, and non-spore forming. They are able grow in both aerobic and anaerobic conditions, known as facultatively anaerobic and are mostly motile, due to their flagella (Giannella, 1996). *Salmonella* possesses a ~4.8Mb genome, with a GC content of approximately 52% (Papanikolaou *et al*., 2009). Biochemically, *Salmonella* are oxidase negative and catalase positive (Ryan *et al.*, 2017). *Salmonella* can grow at a range of temperatures between 2°C and 54°C, however the optimum growth temperature is 37°C. Similarly, a wide range of pH's are suitable for growth of *Salmonella* (pH 3.8-9.5), although the optimum pH is between 6.5 and 7.5 (Jean-Yves, 1989).

The *Salmonella* genus belongs to the Enterobacteriaceae family and is further classified into two species, *Salmonella enterica* (*S*. *enterica*) and *Salmonella bongori* (*S*. *bongori*) (Eng *et al.*, 2015). *Salmonella enterica* is categorised into six subspecies on phylogenetic basis, which are *S. enterica* subspecies *enterica* (or I), *S. enterica* subspecies *salamae* (or II), *S. enterica* subspecies *arizonae* (or IIIa), *S. enterica* subspecies *diarizonae* (or IIIb), *S. enterica* subspecies *indica* (or IV) and finally, *S. enterica* subspecies *houtenae* (or VI). These six subspecies are further divided into more than 2650 serovars on antigenic basis, with 1547 serovars designated to the subspecies *enterica* alone, compared to only 22 serovars for *Salmonella bongori* (Issenhuth-Jeanjean *et al.*, 2014). Serovars belonging to subspecies I are adapted to mammals and avian species and are the causative agents of the majority of human cases of salmonellosis (Aviv *et al.*, 2014).

Salmonella is closely related to *Escherichia coli* (*E. coli*) and these two organisms are thought to have diverged over 100 million years ago (Ochman and Wilson, 1987). *Salmonella* was first discovered in 1885 by Daniel Elmer Salmon and Theobald Smith in the intestines of pigs showing signs of cholera disease (Fedorka-Cray *et al.*, 2000). *Salmonella enterica* is the best studied species of *Salmonella* and within this species, subspecies *enterica* receives the most attention due to the high proportion of human infections it causes, predominantly by serovars *S.* Typhimurium and *S.* Enteritidis (Grimont and Weill, 2007). The most commonly reported serovars infecting individuals from EU member states in 2016 were *S.* Typhimurium (including monophasic 1,4,[5],12:i:-) and *S.* Enteritidis (EFSA and ECDC, 2017).

1.2 Typhoidal and Non-Typhoidal *Salmonella*

Salmonella can be classified as either typhoidal or non-typhoidal *Salmonella* (NTS), depending on the pathology of the disease in humans. Typhoidal *Salmonella* includes *S. enterica* serovars Typhi and Paratyphi A, B and C, which cause an enteric fever called typhoid fever and paratyphoid fever, respectively (Crump *et al.*, 2015). Serovar Typhi is host restricted to humans and it can breach the intestinal barrier, travelling to the host's internal organs, such as the gall bladder, where it can survive and replicate (Dougan and Baker, 2014). Prevalence of typhoid and paratyphoid fever is high in low- and middle-income countries due to food and water contaminated with faecal matter (Mogasale *et al.*, 2014). In 2000, it was estimated that typhoidal *Salmonella* caused nearly 22 million illnesses and over 210,000 deaths worldwide (Crump *et al.*, 2004). The highest burden of disease occurs in developing countries with poor hygiene infrastructure, such as Africa and South-East Asia, however, individuals in high-income countries are still at risk of contracting salmonellosis due to contaminated food products and enteric fever associated with travel to countries where typhoid/paratyphoid fever is endemic (Crump *et al.*, 2004). Individuals can also contract typhoid fever if they are in contact with someone who sheds the bacterium in their faeces or onto food, such as the infamous Typhoid Mary who was an asymptomatic carrier of *S.* Typhi and unknowingly infected civilians whilst working as a cook in New York (Marineli *et al.*, 2013).

Non-typhoidal salmonellosis is notifiable in most European Union (EU) Member States and in the UK, the causative agent of salmonellosis, *Salmonella* spp., are notifiable to the UK Health Security Agency (UKHSA) as stated in the Health Protection (Notifiable) Regulation (SI 2010/659). In 2016, over 96,000 cases of salmonellosis were reported from EU member states and notification rates were highest in the Czech Republic and Slovakia (EFSA and ECDC, 2017). Incidence of salmonellosis usually presents itself as gastroenteritis and symptoms are usually self-limiting; diarrhoea, nausea, abdominal pains and fever (Crum-Cianflone, 2008). In healthy individuals, these symptoms usually manifest between 6 and 48 hours after ingestion of food and drink contaminated with *Salmonella.* The minimum infective dose is estimated to be between 10^5 and 10^{10} cells, depending on serovar and associated food type (McCullough and Eisele, 1951a; McCullough and Eisele, 1951b; McCullough and Eisele, 1951c; Eisele and McCullough, 1951). In severe cases, individuals may be hospitalised if bloody diarrhoea is present, indicative of an invasive blood stream infection, or if

dehydration occurs. Albeit rare, death can occur in immunocompromised patients if not treated effectively (Hardy, 2004).

1.3 *Salmonella* Nomenclature

Serovars are classified using the Kauffman-White scheme, which differentiates *Salmonella* subtypes according to their antigenic composition: phase 1 and phase 2 flagella (H), somatic (O) and occasionally capsular (Vi). The O-antigen is comprised of 5-6 sugar units that form a polysaccharide situated in the bacterial cell outer membrane in the form of a lipopolysaccharide. The O-antigen is encoded by *rfb* genes and the H-antigens are encoded by *fliB* and *fliC* genes (Achtman *et al.*, 2012). Each serovar is given an antigenic formula, written as O:H1:H2 and is unique depending on the agglutination reaction between antisera specific to epitopes situated on the antigens (Chattaway *et al.*, 2021). Currently, there have been 46 O-antigens and 114 H-antigens identified, which gives rise to >2600 unique serotypes (Issenhuth-Jeanjean *et al.*, 2014). Typing serovars using the Kauffman-White scheme can be inconsistent, especially when sub-typing biovars (or biotypes) due to biochemical differences, and the scheme hasn't been updated since 2007 (Grimont and Weill, 2007; Chattaway *et al*., 2021). Furthermore, it requires more than 150 specific antisera and highly trained personnel to phenotype isolates (Wattiau *et al.*, 2011). Molecular subtyping methods and phage typing can also be used to distinguish serotypes of *Salmonella* (Ricke *et al.*, 2013).

A nucleotide sequence-based method, termed multi-locus sequence typing (MLST), assigns a sequence type (ST) to each isolate according to seven species dependent housekeeping genes. MLST was first developed for use in *Neisserria meningitidis*, a Gram-negative pathogen which causes meningococcal disease, however more recently it was found to be advantageous in the categorisation of other pathogenic species (Maiden *et al.*, 1998). Isolates with identical housekeeping gene fragments will be assigned the same sequence type (Turner and Feil, 2007). In *Salmonella,* the seven housekeeping genes are *dnaN*, *hemD*, *hisD, aroC*, *sucA, purE and thrA* (Leekitcharoenphon *et al.*, 2012). More recently, whole genome sequencing (WGS) has been used to classify different *Salmonella* isolates into serovars based on their sequence type. Each year ~8,000 isolates are transferred to the *Salmonella* reference service (SRS) at the UKHSA from hospitals around the country to undergo whole genome sequencing. Whole genome sequencing replaced traditional typing methods at UKHSA (i.e Kauffman-White Scheme) in 2015 and is the current method used for surveillance of *Salmonella* infections (Ashton *et al.*, 2016).

1.4 *Salmonella* Pathogenicity

S. Typhimurium pathogenicity was first described in the 1960's using fluorescent microscopy in Guinea Pigs (Kent *et al*., 1966; Takeuchi, 1967). *S.* Typhimurium is often studied in mice to act as model organism for Typhoid infection in humans (Carter and Collins, 1974). In mice, *S.* Typhimurium passes through the intestinal mucosa from the lumen in the small intestine, via enterocytes or microfold cells (M Cells) (Takeuchi, 1967). The bacterial cells then enter the reticuloendothelial system, where they evade killing by neutrophils by residing inside macrophages and dendritic cells (Mills and Finlay, 1994). Specific virulence factors in *S.* Typhimurium enable the bacteria to survive and thrive in these stressful environmental conditions, primarily encoded on two pathogenicity islands, SPI-1 and SPI-2.

Salmonella strains possess a range of virulence factors making them pathogenic to a variety of host species. Most virulence genes are co-located in areas of the chromosome or plasmid and are termed *Salmonella* pathogenicity islands (SPI), which are acquired from bacteriophage or plasmids via horizontal gene transfer, and contribute to the evolution of the species (Que *et al.*, 2013). There have been over 10 SPI's characterised, however SPI-1 and SPI-2 are the most studied. *Salmonella* infects host intestinal epithelial cells by secreting effectors through a type III secretion system (T3SS) encoded on SPI-1 and in some serovars, *Salmonella* pathogenicity island 2 (SPI-2). SPI-1 can be situated on the chromosome or plasmid and contains approximately 40 genes, encoding T3SS-1, chaperone proteins, effector proteins and regulators. The T3SS-1 is comprised of proteins, including InvG, PrgH and PrgK, which work together to deliver effector proteins to the host (SPI-1 dependent invasion) (Que *et al.*, 2013). These effectors are translocated into the cytoplasm of the host cell and causes inflammation as a consequence of bacterial invasion (Hobbie *et al.,* 1997). SPI-1 is not present in *E. coli* (or related organisms) but is found in both *Salmonella* species and all serovars, indicating that SPI-1 was acquired from an ancestral lineage shared between all serovars after the divergence of *Salmonella* from *E. coli*, whereas SPI-2 is only present in S. *enterica*, which suggests that SPI-2 was acquired after the divergence of the two species (Groisman and Ochman, 1997). The SPI-2 T3SS comprises 44 genes and is essential for growth in different hosts and plays a role during both intestinal and disseminated infection (Bispham *et al*., 2001; Hansen-Wester and Hensel, 2001).

1.5 *Salmonella* Epidemiology

Ingestion of contaminated food caused almost 600 million cases of diarrheal disease and 420,000 deaths worldwide in 2010 and over half of these cases, where the aetiological agent could be identified, were caused by pathogenic bacteria such as *E.coli, Campylobacter* and non-typhoidal *Salmonella* (NTS) (WHO, 2015). It has been estimated that almost 94 million cases of gastroenteritis occur globally each year due to *Salmonella* species, 80.3 million of which were predicted to be foodborne, accounting for approximately 155,000 deaths annually (Majowicz *et al.*, 2010). Typically, food poisoning outbreaks are more commonly linked to animal products, such as meat and eggs, with poultry being one of the major reservoirs of pathogenic bacteria (~20% of cases) (Sanchez *et al.*, 2002). However, more recently there has been a significant increase in foodborne illnesses associated with fresh produce (Heredia and García, 2018).

1.6 Sources of *Salmonella* Contamination

Salmonella has been implicated as the causative bacterium in a variety of food poisoning cases relating to different types of food products. Traditionally, *Salmonella* was primarily thought to infect poultry and eggs, however, more recently a number of other meats and fresh produce have all been responsible for salmonellosis (Jarvis *et al.*, 2016). *Salmonella* is a zoonotic pathogen and can be passed onto humans via both livestock and domestic animals (Schofield, 1945). The bacterium usually lives in the intestinal tract of warm and cold blooded animals, and can be shed into the environment through defecation (Lamas *et al*., 2018). Enteropathogens, including *Salmonella*, can be found contaminating water sources used for crop irrigation. The bacteria often come from animal faeces, soil and sewage overflow, and it has been estimated that 71% of irrigation water in the UK comes from these surface water reservoirs (Tyrrel *et al*., 2006).

According to the Centres of Disease Control and Prevention (CDC), over 40% of foodborne illnesses in the US can be traced back to fresh produce being the source of infection (Painter *et al.*, 2013). Recent foodborne outbreaks of *Salmonella* linked to fresh produce include; pre-cut melon contaminated with *Salmonella* Adelaide infecting 77 people in multiple US states in 2018 (CDC, 2018a), Maradol papayas contaminated with various *Salmonella* serovars isolated from the fruit imported from Mexico, of which, *Salmonella* Anatum caused 1 death (CDC, 2017a) and cucumbers contaminated with *Salmonella* Poona in 40 US states in

2015/2016 resulting in 907 cases, 204 hospitalisations and 6 deaths (CDC, 2016). Fresh produce is a high-risk food commodity because it is commonly eaten raw or with minimal processing to eradicate pathogens. Infants, immunocompromised, elderly and pregnant individuals are at the highest risk of contracting a foodborne illness (WHO, 2015).

In 2005, an outbreak of S. Typhimurium in the UK and Finland was associated with iceberg lettuce imported from Spain that had been irrigated with wastewater (Takkinen *et al.*, 2005). *Salmonella* cells can adhere to plant material and penetrate the plants internal organs, leading to colonisation and suppression of the plant's immune system (Klerks *et al*., 2007; Schikora *et al.*, 2012). Fertilisation of crops using animal manure/ slurry, abattoir waste and sewage will directly contaminate the soil and crops to which it is applied to with potentially pathogenic bacteria (Natvig *et al.*, 2003; Krzyzanowski *et al.*, 2014; Heaton and Jones, 2008).

The UKHSA reported that *Salmonella* species were responsible for nearly 50% of all foodborne disease outbreaks between 1992 and 2008 (Gormley *et al.*, 2011). The consumption of poultry meat and hen's eggs have been identified the most frequently as containing *Salmonella* Enteritidis PT4 in epidemiological studies (Coyle *et al.*, 1988; Kessel *et al*., 2001). In 1988, a report about the risk of consuming raw eggs or undercooked food containing eggs was released due to the correlation between *S.* Enteritidis and chicken products (Desin *et al.*, 2013). The advice was given to elderly people, infants, pregnant women and immunocompromised individuals to only consume eggs which had been properly cooked. Recommendations that eggs were to be considered a short-life product were given, and therefore eggs should be refrigerated below 8°C throughout the production chain and be consumed within 3 weeks of being laid. Following on from these recommendations, a zoonoses order was established in 1989 that required all *Salmonella* isolates from animals or birds to be reported (ACMSF, 1993).

1.7 Control of *Salmonella* in the Food Chain

A voluntary, industry-led vaccination scheme was introduced in 1994 and 1998 for broiler and laying flocks, respectively (Desin *et al*., 2013; Ward *et al*., 2000). Eggs that had been laid by vaccinated chickens were stamped with a lion mark, which certifies that the eggs have been produced in accordance with UK and EU law. This law stipulates that there should be full traceability of hens, eggs and feed, and best-before dates should be stamped onto the egg shells and boxes, in addition to vaccination against *Salmonella* of all young hens, in order

to receive “Lion stamp” approval (Gray, 2018). In the 2016 ACMSF (Advisory Committee on the Microbiological Safety of Food) report, the advice for vulnerable individuals not to eat runny eggs was updated, and it is now considered safe for these individuals to consume partly cooked eggs. This is because the prevalence of *Salmonella* in UK eggs has dramatically reduced in the past few years, and nearly 90% of all eggs in the UK are produced according to the British Lion code of practice (ACMSF, 2016) .

Thermal processing and pasteurisation are common methods often used to eradicate food spoilage microorganisms in food products, including *Salmonella* (Silva and Gibbs, 2012). A mild heat treatment (<95°C) is applied to the food product for a specified amount of time to inactivate vegetative pathogenic cells. High-risk food products (generally those from animal origin) are stored, transported and sold at temperatures below 7°C in refrigerated conditions to minimise the growth of pathogenic bacteria (Silva and Gibbs, 2012). As consumer demand for minimally processed food products increases, alternative processing methods are emerging. Methods such as high-pressure processing, use of pulsed electric field/ X-ray or Ultra-Violet (UV) light, use of ozone and the use of extremely low frequency magnetic fields (ELF-MF) could all replace traditional thermal processing methods (Argyri *et al*., 2018; Korolczuk *et al*., 2006; Lim and Harrison, 2016; Torlak *et al*., 2013; Sudarti, 2016). Some of these alternative methods are already being used, including the use of chlorinated compounds, ozone, UV light and irradiation to control the prevalence of spoilage microorganisms on fresh products (Lee *et al*., 2018; Alwi and Ali, 2014; Kim *et al*., 2013; Mukhopadhyay *et al*., 2014; Palekar *et al*., 2015; Mahmoud, 2010). Even though there are measures in place to reduce the prevalence of *Salmonella* in the food chain, complete eradication is unlikely. However, improved farming practices such as correctly storing and using animal excrement, and increased biosecurity will help improve food safety (Humphrey, 2004).

1.8 Stress Response in *Salmonella*

Salmonella are able to survive and thrive in a variety of conditions and adapt to numerous hosts because of internal mechanisms that react to external environmental stressors (Humphrey, 2004). *Salmonella* is often ingested and therefore must evade multiple defence mechanisms in the host. The first mechanism that must be evaded is the acidic environment of the stomach. Next, surviving cells must be able to adapt to a more anaerobic environment in the intestines and must also be able to cope with exposure to bile salts, antimicrobial

peptides and increased osmolarity, whilst also competing with the gut microbiota for nutrients and space (Rychlik and Barrow, 2005). One example of a stress response regulatory mechanism is the production of stress proteins during transcription, by sigma factors, which help the bacteria survive environmental shifts, such as temperature and pH. Other stress responses and survival strategies include starvation stress, acid stress, oxidative stress, thermal stress, antimicrobial peptide stress, osmotic stress, desiccation stress and iron stress (Spector and Kenyon, 2012). In Gram-negative bacteria, stress response is regulated by the alternative sigma factors rpoE and rpoS, but also by CpxRA, BaeSR and ZraSR (two-component systems), the phage shock response and the Rcs phosphorelay system, which all form part of the envelope stress response (ESR) and are activated by various conditions in *S*. Typhimurium (**Table 1**) (Rowley *et al.*, 2006).

Envelope Stress Response	Stress/Condition
Extracytoplasmic function (ECF) σ-mediated	Heat Shock Cold Shock Oxidative Stress Carbon energy-source starvation Glucose to maltose, citrate or succinate shifts Stationary phase (LB medium) Overexpression of OMPs Growth *in vivo* (pathogenesis)
CpxRA-mediated	Pathogenesis
Phage shock response	Dissipation of proton-motive force High temperature Pathogenesis

Table 1. **Stresses and conditions known to activate the envelope stress response in *S*. Typhimurium**. Adapted from Rowley *et al*. (2006).

1.8.1 Sigma-mediated stress response

1.8.1.1 RpoE

The outer membrane of the cell is maintained by the extracytoplasmic sigma factor *rpoE* (σ^E), which senses damaged proteins situated in the outer membrane and in the area between the cell wall and plasma membrane (periplasmic space) (Rouvière *et al.*, 1995). In the absence of envelope stress, RpoE function is inhibited by an inner membrane protein (RseA) and prevents attachment to RNA polymerase. During envelope stress, RseA is cleaved by proteases *DegS* and *RseP* (also known as *yaeL* and *ecfE*). In the cytoplasm, SspB recruits the ATP-dependent protease ClpXP to bind to the RpoE-RseA complex, resulting in the separation of RpoE from RseA. RpoE is then able to bind to RNA-polymerase as a cofactor and initiates transcription of RpoE-dependent genes and expression of the σ^E-regulon (**Figure 1**) (Humphreys *et al*., 1999; Alba *et al.*, 2002). *RpoE* is an essential gene in the absence of stress in *E. coli*, but not *Salmonella,* however lack of *rpoE* causes *S*. Typhimurium to become more sensitive to reactive oxygen species (ROS) and antimicrobials (Humphreys *et al*., 1999; Testerman *et al*., 2002). A recent study discovered that loss of the lipopolysaccharide (LPS) O-antigen renders a *rpoE* mutant toxic in *Salmonella* (Amar *et al.*, 2018). Several genes are regulated by *rpoE*, including *htrA* which is an important gene for pathogenesis in *Salmonella* (Johnson *et al.*, 1991). Additionally, SurA and FkpA are involved in protein folding in *S*. Typhimurium and are also regulated by RpoE (Dartigalongue *et al*., 2001; Humphreys *et al*., 2003).

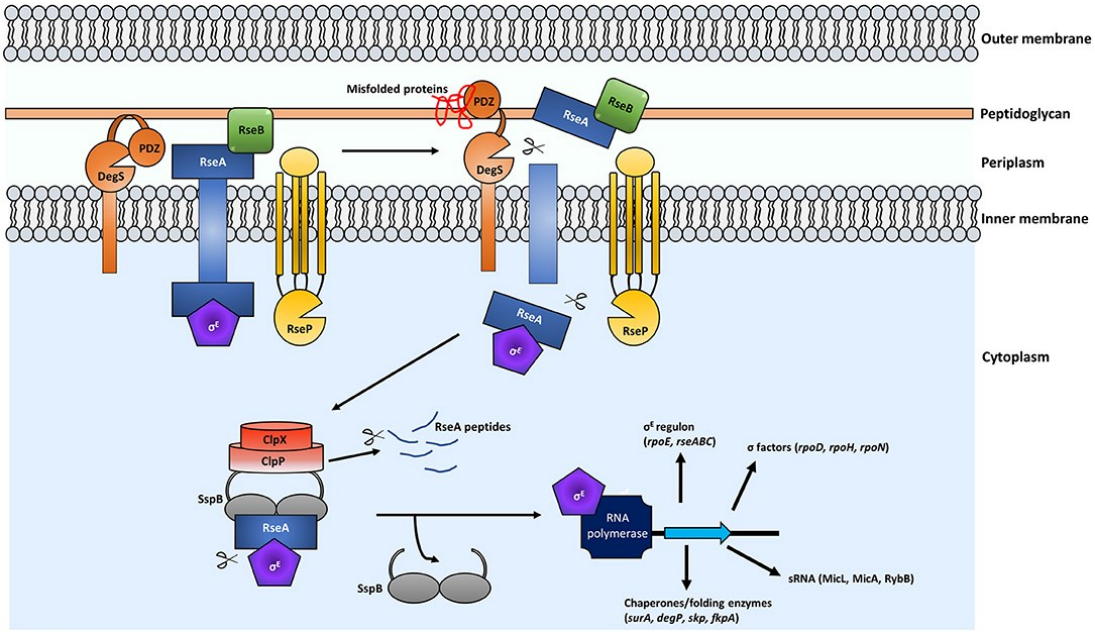


Figure 1. Schematic diagram of bacterial sigma-mediated stress response. σ^E-mediated stress response in Gram-negative bacteria occurs in response to misfolded proteins in the outer membrane, and cleavage of σ^E from RseA, results in transcription of σ^E-dependent genes. Taken from Hews *et al*. (2019).

1.8.1.2 RpoS

Non-specific stress response mechanisms in *Salmonella* are well defined and include the master alternative sigma factor σ^S (or σ^{70}), commonly referred to as RpoS. RpoS expression is induced in bacterial cells when adverse environmental conditions are sensed and upon entering stationary phase of growth, however low levels of RpoS can also be detected during exponential growth phase (Lange and Hengge-Aronis, 1991; Baptista *et al*., 2022). RpoS encodes a sigma factor (sigma s) and controls the expression of a large number of genes involved in osmotic stress, heat shock, oxidative DNA stress and starvation (Loewen *et al.*, 1998). Additionally, RpoS regulation has been extensively studied in *E*. *coli* and *S*. Typhimurium, and σ^S is essential for biofilm formation and virulence (Hengge, 2011; Dong and Schellhorn, 2010). Previously, RpoS stress response was thought to mainly involve transcription, however a large number of genes have been identified recently that are down-regulated at the protein level, rather than at the transcript level, which indicates that post-transcriptional regulation is more important for regulation of RpoS than originally suggested (Lago *et al.*, 2017). In *S*. Typhimurium, virulence plasmid genes (*spv*) are controlled by σ^S and are required for systemic host infection (Heiskanen *et al.*, 1994). Similarly, RpoS enables the expression of genes which increase tolerance to stress, including *katE* (catalase), *poxB* (pyruvate oxidase), and *ogt* (methyltransferase) (Chen *et al*., 1996; Ibanez-Ruiz *et al*., 2000).

1.8.1.3 RpoH

Th sigma factor RpoH (σ^{32}) is one of the main regulators of heat shock response in *E. coli* and *Salmonella* during log-phase, aerobic growth, but not during stationary phase or anaerobic growth (Díaz-Acosta *et al.*, 2006). During elevated temperatures, *rpoH* causes RNA polymerase to initiate transcription in more than 30 heat shock proteins (HSPs). However, at 30°C, intracellular levels of *rpoH* is low and is inhibited through interaction with the DnaK chaperone system (DnaK, DnaJ and GrpE) during non-stress conditions, although GrpE has been shown to reverse this inhibition (Gamer *et al.*, 1996). At high temperatures, RpoH has a higher affinity for RNA polymerase than RpoS (σ^{70}).

1.8.2 Two component system- mediated stress response

1.8.2.1 CpxRA

The conjugative pilus expression (Cpx) response is well characterised in Gram-negative bacteria and is a canonical two-component system acting through the histidine kinase sensor CpxA and cognate response regulator CpxR (Humphreys *et al.*, 2004). During envelope stress, CpxA phosphorylates and transfers a phosphate group to CpxR, however CpxA activity can be inhibited by CpxP (Danese *et al*., 1995; Raivio and Silhavy, 1997). Numerous stresses have been shown to active CpxRA, including alkaline pH, antimicrobials and copper, and mutations in CpxRA increased sensitivity to these stresses (Danese and Silhavy, 1998; Audrain *et al*., 2013; Yamamoto and Ishihama, 2006). Deletion of *cpxA* decreases the expression of genes encoded by SPI-1 and affects cell invasion by *S*. Typhimurium (Nakayama *et al.*, 2003).

1.8.2.2 PhoPQ

PhoPQ is a critical two-component system which allows *Salmonella* to adapt to the intracellular vacuole environment, after being taken up by macrophages following invasion of epithelial cells. PhoPQ consists of a membrane bound sensor kinase, PhoQ and a cytosolic response regulator, PhoP. Initiation of the PhoPQ cascade results in autophosphorylation of PhoQ, which leads to phosphorylation of PhoP (Bader *et al.*, 2005).The PhoPQ system in *Salmonella* can be activated during growth in an acidic pH or exposure to antimicrobial peptides, but can also be repressed when exposed to high concentrations of divalent

cations, such as Mg^{2+} (Prost *et al*., 2007; Véscovi *et al*., 1996). In Gram-negative bacteria, the outer membrane of the cell is composed of lipopolysaccharide (LPS) and glycerophospholipids (GPL), and the hydrophobic interaction between the membrane component of LPS (lipid A) and GPL, anchors the LPS to the surface of the outer membrane (Nikaido, 2003). The outer membrane protects the cell from antibiotics and the innate immune system by regulating lipid A structure and altering membrane permeability. PhoPQ regulates lipid A modification and results in the addition or removal of an acyl group from lipid A via upregulation of the genes encoding PagP and PagL (Needham and Trent, 2013).

1.8.2.3 OmpR/EnvZ

OmpR/EnvZ is a two-component system which responds to changes in osmolarity, pH, temperature, and growth phase. EnvZ, is a sensor kinase which detects environmental stress and is phosphorylated by adenosine triphosphate (ATP). The response regulator, OmpR, then catalyses the transmission of the phosphate group from histidine to a conserved aspartic acid residue (Kenney and Anand, 2020). The two-component system regulates the expression of OmpF and OmpC (outer membrane proteins), which differ from one another due to their pore size and subsequent flow rate (Nikaido and Vaara, 1985). In low osmotic environments, the outer membrane porin is OmpF, whereas in high osmotic environments, transcription of *ompF* is inhibited and the major porin becomes OmpC (Kenney and Anand, 2020). In *S*. Typhimurium, OmpR aids cytoplasmic acidification by repressing the *cadC/BA* operon, preventing pH neutralisation (Chakraborty *et al.*, 2015). Additionally, acidification in response to osmotic stress also occurs through repression of *rpoS* by OmpR *(*Chakraborty *et al.*, 2017).

1.9 Project Rationale

The main aim of this project is to investigate the diversity in stress response observed in strains of *Salmonella enterica*, specifically in food chain related stresses, and identify the mechanisms used to survive under stresses of heat, desiccation, refrigerated storage, NaCl and organic acids. The central hypothesis is that genetic variation of pathogenic strains of *Salmonella* results in differences in the ability to survive stress and therefore the associated risk to food safety and consumer health will be identified.

Currently, there is a gap in scientific knowledge regarding the control of *Salmonella* in vegetarian food products. Pathogen inactivation data is crucial in food production to ensure food safety, and an understanding of the genetic basis of this phenotypic variation in response to stresses commonly used to prevent foodborne contamination will result in a rational selection of target strains for process validations, improved processing, and more reliable risk assessments. At present, food challenge tests rely on specific test strains for which data and clear relevance are missing, especially regarding low moisture foods, and do not include strains with an increased tolerance to stress. The hypothesis is based on preliminary data indicating considerable variation in response to processing stresses, even in closely related *Salmonella* variants.

Understanding phenotypic variation and stress responses amongst *Salmonella* strains will contribute to the development of milder processing techniques that maintain food safety integrity, which not only satisfies consumer demand for minimally processed foods, but also abides by governmental efforts to warrant the production of 'healthier' foods, especially in the UK as the government has numerous schemes in place to reduce the salt, sugar, and fat content in food products. Changes to agricultural practices, for example the abolishment of antibiotic use as growth promoters in animal husbandry, may also affect *Salmonella* survival in the food chain, and further exemplifies the need for a study understanding phenotypic diversity. Additionally, it is important to understand how strain variation is contributing to the continued high incidence of *Salmonella* as a foodborne contaminant, which will further aid food safety. The project impact is that scientific data that will be translated into recommendations for consumers, regulators, and industry to ensure safe supply and consumption of plant protein products. Overall, a better understanding of the mechanisms underpinning food chain related stress response in *Salmonella* will be achieved.

1.9.1 Objectives

1. Determine the genetic variability amongst diverse strains of *Salmonella enterica*
2. Assess phenotypic variation in survival of *Salmonella enterica* in response to heat inactivation, desiccation, refrigerated storage, NaCl and organic acids
3. Investigate the molecular mechanism of food chain related stress response by whole genome functional screen using saturating transposon mutagenesis and transposon directed insertion site sequencing (TraDIS)

Chapter Two

Phenotypic variability of *Salmonella enterica* in food chain related stress

2.1 Introduction

The ability of *Salmonella* to survive and replicate when exposed to stress can result in persistence of the bacterium in food products. Bacterial stress is often encountered during food processing and storage, and can be defined as any physical, nutritional or chemical process which can result in sub-lethally injured bacteria or cell death (Wesche *et al.*, 2009). Stresses commonly encountered throughout the food chain include chemical and physical agents, such as high and low temperatures, desiccation, and preservatives such as organic acids and salt. Exposure to certain stresses in the food chain can also contribute to cross-protection of *Salmonella* against other stresses, for example, exposure to acid and salt stress can increase the heat resistance of *S.* Enteritidis (Kang *et al.*, 2018).

Desiccation can be used as a food preservation technique to extend the shelf-life of products and consequently, these products typically have a low-water activity (a_w < 0.85). Water activity is the ratio of water vapour pressure in a food product compared to the water vapour pressure of pure water at a specified temperature (Finn *et al*., 2013a; Labuza, 1980). Some food products have a naturally low-water activity, such as nuts and honey (Finn *et al.*, 2013a). Tolerance to desiccation enables bacterial cells to survive extreme dehydration and overcome the structural, physiological, and biochemical changes that result from exposure to this environmental stress. *Salmonella's* ability to survive in low-water activity has resulted in multiple foodborne outbreaks such as in puffed-wheat cereal and dried coconut caused by *S.* Mbandaka and *S.* Typhimurium, respectively (CDC, 2018b; CDC, 2018c). Salt is also commonly used as a preservative, and it works by preventing bacterial growth by lowering the water activity of food. The addition of salt to food products disrupts the osmolarity of the cells, by causing water to flow into or out from the cell. This causes damage to the cell membrane and results in cell death (Davidson *et al.*, 2012).

Thermal treatment, using water, steam, or thermal radiation, is one of the most effective methods of eliminating foodborne pathogens. During pasteurisation, a heat treatment of between 65°C and 95°C is applied to the food product for a specified amount of time to inactivate vegetative pathogens (Silva and Gibbs, 2009). Exposure to these high temperatures causes small cytoplasmic ions to leak out of the cell, and irreversible damage to the cell membrane (Ebrahimi *et al.*, 2018). Understanding the thermal characteristics of bacterial strains is important when establishing cooking requirements of a food product. The decimal reduction time (D-value) is the time needed to reduce cell viability by 1-log (or 90%)

at a specific temperature. A linear, first-order kinetics model is typically used to determine D-values for each foodborne pathogen, which can predict the linear inactivation of cells and deduce the time and temperature combination required to kill all vegetative pathogens in a food product (Berk, 2009). The D-value can be calculated from the thermal death curve of the bacterium at a constant temperature by using the negative reciprocal of the slope of the linear regression line (Mazzola *et al.*, 2003).

Refrigeration is another common manufacturing method to prevent the growth of food spoilage organisms. Cold-chain management (CCM) involves implementing the control, transportation, and storage of perishable goods at refrigerated temperatures, to guarantee food safety by inhibiting the growth of foodborne pathogens and minimise waste (Singh *et al.*, 2018).

Organic acids are used as preservatives in food manufacturing and can be defined as organic compounds that retain acidic properties, such as lactic, acetic and citric acid (Sauer *et al.*, 2008). These acids inhibit the growth of bacteria by increasing the permeability of the cell membrane, which lowers the pH inside the cell and causes loss of intracellular components (Sundberg and Jönsson, 2005).

Modern food manufacturing practices rarely rely on one form of preservation to inhibit microbial growth in food products, rather, a combination of multiple techniques are commonly used to provide robust protection against food spoilage and pathogenic microorganisms (Leistner, 2000). However, consumer demand for minimally processed food is increasing, along with increasing pressure from the government for food manufacturers to produce healthier food products. Therefore, an understanding of how pathogens behave under stress will aid in the development of processing techniques which satisfy these requirements for 'healthier' products, without compromising food safety. To assess the effectiveness of preservation and exclusion of pathogens, typically, multiple *Salmonella* strains are used together in a strain cocktail to assess survival during food challenge tests, but certain strains possess an increased resistance to stress, thus posing a greater risk to food safety. Understanding strain variation will improve modelling of consumer risk of contamination of *Salmonella* in food products (Whiting and Golden, 2002). Furthermore, determining the phenotypic variability of *Salmonella* in food chain related stress will result in a verified selection of target strains for process validations, improved processing, and more reliable risk assessments.

2.1.1 Aims

1. Explore genetic variability amongst *Salmonella* strains
2. Determine phenotypic variation amongst *Salmonella* strains in response to heat, desiccation, refrigeration, NaCl and organic acids
3. Identify *Salmonella* strains exhibiting increased tolerance or sensitivity to food chain related stress

2.2 Methods

2.2.1 Preparation of media

Luria-Bertani (LB) broth (10g/L NaCl, 10g/L Tryptone, 5g/L Yeast Extract) (Formedium, LMM0102) was prepared by suspending 25g in 1L of distilled water and sterilised by autoclaving at 121°C for 15 minutes. LB agar (10g/L NaCl, 10g/L Tryptone, 5g/L Yeast Extract, 15g/L agar) (Formedium, LMM0102) was prepared by mixing 25g powder with 1L distilled water and sterilised by autoclaving for 15 minutes at 121°C. Phosphate Buffered Saline (PBS) (Oxoid, BR0014) was prepared by dissolving 1 tablet in 100mL distilled water and sterilised by autoclaving at 121°C for 15 minutes.

2.2.2 Bacterial strains and culture

Fourteen *S. enterica* strains were used in the present study, isolated from human salmonellosis cases, animals, and food. Strains included in the study were acquired from Ken Sanderson at the *Salmonella* Genetic Stock Centre (SGSC) at the University of Calgary. These strains belonged to the serovar Typhimurium (5 strains), Kedougou (1 strain), Newport (2 strains), Infantis (1 strain), Heidelberg (1 strain), Enteritidis (1 strain), Kentucky (1 strain), Gallinarum (1 strain) and Schwarzengrund (1 strain). Stock cultures of each strain were stored in individual Cryovials (Corning) at -80°C in 50% Glycerol. Working cultures were prepared by scraping the frozen stock into a 5mL LB broth bottle and incubating overnight at 37°C with shaking set to 200rpm. Serovars to be included in this study were selected due to their invasiveness, their ability to cause disease in humans and food production animals, or because they were isolated from food or food production environment (**Table 2**). A range of serovars were also chosen to be included from across the phylogeny, to incorporate as much genetic diversity as possible.

Serovar	Sequence Type (ST)	Phage Type*	Strain name	Alternative names*	Source
Typhimurium	ST19	ND	ST4/74	NA	Cattle
Typhimurium	ST568	DT56	S07676-03	NA	Avian
Typhimurium	ST19	U288	S01960-05	NA	Pig
Typhimurium	ST34	DT193	S04698-09	NA	Cattle
Typhimurium	ST34	ND	B54Col9	NA	Chicken
Kedougou	ST1543	NA	B37Col19	NA	Cattle
Infantis	ST32	NA	S1326/28	SGSC4905	Chicken
Heidelberg	ST15	NA	SL476	SGSC4915, CVM30485	Turkey
Enteritidis	ST11	PT4	P125109	SGSC4901, BA394	Human
Schwarzengrund	ST322	NA	SL480	SGSC4919, CVM35940	Human
Gallinarum	ST331	NA	287/91	SGSC4691, BA395	Chicken
Newport	ST45	NA	SL254	SGSC4910, E20002725	Human
Newport	ST118	NA	SGSC4157	NA	Unknown
Kentucky	ST152	NA	SL479	SGSC4918, CVM35942	Human

* ND – not determined, NA – not applicable

Table 2. ***Salmonella enterica*** **strains used during this study**. Including information regarding their sequence/phage type, and their source of isolation (if known).

2.2.3 Preparation and storage of the vegetarian food product

Individual packets of a wheat and pea protein-based vegetarian product (each pack totalling 210g) were subjected to an in-pack irradiation treatment and were supplied by Nestlé for use throughout the study. The 210g packets of food product remained at 4°C until opened, and once opened, each pack was divided into 13g portions and transferred to individual sterile plastic bags inside a microbiological safety cabinet (Herasafe). Each 13g portion of food product was frozen at -20°C and thawed at room temperature prior to use.

2.2.4 Whole genome sequencing and strain phylogeny

Salmonella strains were cultured overnight at 37°C in LB broth and DNA was extracted using a Maxwell RSC 48 instrument (Promega, AS4500) and associated Maxwell RSC cultured cells DNA kit (Promega, AS1620). DNA concentration was quantified using a Qubit 3.0 fluorometer (Invitrogen) and dsDNA broad range assay kit (Invitrogen, Q32850). DNA sequencing was conducted in house by the QIB sequencing team. Genome libraries were prepared using the Nextera XT index kit (Illumina) and whole genome sequencing was performed using a NextSeq500 (Illumina). Data was uploaded to IRIDA (Matthews *et al.*, 2018) and the quality of paired-end reads was evaluated using FastQC (Andrews, 2010). Antigenic formula was predicted using SeqSero2 (version 1.2.1) using paired-end short read fastq sequences as the input to identify serotype (Zhang *et al.*, 2019). SNIPPY (version 4.3.6) (Seemann, 2015) was used to identify single nucleotide polymorphisms (SNPs) between *Salmonella* strains used in this study and the *S. bongori* N268-08 reference, and snippy-core produced a core alignment file from all of the sequences included. The core alignment from snippy-core was used as the input for RaxML (Stamatakis, 2014) to construct a maximum likelihood phylogenetic tree of the 14 *Salmonella* strains used in the study and the tree was plotted using ggtree (Yu *et al.*, 2017) in R (version 4.1) (Team, 2021).The tree was rooted using *S. bongori* N268-08 as an outgroup and the tip was dropped when generating the tree image in R. Pairwise SNP differences between strains was determined using snp-dists (Seemann *et al.*, 2018) and plotted as a matrix with pheatmap (Kolde, 2012) in R (version 4.1) (Team, 2021). SNP-sites (version 2.3.3) (Page *et al.*, 2016) was used to determine the number of nucleotides covered by all isolates included in the study and was executed using the core full alignment file generated from the SNIPPY output, including the options to output monomorphic sites and

columns containing ACGT nucleotides only. FastANI (version 1.3) was used to determine the average nucleotide identity between strains, using the many-to many method, where multiple query and reference genomes were used (Jain *et al.*, 2018).

2.2.5 Heat Inactivation

2.2.5.1 Submerged Tube Method

The submerged tube method was used to determine the thermal death of *Salmonella* serovars, based on a method previously described (Peck *et al.*, 1992). Phosphate Buffered Saline (PBS) (9.9mL, pH 7.4, 0.01mM) was dispensed into glass Hungate tubes (Sciquip, 2047-16125) and sterilised by autoclaving (121°C, 15 minutes). Hungate tubes containing PBS were submerged in a water bath at 45°C-63°C (in 2°C increments) and 100µL of *S.* Typhimurium strain SL1344 at a concentration of $5x10^8$ CFU/mL (adjusted using PBS) was injected into to each tube using a Gas Tight syringe (Hamilton, 26203) and 21Gx 1.5" needle (Terumo, NN2138R). Duplicate Hungate tubes were heated for 30 seconds at each temperature and immediately plunged into an iced water bath to rapidly cool. For a control, a 100µL aliquot of *S*. Typhimurium strain SL1344 was injected into a Hungate tube containing 9.9mL PBS but remained at room temperature. The contents of the tubes were serially diluted (10^0-10^{-5}) using PBS (1 in 10) and 100µL of each dilution was spread onto an LB agar plate, in duplicate, and incubated overnight for 16 hours at 37°C. Surviving colonies on each LB agar plate were enumerated and the CFU/mL calculated.

To determine the decimal reduction time (D-value) of *S*. Typhimurium strain SL1344 at 63°C, sterile glass Hungate tubes containing 9.9mL PBS were inoculated with 100µL of *S*. Typhimurium strain SL1344 at a concentration of $5x10^8$ CFU/mL. Tubes were removed from the water bath at 10, 20, 30, 40 and 50 seconds after inoculation and plunged into iced water, with a swirling motion to rapidly cool. A control was prepared by inoculating a Hungate tube containing 9.9mL PBS with 100µL of *S*. Typhimurium strain SL1344 at room-temperature (~25°C). After heat treatment, the contents of the Hungate tubes were serially diluted (10^0-10^{-5}) in PBS and 100µL of each dilution was spread onto LB agar (in duplicate) and incubated for approximately 16 hours overnight at 37°C. Colonies were enumerated after 16-hour incubation and the viable count (CFU/ml) was determined. The D-value was calculated from

the linear regression equation between 0 and 30 seconds at 63°C and between 0 and 50 seconds for 60°C in *S*. Typhimurium strain SL1344.

2.2.5.2 Thermal Cell Heating Method

Thin-walled, aluminium thermal cells (**Figure 2**) were supplied by Nestlé to imitate the thermal inactivation experiments conducted in their research centre. An overnight culture of the test strain (**Table 2**) was prepared in 5mL LB broth and incubated at 37°C with shaking at 200rpm. A 1mL aliquot of the overnight culture was dispensed into a microcentrifuge tube and pelleted by centrifugation at room temperature for 4 minutes at 13,300rpm. The supernatant was discarded, and the pellet resuspended in 1mL PBS. The culture was adjusted to a concentration of $5x10^8$ CFU/mL with PBS and stored in a refrigerator at 2-4°C for a maximum of 2 hours.

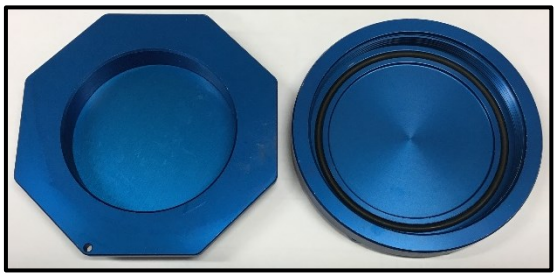


Figure 2. **Photograph of aluminium thermal cells provided by Nestlé for heat inactivation experiments**. The chamber holds up to 1g of food product and the O-rings create a water-tight seal.

A 750mg portion of vegetarian food product was measured into the centre of the thermal cell in sterile conditions and a 5µL aliquot of culture at a concentration of $5x10^8$ CFU/mL was inoculated into the food sample in 3 individual spots. The thermal cells were sealed using the O-ring, metal disk and lid provided (**Figure 2**). The inoculum was left to equilibrate within the food sample for 1 hour at 4°C. For each experimental group, one thermal cell was attached to a type K thermocouple (RS Pro, 363-0250) to monitor the temperature over the duration of the experiment. The thermocouple was attached to a TC-08 thermocouple data logger (Pico Technology, PP222) and the temperature was recorded for the duration of the experiment to monitor reproducibility of conditions between replicates. Thermal cells were simultaneously placed into a water bath maintained at 60°C. The temperature inside the thermal cells (as measured by the thermocouple) increased to the 60°C in approximately 45

seconds and this was treated as time=0. Thermal cells were removed in 10-second increments and immediately plunged into an iced water bath to rapidly cool. Once cooled, the food sample was transferred to a sterile 15mL centrifuge tube (Corning, CLS430055) and mixed with PBS in a 1 in 10 dilution (w/v) and vortexed for 10 seconds. A 300µL aliquot of the supernatant was diluted 1 in 10 with PBS in a CytoOne 96-well plate (Starlab, CC7672-7596). To determine viable colony forming units, 10µL of each dilution was pipetted onto a square 12x12cm LB agar plate (Scientific Laboratory Supplies (SLS), PET3008) using a multichannel pipette and incubated inverted at 30°C overnight. Surviving colonies were enumerated after overnight incubation for 18 hours at 30°C and the log ratio survival determined. Five independent experiments were conducted for each strain at 60°C.

2.2.6 Long Term Survival at Refrigerated Temperatures

Strains were grown to stationary phase for 18 hours at 37°C in 5mL LB broth and a 1mL aliquot was pelleted using centrifugation at 13,300rpm for 4 minutes. The supernatant was discarded, and the pellet resuspended in an equal volume of PBS. Cultures were adjusted to a concentration of $2.5x10^9$ CFU/mL and refrigerated at 4°C. Wells of a CytoOne 24-well plate (Starlab, CC7672-7524) were filled with 750mg thawed vegetarian food product and inoculated with 50µL of each strain at $2.5x10^9$ CFU/mL. Plates were maintained at 4°C for 5 weeks. An initial assessment of viable counts that could be recovered for each strain was determined by immediately transferring the well contents into 5mL LB broth and viable counts determined by plating serial dilutions (1 in 10) using PBS. For CFU/mL counts, 5µL of each dilution was spot plated (in triplicate) onto LB agar and incubated at 30°C for 18 hours. After 5 weeks, the contents of the wells from the experimental plates were transferred to 5mL LB broth, and viable counts enumerated by plating 5µL of each serial dilution onto an LB agar plate. Plates were incubated at 30°C for 18 hours. Surviving colonies were enumerated and the log ratio survival calculated. Four independent experiments were conducted for each strain from 2 biological replicates.

2.2.7 Desiccation

Strains were cultured for 18 hours in 5mL LB broth at 37°C with shaking at 200rpm. Overnight cultures were centrifuged at 13,300rpm for 4 minutes and the supernatant discarded. The

pellet was resuspended in an equal volume of PBS and adjusted to a concentration of approximately $5x10^8$ CFU/mL with PBS. The first column of a 96-well CytoOne plate was filled with 50µL of each strain at a concentration of ~$5x10^8$ CFU/mL. Plates were left to desiccate in a safety cabinet for 24-hours and the temperature and relative humidity was measured using a thermohygrometer. Control wells containing 50µL of each strain at ~$5x10^8$ CFU/mL were mixed with 150µL PBS per well, and 5µL of each serial dilution was spot plated, in triplicate, onto LB agar plates. Plates were incubated at 30°C for 18 hours and surviving colonies were enumerated the following day. After 24-hours, desiccated wells were rehydrated with 200µL PBS and serially diluted (1 in 10) with PBS. Each dilution was spot plated onto a square LB agar plate (in triplicate) and incubated overnight at 30°C. Surviving colonies were counted, and the log ratio survival calculated. Three independent experiments were conducted for each strain.

2.2.8 Growth in the presence of organic acids

Salmonella enterica strains were cultured for 18 hours in 5mL LB broth at 37°C with shaking at 200rpm. A 14mM citric acid (Thermo Fisher Scientific, 110450250) solution and a 12mM acetic acid (SLS, CHE1012) solution were prepared in LB broth and filter sterilised using a 0.2µM Minisart PES Syringe Filter (Sartorius, 16532K). Two 100mL bottles of LB broth were adjusted to pH 5.8 with 14mM and 12mM citric acid and acetic acid, respectively, using a benchtop pH meter (Mettler Toledo, 30046240). A 1mL aliquot of overnight culture of each strain was mixed with 4mL LB broth supplemented with either citric or acetic acid at pH 5.8 for 30 minutes, to initiate the acid shock response. 5mL aliquots of 14mM citric acid and 12mM acetic acid-supplemented LB broth solutions were inoculated with 5µL of each test strain that had been pre-adapted to pH 5.8 at a concentration of approximately $5x10^8$ CFU/mL. A 200µL aliquot of inoculated organic acid solution for each strain was transferred to a 96-well U-Bottom plate (Greiner, 163320), in triplicate wells. LB broth (positive) controls for each strain and non-inoculated (negative) controls were both included. Growth was measured at OD600nm using a Fluostar Omega plate reader (BMG Labtech) for 22 hours, with measurements taken every 5 minutes at 37°C with pre-measurement shaking. Three independent experiments were conducted for each strain.

2.2.9 Growth in the presence of salt

Salmonella enterica strains were grown to stationary phase for 18 hours at 37°C in 5mL LB broth. Cultures were diluted to a concentration of approximately $5x10^8$ CFU/mL using LB broth. 5mL LB broth containing 6% NaCl was aliquoted and inoculated with 5µL of each strain at a concentration of approximately $5x10^8$ CFU/mL. Each well of a 96-well U-Bottom plate was filled with 200µL of inoculated salt solution for each strain (in triplicate). Non-inoculated LB broth controls (with and without NaCl), and inoculated LB broth controls were included. Growth was measured at OD600nm using a Fluostar Omega plate reader at 37°C for 22 hours, with measurements taken every 5 minutes with pre-measurement shaking. Three independent experiments were conducted for each strain.

2.2.10 Statistical Analysis

A one-way ANOVA, with an uncorrected Fisher's least significant difference (LSD) test, was conducted in Graphpad prism (version 8.0.2) on the log ratio survival data of each replicate mean compared to the mean of *S.* Typhimurium strain ST4/74. An area under the curve analysis (AUC) was conducted on each replicate for each strain during growth in NaCl and organic acids using Graphpad prism (version 8.0.2). The difference in AUC was calculated by deducting the AUC in the stress condition from the AUC in LB broth. A two-way ANOVA with multiple comparisons and uncorrected Fisher's Least significant difference test was conducted on the difference in AUC analysis in Graphpad Prism (version 8.0.2) comparing the mean difference in AUC of each strain compared to the mean of *S.* Typhimurium strain ST4/74.

2.3 Results

2.3.1 Establishment of a strain collection of diverse serovars

A collection of 14 strains from nine different serovars of *Salmonella enterica* with distinct epidemiology and risk to food safety were chosen to be included in this study. Strains of serovars Enteritidis, Typhimurium, Newport, Infantis and Kentucky were chosen as they are amongst the top ten serovars most frequently isolated from human infections in the UK (UKHSA, 2021). One strain of *S*. Enteritidis was included due to the serovars association with asymptomatic infection of poultry and because it is a frequent contaminant of eggs. This serovar remains the most frequently isolated from human infection in the UK, however *S*. Infantis and *S*. Kentucky are increasingly isolated from poultry samples in some countries. Serovars were also chosen if they had previously been associated with *Salmonella* outbreaks. Outbreaks of *S*. Kentucky have occurred in Europe and therefore *S*. Kentucky strain SL479 was chosen to be included in the current study. Two strains of *S*. Newport were included due to their frequent association with outbreaks in fruits and vegetables, such as lettuce, papaya, and tomatoes (Lienemann *et al.*, 2011; CDC, 2017b; Greene *et al.*, 2008). *S*. Typhimurium is the second most isolated serovar in human infections and is widely distributed in livestock and wild animal zoonotic reservoirs. Additionally, a *S*. Typhimurium strain had previously been implicated during an outbreak in lettuce in England and Wales and therefore representative strains of *S*. Typhimurium were also included in the current study (Horby *et al.*, 2003).

All strains included in the study were subjected to whole genome sequencing and serovar classification using Seqsero2, a computational method used to predict serotype from genomic sequencing data. Seqsero2 predicted one strain in the collection, originally denoted as *S*. Dublin strain SGSC4157, as a *S*. Newport strain, and reviewing the literature revealed that this strain is frequently variably reported as either a Dublin strain or a Newport strain (Sangal, 2009; Tullio, 2018). For simplicity, this strain will be classified as a Newport strain in the present study, based on the *in-silico* analysis. Host restricted serovars, including *S*. Gallinarum and *S*. Typhimurium U288 were included in the present study, as well as strains of broad host range, such as other *S*. Typhimurium strains. Two monophasic *S*. Typhimurium strains were included in the strain collection, as over 50% of all human *S*. Typhimurium infections in the UK were caused by the monophasic ST34 clone during the past 10 years (EFSA, 2010; Moreno Switt *et al.*, 2009). Furthermore, there was recent large, multi-country

outbreak of *Salmonella* in chocolate products associated with monophasic *S*. Typhimurium ST34 strains (ECDPC and EFSA, 2022).

2.3.2 Genomic diversity of *Salmonella enterica*

The diversity of *Salmonella enterica* strains used throughout this study was assessed using the core SNP alignment from SNIPPY to construct maximum likelihood phylogenetic tree to explore genetic relatedness between strains (**Figure 3A**). The genome of the reference strain, *S*. *bongori* strain N268-08, was 4.83Mb in size. The core alignment was 2,297,416bp, which is approximately 48% of the reference genome, with 194,239 variable sites. In the shared genome, strains of *S*. Typhimurium differed by no more than 600 SNPs, whereas strains of different serovars, such as *S*. Gallinarum and *S.* Kentucky differed by more than 20,000 SNPs (**Figure 3B**). It is evident that *S*. Gallinarum and *S.* Enteritidis are closely related serovars and the two strains used during this study only differed by 2,591 SNPs in this analysis (**Figure 3B**). Strains to be used during the study were selected to incorporate as much genetic diversity as possible within *Salmonella enterica* subspecies I, but also because they were isolated from either humans or animals.

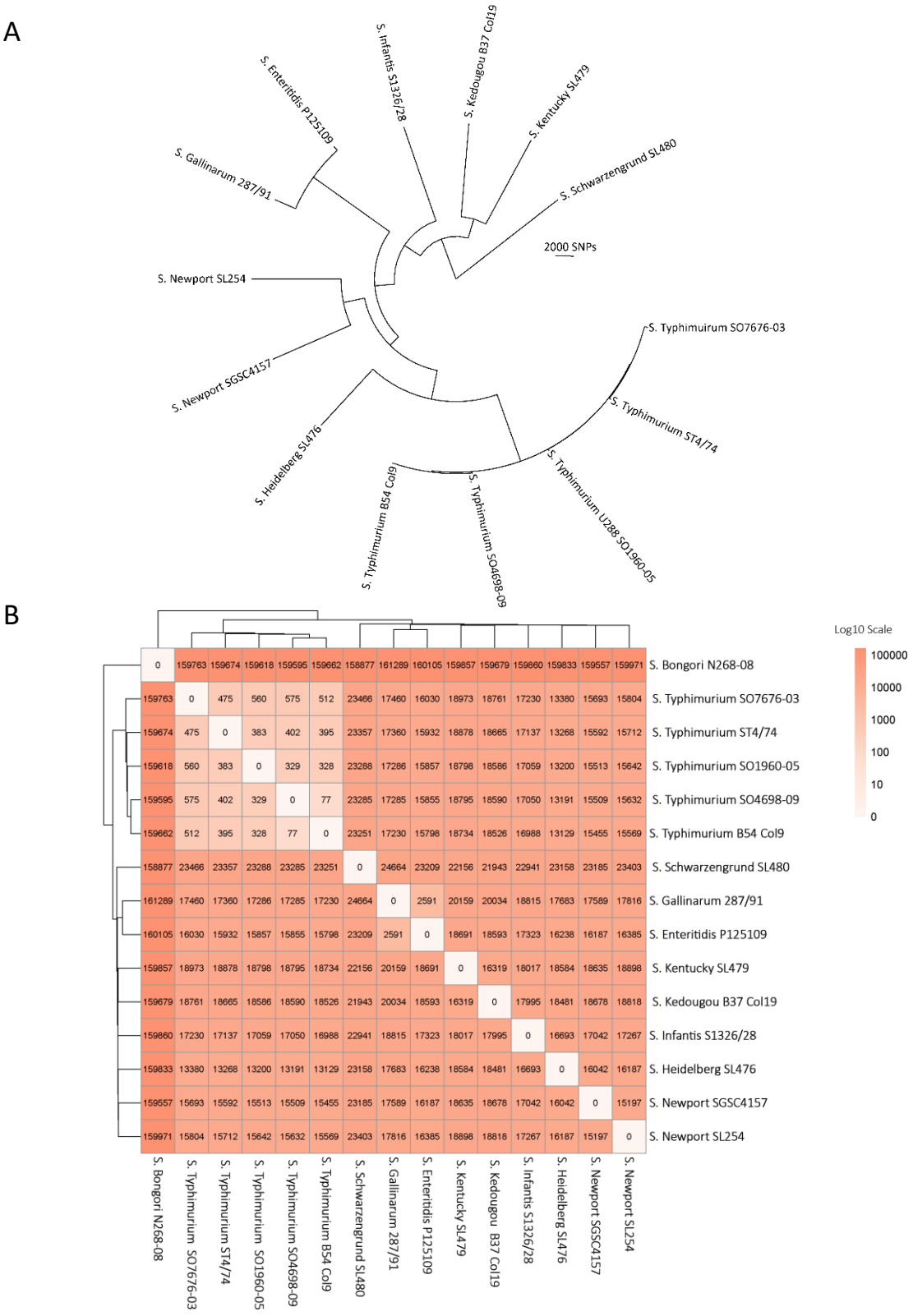


Figure 3. Phylogenetic relatedness of *Salmonella* strains. (A) Maximum likelihood phylogenetic tree of *Salmonella enterica* subspecies I serovars used during study generated from core alignment. The tree is rooted using *S. bongori* strain N268-08 as an outgroup. (B) Heatmap showing SNP distance between each strain used during study on a log10 scale. *S. bongori* strain N268-08 was used as the reference.

2.3.3 *Salmonella* strains had an average nucleotide identity > 98%

The average nucleotide identity (ANI) was computed for strains included in the study as another method to determine the genetic relatedness between strains (**Figure 4**). ANI is a computational method that measures the similarity in nucleotide sequences in the coding regions of two genomes, and the similarity is expressed as a percentage. The species threshold is typically set to 95%, so any ANI values <95% would suggest different species (Goris *et al.*, 2007). All 14 strains were included in both the reference and query lists, ensuring that all strains were compared to one another. *S.* Schwarzengrund strain SL480 was the strain least similar to all of the other strains included in the study, with an ANI range of 98.13-98.34%. The most similar to *S.* Schwarzengrund strain SL480 was *S*. Kentucky strain SL479 whereas the most distantly related strain was *S.* Newport strain SGSC4157. The two *S*. Newport strains (SL254 and SGSC4157) had an ANI of 98.93%. *S.* Enteritidis strain P125109 and *S.* Gallinarum strain 287/91 were most similar overall and had an ANI of 99.77%. *S.* Heidelberg strain SL476 and *S.* Typhimurium strain SO1960-05 were 99.01% similar, closely followed by the other *S.* Typhimurium strains. *S.* Infantis strain S1326/28 had the greatest ANI with *S.* Typhimurium strain ST4/74 at 98.78% and was also most like the other *S.* Typhimurium strains, than any other serovar. *S.* Kedougou strain B37 Col19 had an ANI of 98.81% with *S*. Kentucky strain SL479. All *S.* Typhimurium strains had an ANI >99.8% to one another, and monophasic strains were more closely related to each other, than to the other *S.* Typhimurium strains included (**Figure 4**).

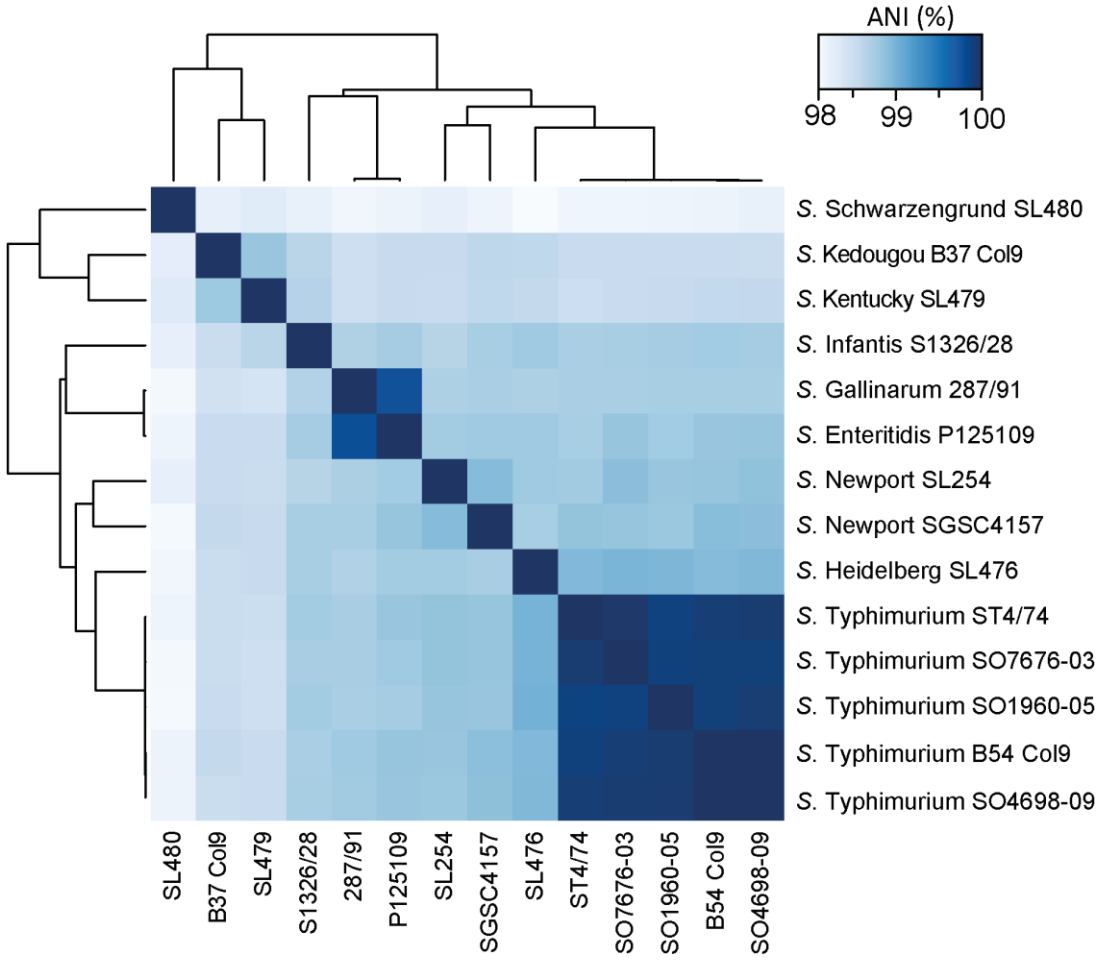


Figure 4. Average nucleotide identity of *Salmonella* strains used in the study. Average nucleotide identity was determined using FastANI. Smaller ANI values, represented by a paler shade of blue, signify a lower homology and a greater phylogenetic distance between strains.

2.3.4 Heating *S*. Typhimurium SL1344 for 30 seconds at 60°C delivers a 4.6-log reduction in cell viability

To evaluate the survival of *S*. Typhimurium strain SL1344 during inadequate inactivation temperatures e.g. to simulate issues with cooking or processing and to quantify the temperature at which rapid cell death starts to occur, the heat inactivation kinetics of *S*. Typhimurium strain SL1344 was assessed at 45-63°C in PBS using the submerged tube method (**Figure 5**). There was minimal cell death between 45°C and 57°C when cells were heated for 30 seconds. At 59°C, there was a 0.5-log reduction in cell viability after 30 seconds, which increased as temperature increased. At 61°C, there was a 3.2-log reduction in cell viability after 30 seconds of heating. The greatest log reduction, at 4.6-log, was seen at 63°C for *S*. Typhimurium strain SL1344 (**Figure 5**).

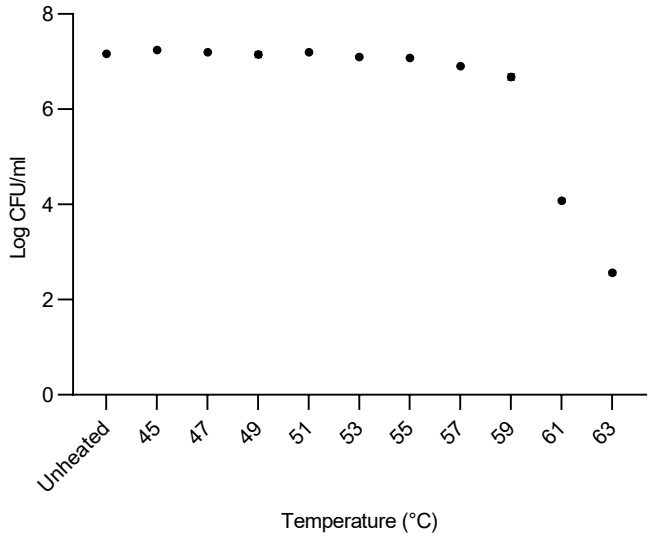


Figure 5. Inactivation of *S.* Typhimurium strain SL1344 during heating between 45°C and 63°C. The log CFU/mL of SL1344 heated for 30 seconds at different temperatures in PBS buffer was determined using the submerged tube method. Each point represents the mean (± SE) of two technical replicates.

2.3.5 The vegetarian food product protects *S*. Typhimurium strain SL1344 cells when heated at 60°C

To determine the decimal reduction time (D-value) at 60°C and 63°C in PBS, which is the time taken to achieve a 1-log reduction in cell viability, cells of SL1344 were heated in 10 second increments from 0-50°C in PBS (**Figure 6**). After heating SL1344 for 20 seconds at 63°C, there was a 3.5-log reduction in cell viability. At 30 seconds, the log-reduction increased by ~1-log, to a 2.4-log reduction in cell viability. There were no cells recovered after 40 and 50 seconds of heating at 63°C (**Figure 6**). SL1344 was also heated at 60°C in 10-second increments, and cells were still recovered after 50 seconds of heating (**Figure 6**). A ~1-log reduction was observed after 10 seconds of heating at 60°C and a 3.2-log reduction was achieved after 20 seconds. The greatest log reduction occurred at 50 seconds of heating at 60°C, at a 4.7-log reduction (**Figure 6**). The resulting D-values calculated from the linear regression of SL1344 at 60°C and 63°C were 10.6 and 5.9 seconds, respectively.

S. Typhimurium strain SL1344 was heated in both PBS and the vegetarian food product using the submerged tube method, to compare both heating matrices (**Figure 6**). Heating the cells of SL1344 at 60°C for 50 seconds in the vegetarian food product delivered a 3.5-log reduction in cell viability (**Figure 6**). There was only a one second difference between the D-values

observed at 60°C for PBS and the vegetarian food product at 10.56 and 11.48 seconds, respectively.

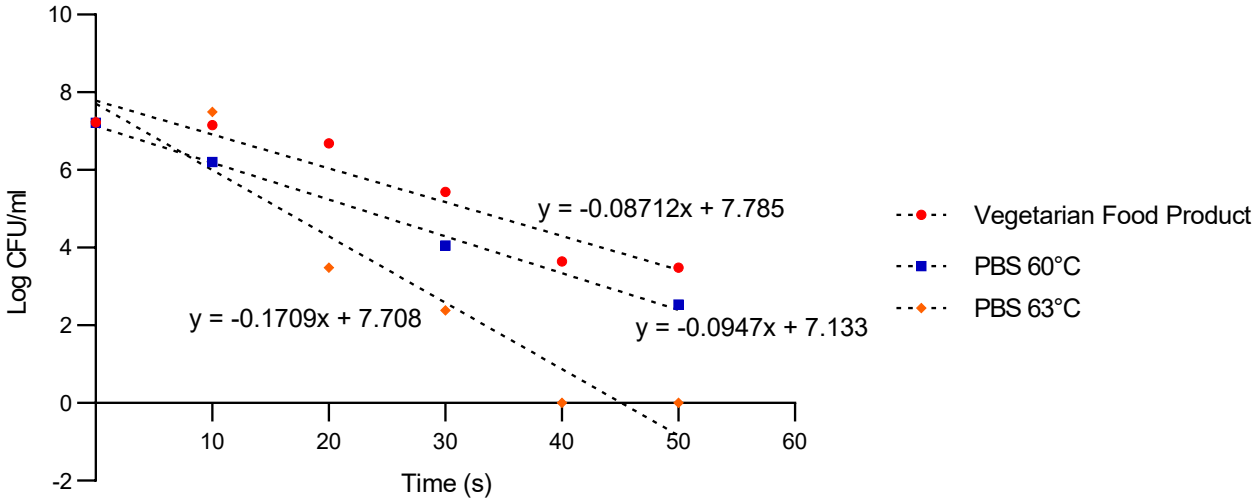


Figure 6. Difference in inactivation kinetics of *S*. Typhimurium strain SL1344 in PBS and the vegetarian food product. Reduction in log CFU/mL of *S.* Typhimurium strain SL1344 in a vegetarian food product heated at 60°C, and PBS buffer heated at both 60°C and 63°C at different time intervals. Each point represents the mean of two technical replicates. The linear regression equation is calculated using the individual replicate y-values.

2.3.6 *S.* Gallinarum strain 287/91 is most sensitive to refrigerated storage

Sub-optimal heat treatment or cross contamination during storage of vegetarian foods may result in survival or even replication of *Salmonella*. To investigate whether the strains of *Salmonella* included in this study were able to replicate and persist in the vegetarian food product, the food product was inoculated with *Salmonella* and incubated at 4°C for 5 weeks (**Figure 7**). The incubation period mimics the typical shelf-life of the vegetarian food product, so it was important to assess *Salmonella* survival over this time-period. On average, there was no increase in viable counts for any strains during the 5-week experiment. *S*. Gallinarum strain 287/91 was the strain most affected by 5-week storage at refrigerated temperatures and resulted in a ~0.6-log reduction in cell viability and was significantly different ($p < 0.0001$) to the control strain *S*. Typhimurium strain ST4/74 (**Figure 7**). The strain showing the greatest tolerance to long term storage at refrigerated temperatures was *S*. Infantis strain S1326/28, with only a ~0.04-log reduction in cell viability. *S*. Infantis strain S1326/28, *S.* Kedougou strain B37 Col19, *S*. Typhimurium strain SO1960-05 and *S*. Schwarzengrund strain SL480 all showed a significant increased ability to survive long periods of time at 4°C compared to ST4/74 (**Figure 7**).

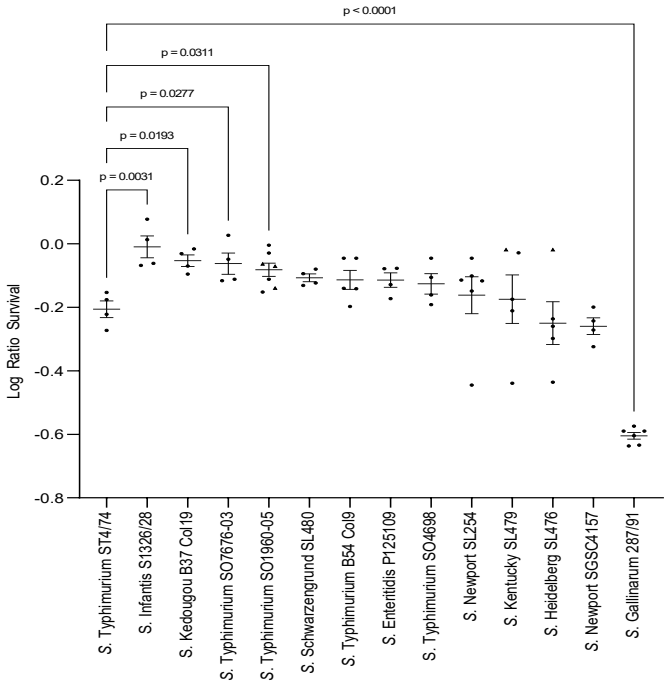


Figure 7. Variability in *Salmonella* survival during long term refrigerated storage. The log ratio survival of 14 *Salmonella* strains used during this study was determined after storage at refrigerated temperature (4°C) for five weeks, in the vegetarian food product. Individual data points represent each technical replicate, and the symbol represents the biological replicate.

2.3.7 *S.* Gallinarum strain 287/91 is most sensitive to heat inactivation

To determine the variation in survival of *Salmonella* strains in response to heat stress, strains were heated in the vegetarian food product and viable counts determined (**Figure 8**). *Salmonella* strains were subjected to heat inactivation at 60°C for 30 seconds using a thermal cell (**Figure 2**) similar to what is used by the food production industry during food pathogen challenge tests. Reduction in viable counts in the food matrix ranged from 0.4-log to 2-log. Viable counts of *S*. Gallinarum strain 287/91 reduced by approximately 2-log and was the most heat sensitive strain used during this study. The cell viability of *S.* Gallinarum strain 287/91 after heating was significantly different to the control strain *S*. Typhimurium ST4/74 at $p < 0.0001$. Conversely, the most heat resistant strain was *S*. Kedougou strain B37 Col19 (**Figure 8**). *S*. Typhimurium strain SO1960-05 also exhibited higher cell death when heated at 60°C for 30 seconds and was also significantly different to the control strain ($p = 0.0004$). The log-ratio survival of *S*. Kentucky strain SL479 and *S.* Schwarzengrund strain SL480 was variable, and therefore it's difficult to conclude whether these serovars were more heat sensitive than some of the other serovars included in this study (**Figure 8**).

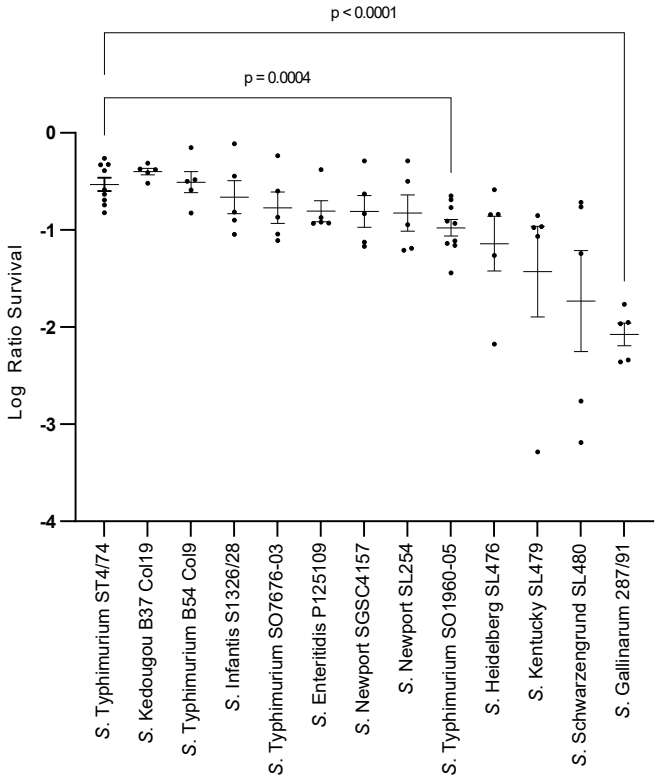


Figure 8. Variability in heat resistance of *Salmonella* strains in a vegetarian food product. Log ratio survival of *Salmonella* strains after heat inactivation at 60°C for 30 seconds in a thermal cell containing 750mg vegetarian food product. Each Individual data point represents a biological replicate.

2.3.8 *S*. Typhimurium strain SO1960-05 is most sensitive to desiccation

To assess the risk to food safety due to the ability of *Salmonella* to survive in low-moisture food, the variation in survival of *Salmonella* strains during desiccation was assessed for 24-hours at an average relative humidity and temperature of 39% and 21°C, respectively (**Figure 9**). All strains decreased in cell number during 24-hour desiccation to different extents (**Figure 9**). *S.* Typhimurium strain SO1960-05 had an average log reduction in cell viability of 2.50 after 24-hour desiccation. This was significantly different ($p = 0.0014$) to the control strain, *S.* Typhimurium strain ST4/74, which had an average log reduction in cell viability of 1.74. *S*. Kentucky strain SL479 was also significantly different to the control strain ($p = 0.0027$) and had an average log reduction in cell viability of 2.49 (**Figure 9**). There were two strains which showed an increased tolerance to desiccation, *S*. Typhimurium strain B54 Col9 and *S*. Typhimurium strain SO4698-09. Both these strains were significantly different to the control strain ($p = 0.0301$ and $p = 0.0358$, respectively), and each had an average log reduction in cell viability of ~1.2. There was a lot of variation in cell viability observed between biological replicates, but also between technical replicates.

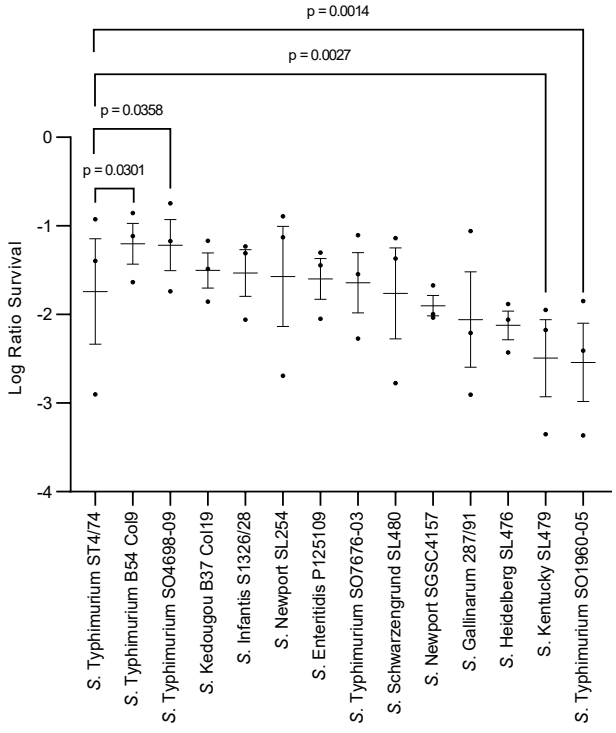


Figure 9. Variability of desiccation tolerance in *Salmonella* strains. Log ratio survival of *Salmonella* strains after desiccation in a safety cabinet for 24 hours at an average relative humidity of 39% and an average temperature of 21°C. Each biological replicate is plotted as a separate point and represents the mean of five technical replicates.

2.3.9 *S*. Typhimurium strain ST4/74 growth is inhibited by 10% NaCl

Sodium chloride (NaCl) is commonly used as a preservative in food products, so to determine the effect NaCl has on the growth and survival of *Salmonella, S.* Typhimurium strain ST4/74 was subjected to increasing NaCl at industry relevant concentrations (1-13%) in LB broth and growth kinetics were measured as optical density (OD600nm) (**Figure 10**). In LB broth, which contains 1% NaCl as standard, growth was initiated after approximately 3 hours of incubation and followed a sigmoidal growth curve. At a NaCl concentration of 2-4%, *S*. Typhimurium strain ST4/74 growth was like that observed in the LB broth control. At 5% NaCl, lag time was increased, and growth began at ~5 hours after initial incubation (**Figure 10**). At 6% NaCl, lag time was longer than observed in 1-5% NaCl, and growth began at ~7 hours. The maximum OD600nm reached with 6% NaCl was ~1.1. Growth of *S*. Typhimurium strain ST4/74 in 7% NaCl began at about 9 hours, indicating a longer lag time than the other salt concentrations. The maximum OD600nm reached for *S*. Typhimurium strain ST4/74 in 7% NaCl was ~0.9 (**Figure 10**). Minimal growth was observed within 20 hours at 8% and 9% NaCl, with OD600nm barely exceeding 0.2 for both concentrations. There was no growth observed during the 20-hour experiment in LB broth supplemented with 10% NaCl and above. LB broth containing 6% NaCl was identified as the concentration in which *Salmonella* begins to be affected by the addition of NaCl, and is therefore in a stressed state, but can still proliferate, with cells remaining viable.

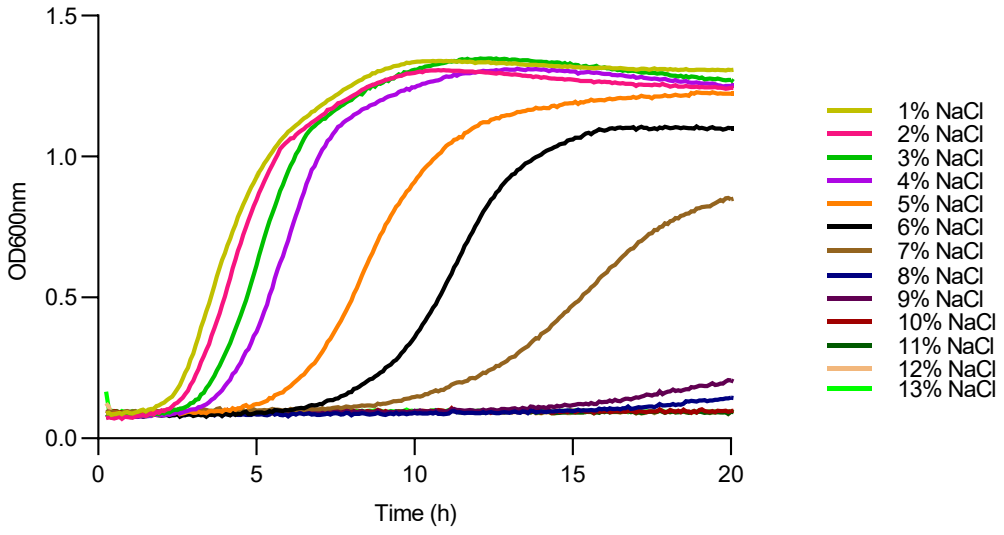


Figure 10. **Growth of *S.* Typhimurium strain ST4/74 in different salt concentrations.** Growth was measured as OD600nm in LB broth containing various NaCl concentrations (as %). Each line represents the mean of three biological replicates, each containing three technical replicates.

2.3.10 *S*. Gallinarum strain 287/91 grows poorly in 6% NaCl

To assess the variability in NaCl stress response for various *Salmonella enterica* serovars, strains were grown in LB broth containing 6% NaCl and growth kinetics were measured using OD600nm (**Figure 11A**). Strains were also grown in LB broth, which typically contains 1% NaCl, for comparison (**Figure 11B**). A 6% concentration of NaCl was chosen due to preliminary studies in *S*. Typhimurium strain ST4/74 which showed 6% NaCl as an ideal concentration to begin to see a decrease in survival but not complete cell death. The lag times differed between strains, and *S*. Gallinarum strain SL287/91 had the longest lag phase in 6% NaCl out of all the serovars tested and growth occurred after ~12 hours incubation in 6% NaCl, whereas growth was initiated between 5 and 9 hours for other serovars (**Figure 11A**). Growth of all strains in LB broth started within 5 hours of incubation, however *S*. Typhimurium strain SO1960-05 exhibited a very short log-phase and proceeded to stationary phase, where the graph plateaued, at ~4 hours (**Figure 11B**). *S*. Gallinarum strain 287/91 had a longer lag time compared to the other strains when grown in LB broth, however after this initial delay, this strain had a similar sigmoidal growth curve to the other strains (**Figure 11B**).

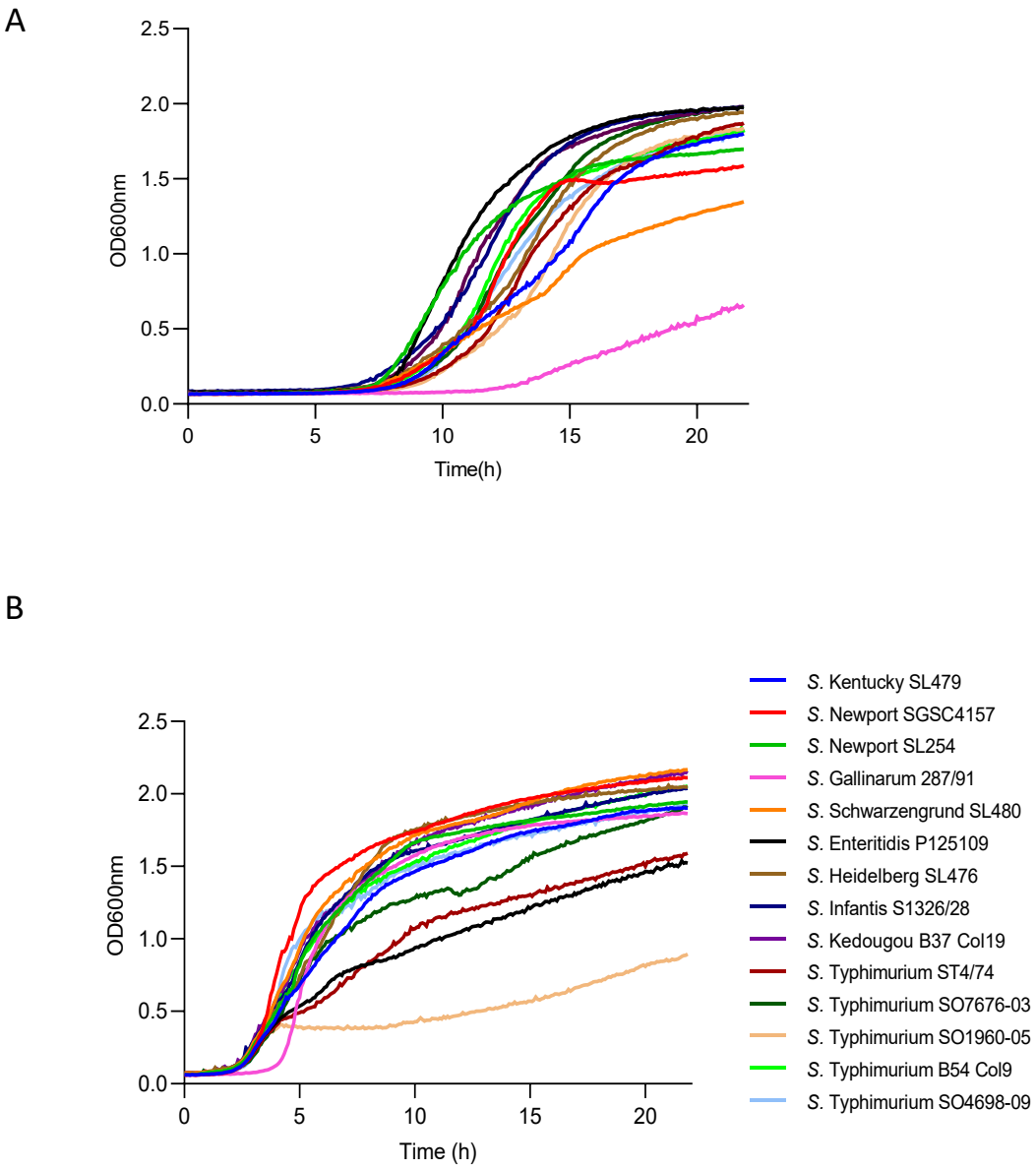


Figure 11. Variation in the growth of *Salmonella* strains when exposed to 6% salt. Growth was measured as OD600nm in 14 different strains *Salmonella* in (A) LB broth containing 6% NaCl and (B) LB broth (1% NaCl). Each data point represents the mean of 3 biological replicates and each biological replicate consisted of three technical replicates.

An R-package, called Growthcurver, was used to determine the growth rate (r-value), maximum culture density and doubling time for each strain grown in LB broth containing 6% NaCl and in typical LB broth (1% NaCl) (**Table 3**). To quantify the carrying capacity and growth rate (r-value), Growthcurver fits a logistic equation to the growth curve data provided and the equation can be defined as:

$$Nt = \frac{K}{1 + (\frac{K - N0}{N0})e^{-rt}}$$

In this equation, the number of cells at time *t* is defined by *Nt*, the maximum population size (maximum OD600nm) is given by *K*, and the intrinsic growth rate of the population is denoted by *r*. The intrinsic growth rate of the population (*r*) is the growth with no restriction on population size. The Levenberg-Marquardt algorithm within the minpack.lm package in R is executed within Growthcurver to find the best values for *K*, *r* and *N0*. The generation time (t_gen) is the time taken for the amount of cells (or optical density) to double and Growthcurver calculates the fastest possible value, with no restrictions on growth, for this parameter (Sprouffske and Wagner, 2016).

S. Schwarzengrund strain SL480 reached the greatest maximum OD600nm (*K*) compared to the other strains in LB broth, whereas in LB broth containing 6% NaCl, *S*. Heidelberg strain SL476 and *S*. Infantis strain S1326/28 reached the greatest OD600nm at 1.87 and 1.88, respectively. *S*. Gallinarum strain 287/91 had an *r* value closest to 1, meaning the steepest log-phase and hence the quickest generation time (~50 minutes). Comparatively, in LB broth with 6% NaCl, the *r* value decreased to 0.48, generation time (t-gen) increased to 1 hour and 27 minutes and the maximum OD600nm reached was 0.629 (**Table 3**). In LB broth with 6% NaCl, *S*. Newport strain SGSC4157 had the shortest generation time (t_gen) at 48 minutes, whereas *S*. Schwarzengrund strain SL480 had the greatest generation time (1 hour 39 minutes). *S*. Typhimurium strain SO1960-05 had an *r* value of only 0.13, which is reflected by an extremely long generation time of 5 hours and 28 minutes (**Table 3**).This is consistent with the shape of the graph of *S*. Typhimurium strain SO1960-05 observed in LB broth without additional NaCl (**Figure 11**). In LB broth with 6% NaCl, *S*. Typhimurium strain SO1960-05 had an increased *r* value and a generation time more like the other strains at 1 hour and 8 minutes (**Table 3**).

Strain	LB Broth			LB Broth + 6% NaCl		
	Max. OD600nm (*K*)	*r*	t_gen (hh:mm)	Max. OD600nm (*K*)	*r*	t_gen (hh:mm)
S. Kentucky SL479	1.74	0.48	01:26	1.79	0.45	01:31
S. Newport SGSC4157	1.89	0.73	00:57	1.48	0.86	00:48
S. Newport SL254	1.78	0.59	01:10	1.57	0.70	00:59
S. Gallinarum 287/91	1.71	0.82	00:50	0.62	0.48	01:27
S. Schwarzengrund SL480	1.93	0.54	01:16	1.28	0.42	01:39
S. Enteritidis P125109	1.37	0.29	02:22	1.84	0.73	00:56
S. Heidelberg SL476	1.90	0.60	01:09	1.88	0.55	01:16
S. Infantis S1326/28	1.81	0.56	01:14	1.87	0.64	01:04
S. Kedougou B37 Col19	1.92	0.49	01:25	1.85	0.69	01:00
S. Typhimurium ST4/74	1.40	0.36	01:54	1.72	0.61	01:07
S. Typhimurium SO7676-03	1.65	0.37	01:52	1.87	0.64	01:04
S. Typhimurium SO1960-05	1.24	0.13	05:28	1.78	0.61	01:08
S. Typhimurium B54 Col9	1.82	0.47	01:28	1.66	0.72	00:57
S. Typhimurium SO4698-09	1.69	0.55	01:15	1.68	0.61	01:07

Table 3. Growth rate properties of *Salmonella* strains in LB broth compared to LB broth supplemented with 6% NaCl. The maximum OD600nm (*K*), intrinsic growth rate of population (*r*) and generation time (t_gen) was determined for 14 *Salmonella* strains grown in LB broth and LB broth containing 6% NaCl over a 22-hour period. The output contains the results from the Growthcurver package in R on the mean data of three biological replicates.

2.3.11 *S.* Typhimurium strain ST4/74 grown in differing concentrations of citric and acetic acid

Citric and acetic acid are both common organic acids used to control pathogens in food products. To establish the minimum inhibitory concentration of each acid on *S*. Typhimurium strain ST4/74, growth kinetics were measured using OD600nm in concentrations of citric and acetic acid relevant to the food industry (between 0 and 30mM of each acid) (**Figure 12**). Cells were subjected to a 30-minute pre-adaptation step at 37°C in LB broth adjusted to pH 5.8 with either citric (30mM) or acetic acid (30mM). The pH of each concentration of acetic and citric acid was measured (**Table 4**), and generally, citric acid has a more acidic pH than acetic acid at the same concentration. *S*. Typhimurium strain ST4/74 reached the greatest OD600nm in LB broth containing 10mM citric acid and acetic acid, however the lag phase was extended compared to the lower concentrations of acids. In 14mM citric acid, growth of ST4/74 began after 10 hours of incubation, whereas in 14mM acetic acid growth was initiated after 13 hours incubation, and ST4/74 only reached a maximum OD600nm of ~0.5 in acetic acid (within 22 hours incubation) compared to an OD600nm of ~1.4 in citric acid. Generally,

the duration of lag phase was longer in the presence of acetic acid than citric acid at concentrations above 10mM (**Figure 12**). Growth of *S*. Typhimurium strain ST4/74 was inhibited at concentrations above 18mM and 16mM for citric acid and acetic acid, respectively, although growth of ST4/74 in 16mM citric acid is minimal.

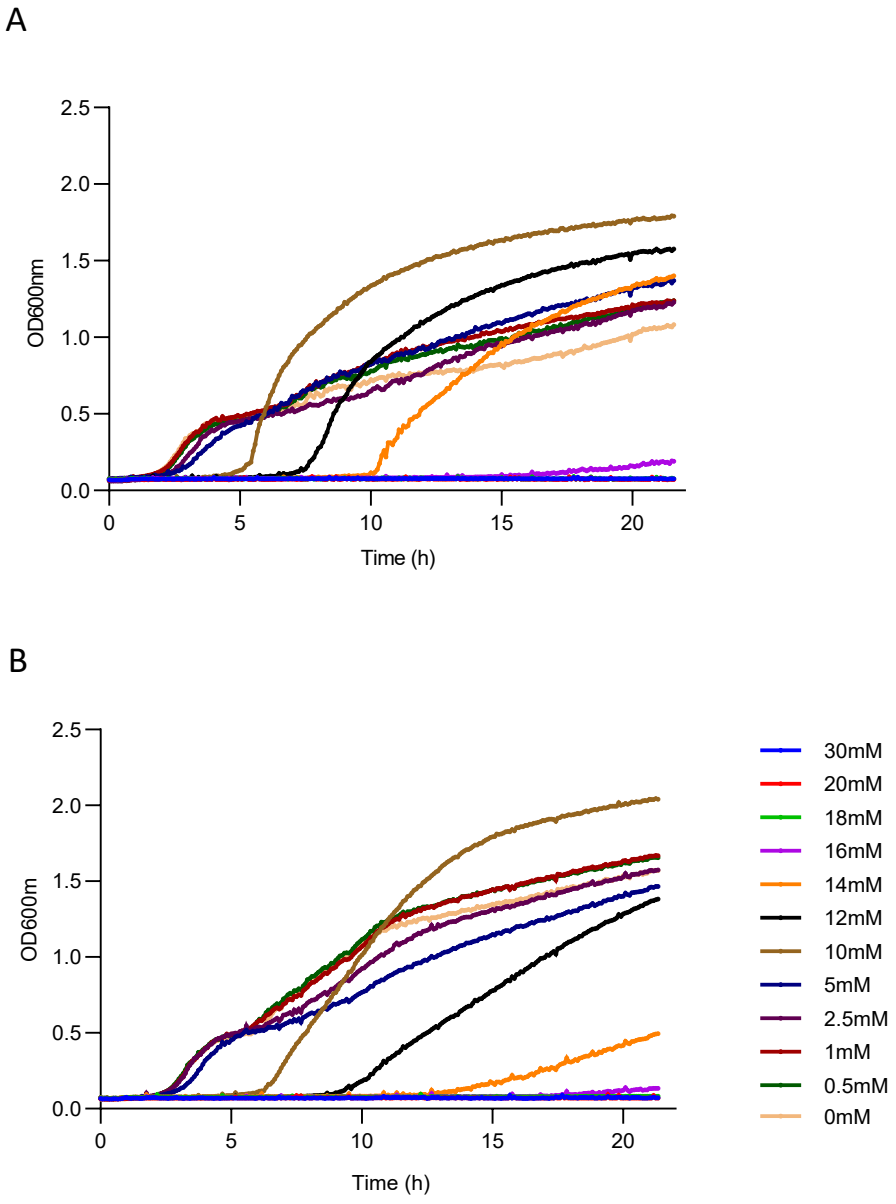


Figure 12. Growth of *S*. Typhimurium strain ST4/74 in different concentrations of citric acid and acetic acid. Growth, measured as OD600nm, of *S*. Typhimurium strain ST4/74 in LB broth containing different concentrations (mM) of (A) citric acid and (B) acetic acid. One representative experiment is shown from two biological replicates. Each data point represents the mean of four technical replicates.

Organic Acid	Concentration (mM)	Concentration (%)	pH
Acetic	30	1.72	4.4
	20	1.15	4.6
	18	1.03	4.7
	16	0.92	4.8
	14	0.80	4.9
	12	0.69	5
	10	0.57	5.1
	5	0.29	5.9
	2.5	0.14	6.4
	1	0.06	6.7
	0.5	0.03	6.8
	0	0.00	6.9
Citric	30	0.57	3.4
	20	0.38	3.7
	18	0.34	3.8
	16	0.31	3.9
	14	0.27	4
	12	0.23	4.1
	10	0.19	4.3
	5	0.10	4.9
	2.5	0.05	5.7
	1	0.02	6.5
	0.5	0.01	6.8
	0	0.00	7

Table 4. Concentration of organic acid used during growth experiments. The average pH measurements of acetic acid and citric acid concentrations (in mM and %) used during growth experiments of *S*. Typhimurium strain ST4/74.

2.3.12 *S*. Gallinarum strain 287/91 grows poorly in 12mM acetic acid and 14mM citric acid

The variation in growth of different *Salmonella* strains in LB broth supplemented with 12mM acetic acid and 14mM citric acid was assessed by monitoring the OD600nm over a 24-hour period (**Figure 13** and **Figure 14**). The concentration of each acid was chosen from the results of preliminary experiments in *S*. Typhimurium strain ST4/74, which showed 12mM and 14mM as the concentrations at which growth begins to be affected by the addition of each acid, but cells are still viable and able to grow. For all strains featured, the duration of lag phase was increased in acetic acid, compared to the same strains in LB broth (**Figure 13**). *S*. Gallinarum strain 287/91 and *S.* Typhimurium strain SO1960-05 log phase began after 8- and

10-hours incubation respectively, when grown in LB broth containing 12mM acetic acid. This was later than the other strains included in this study, which all entered log phase after ~5 hours incubation (**Figure 13A**). *S*. Typhimurium strain SO1960-05 entered log phase after ~8 hours incubation with LB agar containing 12mM acetic acid and reached a maximum OD600nm of ~1.5, which was a greater OD600nm reached than in LB broth alone (**Figure 13**). In LB broth, *S*. Typhimurium strain SO1960-05 experienced a short log phase, and only reached a maximum OD600nm of ~1 (**Figure 13B**). *S*. Typhimurium strain SO7676-03 and *S*. Typhimurium strain ST4/74 exhibited a similar growth pattern in LB broth and reached maximum OD600nm of ~1.5 (**Figure 13B**).

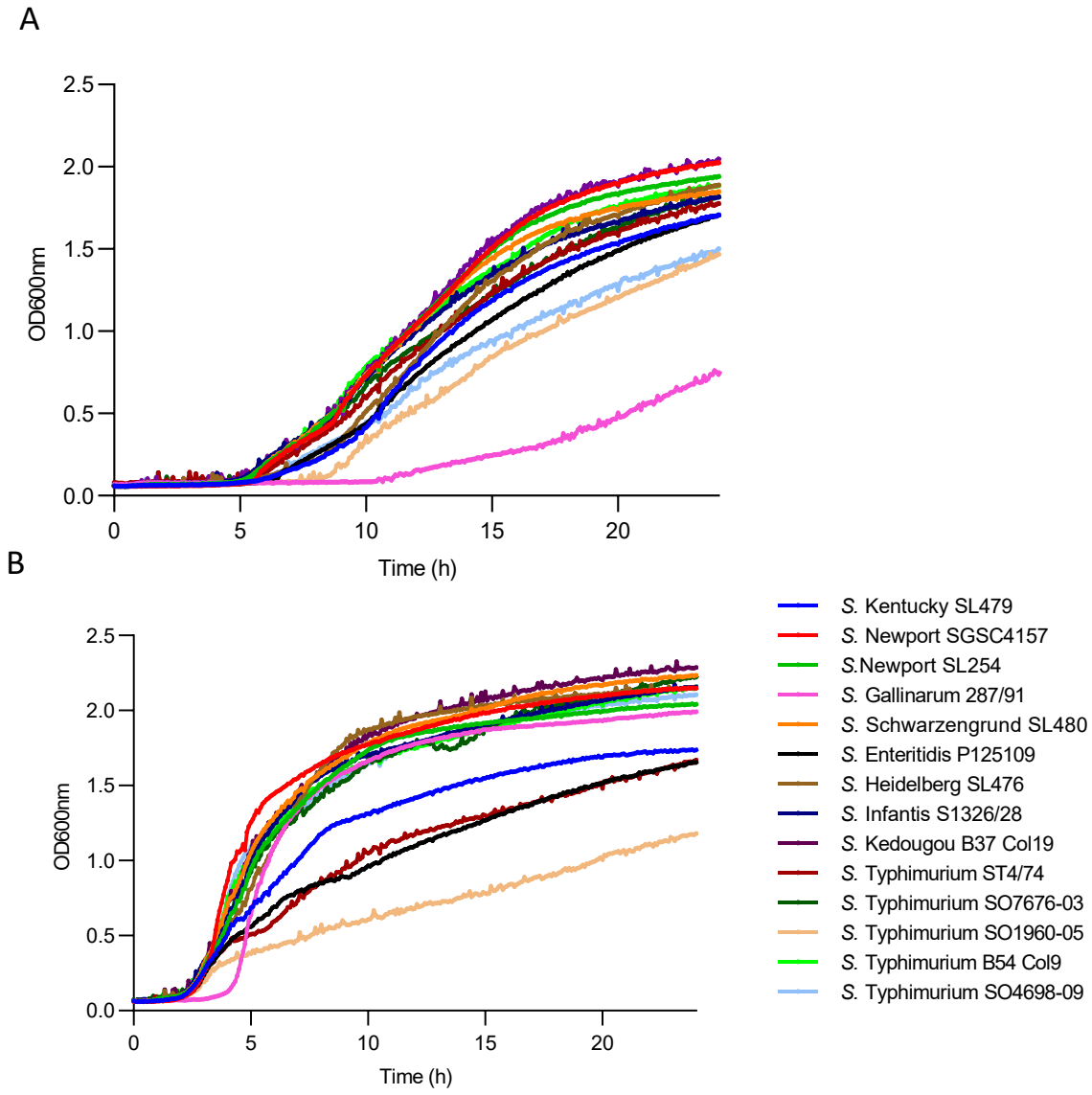


Figure 13. **Variation in the growth of *Salmonella* strains when exposed to acetic acid**. Growth was measured as OD600nm in 14 different strains of various *Salmonella* serovars (pre-adapted to pH 5.8) in (A) LB broth containing 12mM acetic acid and (B) LB broth (no acetic acid). Each line represents the mean of three biological replicates and each biological replicate consisted of three technical replicates.

Similarly, to acetic acid, the addition of citric acid increased the lag phase for all strains featured, in comparison to the same strains in LB broth (**Figure 14**). The initiation of log phase seemed more variable for citric acid than acetic acid, with strains beginning to grow between 5 and 8 hours in LB broth with 14mM citric acid (**Figure 14**). *S*. Gallinarum strain 287/91 had the longest lag phase and entered log phase after ~9 hours after initial incubation, which was similar to that observed in acetic acid. The lag phase duration for *S*. Gallinarum strain 287/91 in LB broth with cells pre-adapted to citric acid, was longer than in other strains, which was observed with cells pre-adapted to acetic acid also (**Figure 14**). Cells of *S.* Typhimurium strain SO1960-05, ST4/74 and SO7676-03 pre-adapted to citric acid, all behaved similar to when pre-adapted to acetic acid in LB broth (**Figure 14**).

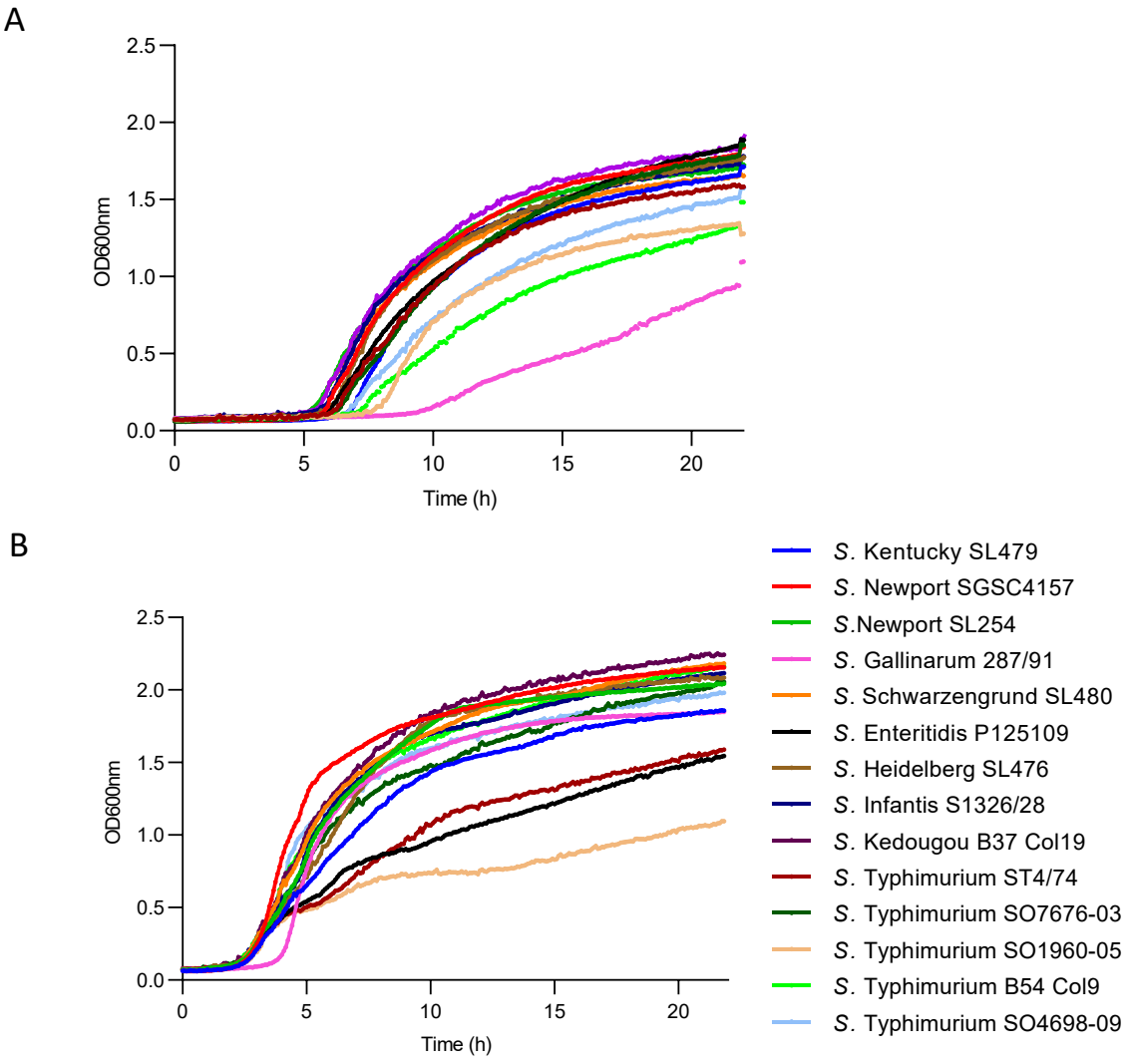


Figure 14. **Variation in the growth of *Salmonella* strains when exposed to citric acid.** Growth was measured as OD600nm in 14 different strains of various *Salmonella* serovars (pre-adapted to pH 5.8) in (A) LB broth containing 14mM citric acid and (B) LB broth (no citric acid). Each line represents the mean of 3 biological replicates and each biological replicate consisted of 3 technical replicates.

2.3.13 Variation in growth rate

To determine the amount of variability observed in strains of *Salmonella* when grown in LB broth supplemented with 12mM acetic acid or 14mM citric acid, the Growthcurver package in R was used to quantify the growth rate, doubling time and maximum OD600nm of each strain. The intrinsic growth rate of the population (*r*), maximum OD600nm reached (*K*) and doubling time (t_gen) were calculated from growth curves for each *Salmonella* strain (**Table** 5). *S*. Gallinarum strain 287/91 grown in LB broth had the highest *r* value (~0.82) in LB broth, when pre-adapted to pH 5.8 in both acetic and citric acid, and the quickest doubling time out of all the trains tested, at ~50 minutes (**Table 5**). *S*. Kedougou strain B37 Col19 reached the greatest OD600nm in LB broth (cells pre-adapted to acetic and citric acid) and in LB broth containing 12mM acetic acid, however *S.* Enteritidis strain P125109 reached the greatest OD600nm in LB broth containing 14mM citric acid (**Table 5**). *S*. Typhimurium strain SO1960-05 exhibited the longest doubling time in LB when pre-adapted to both acids (**Table 5**). The doubling time for *S*. Typhimurium strain SO1960-05 decreased with the addition of acetic acid and citric acid and was more similar to the doubling time observed in other strains (**Table 5**).The doubling time for *S*. Typhimurium strain SO1960-05 was the quickest in 14mM citric acid (pH 4.0) at 1.03 hours (**Table 5**). *S.* Kentucky strain SL479 exhibited the lowest *r* value and the quickest doubling time in LB broth with 12mM acetic acid (**Table 5**).

Strain	LB Broth (cells pre-adapted to pH 5.8 with Acetic Acid)			LB Broth + 12mM Acetic Acid			LB Broth (cells pre-adapted to pH 5.8 with Citric Acid)			LB Broth + 14mM Citric Acid		
	Max. OD600nm (*K*)	*r*	t_gen (hh:mm)	Max. OD600nm (*K*)	*r*	t_gen (hh:mm)	Max. OD600nm (*K*)	*r*	t_gen (hh:mm)	Max. OD600nm (*K*)	*r*	t_gen (hh:mm)
S. Kentucky SL479	1.59	0.44	01:35	1.59	0.42	01:40	1.70	0.48	01:27	1.53	0.55	01:15
S. Newport SGSC4157	1.94	0.69	01:00	1.94	0.39	01:46	1.94	0.77	00:54	1.65	0.54	01:16
S. Newport SL254	1.89	0.59	01:10	1.86	0.40	01:44	1.90	0.61	01:07	1.57	0.56	01:13
S. Gallinarum 287/91	1.82	0.82	00:50	0.95	0.27	02:31	1.69	0.83	00:49	1.33	0.27	02:34
S. Schwarzengrund SL480	2.03	0.52	01:19	1.76	0.40	01:43	1.94	0.54	01:16	1.52	0.52	01:19
S. Enteritidis P125109	1.53	0.26	02:39	1.62	0.34	02:01	1.37	0.30	02:18	1.72	0.42	01:38
S. Heidelberg SL476	2.01	0.61	01:07	1.79	0.40	01:42	1.93	0.59	01:10	1.59	0.53	01:18
S. Infantis S1326/28	1.92	0.55	01:15	1.71	0.37	01:53	1.88	0.58	01:11	1.57	0.53	01:18
S. Kedougou B37 Col19	2.09	0.51	01:21	1.95	0.39	01:47	2.04	0.56	01:14	1.69	0.53	01:18
S. Typhimurium ST4/74	1.48	0.31	02:13	1.68	0.35	01:59	1.40	0.37	01:53	1.46	0.55	01:15
S. Typhimurium SO7676-03	1.97	0.43	01:36	1.75	0.32	02:09	1.80	0.45	01:33	1.67	0.47	01:28
S. Typhimurium SO1960-05	1.13	0.19	03:34	1.41	0.33	02:05	0.88	0.34	02:01	1.20	0.65	01:03
S. Typhimurium B54 Col9	1.91	0.50	01:22	1.79	0.36	01:55	1.92	0.51	01:22	1.27	0.38	01:50
S. Typhimurium SO4698-09	1.90	0.50	01:23	1.41	0.32	02:09	1.76	0.60	01:09	1.41	0.45	01:33

Table 5. Growth rate properties of *Salmonella* strains in LB broth compared to LB broth supplemented with acetic and citric acid. Maximum OD600nm, intrinsic growth rate of population (r-value) and generation time (t_gen) of 14 *Salmonella* strains grown in LB broth and LB broth supplemented with either 12mM acetic acid or 14mM citric acid over a 24-hour period. The output contains the results from the Growthcurver package in R on the mean data of 3 biological replicates.

2.3.14 *S*. Gallinarum strain 287/91 had the greatest difference in area under the curve in all food chain related stresses

To assess the variation in growth of each strain in LB broth compared to LB broth supplemented with either 6% NaCl, 12mM acetic acid or 14mM citric acid, the difference in area under the curve was calculated (**Figure 15**). *S*. Gallinarum strain 287/91 exhibited the greatest difference in the area under the curve for all three stresses and were significantly different from *S*. Typhimurium strain ST4/74 ($p < 0.0001$) (**Figure 15**). *S*. Enteritidis strain P125109 and *S*. Typhimurium strain SO1960-05 grew better, as indicated by a greater area under the curve, in LB broth containing NaCl and 14mM citric acid, compared to LB broth with no supplementation (**Figure 15**). The difference in area under the curve for *S*. Typhimurium strain B54 Col9 was significantly different to the control strain in LB broth containing 14mM citric acid (**Figure 15**).

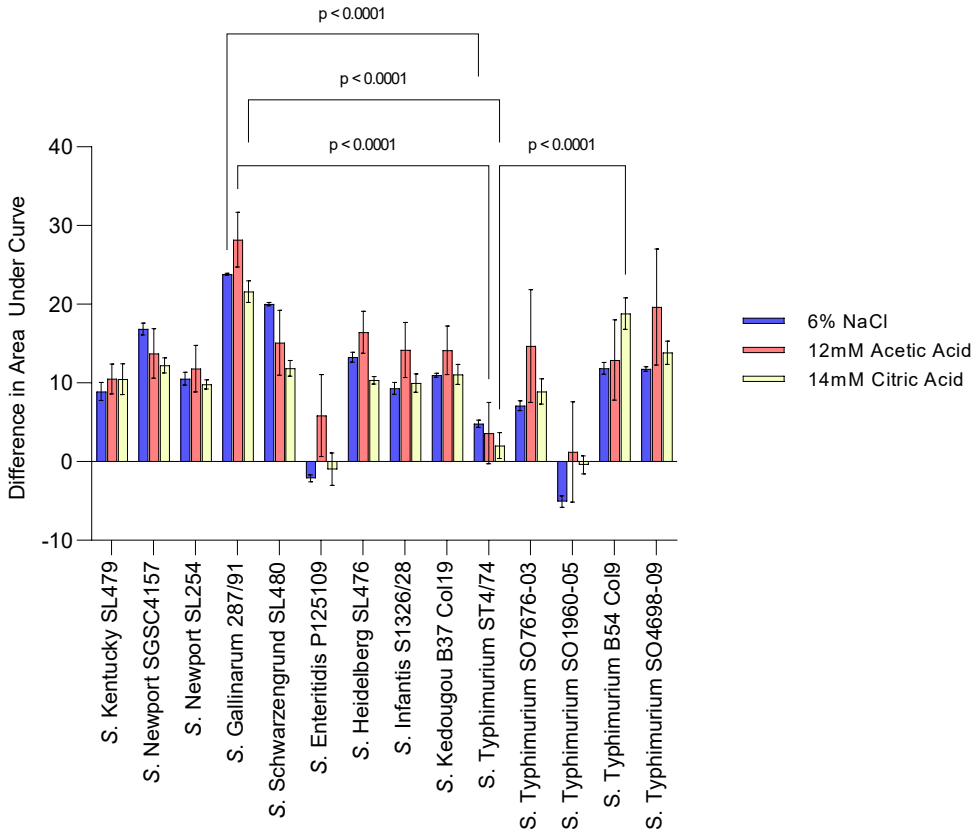


Figure 15. Difference in area under the curve for *Salmonella* strains after chemical stress induction. The difference in area under the curve for 14 *Salmonella* strains grown in LB broth compared to LB broth supplemented with 6% NaCl, 12mM acetic acid or 14mM citric acid was determined. Bars represent the mean of 3 biological replicates (±SE).

2.3.15 Summary of food chain related stress phenotype for 14 *Salmonella enterica* strains

To summarise the phenotypic results obtained for each strain of *S*. *enterica* used in the present study, radial plots were generated from the log ratio survival data for heat inactivation, desiccation and 5-week refrigerated storage (**Figure 16**), and the difference in area under the curve data during growth in LB broth supplemented with 6% NaCl, 14mM citric acid and 12mM acetic acid (**Figure 17**). A web-based tool (RAWGraphs 2.0) was used to create the plots for each strain. It is evident from the radial plots that *S*. Gallinarum strain 287/91 is most sensitive to both the physical stressors (heat inactivation, desiccation, and refrigerated storage) and chemical stressors (6% NaCl, acetic acid, and citric acid) (**Figure 16** and **Figure 17**). *S*. Typhimurium strain SO4698-09 was most resistant to physical stressors (**Figure 16**), however *S*. Typhimurium strain SO1960-05 had the smallest difference in area under the curve for each chemical stress, and therefore was the most resistant strain to chemical stressors in this study (**Figure 17**).

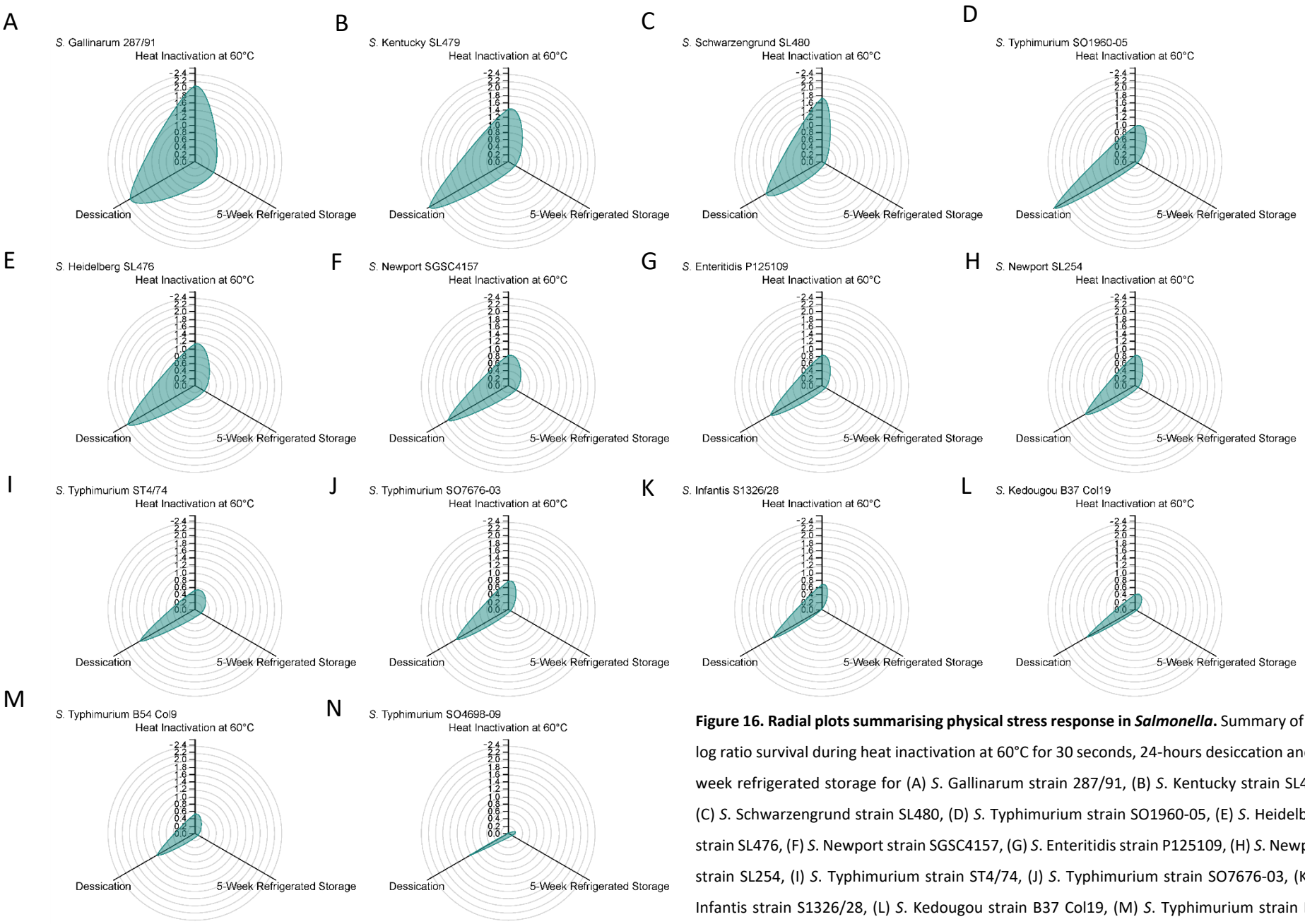


Figure 16. Radial plots summarising physical stress response in *Salmonella*. Summary of the log ratio survival during heat inactivation at 60°C for 30 seconds, 24-hours desiccation and 5-week refrigerated storage for (A) *S*. Gallinarum strain 287/91, (B) *S*. Kentucky strain SL479, (C) *S*. Schwarzengrund strain SL480, (D) *S*. Typhimurium strain SO1960-05, (E) *S*. Heidelberg strain SL476, (F) *S*. Newport strain SGSC4157, (G) *S*. Enteritidis strain P125109, (H) *S*. Newport strain SL254, (I) *S*. Typhimurium strain ST4/74, (J) *S*. Typhimurium strain SO7676-03, (K) *S*. Infantis strain S1326/28, (L) *S*. Kedougou strain B37 Col19, (M) *S*. Typhimurium strain B54 Col9 and (N) *S*. Typhimurium strain SO4698-09.

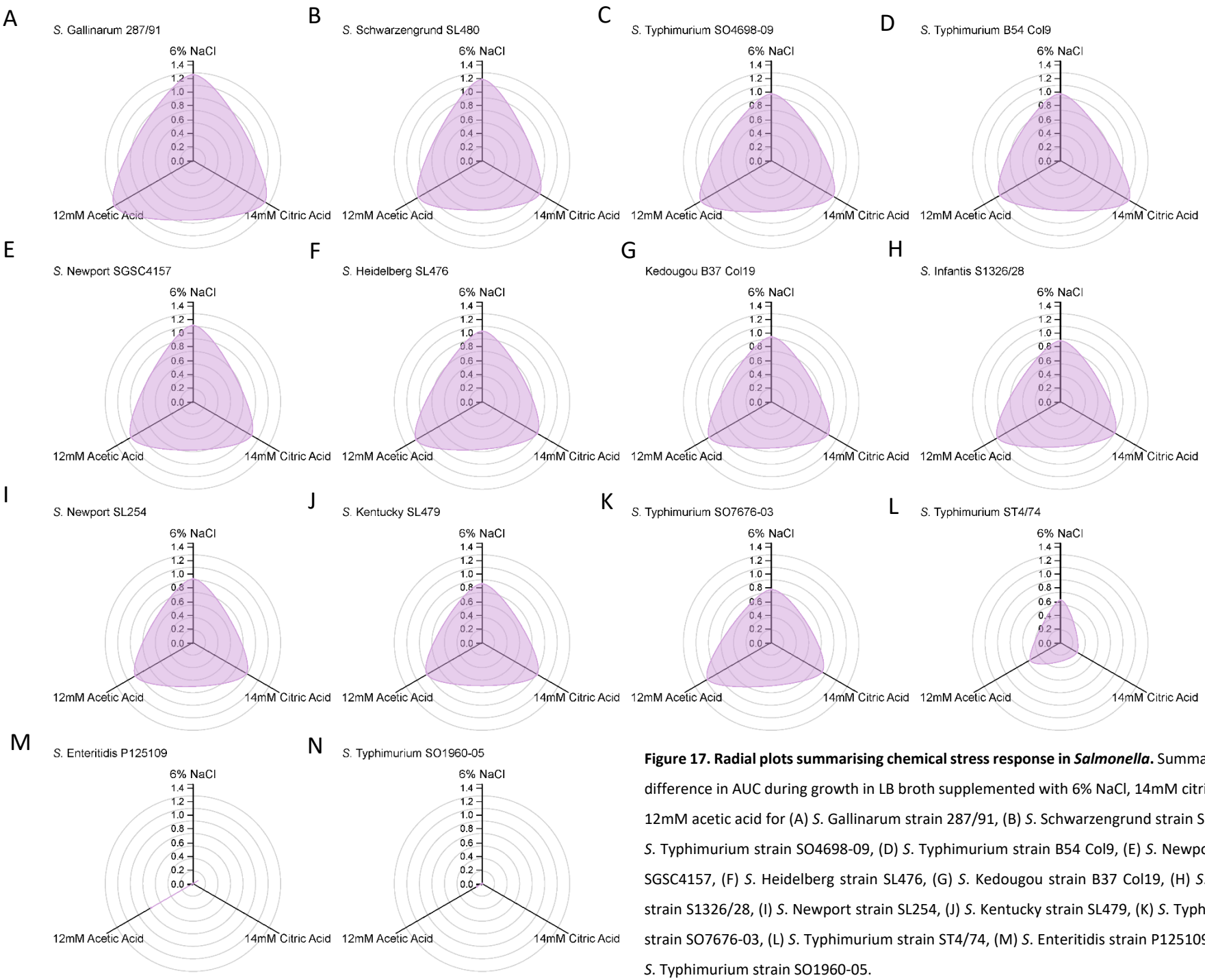


Figure 17. Radial plots summarising chemical stress response in *Salmonella*. Summary of the difference in AUC during growth in LB broth supplemented with 6% NaCl, 14mM citric acid or 12mM acetic acid for (A) *S*. Gallinarum strain 287/91, (B) *S*. Schwarzengrund strain SL480, (C) *S*. Typhimurium strain SO4698-09, (D) *S*. Typhimurium strain B54 Col9, (E) *S*. Newport strain SGSC4157, (F) *S*. Heidelberg strain SL476, (G) *S*. Kedougou strain B37 Col19, (H) *S*. Infantis strain S1326/28, (I) *S*. Newport strain SL254, (J) *S*. Kentucky strain SL479, (K) *S*. Typhimurium strain SO7676-03, (L) *S*. Typhimurium strain ST4/74, (M) *S*. Enteritidis strain P125109 and (N) *S*. Typhimurium strain SO1960-05.

2.4. Discussion

Understanding variation in stress response of diverse *Salmonella enterica* serovars will enable the development of processing techniques which satisfies increasing demand for minimally processed foods due to government regulation and consumer habits, which contain fewer preservatives, without compromising food safety. Multiple hurdles are implemented in the manufacturing process to control food safety and quality of food products, yet there is still the risk of post-process and post-sale contamination. Currently, all *Salmonella* strains and serovars are treated the same in risk assessments, however certain strains may pose an increased risk to food safety. To assess the risk different *Salmonella* strains have on food safety, strains of diverse serovars were subjected to food chain related stress in the current study, and the differences in ability to survive these stresses analysed.

The strains chosen to be included in the study were selected due to their source of isolation (human, animal, or food) and virulence, however the number of strains included in the study was constrained due to the minimal number of strains of diverse serovars available in the culture collection obtained from the SGSC. Defining the thermal inactivation kinetics of a target pathogen in a food product is an important step towards determining the thermal processing requirements of the finished food product. In the current study the time required to reduce cell viability by 1-log, the D-value, was determined for *S*. Typhimurium strain SL1344. The D-value at 60°C in PBS was 10.6 seconds, which a greater D-value than reported for different *S*. Typhimurium strains in a publication by Monu *et al*. (2015), which ranged from 0.16 to 0.23 seconds at 60°C in *S*. Typhimurium strains 2576, 2486 and ATCC 23564. The differences in D-values at 60°C in PBS could be due to the different strains that were used to conduct each experiment, but also due to the method of heating. A key objective in the current study was to assess the survival of different *Salmonella* strains directly in the food product, to recommend a time and temperature combination to effectively eradicate any potential *Salmonella* contaminants in the food.

First, it was important to compare the D-values for SL1344 in PBS and the vegetarian food product, to determine whether the food product increases the decimal reduction time. There was only a one second difference between the D-values observed at 60°C for PBS and vegetarian food product at 10.5 and 11.5 seconds, respectively. The amount of fat, sucrose, glycerol and the addition of additives in food can affect the heat resistance of pathogenic microorganisms that may be present in the food product, and hence a greater difference in

D-value was originally hypothesised for thermal death of *S*. Typhimurium strain SL1344 in PBS and the vegetarian food product (Sallami *et al.*, 2006). Previous studies have shown that a high fat content protects the bacterial cells from heat by reducing the amount of free water in the food matrix and altering the heat transfer through the food product. For example, Ahmed *et al.* (1995) reported that *E. coli* O157:H7 heated in food products with a high fat content had higher D-values than when heated in foods with a lower fat content.

The small difference in D-value observed in PBS and the food matrix reported in the current study could be due to the method used to heat the *Salmonella* in the food product, which is known as the submerged tube method. The submerged tube method involves homogenising the food matrix in PBS thus changing the structure of the food by increasing the water content, with the potential to alter the D-value. A greater difference in D-value might be observed if *Salmonella* was heated in the food product without the addition of PBS. As a result, future experiments involving heating the food product to assess the variation in survival of different *Salmonella s*erovars were conducted in a thermal cell, supplied by Nestlé, which not only mimics the heat inactivation tests conducted at their research facility, but also enabled the food product to be heated without manipulation.

From this study, it is evident that survival under stress differs in strains of various serovars. Furthermore, investigation of multiple strains of *S*. Typhimurium indicated that variation also exists amongst strains of the same serovar. *S*. Gallinarum strain 287/91 was the strain most sensitive to temperature stress, specifically heat inactivation at 60°C and long term refrigerated storage at 4°C. The *S*. Gallinarum strain also exhibited the greatest sensitivity to the addition of salt, acetic acid and citric acid, which are all common preservation techniques. *S.* Gallinarum strain 287/91 resulted in an approximate 2-log reduction when heated for 30 seconds at 60°C in the vegetarian food product, which was the greatest reduction in cell viability observed for all strains tested during the present study. In a study by Jones-Ibarra *et al*. (2017), *S.* Gallinarum strain SG60 had a D-value of 0.41±0.37 min at 60°C in peptone buffer, and exhibited an initial steep death phase, which later plateaued indicating no further inactivation. In this previously published study, the D-values of *S.* Gallinarum strains SG60, SG70 and SG98 at 60°C were not significantly different to an *S.* Enteritidis strain, however there was an initial rapid 3-4 log reduction in mean CFU/mL during the first 30 seconds of heating at 60°C, which was not observed in the other two strains included in the study, an *S.* Enteritidis and *S.* Senftenberg strain, which only reduced mean CFU/mL by ~1-log in 30 seconds (Jones-Ibarra *et al.*, 2017). The rapid initial decrease in CFU/mL in *S*. Gallinarum was

also observed in the current study, whereby *S.* Gallinarum strain 287/91 resulted in the greatest log reduction within 30 seconds of heating compared to the other strains tested.

S. Gallinarum is highly restricted to avian species, and causes fowl typhoid with a high mortality rate in birds (Barrow and Neto, 2011). The ability of a serovar to be adapted to a specific niche may increase sensitivity to environmental stress. Furthermore, typhoid-like diseases have different transmission strategies than non-typhoidal *Salmonella.* Typically, Fowl typhoid is transmitted between individuals within a flock (horizontal transmission), but some studies have also indicated that vector-mediated transmission via mites (*Dermanyssus gallinae*) can occur (Pugliese *et al*., 2019; Cocciolo *et al*., 2020). Whereas, non-typhoidal *Salmonella* is often acquired through ingesting contaminated food, or direct contact with infected animals (de Freitas Neto *et al.*, 2010). A comparative analysis of *S.* Gallinarum strain 287/91 and *S.* Enteritidis strain PT4, revealed that *S.* Gallinarum is a direct descendent of *S.* Enteritidis and strain 287/91 harbours 309 pseudogenes (Thomson *et al.*, 2008). These pseudogenes in 287/91 are responsible for loss of gene function in many metabolic pathways including, motility, metal/ drug resistance, amino acid catabolism and cellulose biosynthesis, and although not strictly related to the stresses included in this study, they could explain *S.* Gallinarums' general sensitivity to environmental stress. For instance, 287/91 has a mutation in *bcsG* which may affect cellulose production, and therefore a lack of cellulose production also affects the cells' ability to form biofilm, rendering it more sensitive to chemical and mechanical stress (Thomson *et al.*, 2008).

Inappropriate food storage temperature is one of the most common causes of foodborne contamination (FAO and WHO, 2009). The recommended temperature for a domestic refrigerator is 2°C to 4°C to prevent microbial growth, however research into consumer refrigeration habits has revealed that the majority of household fridges exceed this temperature (FAO and WHO, 2009; Ovca *et al*., 2021). None of the 14 strains tested exhibited a net increase in CFU over a 5-week period (positive log ratio), indicating that the replication rate did not exceed the death rate in the food product at 4°C. This is unsurprising as *Salmonella* typically grows between 7°C and 48°C, with an optimum temperature of 37°C, although some studies have shown that *Salmonella* is able to grow at 2-4°C (Cox and Pavic, 2014; D'Aoust, 1991; Matches and Liston, 1968). *S*. Infantis strain S1326/28, *S*. Kedougou strain B37 Col19, *S.* Typhimurium strain SO1960-05 and *S*. Schwarzengrund strain SL480 all showed an increased capability of surviving at 4°C during the 5-week period compared to *S.* Typhimurium strain ST4/74, however it is important to note that the total decrease in cells over the 5-weeks for all strains included in this study was no more than 0.6-log.

A similar study in egg yolks showed that *S*. Infantis cell counts at 5.5°C decreased by about 1-log over a 4-week period, which is more than observed in the current study, although this may be due to the difference in temperature or food matrix used (Lublin *et al.*, 2015). *Salmonella* is particularly problematic in low moisture foods such as dried fruit, peanut butter, and flour as it can survive for long periods of time in these conditions (Finn *et al*., 2013a). In the present study, resistance to desiccation was variable amongst the 14 strains tested, which agrees with a recent study elucidating desiccation resistance of 37 strains of *Salmonella* in soybean meal (Norberto *et al.*, 2022). In the current study, both *S*. Typhimurium strain SO1960-05 and *S*. Kentucky strain SL479 exhibited an increased sensitivity to desiccation and the number of cells recovered after 24 hours were significantly lower than *S*. Typhimurium strain ST4/74. Tolerance to desiccation in U288 strains was also observed in a study by Kirkwood *et al*. (2021), whereby only 0.1% of cells of U288 strains remained viable after 24-hour desiccation.

In the present study, an increased resistance to desiccation was observed in two monophasic *S.* Typhimurium strains, B54 Col9 and SO4698-09, and the average log reduction for both strains was approximately 1.2. This equates to about a 90% reduction in cell viability after desiccation, indicating more resistance to desiccation than that observed by Kirkwood *et al*. (2021) in three ST34 strains tested (98% reduction). In another study following *Salmonella* desiccation survival, *S*. Enteritidis had the highest survival rate (80% ± 9%) and *S*. Newport had the lowest survival rate (36% ± 3%) during 22-hour desiccation in a 96-well plate, which disagrees with the results from the present study where the mean log ratio survival for *S*. Enteritidis strain P125109 and *S.* Newport strain SL254 were similar, suggesting that tolerance to desiccation is strain specific (Gruzdev *et al.*, 2011).

For the heat inactivation, desiccation and refrigerated storage experiments, strains were grown to stationary phase prior to exposure to stress to allow for consistent control of input across multiple stress conditions. Using a single growth phase could be a limitation of the current study, as cells undergo different physiological and metabolic changes during stationary phase compared to log phase, so it is likely that their response to stress in different growth phases would also be different (Jaishankar and Srivastava, 2017). To reduce the effect growth phase has on food chain related stress response in future experiments, strains could be grown to both log-phase and stationary phase prior to exposure to stress, and then the survival compared.

Sodium chloride is often used as a preservative to prevent microbial growth in food products, however certain *Salmonella* strains may be able to tolerate higher salt concentrations, resulting in an increased risk to food safety. Growth of *S*. Typhimurium strain ST4/74 was inhibited in LB broth containing 10% NaCl, and minimal growth was observed in LB broth containing 8-9% NaCl. The MIC previously reported for *S*. Typhimurium strain ST4/74 in NaCl was 7.5% (w/v) but ranged from 7.5-10% depending on strain (Finn *et al.*, 2013c). In *S*. Typhimurium strain ST4/74, lag time increased with an increase in concentration of NaCl, which was also observed in an early study by Matches and Liston (1972). In the current study, growth of *S*. Typhimurium strain ST4/74 occurred after a 7-hour lag phase in LB broth containing 6% NaCl, whereas in another *S*. Typhimurium strain (ATCC 6994) growth was initiated after 11 hours incubation with 6% NaCl (Matches and Liston, 1972). This difference in lag phase duration between the two *S*. Typhimurium strains could be due to the growth media used, and hence difference in pH, as nutrient broth was used instead of LB broth in the study by Matches and Liston (1972). Minimal growth was observed for ST4/74 in LB broth containing 8% NaCl or more, whereas in *S*. Typhimurium strain ATCC 6994, no growth occurred at NaCl concentrations of 8% or above at 37°C (Matches and Liston, 1972).

In the current study, 14 *Salmonella* strains were subjected to 6% NaCl, and growth was measured over a 24-hour period at 37°C. *S*. Gallinarum strain 287/91 displayed the greatest tolerance to salt stress out of the strains tested, whereas *S*. Enteritidis strain P125109 and *S*. Typhimurium strain SO1960-05 both exhibited an increased resistance to 6% NaCl, as evidenced by a greater area under the curve when grown in 6% NaCl compared to the LB broth control. This is a similar finding to a previous study, in which a *S*. Enteritidis strain showed the smallest decrease in cell number during incubation with salt over a 3-hour period, and could resist the addition of salt concentrations as high as 8% (Wang *et al.*, 2020). The ability for *S*. Enteritidis to tolerate high salt concentrations poses an increased threat in high-salt foods, such as soy sauce and seafood (Brown *et al.*, 2009). Unexpectedly, *S.* Typhimurium strain SO1960-05 exhibited enhanced growth in LB broth containing 6% NaCl. This suggests that *Salmonella* strains may be able to tolerate NaCl in higher quantities than originally proposed and, in some cases, may actually boost growth.

Organic acids are able to inhibit microbial growth and are often used in food products as a preservative due to their cost effectiveness and ease of use (El Baaboua *et al.*, 2018). In this study, strains were pre-adapted to each acid at pH 5.8 for 30 minutes at 37°C to induce the acid tolerance response mechanism. This methodology was introduced to improve

consistency of results between biological replicates and to condition cells to survive in a low pH, which would be similar to the pH used in food products to prevent microbial growth. The acid tolerance response (ATR) is a mechanism by which bacterial cells become more resistant to low, acidic pH environments, after exposure to a moderate pH of between 5.5-6.0, in turn protecting the cells from severe acid stress. In the first study to describe this phenomenon, cells grown to logarithmic phase at pH 7.4 and then transferred to pH 5.8 for one generation, were 100-1000 times more resistant to low pH than unadapted cells (Foster and Hall, 1990).

In the present study, *S*. Typhimurium strain ST4/74 reached the greatest OD600nm when grown in LB broth supplemented with 10mM citric acid (pH 4.3) and acetic acid (pH 5.1), indicating that *S*. Typhimurium strain ST4/74 prefers a more acidic environment than what is typically used (pH 7). Lag time increased, as concentration of acid increased for both acetic and citric acid, with acetic acid being most effective at inhibiting *S.* Typhimurium strain ST4/74 at lower concentrations. This is a similar finding to what was discussed in the paper by Álvarez-Ordóñez *et al*. (2010), where generation time, lag-phase duration and time needed to reach stationary-phase all increased as pH became more acidic, whereas the maximum population density and maximum growth rate decreased. In the present study, *S*. Typhimurium strain ST4/74 was inhibited at 18mM (pH 4.7) and 16mM (pH 3.9) citric and acetic acid, respectively. Previously, *S*. Typhimurium has been reported to be inhibited by acetic acid at pH ≤ 6.4 and citric acid at pH ≤ 5.4, which is a higher pH than the minimum inhibitory concentrations for both acids in the present study (Álvarez-Ordóñez *et al.*, 2010). For comparison, the minimum inhibitory concentration of acetic and citric acid in the current study were 1.03% and 0.34%, respectively. This agrees with the MIC found in another study, which reported the MIC for acetic acid as 1% for multiple *Salmonella* strains. However, the MIC reported for citric acid was greater (1.5%) than that found in the present study, but this could be due to the difference in strains used (Amrutha *et al.*, 2017). Results from this study, and those published previously, indicate that *Salmonella* growth in organic acids is dependent on the acid molecule itself, rather than on pH (Chung and Goepfert, 1970).

2.5. Conclusion

In this study, the variation in food chain related stress response for 14 *Salmonella* strains from 9 different serovars was determined. *S.* Gallinarum strain 287/91 was most sensitive to heat, organic acids, NaCl and refrigerated storage whereas *S*. Typhimurium strain SO1960-05 had the greatest sensitivity to desiccation, but an increased resistance to NaCl. Monophasic *S*. Typhimurium strains exhibited an increased tolerance to desiccation. *S*. Enteritidis strain P125109 showed an increased resistance to NaCl and citric acid, but sensitivity to acetic acid. The phenotypic variability observed for strains in different stress conditions indicate that response to stress is strain specific, and thus strains exhibiting an increased resistance towards food chain related stress must be included in food challenge tests, to improve risk assessment and product safety.

Chapter Three

Construction of a transposon mutant library in *S*. Typhimurium strain ST4/74 and determination of essential genes using transposon directed insertion site sequencing (TraDIS)

3.1 Introduction

Transposon mutagenesis was developed in the 1980's by Simon *et al.* (1983) and has been used to study gene essentiality in many different bacterial species (Reznikoff and Winterberg, 2008; Choi and Kim, 2009). Several approaches have been used to apply the technique to study gene function in a high-throughput manner (Langridge *et al*., 2009; Christen *et al*., 2011; Eckert *et al*., 2011; Khatiwara *et al*., 2012). One of the main advantages of this high-throughput method of transposon mutagenesis is that pooled mutant libraries are assayed simultaneously, relating phenotype to genotype in identical conditions (Cain *et al*., 2020). In Gram-negative bacteria, the Tn5 transposon has been widely used as it inserts randomly into the chromosome with high efficiency with few hotspots (Kirby, 2007). The Tn5 transposon consists of 19bp repeated sequence either side of any sequence that creates the Tn5 mosaic end (ME) (Goryshin and Reznikoff, 1998). The repeated sequences are the substrate for a specific transposase that can be *cis* or *trans* encoded and results in transposition.

Transposon insertion sequencing (TIS) is an approach which combines transposon mutagenesis with next generation sequencing to determine the position of transposon insertion sites. By comparing the frequency of transposon insertions before and after selection it is possible to assess gene essentiality and/or role in fitness during a defined condition. To date, there have been four variations of transposon insertion sequencing developed; transposon sequencing (Tn-Seq), insertion sequencing (INseq), high-throughput insertion tracking by deep sequencing (HITS) and transposon directed insertion site sequencing (TraDIS) (van Opijnen *et al*., 2009; Goodman *et al*., 2009; Gawronski *et al*., 2009; Langridge *et al*., 2009). Transposon directed insertion site sequencing (TraDIS) was developed to define essential genes in *S*. Typhi, and has since been used to study gene essentiality in various conditions in numerous bacterial species, including *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Clostridium difficile* (Langridge e*t al.*, 2009; Christiansen *et al.*, 2014; Dembek *et al.*, 2015; Nolan *et al*., 2018; Goodall *et al*., 2018). Most recently, TraDIS has been used to determine the antibacterial mechanism of honey in *E*. coli strain K-12 and the genes required for *in vitro* growth and macrophage infection in two strains of *S.* Enteritidis (Masoura *et al*., 2022; Fong *et al*., 2022). TraDIS was used in the current study to determine the genes essential for survival when exposed to food chain related stresses, in *S*. Typhimurium strain ST4/74. Historically, the transposase function has been provided by a transposase gene (*tnp*) on a plasmid. An alternative to this is the use of

commercially available purified Tn5 transposase that when added to the Tn5 transposon DNA sequence *in vitro* binds to the mosaic ends forming a transposase-transposon complex called a transposome. Transformation of the target bacterial strain with the transposome by electroporation results in random transposition of the Tn5 transposon into the genome. TraDIS utilises Illumina sequencing technology to sequence reads across the transposon bounds of each insertion, which allows the location of the insertion to be mapped accurately against the reference genome sequence (Luan *et al*., 2013).

To analyse TraDIS data, Bio-Tradis software was developed to aid in the identification of essential genes (Barquist *et al*., 2016). The Bio-Tradis pipeline script bacteria_tradis, identifies reads with 8-10 base transposon sequence from a fastq file, which verifies that the read comes from a genuine insertion, discarding reads that do not have this sequence. The sequence is then trimmed to remove Tn5 sequence and the remaining genome sequence that flanks the transposon insertion site is aligned to the reference genome sequence using SMALT to identify the insertion site. Visualisation of the insertion sites and the number of reads from BAM files can then be used to generate a plot file that can be browsed using the Artemis software (Carver *et al*., 2012). An R script, tradis_essentiality.R uses the insertion site frequency data (plot file) to determine essential genes based on genes with fewer insertions than expected based on the bimodal distribution of insertions per gene, after gene length normalisation (Robinson *et al*., 2009; Barquist *et al*., 2016).

3.1.1 Aims

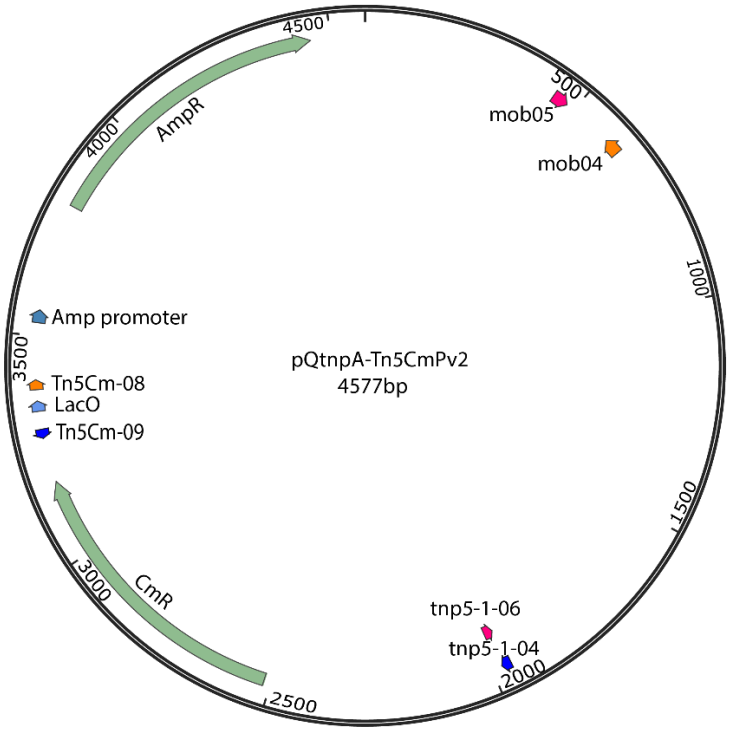
1. To generate a saturated transposon insertion mutant library in *S*. Typhimurium strain ST4/74
2. To determine the genes essential for survival during laboratory growth at 37°C in LB broth using TraDIS
3. Assess the library by comparing the essential genes required during laboratory growth in ST4/74 to previously published studies

3.2 Methods

3.2.1 Bacterial strains and plasmids

Stocks of *E. coli* MFD*pir* used during the study were provided by Keith Turner on LB agar plates containing 5.2mM diaminopimelic acid (DAP) and supplemented with chloramphenicol (15µg/mL) and ampicillin (100µg/mL) for MFD*pir* pQtmpA-Tn5CmPv2 (**Figure 18**) or chloramphenicol (15µg/mL) and kanamycin (25µg/mL) for MFD*pir* pQ5χmobRP4.1-Ptac (**Figure 18**). A scoop of colonies of each strain were picked and used to inoculate 5mL LB broth containing 5.2mM DAP and either kanamycin (25µg/mL) or ampicillin (100µg/mL) and incubated at 37°C for approximately 18-hours.

A



B

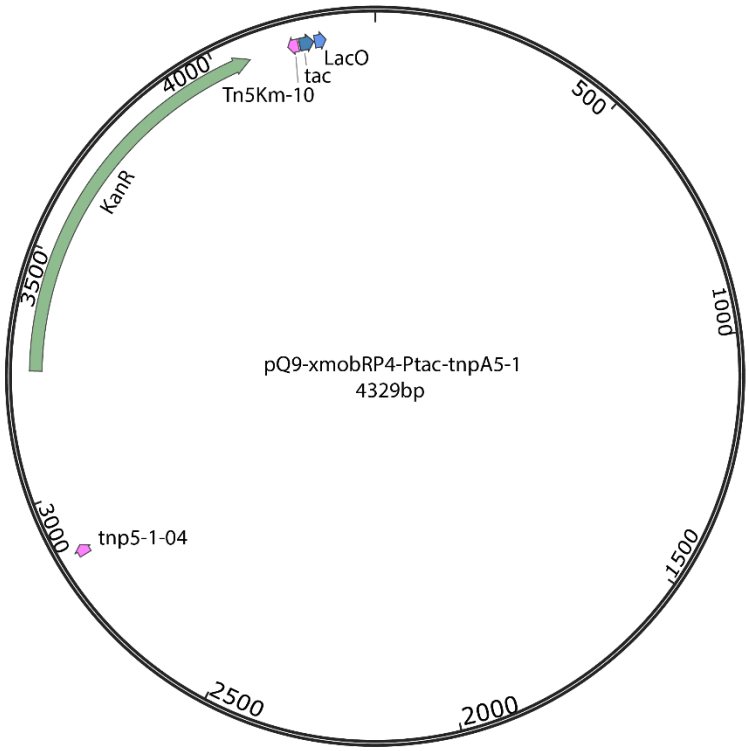


Figure 18. Plasmid maps of pQtmpA-Tn5CmPv2 and pQ5χmobRP4.1-Ptac. Template plasmid of A) pQtmpA-Tn5CmPv2 and B) pQ5χmobRP4.1-Ptac harbouring ampicillin and chloramphenicol resistance and kanamycin resistance, respectively. The coloured arrows denote oligo pairs. Maps were constructed using SnapGene.

3.2.2 Template plasmid extraction and restriction enzyme digest

To construct a new plasmid harbouring ampicillin and kanamycin resistance cassettes, whilst simultaneously removing the tac-like promoter, template plasmids (pQtmpA-Tn5CmPv2 and pQ5χmobRP4.1-Ptac) (**Figure 18**) were extracted from stationary phase cultures after ~18 hours of growth using a QIAprep Spin Miniprep Kit (Qiagen, 27104) and eluted in 30µL elusion buffer (Qiagen, 19086). Digestion of pQtmpA-Tn5CmPv2 occurred with EcoRI-HF (NEB, R3101S) and pQ5χmobRP4.1-Ptac was digested with AgeI-HF (NEB, R3552S) (**Table 6**) and incubated at 37°C for 15 minutes. Once digested, linearised plasmid sizes were confirmed by 1% Agarose gel electrophoresis at 120V for 40 minutes. Bands were stained with Midori green direct DNA stain (Geneflow) and visualised under blue light.

Component	Volume
DNA	1µL
10X CutSmart Buffer (NEB)	2µL
Restriction Enzyme	0.4µL
Nuclease Free Water	16.6µL
Total	20 µL

Table 6. Plasmid digestion reaction mix. General protocol for digestion of pQtmpA-Tn5CmPv2 and pQ5χmobRP4.1-Ptac plasmids using restriction enzymes.

3.2.3 Amplification of resistance cassettes using PCR

PCR reactions were set up in 20µL aliquots to amplify each part of the template plasmid DNA to be used for Gibson Assembly, containing either a kanamycin resistance cassette, an ampicillin cassette, or the plasmid backbone with the tac-like promoter removed (**Table 7**) For amplification of the ampicillin cassette, pQtmpA-Tn5CmPv2 template DNA was used with Tn5Cm-08 and mob-04 primers (**Table 8**). The plasmid backbone was amplified using template DNA from pQtmpA-Tn5CmPv2 (mob-05 and Tnp5-1-06 primers) and amplification of the kanamycin cassette and Tn5Km transposon occurred using pQ5χmobRP4.1-Ptac template DNA with Tn5Km-10 and Tnp5-1-04 primers (**Table 8**). PCR amplification was carried out in a DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad Laboratories) using the protocol described (**Figure 19**). Amplicons were analysed by 1% Agarose gel electrophoresis at 120V for 30 minutes. Bands were stained with Midori green direct DNA stain (Geneflow, MG05) and visualised under blue light. Amplified DNA was quantified using the Qubit dsDNA

Broad Range (BR) reagent kit (Invitrogen, Q32850). A working solution was prepared by mixing 199µL dsDNA BR buffer with 1µL dye for each reaction to be quantified. Standard reagents were prepared by mixing 190µL working solution with 10µL standard 1 or 2 in a Qubit tube. For each PCR product to be quantified, 195µL working solution was mixed with 5µL PCR product and left to incubate at room temperature for 5 minutes before being measured using a Qubit 3.0 Fluorometer (Invitrogen).

Component	**Volume**
Q5 HF 2x Master Mix (NEB, M0492S)	10µL
Forward Primer (0.25µM)	1µL
Reverse Primer (0.25µM)	1µL
DNA	0.075µL
Nuclease Free Water	7.925µL
Total	20µL

Table 7.**Plasmid fragment amplification reaction mix**. General PCR protocol for amplification of resistance cassettes and fragments needed for Gibson Assembly.

Primer	Sequence
Tn5Cm-08	CGTGGCAAAGTAGGTGTTTTCACGAGCACTG
mob-04	ATTGTCCACAATTTCTTATCAACATAAAGCTAGCGG
mob-05	TTCAGAGCTAGCCTCGCAGAGCAGGATTCCCGTTG
tnp5-1-06	TGTAAGCCCACTGCAAGCTAC
Tn5Km-10	AAAACACCTACTTTGCCACGCTTCAACTCAGCAAAAGTTCG
tnp5-1-04	GAAAGCAGGTAGCTTGCAGTG

Table 8. **PCR primers for amplification of fragment for Gibson Assembly**. Primers were supplied by Keith Turner.

96°C	55s
96°C	5s
58°C	40s
72°C	60s
72°C	2 mins
18°C	∞

X 30 cycles

Figure 19. Thermocycling conditions for amplification of individual fragments to be used in Gibson Assembly.

3.2.4 Construction of plasmid containing Tn5 transposon and kanamycin resistance cassette using Gibson Assembly

A Gibson Assembly Cloning Kit (NEB, E5510S) was used to create the pHPTn5Km plasmid from fragments containing antibiotic resistance cassettes amplified by PCR. To assemble the pHPTn5Km plasmid, 1µL of each fragment was mixed with 10µL 2x Gibson Assembly Master Mix and 6µL deionised RNA free water. Assemblies were incubated in a thermocycler at 50°C for 15 minutes and then stored at -20°C until required. The assembled pHPTn5Km plasmid was transformed into an *E. coli* CC118λ*pir* strain for long term storage at -80°C in 25% glycerol. Electrocompetent *E. coli* CC118λ*pir* cells were prepared by inoculating 5mL LB broth with culture from a -80°C glycerol stock and incubated at 37°C overnight. 50mL of 2x YT broth were inoculated with 500µL of the bacterial culture. The culture was incubated at 37°C in a shaking incubator until the OD600nm reached 0.2-0.25. Once the required optical density was reached, the entire contents of the conical flask was transferred to a 50mL centrifuge tube and pelleted by centrifugation at 4000rpm for 10 minutes at room temperature. The supernatant was discarded, and the pellet resuspended in 1mL sterile ultra-pure water before being transferred to a sterile 1.5mL microcentrifuge tube. The cells were pelleted by centrifugation at 4000rpm for 5 minutes at room temperature and the resulting supernatant discarded. The pellet was resuspended in 1mL sterile ultra-pure water, and washed 5x in 1mL sterile ultra-pure water by spinning at 9000g for 2 minutes in a centrifuge each time at room temperature. After the final wash, the supernatant was discarded, and the resulting pellet resuspended in 100µL sterile ultra-pure water.

A 20µL aliquot of clean bacterial cells was transferred to each individual 1.5mL microcentrifuge tube and 2µL of the required pHPTn5Km plasmid or the positive control DNA (pUC19) was added to the cells. A negative control contained 20µL bacterial cells and 2µL elusion buffer (Qiagen, 19086). The contents of the microcentrifuge tube were transferred

to individual sterile electroporation cuvettes (2mm gap) (Cell Projects, E6-0060) and electroporated on setting Ec2 on a MicroPulser Electroporator (Bio-Rad Laboratories), then immediately resuspended in 200µL super optimal broth medium (S.O.C) (prewarmed to 37°C). The bacterial cells were placed in a shaking incubator for 45 minutes at 37°C to recover, before being plated onto LB agar plates containing; kanamycin (50µg/mL) for pHPTn5Km transformants, and ampicillin (100µg/mL) for positive control transformants. The negative control was plated on all antibiotic plates. Plates were incubated overnight at 37°C and enumerated the following day.

3.2.5 Confirmation of pHPTn5Km transformants

Individual colonies harbouring the pHPTn5Km plasmid were subjected to colony PCR to confirm that the cells contained the required plasmid. Colonies of each transformant were resuspended in 100µL RNAse free water (Albion, AM9938) and transferred to a fresh 5mL LB broth bottle containing kanamycin (50µg/mL). A 1µL aliquot of the diluted colony in water was used as template DNA in a PCR reaction (**Table 9**) with primers (Tn5Cm-10 and Km-01) (**Table 10**). Negative controls contained 0.3µL of the original plasmid DNA (either pQtmpA-Tn5CmPv2 or pQ5χmobRP4.1-Ptac). *Taq* polymerase was used to prevent any proof-reading of the Tn5Cm-10 primer. PCR amplification was carried out in a DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad Laboratories) using the thermocycling conditions outlined (**Figure 20**). Amplicons were analysed by 1% Agarose gel electrophoresis at 120V for 30 minutes. Bands were stained with Midori green direct DNA stain (Geneflow, MG05) and visualised under blue light. Glycerol stocks were made of each colony using 500µL 50% glycerol and 500µL overnight culture in a cryogenic vial (Corning, 10340412).

Component	**Volume**
10X Standard *Taq* reaction Buffer (NEB, M0273S)	2.5µL
10mM dNTPs	0.5µL
Forward Primer (10µM)	0.5µL
Reverse Primer (10µM)	0.54µL
Template DNA (pHPTn5Km)	1µL
Taq DNA Polymerase (NEB, M0273S)	0.125µL
Nuclease Free Water	19.875µL
Total	25µL

Table 9. **Reaction mix for amplification of 322bp recombinant in pHPTn5Km Gibson Assembled Plasmid.**

Primer	Sequence
Tn5Cm-10	GTGCTCGTGAAAACACCTACTT
Km-01	CTCCTTCATTACAGAAACGGC

Table 10. Primers used for amplification of 322bp recombinant in pHPTn5Km Gibson Assembled Plasmid.

95°C	2 mins
95°C	15 secs
55°C	15 secs
68°C	30 secs
68°C	2 mins
10°C	∞

X 30

Figure 20. Thermocycling conditions for amplification of 322bp recombinant in pHPTn5Cm and pHPTn5Km.

3.2.6 Sequencing of pHPTn5Km plasmid

Cultures were prepared from pHPTn5Km glycerol stocks in 5mL LB broth bottles containing kanamycin (50µg/mL) and grown for 18 hours overnight at 37°C with shaking at 200rpm. Plasmids were extracted using a Qiagen QIAprep Spin Miniprep kit (Qiagen, 27104). 3mL of overnight culture of each plasmid was used initially and the quick start protocol followed. The plasmid DNA was eluted in 30µL elusion buffer and the amount of pDNA quantified using the Qubit Broad Range (BR) dsDNA assay kit (Invitrogen, Q32850) and a Qubit 3.0 Fluorometer. Plasmid DNA was normalised to ~0.5ng/µL with ultra-pure water using the Qubit High Sensitivity (HS) dsDNA assay kit (Invitrogen, Q32851). Plasmids were sequenced using Illumina (Nextera XT library preparation and NextSeq 500) platform to generate paired end reads. Read lengths were a minimum of 51bp and a maximum of 151bp for forward and reverse reads for pHPTn5Km, respectively. A bioinformatic tool, plasmidSPAdes (v3.8.0) (Antipov *et al*., 2016) was used to assemble plasmids from forward and reverse reads. Assembled plasmids were then annotated using SnapGene software (from GSL Biotech; available at snapgene.com). To check whether the tac-like promotor had been successfully deleted from pHPTn5Km, a simulated pHPTn5Km template was constructed using the Gibson Assembly programme on SnapGene software. The simulated plasmid sequence was then aligned to the assembled pHPTn5Km plasmids, using BRIG (BLAST Ring Image Generator) (Alikhan *et al*., 2011).

3.2.7 Tn5 Transposon amplification

To prepare the template DNA for amplification of the transposon ready for the generation of a transposon mutant library in *S.* Typhimurium strain ST4/74, the pHPTn5Km plasmid was digested using restriction enzymes, ScaI-HF (NEB, R3122S), MfeI-HF (NEB, R3589S) and AgeI-HF (NEB, R3552S) at 37°C for 15 minutes (**Table 11**). The transposon was amplified using the Q5 2X Master Mix (**Table 12**) and associated primers (**Table 13**). For a negative control, 1µL of water was used instead of DNA. A pKD4 positive control was also included using primers KmrF and KmrR (**Table 13**). PCR amplification was carried out in a DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad Laboratories) using the thermocycling conditions outlined (**Figure 21**). Transposons were analysed by 1% Agarose gel electrophoresis at 120V for 30 minutes. Bands were stained with Midori green direct DNA stain (Geneflow, MG05) and visualised under blue light.

Component	Volume
Plasmid DNA (pHPTn5Km)	10µL (@ 1µg)
10X CutSmart Buffer	5µL
ScaI-HF	1µL
MfeI-HF	1µL
AgeI-HF	1µL
Nuclease Free Water	32µL
Total	50µL

Table 11. **Restriction enzyme digestion reaction mix for pHPTn5Km.**

Component	Volume
Q5 2X Master Mix	25µL
10µM Forward Primer (P-Tn5Km-01)	2.5µL
10µM Reverse Primer (P-Tn5Cm-04)	2.5µL
Digested Plasmid DNA (pHPTn5Km)	1µL
Nuclease Free Water	19µL
Total	50µL

Table 12. Reaction mix for amplification of kanamycin transposon in pHPTn5Km.

Primer	Sequence
P-Tn5Km-01	P-CTGTCTCTTATACACATCTTCTAGACAACC
P-Tn5Cm-04	P-CTGTCTCTTATACACATCTGACGC
KmrF	GAATGAACTGCAGGACGAGG
KmrR	AGCAATATCACGGGTAGCCA

Table 13. Primers used for Tn5Km transposon amplification. Primers were supplied by Keith Turner.

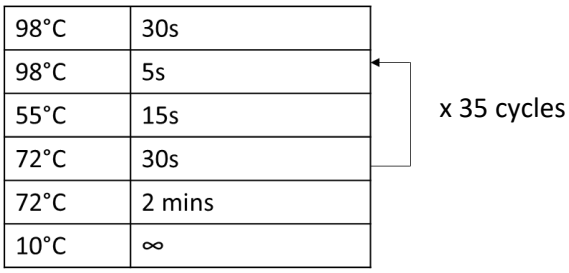


Figure 21. **Thermocycling conditions for amplification of kanamycin transposon in pHPTn5Km.**

3.2.8 Transposon mutant library construction in *S*. Typhimurium strain ST4/74

An overnight culture of *S.* Typhimurium strain ST4/74 was prepared in 5mL LB broth and incubated at 37°C for ~18 hours, with shaking set to 200rpm. A 500µL aliquot of the overnight culture was added to a sterile 250mL conical flask containing 50mL 2x YT broth. The flask was incubated at 37°C with shaking set to 200rpm and the OD600nm of the culture measured until the OD600nm reached 0.20-0.25. Once the culture had reached the required optical density, the contents of the flask was transferred to a 50mL centrifuge tube and pelleted by centrifugation for 10 minutes at 3500g and 4°C. The supernatant was discarded, and the pellet resuspended in 1mL 10% glycerol before being topped up to 25mL with 10% glycerol. The bacterial cells were washed twice with 25mL of 10% glycerol, spinning down at 3500g for 10 minutes at 4°C each time, and on the final wash the pellet was resuspended in 1mL 10% glycerol. The resuspended cells were transferred to a sterile 1.5mL microcentrifuge tube and pelleted by centrifugation at 3500g for 5 minutes at 4°C. The supernatant was discarded, and the pellet resuspended in 600µL 10% glycerol.

Transposomes were prepared by mixing 2µL transposon DNA (100ng/µL), with 2µL 100% glycerol and 4µL transposase (Lucigen, TNP92110). 60µL bacterial cells were added to microcentrifuge tubes containing 2µL sterile nuclease free water (Albion, AM9938), 2µL TypeOne Restriction Inhibitor (Lucigen, TY0261H) and 0.4µL transposome on ice. Two different types of negative control were included; water as a substitute for transposon DNA in the transposome and water substituted for transposase. A positive pUC19 control was included, which was prepared by substituting transposon DNA for pUC19 DNA in the transposome. Cells were transferred to 2mm electroporation cuvettes (Cell Projects, E6-0060) and electroporated at 2.4k using a MicroPulser Electroporator (Bio-Rad Laboratories). Cells were immediately resuspended in 1mL S.O.C (prewarmed to 37°C) and placed in a shaking incubator at 200rpm to recover at 37°C for 1.5 hours. A 10µL and 100µL aliquot of electroporated cells were plated on LB agar containing kanamycin (50µg/mL). The positive control cells were plated on LB agar plates containing ampicillin (100µg/mL). The remaining 890µL electroporated cells were plated in equal aliquots onto four square LB agar plates containing kanamycin (50µg/mL). Plates were incubated at 37°C overnight and colonies enumerated. The transposon efficiency was calculated for pUC19 positive control.

The colonies present on the square LB agar plates were harvested using 1mL LB broth per plate and an L-shaped sterile spreader. The colonies from three individual electroporation's were combined into a 50mL centrifuge tube and 50% glycerol was added to a final concentration of 15% glycerol. Each electroporation batch (containing three individual electroporation's) was stored at -80°C until required. In total, 35 electroporation's were conducted and stored in 13 different batches. A master mix library stock was created by pooling aliquots of each batch (**Table 14**) and mixing thoroughly. The master mix was divided into 50µL aliquots and stored at -20°C until required. In total, there were approximately 762, 000 transposon mutants, which forms the transposon mutant library in *S*. Typhimurium strain ST4/74.

Batch Number	Aliquot for Master Mix (µL)
1	95
2	212
3	130
4	21
5	26
6	53
7	70
8	33
9	23
10	29
11	20
12	26
13	25
Total	763

Table 14. Preparation of the pooled transposon library master mix. The amount, in µL, of each electroporation batch combined to make the transposon library master mix stock.

3.2.9 DNA Extraction of transposon mutant Library

For DNA extraction of the transposon library, 50µL of thawed library was grown to stationary phase in 5mL LB broth for 18 hours at 37°C and 200rpm, 100µL of culture was then deposited directly into a Maxwell RSC cultured cells DNA kit cassette (Promega) and loaded into a Promega Maxwell RSC 48 Instrument. DNA was extracted in duplicate for each sample, following the manufactures standard protocol for DNA extraction from bacterial cells. The DNA concentration was quantified using a Qubit 3.0 fluorometer (Invitrogen) and the Qubit dsDNA BR (Broad Range) Assay Kit. Extracted DNA was stored at -20°C until required.

3.2.10 Sequencing of LB broth control transposon mutant library

To prepare the library ready for sequencing, a MuSeek Library Preparation Kit (Illumina Compatible, K1361) was used. DNA extractions were diluted to ~20ng/µL with elution buffer. MuSeek reactions were prepared in a 96-well plate (**Table 15**). The plate was sealed and incubated at 30°C using a thermocycler (Veriti) for 5 minutes. Sterile ultra-pure water was added to each well so that the final volume was 30µL. To each well, 45µL of AMPure XP (Beckmann, A63880) beads were added (1.5x volume) and mixed by pipetting. The plate was incubated at for 5 minutes at room temperature, and then placed onto a magnetic rack for 5 minutes until the solution became clear. Keeping the plate on the magnetic rack, the

supernatant was discarded and 200µL of freshly prepared ethanol (80%) was added to each well. The plate was incubated at room temperature for 30 seconds, and the supernatant discarded. Another 200µL of freshly prepared ethanol (80%) was added to each well, incubated for 30 seconds, and the supernatant discarded. Any residual ethanol was removed before air drying for 5-15 minutes. Beads were resuspended in 20µL elusion buffer and placed back on the magnetic rack until the solution turned colourless.

In a new 96-well plate, 5µL of Tn5-specific enrichment primer (either N701, N702 or N703) and 5µL of each index primer (either S502, S503, S505 or S507) was added to each well. To each well containing primers, 15µL of DNA solution and 25µL of Q5 High-Fidelity Master Mix (NEB, M0544) was added and mixed thoroughly, to give a final volume of 50µL. The plate was sealed and placed into a thermocycler (Veriti) for PCR (**Figure 22**).

Reagent	Quantity (µl)
MuSeek Fragmentation Buffer	2.5
MuSeek Enzyme Mix	0.5
gDNA (20ng/µL)	4.5

Table 15. Reagents and quantities required for MuSeek reactions for library preparations.

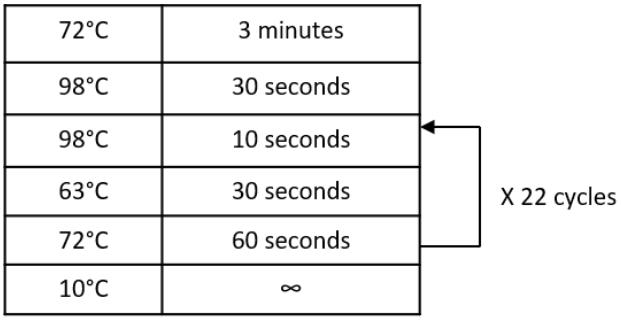


Figure 22. **Thermocycling conditions for transposon fragment enrichment.**

To clean up the transposon fragment enrichment PCR, 30µL of evenly mixed AMPure XP beads were added to each PCR reaction and mixed by pipetting. The PCR reactions were incubated at room temperature for 5 minutes and placed into a magnetic stand for 2 minutes, until the solution became colourless. The supernatant was discarded, and the plate was washed twice with freshly prepared 200µL 80% ethanol, incubating for 30 seconds each time after addition of ethanol on the magnetic stand before discarding the supernatant. Residual ethanol was removed, and the plate left to airdry for 15 minutes. The plate was removed from the magnetic stand and 15µL of elusion buffer added to each well. The beads

were mixed thoroughly and incubated at room temperature for 2 minutes. The plate was placed onto the magnetic stand for the final time until the solution cleared. A 10µL aliquot of supernatant from each well was transferred to a sterile microcentrifuge tube. Prior to sequencing, the concentration of the transposon mutant library DNA was quantified using a Qubit dsDNA High Sensitivity (HS) Assay kit (Invitrogen, Q32851) and Qubit 3.0 fluorometer (Invitrogen). The mutant library was sequenced on a NextSeq 500, using the NextSeq 500/550 High Output kit (75 cycles) (Illumina, 20024906), following the protocol denoted in the Illumina NextSeq Denature and Dilute Libraries Guide (Illumina, version 13).

3.2.11 Bioinformatic Analysis of Mutant Library Sequencing Data in LB Broth Control

The Bio-Tradis toolkit (version 1.4.1) was used for TraDIS data analyses (Barquist *et al.*, 2016). A bacteria_tradis script was used to produce mapped BAM files from fastq files. The script was ran using a singularity created by Martin Lott. In the Bio-Tradis script used, mismatches were not allowed (-mm 0) and the –smalt_r parameter, which specifies whether to allow multimapping, was set to 0 rather than the default (-1) to ensure that reads that mapped to multiple positions were randomly assigned a position to avoid false positive results for gene essentiality, due to repetitive elements such as insertion sequences. The -m option was set to 1, which designates the minimum mapping quality score to use an alignment for further processing, choosing this option allowed for multi-mapping. This script produced insert site plot files for each fastq file listed in the post_stress.txt file and summary statistics. Artemis (version 17.0.1) was used to visualise insert site plots of the ST4/74 transposon mutant library alongside a ST4/74 reference (embl format) (Carver *et al.*, 2011). For further analyses to determine essential genes, the tradis_genes_insert_sites script was used to create a csv file containing information about insertion sites and read counts. The -trim3 option was used as it trims the last 10% of bases at the 3’ end of the coding sequence, because essential genes may tolerate insertions towards this part of the coding sequence. The tradis_essentiality.R script was used to generate a file of essential genes and additional diagnostic plots denoting insertion index. A bespoke python3 script was written by Gaetan Thilliez to compare the essential genes lists from the tradis_essentiality.R output. All bioinformatic analyses was conducted using a high-performance cluster (HPC).

3.3 Results

3.3.1 Construction of the novel pHPTn5Km plasmid

An available Tn5Km-transposon contained an outward orientated promotor (Ptac), which can be useful for determining gene expression changes. The tac-promoter is a synthetic DNA promoter derived from the trp and lac promoters in *E*. *coli*, comprising of the -35 (TTGACA) from trp and -10 (TATAAT) from lac consensus sequences. However, due to the presence of sequence DNA from the lac promoter it cannot be used for food-related studies because the lac promoter initiates transport and metabolism of lactose and therefore, the presence of the tac-promoter may affect any downstream application in food products containing lactose in an unpredictable way. As a result, a new plasmid, termed pHPTn5Km was constructed which did not contain the outward facing promoter. The original plasmid templates containing a chloramphenicol and kanamycin transposon (pQtmpA-Tn5CmPv2 and pQ5χmobRP4.1-Ptac, respectively) were used to construct the new plasmid (pHPTn5Km), containing both a transposon within a kanamycin resistance cassette and an ampicillin resistance cassette, but also with the deletion of the 100bp tac-promotor (5'-AATGAGCTGTGGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTC ACACAGGAAACAGCCAGTCCGTTTAGGTGTTTTC-3'). Furthermore, the Tn5Cm transposon used to generate pHPTn5Km has additional Illumina transposase adapter sequences incorporated, aiding the TraDIS sequencing process, whilst the available Tn5Km transposon did not contain this sequence. Deletion of the promoter from Tn5Km and the concomitant insertion of the adapter sequences from Tn5Cm was achieved using Gibson Assembly to combine PCR fragments from the Tn5Km and Tn5Cm plasmids (**Figure 23**).

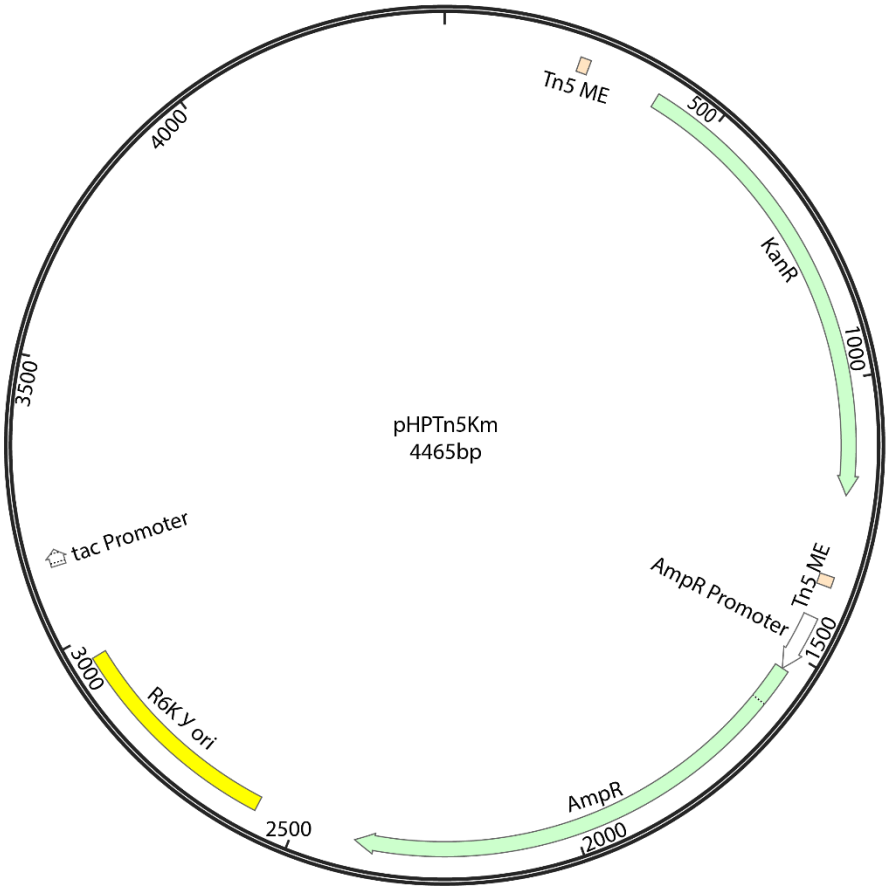


Figure 23. Diagram of the pHPTn5Km plasmid. The pHPTn5Km plasmid was generated using Gibson Assembly and contained a Tn5 transposon, and both kanamycin and ampicillin resistance cassettes. The plasmid map was generated from plasmid sequencing data and visualised using SnapGene (version 5.3.2).

3.3.2 The saturated transposon library created in *S*. Typhimurium strain ST4/74 contained 763, 000 transposon mutants with 609, 000 unique insertions

To determine the genes essential for survival under various food chain related stress conditions, a saturated transposon mutant library was first constructed in *S*. Typhimurium strain ST4/74 using a transposon sequencing method, termed TraDIS. The mutant library was generated by electroporating a transposome containing a Tn5 transposon harbouring kanamycin resistance, alongside transposase to increase transformation efficiency, into *S*. Typhimurium strain ST4/74. This was repeated multiple times until the number of colonies enumerated amounted to over 750,000, stored in separate batches. Batches were pooled and an aliquot of library was grown for 18-hours in LB broth at 37°C, in duplicate, to emulate laboratory growth. Two biological replicates of the resulting library post growth in LB broth were sequenced using illumina sequencing and the data was analysed using the Bio-Tradis

toolkit (Barquist *et al.*, 2016). In total, 762,000 mutants were produced using a Tn5-derived transposon in 35 electroporation's. Sequencing of the mutant library revealed that there were on average ~609, 000 unique insertions between the two transposon library biological replicates (**Table 16**). The library contained an average of 1 insertion every 8bp. The TraDIS sequence data was mapped to the ST4/74 reference, resulting in ~65% and 76% of the reads mapping to the reference genome for replicate 1 and 2, respectively. There were more total reads in replicate 2 compared to replicate 1, which in turn resulted in more reads mapping to the reference genome and more unique insertions (**Table 16**). The reference genome used for mapping contained the chromosome and three plasmids (TY474p1, TY474p2 and TY474p3). For both replicates, the percentage reads matched represents the percentage of reads matching the transposon tag sequence, and in this case 100% of the reads matched the transposon tag. Overall, a high -density transposon library was created in *S*. Typhimurium strain ST4/74.

Transposon Library	Total Reads	% Reads Matched	Reads Mapped	% Mapped	Total Unique Insertion Sites (UIS)	Total Sequence Length/Total UIS
Control (replicate 1)	23171888	100	15133865	65.31	601918	8.42
Control (replicate 2)	25129904	100	19135922	76.15	616245	8.22

Table 16. **Transposon mutant library summary statitsics**. Summary statistics of sequencing data from *S*. Typhimurium strain ST4/74 transposon mutant library grown in LB broth at 37°C for 18 hours (2 biological replicates).

3.3.3 486 genes are essential for growth LB broth at 37°C in *S*. Typhimurium strain ST4/74

Gene essentiality during growth in LB broth at 37°C was determined from analyses of the transposon mutant library sequencing data based on the observed bimodal frequency distribution of insertion indices, as described previously (PHE, 2010; Barquist *et al.*, 2016). The insertion index is the number of unique insertion sites normalised for the length of the gene. For determination of the likelihood ratio that each gene was in each part of the frequency distribution, a cut off was based on a log2-LR (log ratio) of <-2 (essential genes) or >2 (non-essential genes). Genes with insertion indices between these values were classified as ambiguous. There were 522 and 527 genes classified as essential for culture in LB broth,

for replicate 1 and 2, respectively, and 76 genes classified as ambiguous in both replicates. For replicate 1, genes were classified as essential if the insertion index was less than 0.014, whereas in replicate 2, essential genes had an insertion index of less than 0.0161 (**Figure 24**). The calling of essential genes was ambiguous if the insertion index was between 0.014 and 0.0212 for replicate 1, and between 0.0161 and 0.0244 for replicate 2 (**Figure 24)**. In replicate 1, 36 genes were designated as essential that did not appear as essential in replicate 2, and 40 were essential genes in replicate 2 that were not classified as essential in replicate 1. The position of essential genes for replicate 1 were plotted against the ST4/74 coding sequences (CDS) and position and frequency of Tn5 insertions using DNA plotter in Artemis (version 17.0.2) (**Figure 25**). There were few insertions in genes which were essential for survival in growth in LB broth at 37°C (**Figure 25**). In replicate 1, 38% of essential genes had an insertion count of 0, whereas 32% of genes had 0 insertions in replicate 2. In total, there were 486 genes shared between both replicates that were predicted to be essential for laboratory growth in LB broth at 37°C (**Supplementary Table 1**).

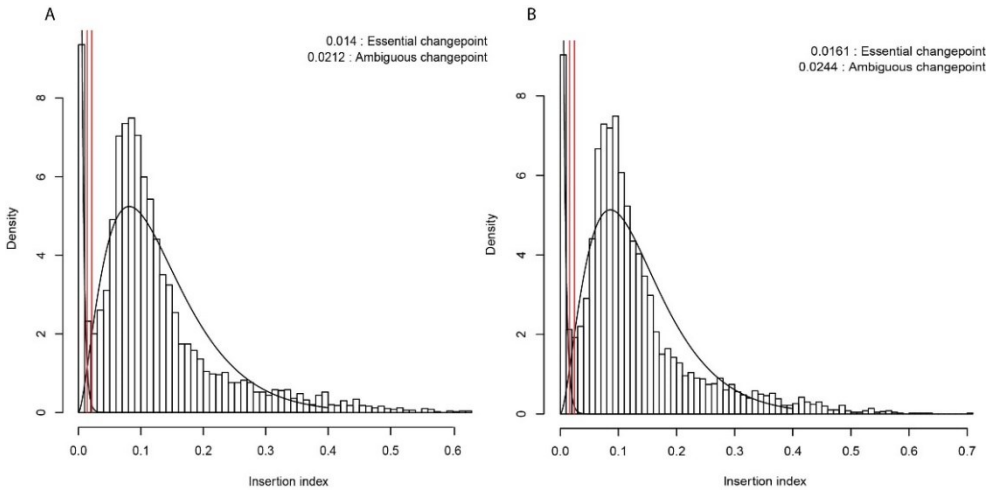


Figure 24. Insertion index and gene density of transposon mutant library. Gamma distribution fitting the relationship of insertion index and gene density for the *S*. Typhimurium strain ST4/74 transposon mutant library for A) replicate 1 and B) replicate 2. Genes are classified as essential if the insertion index < 0.014 and < 0.0161 for replicate 1 and 2, respectively.

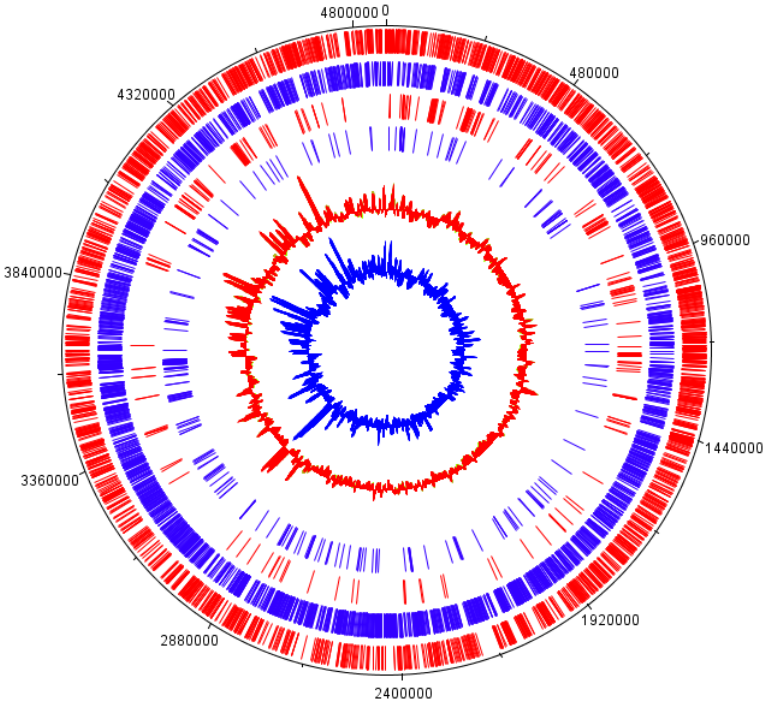


Figure 25. Insertion site map and essential genes of transposon mutant library in *S.* Typhimurium strain ST4/74. The mutant library was constructed using transposon directed insertion site sequencing (TraDIS) under laboratory growth conditions (LB Broth, 37°C). The genome size is denoted in basepairs. The outer two tracks represent the CDS of ST4/74 (forward (red) and reverse (blue)), the next two tracks show the essential genes required for laboratory growth and the inner-most tracks show the position of Tn5 insertions. This plot was generated using DNA Plotter in Artemis (version 17.0.2).

3.3.3 Distribution of functional categories of the genes required for growth in LB broth at 37°C for S. Typhimurium strain ST4/74

The Kyoto Encyclopaedia of Genes and Genomes (KEGG) was used to explore the functional classes of essential genes. KEGG is a database that assigns a high-order biological function to a gene from genomic data by curating knowledge from computational analysis of cellular processes and standardising gene annotations for prokaryotes (Kanehisa and Goto, 2000). The deduced amino acid sequence of coding sequences in the *S.* Typhimurium strain ST4/74 genome were assigned to K numbers (KEGG orthology identifiers) by aligning sequences with the BLAST algorithm using BlastKOALA (version 2.2). Once the *S.* Typhimurium strain ST4/74 genes had K numbers assigned, the file containing essential genes during growth in LB broth at 37°C was compared and each essential gene was assigned a K number. Of the 486 genes identified as essential during growth in LB broth at 37°C in *S.* Typhimurium strain ST4/74, 103 genes could not be assigned a K number. K numbers were assigned to 383 essential genes

(**Figure 26**). The KEGG pathway with the greatest number of classified genes was translation, whereas the fewest number of genes were classified into the cell motility pathway. Overall, genes essential for survival in LB broth at 37°C were involved in translation, metabolism and replication and repair (**Figure 26**).

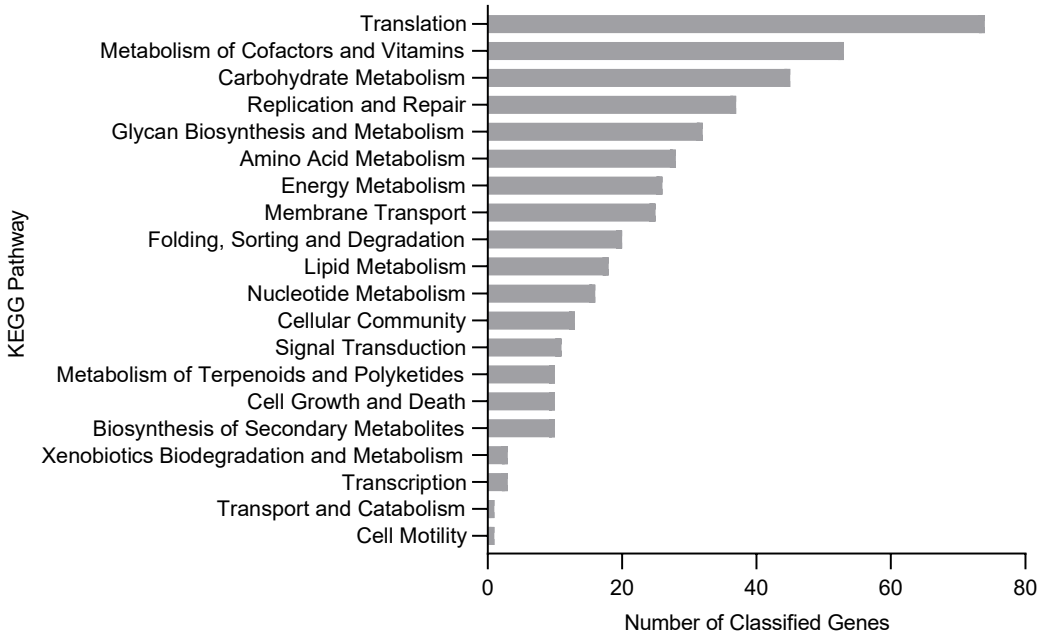


Figure 26.**KEGG pathways involved in growth of *S.* Typhimurium strain ST4/74 in LB broth**. The high-level molecular function of genes predicted to be essential for laboratory growth in LB broth at 37°C in *S.* Typhimurium strain ST4/74 was determined. Each gene was assigned a K number according to BlastKOALA, which relates to a KEGG pathway. Out of the 486 essential genes input into the programme, 103 were unable to be assigned a KEGG pathway. Only the top 12 KEGG pathways are shown.

3.3.4 Cross-strain comparison of genes required for growth in LB broth at 37°C

To validate the transposon insertion mutant library and TraDIS analysis, the essential genes required for growth in LB broth at 37°C were compared with a mutant library in a different *S.* Typhimurium strain under similar conditions, published previously (Barquist *et al.*, 2013). Orthologous genes were identified between the two datasets using Roary (version 3.13.0) that included an all BLAST all approach to build a pangenome of the two strains (Page *et al.*, 2015). *S.* Typhimurium strain ST4/74 was compared to strain SL1344, rather than SL3261, as it was reported in the publication that SL3261 feature annotations were based on SL1344, ignoring the deleted *aroA*, *ycaL* and *cmk* genes. Genes were grouped together if their sequence identity was greater than 95%. Only coding sequences (CDS) were used in the

analysis, hence why fewer essential genes were included for *S*. Typhimurium strain ST4/74 (445), than originally reported (486). A custom python3 script written by Gaetan Thilliez was used to compare the essential genes list from *S*. Typhimurium strain ST4/74 and the essential genes listed in the supplementary data for strain SL3261 (Barquist *et al.*, 2013). The data was visualised using jvenn, an interactive Venn diagram viewer (Bardou *et al.*, 2014).

There were 354 and 445 CDS identified as essential in *S*. Typhimurium strains SL3261 and ST4/74, respectively, and 306 of these CDS were predicted to be essential in both strains (**Figure 27**). There were 48 essential genes specific to SL3261, which did not appear in the essential genes list for strain ST4/74. Of these 48 genes, four ST4/74 CDSs were not processed by Roary as they were misannotated pseudogenes. There were 139 CDS exclusive to strain ST4/74, and 48 of these did not have an equivalent ortholog in the sequence of SL1344 according to the BLAST analysis in the Roary computational pipeline. Some of the genes that were exclusively essential for growth in LB broth at 37°C for *S*. Typhimurium strain ST4/74 included the lipopolysaccharide biosynthesis genes *rfaCDEF*, the transcription termination factors *nusA* and *nusG*, and members of the *tra* genes. Some of the unique essential genes are labelled (**Figure 27**), but a full list can be found in **Supplementary Table 2**, alongside the essential genes common to both strains and the essential genes unique to strain SL3261. Subunits of RNA polymerase including *rpoA*, *rpoB*, *rpoC* and *rpoD* were all considered to be essential genes in both strains during gorwth in LB broth at 37°C. Other examples of genes required by both strains include *ftsZ*, *murG*, *dapE*, *accB* and *ubiG*. The 48 genes specific for growth of strain SL3261 included *sseA*, *sseJ*, *lipB* and *rfbJ*.

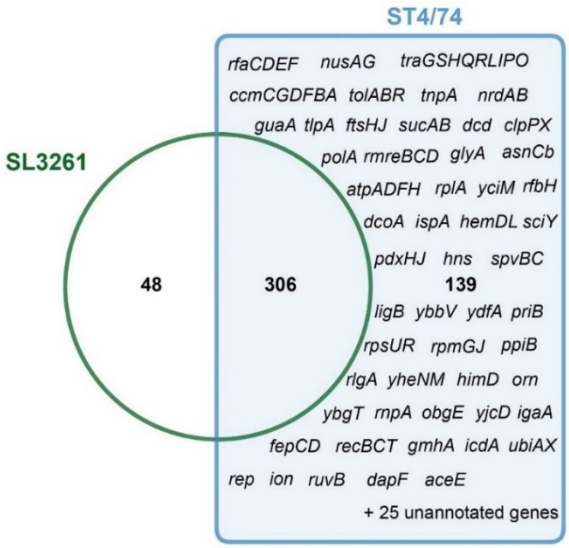


Figure 27. Number of essential genes required for growth at 37°C in LB broth shared between *S*. Typhimurium strain ST4/74 and SL3261. Essential genes exclusive to strain ST4/74 are labelled.

3.4 Discussion

A high-throughput transposon sequencing technique, termed TraDIS (Transposon Directed Insertion Site Sequencing), was used to determine genes that contained zero or only a few transposon insertions in a mutant library and were therefore likely to be essential for growth in LB broth. The transposon used in the current study did not contain the outward facing tac-like promoter due to the presence of lactose in the food matrix used during certain stress experiments in the current study. This is one of the limitations in the current study, as the updated version of TraDIS (TraDIS-Xpress) utilises this outward facing inducible promoter to identify genes which are overexpressed or repressed during a condition (Yasir *et al.*, 2020). This level of detail cannot be achieved using the traditional TraDIS protocol, however, TraDIS does provide information on gene essentiality, which is most useful to decipher genes involved in food chain related stress response in the current study. During the current study, a transposon mutant library was created in *S*. Typhimurium strain ST4/74 and contained approximately 762, 000 mutant colonies, equating to 609, 000 unique insertions after sequencing. This is comparable to a transposon mutant library generated in a different *S*. Typhimurium strain, SL3261, using a similar Tn5-derived transposon method to the one used in the current study, which resulted in 930, 000 mutants containing 548, 086 unique insertions (Barquist *et al*., 2013).

Other mutant libraries generated in *S*. Typhimurium include a 650, 000 mutant library in strain ATCC14028 with 118, 086 unique insertions using transposon directed insertion site sequencing and a library generated in the same strain via insertion-duplication mutagenesis (IDM) (Karash and Kwon, 2018; Knuth *et al*., 2004). More recently, transposon libraries have been constructed in *S*. Enteritidis Global Epidemic Clade (GEC) strain P125109 and Central/Eastern Africa clade (CEAC) strain D7795 using TraDIS resulting in 246, 743 and 195, 646 unique insertions, respectively (Fong *et al*., 2022). This equates to about one insertion in every 19 and 24 nucleotides for *S*. Enteritidis strains P125109 and D7795, respectively (Fong *et al*., 2022). In the present study, the number of unique insertions equates to about one in every 9bp, which is more dense than previous studies, indicating every gene in the genome is likely to have at least one mutation, whereas in libraries with fewer insertions, some genes may not be assayed in the output. Overall, the library generated in *S*. Typhimurium strain ST4/74 during the present study is the highest density transposon library currently available in *S.* Typhimurium.

There were 486 genes predicted to be essential based on their insertion index during growth in LB broth at 37°C. The majority of these genes were involved in translation according to the KEGG pathway database. A pan-genome explorer was used to compare the genes present in two strains of *S*. Typhimurium, and the essential genes identified from a previously published study in strain SL3261 and the current study were compared. The pan-genome explorer identified 445 essential coding sequences (CDS) in *S*. Typhimurium strain ST4/74, and in the study by Barquist *et al*. (2013), 353 CDS were predicted to be essential during growth in LB broth at 37°C in *S*. Typhimurium strain SL3261. The main biological functions of the essential genes in strain SL3261 were cell division, DNA replication, transcription, translation, peptidoglycan, and fatty acid biosynthesis, which is somewhat similar to the main functions observed in strain ST4/74, which were translation, metabolism, replication and repair, and membrane transport.

S. Typhimurium strain SL3261 contains a 2166bp deletion compared to the parent strain SL1344, and the deletion ranges from within the *aroA* gene to the *cmk* gene, resulting in two pseudogenes and the removal of the SL0916 (*ycaL*) gene completely (Barquist *et al*., 2013). The SL1344 whole-genome sequence was used alongside ST4/74 in the pan-genome analysis due to its similarity to SL3261. Furthermore, the publication reported using functional annotations from SL1344 for strain SL3261 during their analysis (Barquist *et al.*, 2013). *S* Typhimurium strain ST4/74, used in the current study, is the parent strain of SL1344, differing by only 8 single nucleotide polymorphisms (SNPs) and results in a histidine mutation in SL1344 (Okoro *et al*., 2015). *S*. Typhimurium strains ST4/74 and SL3261 are therefore closely related. Due to the sequence similarity of the two strains, it was surprising that there were so many unique genes required for strain ST4/74, that were not classified as essential in strain SL3261. There were 306 essential orthologous genes shared between the two strains, including subunits of the 50s ribosomal protein (such as *rplL* and *rplT*), *parC*, *lepB* and *imp*. Although 306 genes were deemed essential in both strains during growth in LB broth at 37°C, there were still 139 and 48 genes unique to strain ST4/74 and SL3261, respectively. The most likely explanation for the high number of unique genes in strain ST4/74 is the difference in density of the libraries or differences in the quality of the sequence, although this was not explored further in this study.

In another study, 257 essential genes were identified in *S*. Typhimurium strain ATCC14028 during growth at 37°C in LB broth, however due to the absence of the whole genome sequence used in the study, it was not possible to include this strain in the comparison of essential genes (Knuth *et al*., 2004). The essential genes in *S*. Typhimurium strain ATCC14028

included *imp*, *murEDGC*, *bamA*, *ipxA*, *ftsWAZI* and *dnaK*, amongst others. Based on gene annotation alone, there were 93 genes considered essential during growth on LB agar at 37°C in *S*. Typhimurium strains ST4/74 and ATCC14028. Some of the conserved essential genes between the strains included, *dapE*, *rpoABCD*, *recC*, *dnaCK*, *clpX*, *rplOL* and *murDEG*, amongst others. DNA replication protein, *dnaC*, has also been determined as essential for LB enriched growth in *E*. *coli* (Gerdes *et al*., 2003). *murE* encodes a UDP-*N*-acetylmuramoylalanyl-D-glutamate 2, 6-diaminopimelate ligase and specifically catalyses the addition of the third amino acid residue to the peptidoglycan monomer unit, and has also been found to be temperature sensitive (Michaud *et al*., 1990; Lugtenberg and v Schijndel-van Dam, 1972). As *murE* is involved in the cytoplasmic synthesis of the peptidoglycan monomer, it has the potential to be an alternative drug target for antimicrobials (Bratkovič *et al*., 2008). The *rplO* gene, for example, which encodes the 50s ribosomal protein L15, has also been identified as essential in *E*. *coli* strain K-12 (Goodall et al., 2018).

There were 139 novel essential genes identified in *S*. Typhimurium strain ST4/74. These included *atpADFH*, *ccmABCDFG*, *fepCD*, *hemDL*, *mreBCD*, *rfaCDEF* and *tolABR*. Nine *tra* genes were also deemed essential for laboratory growth in strain ST4/74, but not SL3261. The *atp* genes are involved in ATP biosynthesis and transport of H^+ ions and the *ccm* gene cluster is involved in cytochrome *c* biogenesis (Thöny-Meyer *et al*., 1995). Certain *tra* genes have been identified as essential for IncC transfer and can aid mobilisation of *Salmonella* genomic island-1 (SGI-1) (Carraro *et al*., 2017). IncC is a large, low-copy number plasmid with a broad host range which frequently contains antimicrobial resistance genes (Harmer and Hall, 2015). Another study identified 336 essential genes in iron-replete conditions (LB broth), 265 of which were shared with the *S*. Typhimurium SL3261 strain previously discussed (Karash and Kwon, 2018). There were 256 genes shared between *S*. Typhimurium strains ST4/74 and 14028s based on gene annotation and comparing only these two strains revealed 220 unique genes required for laboratory growth in ST4/74. A novel fitness gene, *yheM*, was recently identified in *S*. Typhimurium strain 14028s and cells lacking *yheM* accrued more aggregated protein during stress. This gene was identified as essential for growth in LB broth at 37°C for *S*. Typhimurium strain ST4/74 in the current study but did not appear in the essential gene list for SL3261 (Barquist *et al*., 2013; Wang *et al*., 2022).

3.5 Conclusion

A highly saturated transposon mutant library was generated in *S*. Typhimurium strain ST4/74 using transposon directed insertion site sequencing (TraDIS), which contained 609, 000 unique insertions. Growth of the mutant library in LB broth at 37°C identified 486 essential genes, with an insertion index of less than 0.0161. A total of 306 genes were identified as essential during growth in LB broth in two different *S*. Typhimurium strains, suggesting that these are the core genes required for survival in laboratory growth conditions. Together these data suggested that the transposon library was comparable to previously described libraries and was suitable for further analysis to identify conditionally essential genes in stress conditions of the food chain.

Chapter Four

Conditionally essential genes during food chain related stress survival in *S*. Typhimurium strain ST4/74

4.1 Introduction

Salmonella encounters diverse environments throughout the food chain, such as heat, desiccation, and refrigeration/freezing, as well as encountering preservatives, including, salt, sugar, organic acids, and polyphenols. Preservation techniques are becoming milder due to consumer demand for minimally processed products that are less reliant on preservatives, such as salt, nitrite and nitrate (Abee and Wouters, 1999). *Salmonella* has evolved to be able to cope with exposure to stress and evade the hosts defence mechanisms. Understanding the mechanisms behind how S*almonella* can survive during stress and compete for nutrients in food is important, as it would determine the extent at which *Salmonella* is able to cause illness in the host.

Non-specific stress response mechanisms in *Salmonella* are well established and include the master alternative sigma factor σ^S, commonly referred to as RpoS, RpoE and Cpx (discussed in detail in the introduction). However, the specific mechanisms behind food chain related stress tolerance in *Salmonella* remains largely unknown, especially in the stresses investigated during chapter 2, which were desiccation, heat inactivation, organic acids, salt and refrigerated storage. Therefore, this study aims to identify the genes essential for survival in specific food chain related stresses using a high-throughput sequencing technique, called transposon directed insertion site sequencing (TraDIS) which was introduced in chapter three. To determine the genes essential for survival in a particular condition, the transposon mutant library is subjected to stress and then re-sequenced, allowing comparisons to be made between the input (pre-stress) and output (post-stress) libraries (**Figure 28**).

In this study, essential genes are defined as genes required for full tolerance to a defined condition. If a gene has significantly less insertions compared to the input library, then the gene is considered to be essential for survival in that stress condition. Conditionally essential genes are determined by comparing the prevalence of transposon mutants within the input (control) and output (stress) libraries, to ascertain genes essential for survival under a specific condition (Barquist *et al.*, 2016). Mutants exhibiting a fitness advantage will proliferate, whereas mutants in essential genes will not survive, allowing a comparison to be made between the total number of insertions in each mutant library pool, indicating genes of interest (Yasir *et al.,* 2022).

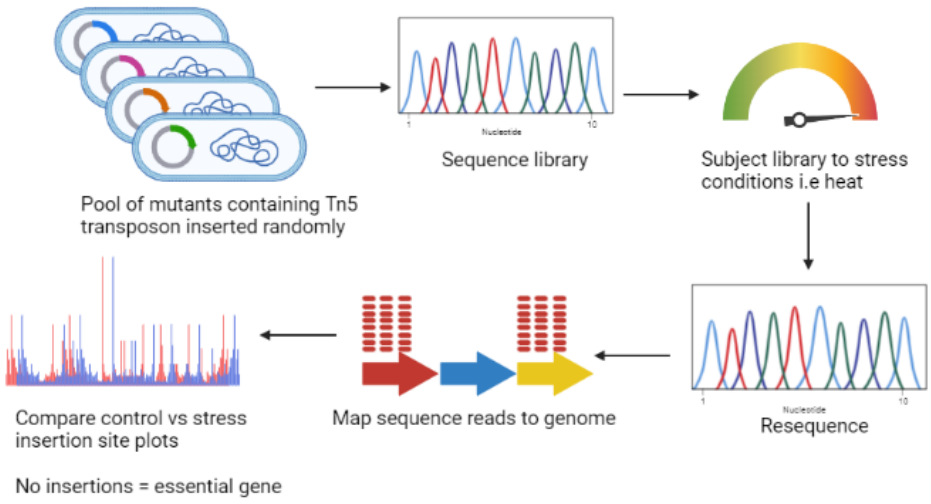


Figure 28. Overview of the transposon directed insertion site sequencing (TraDIS) method.

4.1.1 Aims

1. Determine the genes essential for survival of *S.* Typhimurium strain ST4/74 during;
    a) Heat Inactivation at 60°C for 30 seconds
    b) 24-hour desiccation
    c) 6% NaCl
    d) 8mM acetic acid
    e) 14mM citric acid
    f) 5-week refrigerated storage
2. Identify functional pathways associated with essential genes in each stress condition
3. Identify genes required for survival in multiple food chain related stresses
4. Compare essential genes identified in this study to previously published studies

4.2 Methods

4.2.1 Heat Inactivation of S. Typhimurium strain ST4/74 mutant library

A 50µL aliquot of the *S*. Typhimurium strain ST4/74 mutant library described in chapter 3, was deposited into 5mL LB broth and incubated for approximately 18 hours at 37°C with shaking at 200rpm. A wild type culture of *S*. Typhimurium strain ST4/74 was also prepared from a -80°C glycerol stock in LB broth and incubated simultaneously. Cells were collected by centrifugation at 13,000rpm for 4 minutes at room temperature and the supernatant was discarded. The pellet was resuspended in PBS and the optical density (OD) at 600nm of each culture was diluted to approximately 2.5 x 10^9 CFU/mL, using PBS. The vegetarian food product, previously stored at -20°C, was thawed and 750mg of the food was deposited into the centre of each thermal cell (**Figure 2**) (provided by Nestlé). The food was inoculated with 50µL of each culture (mutant library and wild type) at a concentration of approximately 2.5 x 10^9 CFU/mL and two thermal cells were inoculated per strain. Thermal cells were incubated for 1 hour at 4°C to ensure the culture was fully incorporated into the food sample. Thermal cells were heated in a water bath at 60°C for 30 seconds and immediately plunged into an iced water bath to rapidly cool. One thermal cell was attached to a TC-08 datalogger (Pico Technology) to monitor the temperature of the sample for the duration of the experiment. For a control, thermal cells were incubated for 1 hour at 4°C and then remained at room temperature. Food samples (heated and control) were deposited directly into 5mL LB broth bottles and mixed by vortex, before being serially diluted (1:10) in a 96-well plate with PBS. A 5µL aliquot of each dilution was spotted onto a square LB agar plate, in triplicate. Plates were incubated at 30°C overnight and surviving colonies enumerated. The 5mL LB broth bottles containing the inoculated food samples were incubated for 18 hours overnight at 37°C with shaking at 200rpm, and 50µL of the resulting culture after incubation was used for DNA extraction.

4.2.2 Desiccation of *S*. Typhimurium strain ST4/74 mutant library

S. Typhimurium strain ST4/74 (wild type) and a 50µL aliquot of *S*. Typhimurium strain ST4/74 mutant library were grown to stationary phase in 5mL LB broth by incubating overnight for 18 hours at 37°C with shaking at 200rpm. Cells of both strains were pelleted by centrifugation at 13,000rpm for 4 minutes at room temperature and the supernatant was discarded. The

pellet was resuspended in PBS and the optical density of each culture was diluted to approximately 2.5 x 10^9 CFU/mL, using PBS. Five wells of a 96-well plate were filled with 50µL of *S.* Typhimurium strain ST4/74 (wild type) and mutant library at a concentration of 2.5 x 10^9 CFU/mL. Plates were left to desiccate in a safety cabinet for 24-hours and the temperature and humidity was monitored using a thermohygrometer placed inside the safety cabinet. To determine the number of viable CFU/mL at the start of the experiment prior to desiccation, 50µL of each culture was deposited into a 96-well plate (five wells for each) and mixed with 150µL PBS and dilutions (1:10) with PBS were spotted, in triplicate, onto LB agar and incubated overnight at 30°C. After 24-hours, desiccated cells were resuspended with 200µL PBS and serially diluted (1:10) with PBS, and 5µL of each dilution was spot plated (in triplicate) onto square LB agar plates. Colonies were enumerated and the ratio of the viable counts before desiccation and after desiccation were calculated. The ratio was log10 transformed before statistical analysis. The remainder of the rehydrated cells were deposited into 5mL LB broth bottles and incubated overnight for 18 hours at 37°C with shaking at 200rpm. A 50µL aliquot of overnight culture after 18 hours incubation was used for DNA extraction.

4.2.3 Long term storage at refrigerated temperature of *S*. Typhimurium strain ST4/74 mutant library

Overnight cultures of *S*. Typhimurium strain ST4/74 and a 50µL aliquot of the mutant library was deposited into 5mL LB broth bottles and incubated for 18 hours at 37°C with shaking set to 200rpm. Cells were pelleted by centrifugation at 13,000rpm for 4 minutes at room temperature and the supernatant was discarded. The pellet was resuspended in PBS and the optical density of each culture was adjusted to approximately 2.5 x 10^9 CFU/mL, with PBS. Wells of a CytoOne 24-well tissue culture plate (Starlab, CC7682-7524) were filled with 750mg thawed vegetarian food product and each well was inoculated with 50µL culture (wild type and mutant library) at approximately 2.5 x 10^9 CFU/mL (four wells per strain). Plates were left to incubate for 5 weeks in a cold-room at 4°C. Control plates were prepared by inoculating 750mg portions of the vegetarian food product with 50µL culture, as before, but the inoculated food samples were immediately deposited into individual 5mL LB broth bottles, mixed by vortex and serially diluted (1:10) with PBS, rather than incubating at 4°C. To determine the CFU/mL of the starting inoculum, 5µL of each dilution was spotted onto a square LB agar plate, in triplicate, and incubated overnight for 18 hours at 30°C. Control

colonies were enumerated the following day. After 5-weeks of incubation, inoculated food samples were deposited into individual 5mL LB broth bottles, mixed by vortex and serially diluted (1:10) with PBS. Serial dilutions were spot plated onto square LB agar plates and incubated overnight for 18 hours at 30°C. Surviving colonies were enumerated and the log10 ratio before and after refrigerated incubation was determined. LB broths containing inoculated food samples were incubated overnight for 18 hours at 37°C with shaking at 200rpm. A 100µL aliquot of overnight culture was used for DNA extraction.

4.2.4 Growth of *S*. Typhimurium strain ST4/74 mutant library in 6% NaCl

Overnight cultures of *S*. Typhimurium strain ST4/74 and a 50µL aliquot of mutant library was deposited into 5mL LB broth bottles and incubated at 37°C for 18 hours with shaking set to 200rpm. A 6% NaCl solution was prepared in LB broth (w/v) and sterilised by autoclaving at 121°C for 15 minutes. A 5µL aliquot of overnight culture of both *S*. Typhimurium strain ST4/74 and mutant library at a concentration of approximately 2.5 x 10^9 CFU/mL were deposited into 5mL 6% NaCl solution. For a control, 5µL of each culture at ~2.5 x 10^9 CFU/mL was deposited into 5mL LB broth. 200µL aliquots of each culture in either 6% NaCl or LB broth were dispensed into wells of a Bioscreen honeycomb plate (Thermo Fisher Scientific, 12871511), in triplicate. Growth was measured at an optical density of 600nm using a Bioscreen C Analyser at 37°C, with constant shaking. Measurements were taken every 5 minutes for 24-hours. The following day, 200µL of each well containing the mutant library grown in 6% NaCl was deposited into a 5mL LB broth bottle and incubated overnight for 18 hours at 37°C (200rpm). A 100µL aliquot of turbid culture of mutant library in 6% NaCl was used for DNA extraction.

4.2.5 Growth of *S*. Typhimurium strain ST4/74 mutant library in 14mM Citric Acid

Cultures of *S*. Typhimurium strain ST4/74 and a 50µL aliquot of the mutant library were grown to stationary phase in 5mL LB broth at 37°C for 18 hours with shaking set to 200rpm. A 30mM stock solution of citric acid (Thermo Fisher Scientific, 110450250) was prepared in 250mL LB broth and sterilised using a Minisart polyethersulfone (PES) syringe filter (0.22µM) (Sartorius, 16532K). Working stocks of citric acid were prepared from the 30mM stock solution in LB broth in 2mM increments from 2mM to 16mM. A 5mL portion of each

concentration of citric acid, and an LB broth control (no citric acid), was dispensed into 15mL centrifuge tubes, in duplicate for each strain. A 1mL aliquot of overnight culture (18-hour growth) of *S*. Typhimurium strain ST4/74 and the mutant library was added to 4mL LB broth adjusted to pH 5.8 with 30mM citric acid and incubated for 30 minutes at 37°C (200rpm). Cultures were then adjusted to approximately 5 x 10^8 CFU/mL with LB broth, and 5µL of each strain was inoculated into each concentration of citric acid. 200µL of each citric acid concentration inoculated with either *S*. Typhimurium strain ST4/74 or the mutant library, including an LB broth positive control and a non-inoculated control of each concentration, was dispensed into a 96-well U-Bottom plate (Greiner, 163320) in triplicate wells. The pH of each concentration of citric acid was measured using a benchtop pH meter (Mettler Toledo, 30046240). Absorbance at 600nm was measured for 24 hours at 37°C using a FLUOstar Omega Spectrophotometer (BMG Labtech). After 24 hours, the contents of the wells containing the mutant library in 14mM citric acid was deposited into individual 5mL LB broth bottles and grown overnight at 37°C with shaking at 200rpm. Following growth, a 100µL aliquot from two separate overnight cultures of the mutant library grown for 18 hours in 14mM citric acid was used for DNA extraction.

4.2.6 Growth of *S*. Typhimurium strain ST4/74 mutant library in 8mM Acetic Acid

S. Typhimurium strain ST4/74 and the mutant library were grown in 5mL LB broth and incubated for 18 hours at 37°C with 200rpm shaking. A 60mM stock solution of acetic acid (SLS, CHE1012) was prepared in LB broth and sterilised using a 0.22µM PES filter (Sartorius, 16532K) and syringe. The 60mM acetic acid stock solution was used to prepare working stocks of 2mM to 14mM acetic acid, increasing in 2mM increments, in LB broth. A 5mL aliquot of each dilution of acetic acid was transferred to a 15mL centrifuge tube, in duplicate. Overnight cultures of *S*. Typhimurium strain ST4/74 and the mutant library were pre-adapted to pH 5.8 using 60mM acetic acid, and incubated for 30 minutes at 37°C, with shaking at 200rpm. The centrifuge tubes containing 5mL of acetic acid at each dilution were inoculated with 5µL of *S.* Typhimurium strain ST4/74 and the mutant library. A 200µL aliquot of each acetic acid concentration inoculated with either the wild type strain or the mutant library (including LB broth positive control), and a non-inoculated control of each concentration was dispensed into a 96-well U-Bottom plate (Greiner, 163320), in triplicate wells. The pH of each concentration of acetic acid was measured using a benchtop pH meter (Mettler Toledo, 30046240) and growth was measured at OD600nm for 24 hours at 37°C using a FLUOstar

Omega Spectrophotometer (BMG Labtech). After 24 hours, the contents of the wells containing the mutant library in 8mM acetic acid was deposited into individual 5mL LB broth bottles and grown overnight at 37°C with shaking at 200rpm. Following growth, a 100µL aliquot from each culture was used for DNA extraction.

4.2.7 Statistical analysis

A Welch's t-test was conducted on the nested values of each stress experiment using GraphPad Prism (version 8.0.2), comparing the log10-ratio reduction of wild type cells and cells of the transposon insertion master mix library.

4.2.8 DNA Extraction of mutant library post exposure to stress

For DNA extraction of the mutant library post exposure to stress, 50-100µL of overnight culture was deposited directly into a Maxwell RSC cultured cells DNA kit cassette (Promega, AS1620) and loaded into a Maxwell RSC 48 Instrument (Promega, AS4500). DNA was extracted in duplicate for each sample, following the manufactures standard protocol for DNA extraction from bacterial cells. The DNA concentration was quantified using a Qubit 3.0 fluorometer (Invitrogen) and the Qubit dsDNA HS (High Sensitivity) Assay Kit (Invitrogen, Q32851). Extracted DNA was stored at -20°C until required.

4.2.9 Sequencing of mutant library post exposure to stress

To make the library preparations for sequencing, a MuSeek Library Preparation Kit (Illumina Compatible, K1361) was used. DNA extractions from the mutant library were diluted to 20ng/µL with elution buffer. MuSeek reactions were prepared in a 96-well plate using the reagents outlined (**Table 17**). The plate was sealed and incubated at 30°C using a thermocycler (Veriti) for 5 minutes. Sterile ultra-pure water was added to each well to a final volume of 30µL. To each well, 45µL of AMPure XP beads (Beckmann, A63880) were added (1.5x volume) and mixed by pipetting. The plate was incubated for 5 minutes at room temperature, and then placed onto a magnetic rack for 5 minutes until the solution became clear. The supernatant was discarded and 200µL of freshly prepared ethanol (80%) was added to each well. The plate was incubated at room temperature for 30 seconds,

and the supernatant discarded. Another 200µL of 80% ethanol was added to each well, incubated for 30 seconds, and the supernatant discarded. Any residual ethanol was removed, and the plate was air dried for 5-15 minutes. Beads were resuspended in 20µL elusion buffer and placed back on the magnetic rack until the solution became clear.

In a new 96-well plate, 5µL of Tn5-specific enrichment primer (either N701, N702 or N703) and 5µL of each index primer (either S502, S503, S505 or S507) was added to each well. To each well containing primers, 15µL of DNA solution and 25µL of Q5 High-Fidelity Master Mix was added and mixed thoroughly, to give a final volume of 50µL. The plate was sealed and placed into a thermocycler (Veriti) for PCR, using the thermocycling conditions outlined (**Figure 29**).

Reagent	Quantity (µL)
MuSeek Fragmentation Buffer	2.5
MuSeek Enzyme Mix	0.5
gDNA (20ng/µL)	4.5

Table 17. Reaction mix for MuSeek library preparations.

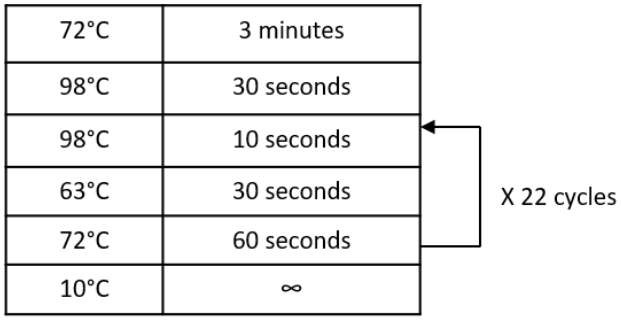


Figure 29. Thermocycling conditions for Tn5 fragment enrichment.

To clean up the Tn5 fragment enrichment PCR, 30µL of evenly mixed AMPure XP beads (Beckmann, A63880) were added to each PCR reaction and mixed by pipetting. The PCR reactions were incubated at room temperature for 5 minutes and placed on a magnetic stand for 2 minutes, until the solution became colourless. The supernatant was discarded, and the plate was washed twice with 200µL 80% ethanol, incubating for 30 seconds each time after addition of ethanol on the magnetic stand before discarding the supernatant. Residual ethanol was removed, and the plate left to airdry for 15 minutes. The plate was removed from the magnetic stand and 15µL of elusion buffer added to each well. The beads were mixed thoroughly and incubated at room temperature for 2 minutes. The plate was placed

onto the magnetic stand for the final time until the solution cleared. A 10µL aliquot of supernatant from each well was transferred to a sterile microcentrifuge tube, ready for sequencing. Prior to sequencing, a Qubit 3.0 fluorometer (Invitrogen) was used to check the concentration of the mutant libraries post-exposure to stress. A Qubit dsDNA High Sensitivity (HS) Assay kit (Invitrogen, Q32851) was used to determine the concentration of a 1µL aliquot of each library. The mutant library was sequenced on a NextSeq 500, using the NextSeq 500/550 High Output kit (75 cycles) (Illumina, 20024906), following the protocol outlined in the Illumina NextSeq Denature and Dilute Libraries Guide (Illumina, version 13).

4.2.10 Bioinformatic analysis of mutant library sequencing data post-exposure to stress

The Bio-Tradis toolkit (version 1.4.1) was used to analyse transposon insertion sequencing data generated using the TraDIS method (Barquist *et al.*, 2016). Essential genes for each condition were identified using the tradis_essentiality.R script (part of the Bio-Tradis package) according to their insertion index. A bespoke python3 script was written by Gaetan Thilliez to compare the essential gene lists generated for each replicate in the stress conditions. The tradis_comparison.R script within the package was used to identity essential genes in each stress condition compared to the control. This script utilises edgeR (Robinson *et al.*, 2009) to identify significant differences in read counts, and insertion frequencies between experimental stress conditions, pre- and post- selection. Normalisation was applied to the dataset using trimmed mean of M values (TMM), and dispersion of transposon tags was estimated as part of the edgeR package. Only genes with more than 20 reads in both replicates were compared for differences in the occurrence of mutants. Significance was corrected for by using the Benjamini-Hochberg procedure, and genes with an adjusted P value, denoted q-value, of <0.05, which equates to a hypothetical false discovery rate (FDR) of 5% and an absolute log fold change (logFC) of >2 or <-2, were considered significant (Dembek *et al.*, 2015).

4.3 Results

4.3.1 Stress conditions were defined in the *S*. Typhimurium strain ST4/74 wild type for use in TraDIS experiments

The stress conditions the mutant library was exposed to in order to determine the conditionally essential genes were selected due to the results from the phenotypic assays in chapter 2. The conditions chosen to subject the mutant library to were heat inactivation at 60°C, 24-hour desiccation, 5-week refrigerated storage, 6% NaCl and 14mM citric acid. After evaluating the results from *S*. Typhimurium strain ST4/74 growth in acetic acid, it was decided that the transposon mutant library should be exposed to a lower concentration of acetic acid, 8mM, due to the difference in phenotype observed at 12mM acetic acid for the wild type strain. Overall, conditions were chosen which resulted in a decrease in cell viability of approximately 1-log for heat inactivation, desiccation and cold storage stresses, and concentrations of organic acid and NaCl which affected growth of the wild type strain in 24 hours, but did not eradicate the cell population entirely.

4.3.2 The *S*. Typhimurium strain ST4/74 mutant library displays a similar phenotype to the wild type strain during stress

In order to test that construction of the mutant library by random insertion of Tn5 had no generalised effects on the survival of *S*. Typhimurium strain ST4/74, the phenotypic response of the mutant library was compared to the wild type strain in each stress condition. After exposure to stress, the mutant library was sequenced and a comparison between the transposon insertions in the control sequencing data and after exposure to stress identified conditionally essential genes. The library was incubated at 60°C for 30 seconds and viable CFUs were enumerated (**Figure 30**). A *S*. Typhimurium strain ST4/74 wild type control was included alongside the mutant library to ensure there was no difference in recovery between the two strains due to the presence of transposons. For all four biological replicates, there was an average log-reduction in cell viability of 1.1 for the ST4/74 wildtype strain, compared to 1.2 for the ST4/74 transposon mutant library (**Figure 30**). There was no significant difference observed between the two strains in response to heat inactivation and therefore, DNA was extracted from the ST4/74 transposon mutant library after overnight recovery in LB broth at 37°C, to be used for sequencing.

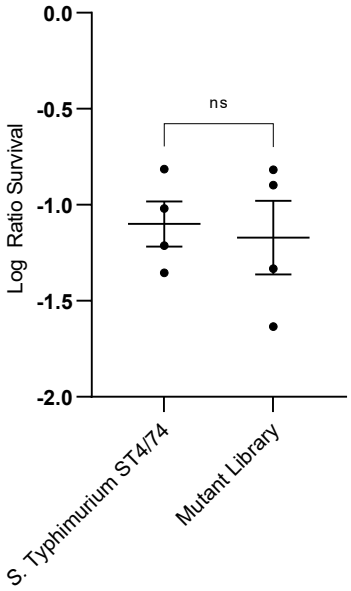


Figure 30. Effect of heat inactivation on the wild type *S.* Typhimurium ST4/74 strain compared to the transposon mutant library. The log ratio survival of *S*. Typhimurium strain ST4/74 wild type strain and mutant library was determined by heating at 60°C for 30 seconds. The mean of three replicates is plotted (±SE) and each data point represents the average of three technical replicates.

The mutant library was desiccated for 24 hours at an average temperature of 21°C and an average relative humidity of 34% alongside the *S*. Typhimurium strain ST4/74 wild type to ensure Tn5 insertions in the mutant library did not affect survival during desiccation (**Figure 31**). After 24-hour desiccation, there was a ~2-log reduction in cell viability in the wild type strain and a ~2.5-log reduction in cell viability for the mutant library (**Figure 31**). The individual replicates for the wild type strain were more varied than that observed in the mutant library, and as a result a Welch's t-test on the values revealed a significant difference between the two strains ($p = 0.0186$). Cells were still recovered after 24-hour desiccation for the mutant library and total cell death did not occur, therefore DNA extraction from overnight recovery of the desiccated cells in LB broth proceeded even though there was a significant difference between the mutant library and wild type strain.

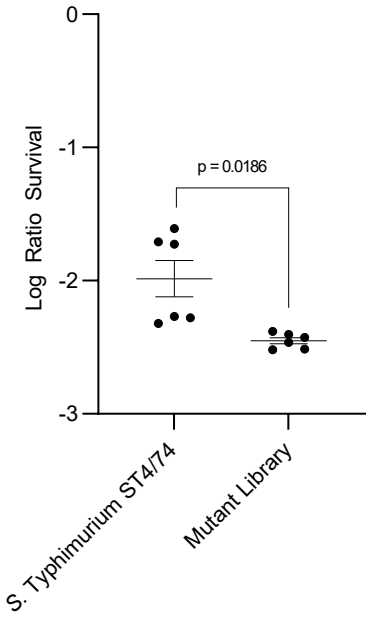


Figure 31. **Effect of desiccation on the wild type *S*. Typhimurium ST4/74 strain compared to the transposon mutant library.** The log ratio survival of *S.* Typhimurium strain ST4/74 and the mutant library after 24-hour desiccation in a safety cabinet was determined at an average temperature and humidity of 21°C and 34%, respectively. The mean of six replicates is plotted (±SE) and each data point represents the average of five technical replicates.

To determine the genes involved in survival during long term refrigerated storage (cold stress), the vegetarian food product was inoculated with mutant library and incubated for 5-weeks at 4°C (**Figure 32**). A *S*. Typhimurium strain ST4/74 wild type control was included in the assay to assess the survivability of the mutant library compared to the wild type strain. A Welch's t-test revealed no significant difference in the log-ratio survival of the wild type strain and the mutant library. The mean log-ratio survival for each strain varied between biological replicates, however overall, there was a ~0.04-log reduction in cell survival for the ST4/74 wildtype strain and a ~0.03-log reduction observed for the ST4/74 mutant library (**Figure 32**). Surviving colonies of the mutant library after 5-weeks incubation at 4°C were recovered in LB broth overnight and DNA was extracted in preparation for sequencing to determine essential genes.

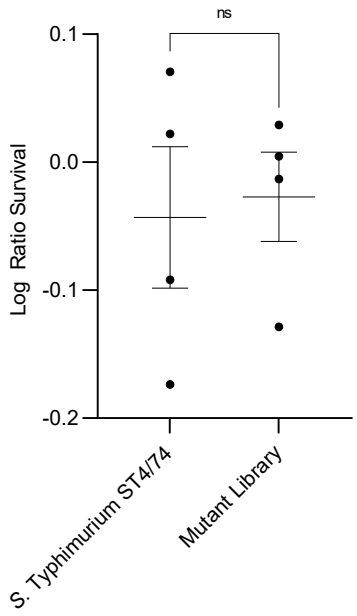


Figure 32. Effect of long term refrigerated storage on the wild type *S*. Typhimurium ST4/74 strain compared to the transposon mutant library. The log ratio survival of *S*. Typhimurium strain ST4/74 wild type and mutant library was determined during long term refrigerated storage at 4°C for 5 weeks. The mean of four replicates is plotted (±SE) and each data point represents the average of three technical replicates.

Growth of the mutant library in 6% NaCl was assessed alongside a wild type control to ascertain the genes involved in surviving salt stress (**Figure 33**). The *S*. Typhimurium strain ST4/74 wild type and mutant library followed a similar sigmoidal growth curve in LB broth and in 6% NaCl. There was an increased lag phase observed for the mutant library in both LB broth and 6% NaCl (**Figure 33**). In 6% NaCl, growth was initiated after ~7-hours for the wild type strain, whereas the mutant library began to grow at about 10 hours post inoculation. The maximum OD600nm reached was ~1.0 for both strains in 6% NaCl. The subsequent mutant library cells grown after 24 hours in 6% NaCl were recovered in LB broth overnight in preparation for DNA extraction and sequencing.

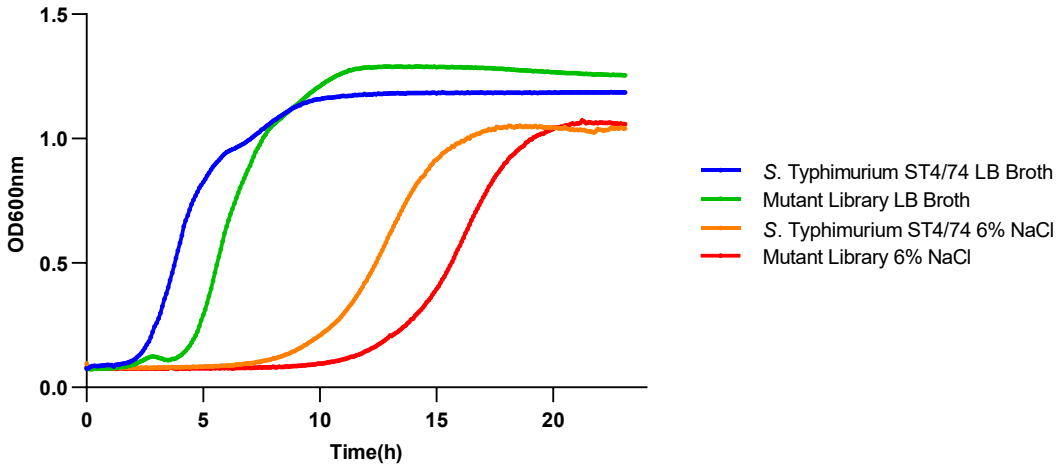


Figure 33. Effect of NaCl on the wild type *S*. Typhimurium ST4/74 strain compared to the transposon mutant library. Growth was measured as OD600nm in the *S*. Typhimurium ST4/74 wild type strain and mutant library in LB broth and LB broth supplemented with 6% NaCl for 24 hours.

To test whether transposon insertion had a non-specific effect on the growth of the mutant library, the mutant library was grown in 8mM acetic acid for 24 hours alongside a *S*. Typhimurium strain ST4/74 wild type control (**Figure 34**). Growth in LB broth and 8mM acetic acid occurred after ~5 hours for the ST4/74 mutant library. There was an increase in lag-time between initial incubation at 37°C and subsequent growth for the mutant library compared to the wild type strain in LB broth. The maximum OD600nm reached for the mutant library was less than the wild type strain in 8mM acetic acid (**Figure 34**). Turbid wells containing the mutant library in 8mM acetic acid were deposited into LB broth bottles and incubated overnight at 37°C, to allow cells to recover and DNA was extracted in preparation for sequencing.

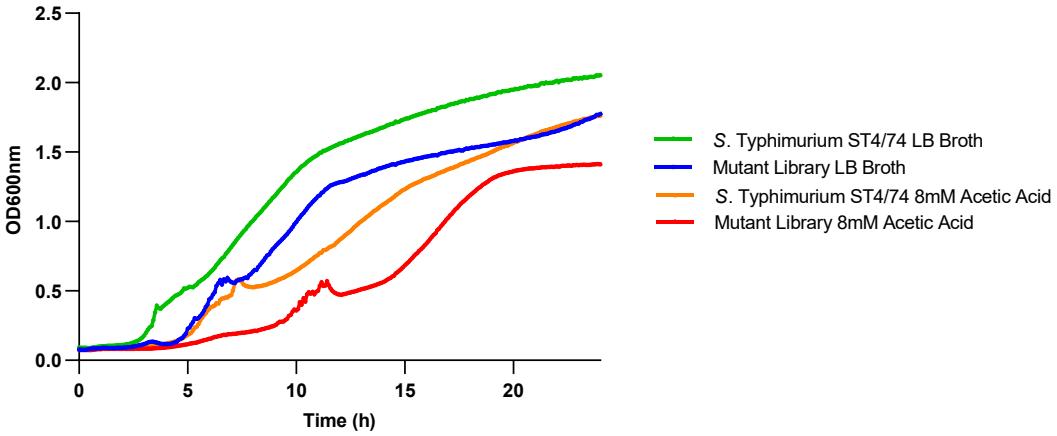


Figure 34. Effect of acetic acid on the wild type *S.* Typhimurium ST4/74 strain compared to the transposon mutant library. Growth was measured as OD600nm in the *S.* Typhimurium ST4/74 wild type strain and ST4/74 mutant library in LB broth and LB broth supplemented with 8mM Acetic Acid for 24 hours.

To test whether the mutant library shared a similar phenotype to the wild type strain during exposure to citric acid, growth of both strains was assessed in 14mM citric acid (**Figure 35**). There was an increased lag-phase observed for the mutant library in both LB broth and 14mM citric acid compared to the wild type strain. Growth occurred after ~10 hours for the wild type strain in citric acid, whereas growth occurred after ~12 hours for the mutant library. The maximum OD600nm reached for both strains in the two types of media was between 1.2-1.3. DNA was extracted from an overnight recovery of mutant library cells in 14mM citric acid, for library preparation and sequencing.

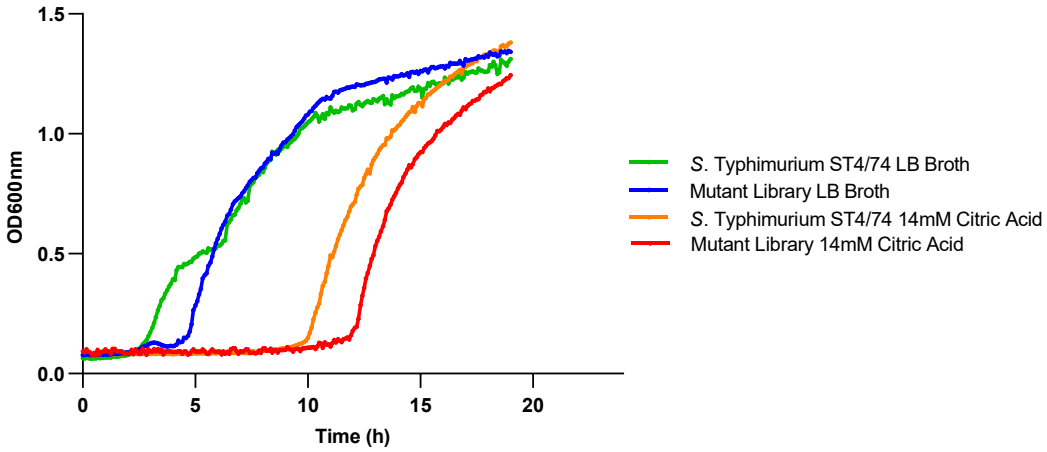


Figure 35. Effect of citric acid on the wild type *S.* Typhimurium ST4/74 strain compared to the transposon mutant library. Growth was measured in OD600nm in the *S.* Typhimurium ST4/74 wild type strain and ST4/74 mutant library in LB broth and LB broth supplemented with 14mM citric Acid for 24 hours.

4.3.3 The transposon inserted randomly within the genome of *S*. Typhimurium strain ST4/74

The high-density saturated transposon mutant library in *S*. Typhimurium strain ST4/74 was subjected to food chain related stress to determine the essential genes in each stress condition. The Bio-Tradis pipeline was used to predict conditionally essential genes according to the transposon insertions in each gene during stress exposure. Genes that are essential for survival in food chain related stresses will have few insertions. The results from executing the Bio-Tradis pipeline on the output library post-stress is summarised (**Table 18**). The percentage of reads mapping to the *S.* Typhimurium strain ST4/74 reference genome for all output libraries was between 53% and 94%, which is similar to other libraries (Yasir *et al.*, 2022). The library with the lowest percentage of reads mapping the reference was the desiccation (replicate 1) output library, with only 53.7% of reads mapping, whereas the highest percentage of reads mapping to the reference was achieved by the 5-week cold storage (replicate 2) output library at 93.5% (**Table 18**). The number of unique insertion sites varied between each output library and between each biological replicate of each output library. There were 317,105 unique insertions in the desiccation output library (replicate 2), which equated to approximately one insertion every 16bp. The 5-week refrigerated storage (replicate 2) output library had the fewest number of insertions at 76,197 equating to an insertion every ~67bp. Overall, the mutant libraries generated using TraDIS were suitable for the identification of conditionally essential genes.

TraDIS Library	Total Reads	% Reads Matched	Reads Mapped	% Mapped	Total Unique Insertion Sites (UIS)	Total Sequence Length/Total UIS
6% NaCl (1)	19955274	100	13080706	65.55	266833	18.99
6% NaCl (2)	15920944	100	12262124	77.02	195868	25.87
5-Week RS (1)	11288709	100	8478554	75.11	115146	44.01
5-Week RS (2)	10580715	100	9893275	93.50	76197	66.50
8mM AA (1)	16888863	100	10225173	60.54	126122	40.18
8mM AA (2)	16817402	100	12672060	75.35	151905	33.36
14mM CA (1)	13888594	100	7748508	55.79	233924	21.66
14mM CA (2)	15827966	100	11546631	72.95	257514	19.68
Heat In. (1)	10061509	100	6068420	60.31	303191	16.71
Heat In. (2)	12088305	100	11337741	93.79	86830	58.36
Desiccation (1)	14404069	100	7740486	53.74	242560	20.89
Desiccation (2)	21694000	100	16933843	78.06	317105	15.98

Table 18. Summary of sequencing results of the ST4/74 transposon mutant library post-stress exposure. The Bio-Tradis pipeline was used to generate summary statistics for the saturating transposon mutant library in ST4/74 after exposure to food chain related stresses (RS=refrigerated storage, AA= Acetic Acid, CA= Citric Acid).

In order to visualise the location of the insertions and the positioning of conditionally essential genes, DNA plotter was used to plot the predicted essential genes from the tradis_essentiality.R script for one replicate of each output library post-stress exposure (**Figure 36**). For all output libraries except 14mM Citric Acid, there was an increase in number of insertions in genes involved in lipopolysaccharide (LPS) biosynthesis, such as *rfaB* and *rfaK.* Insertions were spread across the entirety of the genome and no sequencing bias was observed (**Figure 36**).

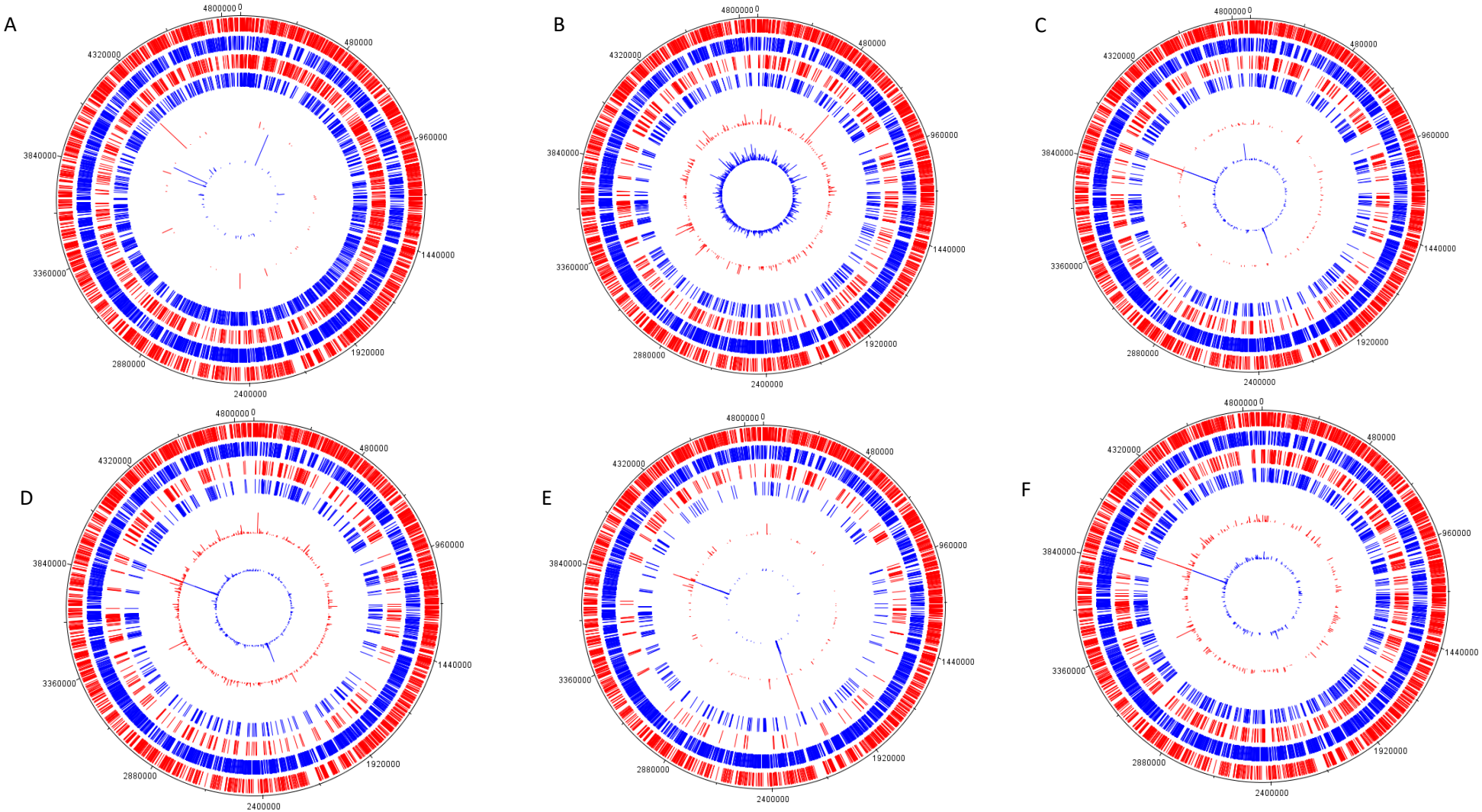


Figure 36. Circular genetic maps of the ST4/74 transposon library post exposure to stress. Each figure represents the *S*. Typhimurium strain ST4/74 transposon mutant library after exposure to stress, where (A) 8mM Acetic Acid, (B) 14mM Citric Acid, (C) Desiccation, (D) Heat Inactivation, (E) 6% NaCl and (F) 5-week cold storage. For each plot, the outer two tracks represent the CDS of ST4/74 (forward (red) and reverse (blue)), the next two tracks show the essential genes required for each stress and the inner-most tracks show the position of Tn5 insertions. Plots were made using DNA Plotter in Artemis.

4.3.4 A positive correlation is observed between biological replicates of the output library in all stress conditions

To test the reproducibility of the genome wide functional analysis using TraDIS, two biological replicates of each stress were compared to the input library and the number of insertions for each gene in the output library were compared using regression analysis (**Figure 37**). A positive correlation was observed between the number of insertions in each replicate for all stresses, with R^2 ranging from 0.82 to 0.95 (**Figure 37B-G).** The control library that was not subjected to stress had the highest R^2 of 0.99, suggesting a small variation in the level of stress between replicates in test experiments (**Figure 37**). There were 4,992 genes in total present in the genome of *S*. Typhimurium strain ST4/74. The number of genes with insertions differed depending on the library. The control library had the greatest number of genes with insertions at 4,774, whereas the 5-week refrigerated storage library had the least number of genes with insertions (**Table 19**). One gene in particular, STM474_4456, had the greatest number of insertions in all libraries (**Figure 37**). This gene was annotated as a putative inner membrane protein and a blastp search of the amino acid sequence revealed 98.98% similarity to an Ig-like domain repeat protein and also a 99.98% similarity to a non-fimbrial adhesin (*SiiE*). STM474_4456 is a large 5,307bp gene, with lots of repetitive regions, which could explain the increased frequency of insertions observed in the library. Overall, the number of insertions for each gene in both replicates for each library was similar and as a result the R^2 value was close to one, indicating points are situated near to the linear regression line.

Library	Number of Genes with Insertions
Control	4,774
Heat Inactivation	4,205
Desiccation	4,474
Acetic Acid	4,082
Citric Acid	4,284
NaCl	4,388
Refrigerated Storage	4,053

Table 19. **Average number of genes with insertions in the transposon mutant library after exposure to stress.**

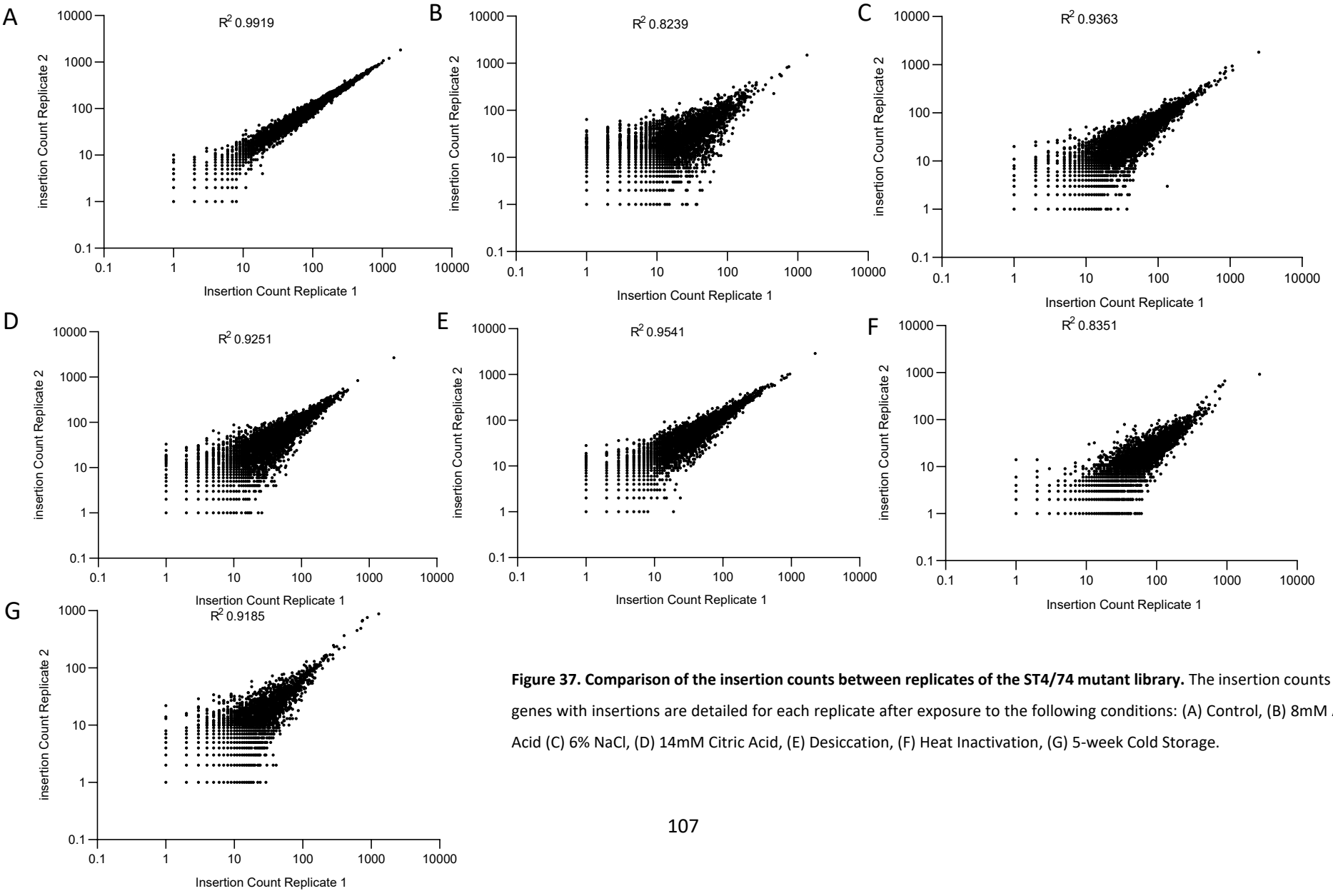


Figure 37. Comparison of the insertion counts between replicates of the ST4/74 mutant library. The insertion counts for all genes with insertions are detailed for each replicate after exposure to the following conditions: (A) Control, (B) 8mM Acetic Acid (C) 6% NaCl, (D) 14mM Citric Acid, (E) Desiccation, (F) Heat Inactivation, (G) 5-week Cold Storage.

4.3.5 The number of essential genes differed between replicates

In order to check the reproducibility of TraDIS, the number of essential genes identified in each replicate according to the insertion index from the tradis_essentiality.R script were compared. Both control replicates had a similar number of genes predicted to be essential and as a result there were 476 genes common to both, and only 36 and 38 genes unique to replicate 1 and 2, respectively (**Figure 38**). The genes identified as essential in the control are the genes involved in growth in LB broth and should be excluded from the essential gene lists during stress. There were more genes assigned as essential in the 5-week refrigerated storage library for replicate 2 than replicate 1, and there were 809 genes shared between both replicates. In the heat inactivation library, there were 2,518 genes uniquely essential in replicate 2, compared to only 8 designated as uniquely essential in replicate 1 (**Figure 38**). In contrast, for the mutant library exposed to 8mM acetic acid more essential genes were assigned in replicate 1, than replicate 2. The number of genes predicted to be essential for desiccation was similar for both replicates at 595 for replicate 1 compared to 550 for replicate 2, with 476 genes common to both. For 14mM citric acid, there were 483 genes essential in both output transposon library replicates and 505 genes essential in both replicates for 6% NaCl (**Figure 38**). In general, the number of essential genes differed between replicates, and therefore only genes predicted to be essential in both replicates were reported in future analyses.

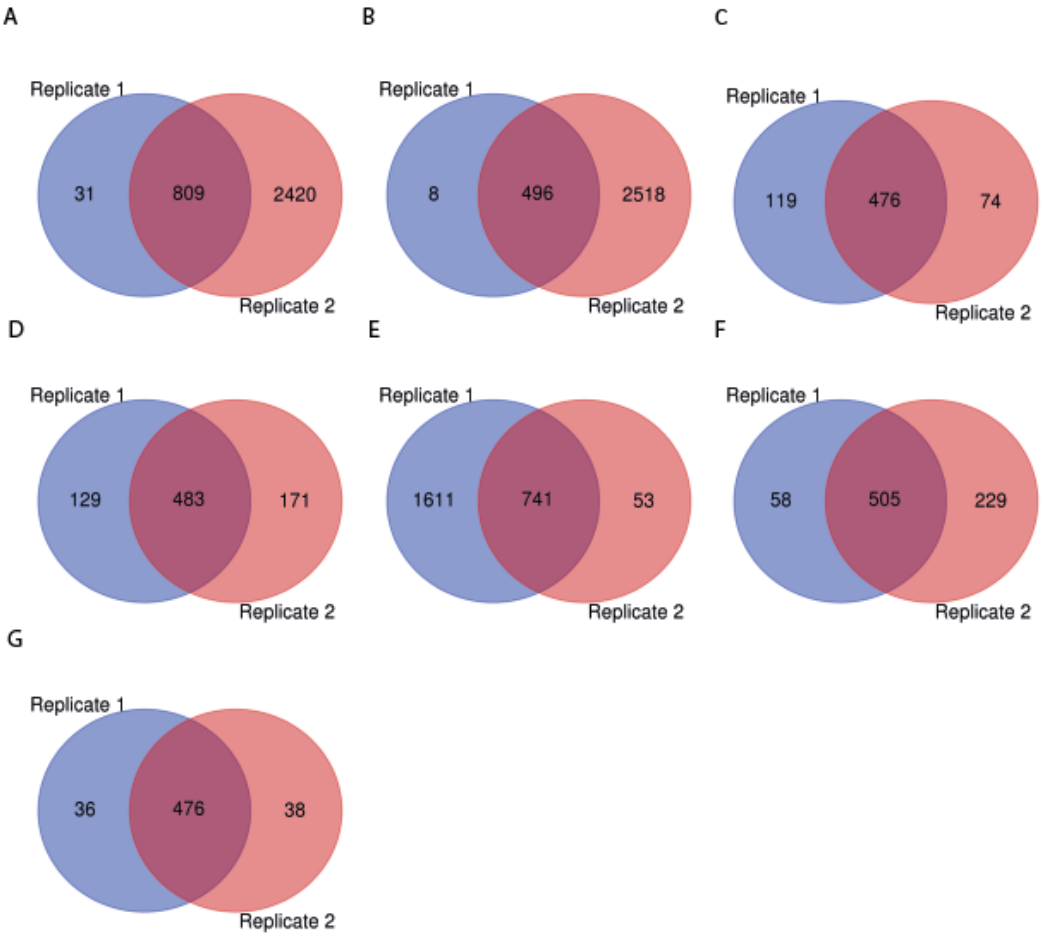


Figure 38. **Venn diagrams comparing the number of essential genes in each replicate, for each condition**. The diagrams pictorialise the number of essential genes in each replicate for *S*. Typhimurium ST4/74 and the number of essential genes shared between replicates for (A) 5-Week Cold Storage, (B) Heat Inactivation, (C) Desiccation, (D) 14mM Citric Acid, (E) 8mM Acetic Acid, (F) 6% NaCl and (G) Control.

4.3.6 Volcano plots showing genes involved in surviving food chain related stress

In analysis described in chapter 3, gene essentiality was predicted by identifying genes with fewer than expected number of insertions per gene. To determine whether genes that are predicted to be essential due to their low number of insertions are also essential when compared to the control dataset, and furthermore, to predict the genes most essential for survival in each stress condition, a script in the Bio-Tradis package (tradis_comparison.R) was used to compare the fitness effect of exposure to stress and to identify differences in mutant frequency between the two conditions (control vs stress). The difference in read counts and insertion frequencies between the two libraries were compared to elucidate the effect each gene has on the fitness of the cell during each experimental stress condition. An arbitrary cut-off for significance was set as a $\log_2$fold-change (logFC) > 2 and < -2, which is commonly used for transposon mutant libraries in the literature, with a q-value (*P* value adjusted for multiple testing) of < 0.05. A positive logFC indicated genes had more insertions in the stress condition than the control, and thus a mutation in this gene provides a benefit to the cell during the stress, whereas a negative logFC indicates fewer insertions in the gene during stress compared to the control and therefore mutations in this gene are detrimental to survival and the gene is therefore described as essential for survival during stress. The genes satisfying the statistical parameters for significance for each food chain related stress condition are displayed as volcano plots (**Figure 39**).

For survival in 8mM acetic acid, 74 genes had a logFC > 2 or < -2 and a q-value < 0.05 (**Figure 39A**). There were not many genes required by *S*. Typhimurium strain ST4/74 in 8mM acetic acid stress that were not required for general growth in LB broth at 37°C. On the contrary, there were many more genes with a negative logFC during 14mM citric acid stress (128 genes), than with a positive logFC (13 genes), indicating that there were 128 genes required for survival during citric acid stress. Genes which had the greatest negative logFC during 14mM citric acid stress and a q-value of < 0.05, included *envZ* and *htrA* (**Figure 39B**). For desiccation stress, 43 genes had a logFC > 2 and a q-value < 0.05. Genes with the greatest positive logFC during 24-hour desiccation included, *rfaK*, *rfaL* and *rfaH*, which are all involved in lipopolysaccharide synthesis (**Figure 39C**). There are very few genes with a logFC < -2 during heat inactivation (3 genes), but more genes with a positive logFC. Genes with a logFC < -2 during heat inactivation included, *tolC*, *clpB* and *rnhA* (**Figure 39D**). For 6% NaCl stress, there were a similar number of genes with a logFC > 2 and < -2. *proP* and *dam* had a logFC < -2 and a q-value < 0.05 during 6% NaCl stress (**Figure 39E**). For 5-week cold storage, there

were more genes with a logFC > 2 (18 genes), with very few significant genes with a logFC < -2 (11 genes) (**Figure 39F**). Mutations in some of some of the lipopolysaccharide genes appear to be beneficial for survival in 6% NaCl, desiccation, heat inactivation and 5-week refrigerated storage (**Figure 39**). The number of genes with a logFC > 2 or < -2 in each condition, with a q-value < 0.05 are summarised (**Table 20**).

Condition	Number of genes with logFC > 2	Number of genes with logFC < -2
Heat Inactivation	43	3
Desiccation	43	105
Refrigerated Storage	18	11
6% NaCl	45	101
14mM Citric Acid	13	128
8mM Acetic Acid	22	52

Table 20. Number of genes with a significant logFC in each stress condition. The number of genes with a logFC > 2 and < -2, with a significant q-value of less than 0.05, in each food chain related stress condition.

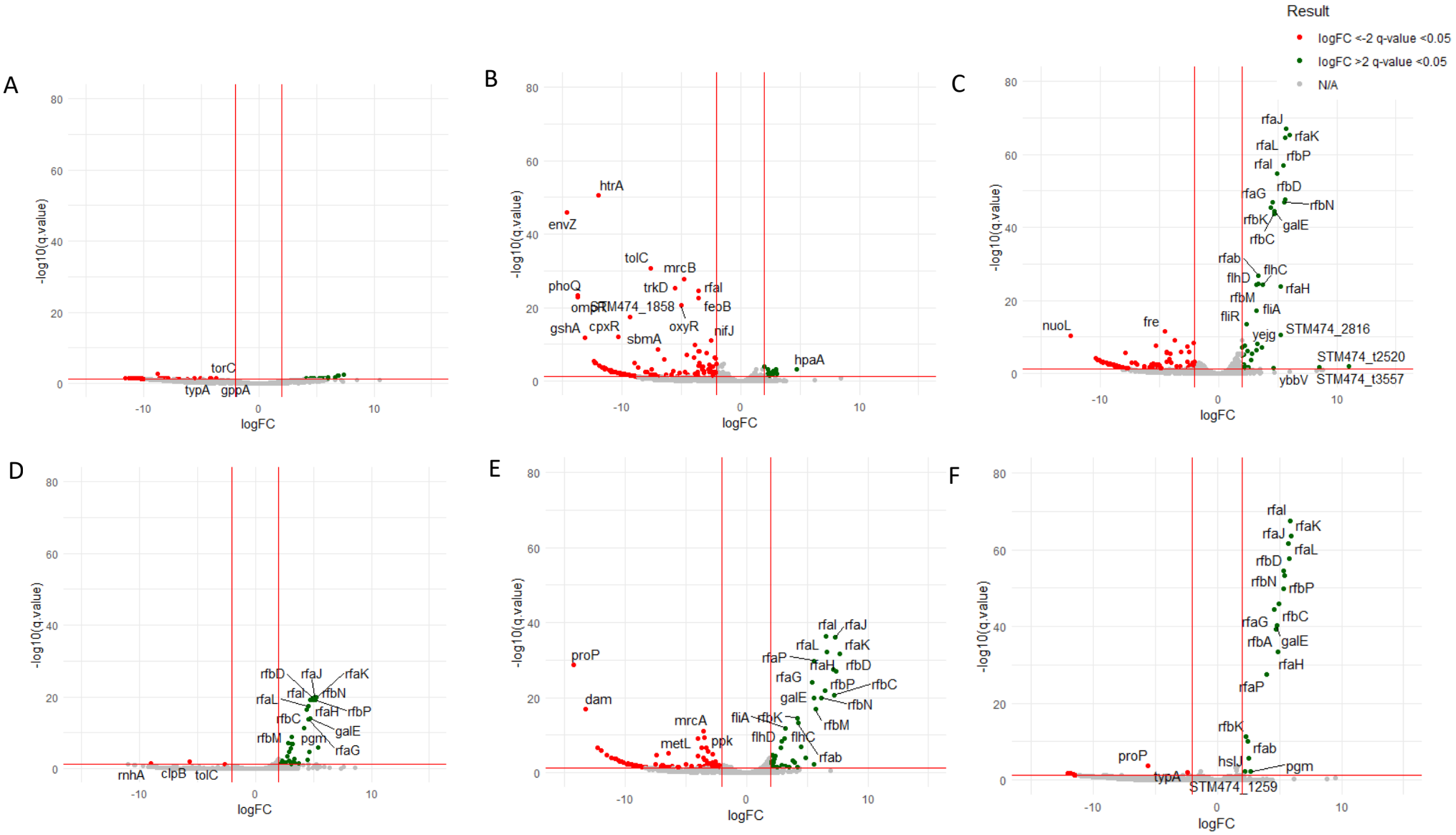


Figure 39. Identification of genes involved in stress response in *S.* Typhimurium strain ST4/74 using volcano plots of TraDIS-based log$_2$fold change. (A) 8mM Acetic Acid, (B) 14mM Citric Acid, (C) Desiccation, (D) Heat Inactivation, (E) 6% NaCl and (F) 5-week cold storage. Red lines represent the cut-off criteria of a q-value < 0.05 (horizontal) and a log$_2$fold change (logFC) of > 2 or < -2 (vertical). Green dots represent genes that have a logFC > 2 and a q-value < 0.05, and a loss of these genes would be beneficial for survival, whereas red dots represent genes with a logFC < -2 and a q-value < 0.05, and a loss of these gene would be detrimental to survival in the associated stress condition. Grey dots represent genes that do not fulfil the selection criteria.

4.3.7 Culture in 14mM citric acid stress required the greatest number of essential genes

To compare the differences in the number of essential genes in each of the six food chain related stresses, the logFC for each gene with a significant q-value of less than 0.05 was plotted (**Figure 40**). Genes with a logFC > 2 were non-essential, as there were more insertions in the output library compared to the control, whereas genes with a logFC < -2 were essential for survival under the associated stress condition, as there were fewer insertions in the output library compared to the control. The genes with a logFC < -2 were of interest in the current study and therefore, the number of essential genes in each stress condition was denoted at the top of the graph (**Figure 40**). A total of 128 genes were essential for survival in 14mM citric acid, which was the highest number of essential genes recorded out of all the stresses included in the study (**Figure 40**). The stress condition with the fewest number of essential genes identified from TraDIS data was heat inactivation, with only 3 genes predicted to be essential (**Figure 40**) The full list of novel essential genes in each stress condition and their associated logFC values are detailed in **Supplementary Table 3** to **Supplementary Table 8**.

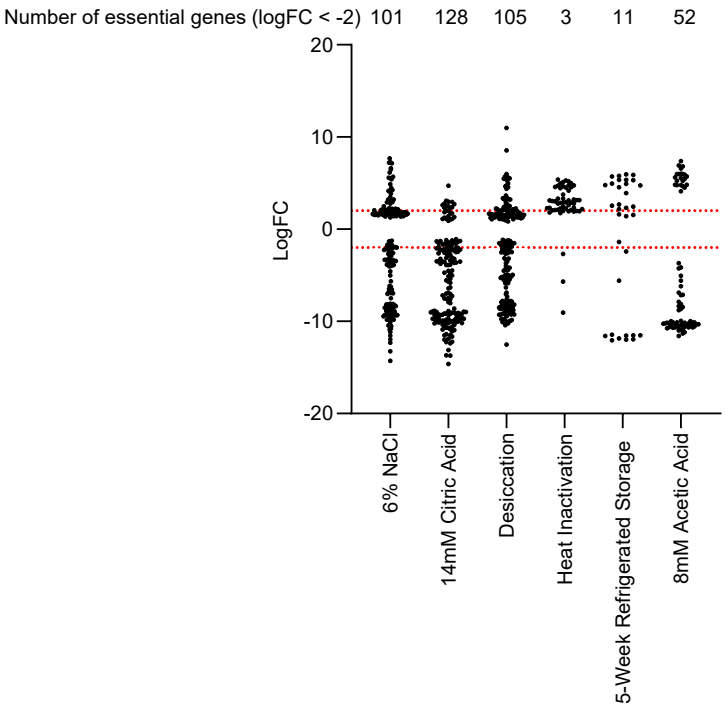


Figure 40. Scatter plot showing the mutant fitness for six stress conditions. The difference in insertions for each gene (black dots) in the test condition compared to the control are plotted for those with logFC < 2 or > 2 (red broken lines) and q-value < 0.05. The number of essential genes is labelled above each dataset. The red lines represent the logFC cut-off of < -2 and > 2.

4.3.8 The majority of essential genes are involved in carbohydrate metabolism

To investigate the main biological functions involved during food chain related stress response in *Salmonella*, the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database was used to assign biological pathways to genes involved in each stress condition. Essential genes with a logFC < -2 were used as the input and were assigned a KEGG orthology (KO) number using the KEGG database. The majority of genes involved in food chain related stress survival were involved in carbohydrate metabolism (**Figure 41**). There were 101 genes identified as essential during 6% NaCl stress and the KEGG analysis assigned a KO number to 97 genes. Most of the genes involved in 6% NaCl stress survival were assigned to metabolism pathways and replication and repair (**Figure 41**).

For 14mM citric acid stress, there were 128 genes predicted to be essential for survival in *S*. Typhimurium strain ST4/74, and 121 genes with an associated KEGG pathway (**Figure 41**). Most genes were assigned to carbohydrate and energy metabolism, and the antimicrobial resistance pathway also had a large number of genes associated (**Figure 41**). For desiccation survival, there were 105 essential genes predicted and out of these 105 genes, 16 genes could not be assigned a KO number (**Figure 41**). Genes involved in desiccation survival were mainly associated with metabolism (carbohydrate, energy, amino acid and glycan biosynthesis), however there were 7 genes categorised into the cellular community (including biofilm formation) and membrane transport pathways.

There were only three genes predicted to be essential for survival during heat inactivation at 60°C for 30 seconds. The three genes were assigned to the following KEGG pathways: replication and repair, membrane transport, signal transduction and antimicrobial resistance (**Figure 41**). There were 11 genes likely to be required for survival during 5-week refrigerated storage and KO numbers were assigned to all 11 genes. The genes were categorised into carbohydrate, energy and amino acid metabolism, and signal transduction (**Figure 41**). For 8mM acetic acid stress, 52 genes were predicted to be essential for survival and the KEGG pathway with the highest number of classified genes was the cellular community pathway, which included biofilm formation and quorum sensing. Overall, genes involved in carbohydrate metabolism seem to be involved in food chain related stress (**Figure 41**).

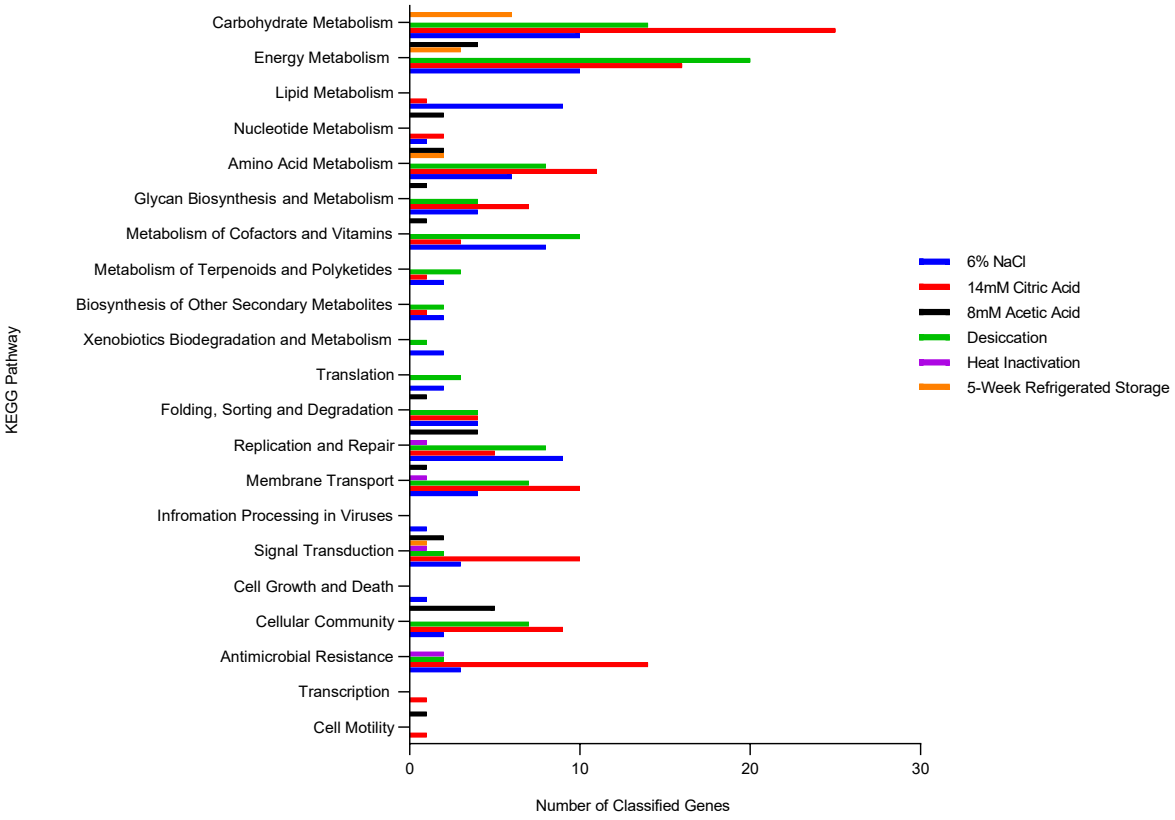


Figure 41. **KEGG pathways involved in each food chain related stress condition in *S*. Typhimurium strain ST4/74**. Only the top pathways are shown for each condition.

4.3.9 *proP* and *dam* are essential for survival in 6% NaCl

The 6% NaCl transposon library output identified 206 genes with a significant q-value compared to the control (q-value < 0.05). Out of these 206 genes, 101 had a logFC < -2, and were likely to be essential for survival in NaCl stress. The genes most likely to be essential for survival in 6% NaCl stress included *proP*, *dam*, *idcA* and *xerC* (**Table 21**). Alignment of the amino acid sequence of STM474_4025, which was predicted to also be essential for survival in 6% NaCl, using blastp revealed a 100% identity with retron St85 family RNA-directed DNA polymerase present in *Salmonella enterica*. The top 10 genes with a positive logFC and thus, beneficial if mutated, belong to the *rfa* and *rfb* operons involved in LPS biosynthesis (**Table 21**).

Gene	Function	logFC	q-value
proP	proline/glycine betaine transporter	-14.29	1.82E-29
dam	DNA adenine methylase	-13.26	1.44E-17
STM474_4025	putative reverse transcriptase	-12.31	1.93E-07
ldcA	L,D-carboxypeptidase A	-11.94	1.48E-06
xerC	site-specific tyrosine recombinase XerC	-11.54	1.98E-05
yhgI	putative DNA uptake protein	-11.14	8.51E-05
lptc	Lipopolysaccharide export system protein lptC	-10.87	0.00019
nuoA	NADH dehydrogenase subunit A	-10.79	0.00025
recA	recombinase A	-10.51	0.00064
ruvA	Holliday junction DNA helicase RuvA	-10.46	0.00086
rfaK	putative hexose transferase	7.68	2.28E-32
rfbD	dTDP-4-dehydrorhamnose reductase	7.34	1.12E-27
rfaJ	lipopolysaccharide glucosyltransferase	7.28	8.75E-37
rfbC	dTDP-4,deoxyrhamnose 3,5 epimerase	7.22	1.85E-21
rfaH	transcriptional activator RfaH	7.15	2.66E-28
rfaL	O-antigen ligase	6.60	8.50E-33
rfaI	lipopolysaccharide-alpha-1, 3-D-galactosyltransferase	6.53	4.35E-37
rfbP	undecaprenol-phosphate galactosephosphotransferase/O-antigen transferase	6.49	1.63E-22
rfbN	rhamnosyl transferase	6.14	1.19E-20
rfbM	mannose-1-phosphate guanylyltransferase	5.67	1.39E-17

Table 21. Top 20 genes involved in 6% NaCl stress for *S*. Typhimurium strain ST4/74. The associated logFC and q-value (adjusted p-value) is denoted for each gene. Genes with a positive logFC have more insertions in the gene during stress compared to the control, indicating that a loss of the gene would be beneficial for survival in 6% NaCl, whereas genes with a negative logFC have fewer insertions in the stress compared to the control, and therefore loss of the gene would be detrimental to survival in 6% NaCl.

4.3.10 *envZ* and the two-component system *phoPQ* are essential for survival in 14mM citric acid

The genes predicted to be essential for survival in citric acid stress with the greatest negative logFC included genes encoding the osmolarity sensor EnvZ and its cognate DNA-binding transcriptional regulator OmpR, and the bifunctional histidine sensor-kinase PhoQ and its cognate transcriptional regulator PhoP (**Table 22**). The unannotated gene STM474_4025 was essential in both citric acid and NaCl survival (**Table 21** and **Table 22**). Another gene essential for survival in citric acid was *htrA* (also known as *degP*) which is a periplasmic protease involved in degrading damaged proteins (**Table 22**). Genes with a logFC > 2, and therefore mutations in these genes appear to be beneficial for survival in 14mM citric acid included, *hpaA*, *yfdZ* and *glpG* (**Table 22**).

Gene	Function	logFC	q-value
envZ	osmolarity sensor protein	-14.60	1.36E-46
phoQ	sensor protein PhoQ	-13.73	3.89E-24
ompR	osmolarity response regulator	-13.70	1.12E-23
gshA	glutamate--cysteine ligase	-13.13	1.57E-12
STM474_4025	putative reverse transcriptase	-12.38	4.01E-06
phoP	DNA-binding transcriptional regulator PhoP	-12.25	1.03E-05
dsbA	thiol:disulfide interchange protein	-12.22	1.27E-05
htrA	serine endoprotease	-11.98	3.14E-51
truA	tRNA pseudouridine synthase A	-11.89	6.35E-05
tatA	twin arginine translocase protein A	-11.65	0.00024
hpaA	4-hydroxyphenylacetate catabolism	4.73	0.00063
yfdZ	aminotransferase	3.08	0.00913
glpG	intramembrane serine protease GlpG	2.97	0.00205
ugpA	glycerol-3-phosphate transporter permease	2.95	0.00057
scsD	suppression of copper sensitivity protein	2.77	0.00193
STM474_2982	putative periplasmic or exported protein	2.72	0.00929
celC	N,N'-diacetylchitobiose-specific PTS system transporter subunit IIA	2.62	0.00864
ybjN	putative cytoplasmic protein	2.47	0.03753
STM474_0030	hypothetical protein	2.47	0.03221
STM474_2629	putative polyferredoxin	2.38	0.0041

Table 22. **Top 20 genes involved in 14mM citric acid stress for *S.* Typhimurium strain ST4/74.** The associated logFC and q-value (adjusted p-value) is denoted for each gene. Genes with a positive logFC have more insertions in the gene during stress compared to the control, indicating that a loss of the gene would be beneficial for survival in 14mM citric acid, whereas genes with a negative logFC have fewer insertions in the stress compared to the control, and therefore loss of the gene would be detrimental to survival in 14mM citric acid.

4.3.11 The *nuo* genes are essential for survival during 24-hour desiccation

The genes most important for 24-hour survival in desiccated conditions based on logFC are *nuoLJE*, *atpBD*, *dnaC*, *efp* and *yfgE* (**Table 23**). The *nuo* genes form part of the inner membrane components of NADH dehydrogenase I. Inner membrane proteins, *atpB* and *atpD*, are also important for desiccation survival. A putative cytoplasmic protein, STM474_3824, had 100% similarity with the protein domain DUF1778 according to a blastp search, and was predicted to be essential for survival during desiccation in *S.* Typhimurium strain ST4/74 (**Table 23**). Other genes with a large negative logFC during desiccation stress include, *dnaC*, which is located in the cytosol and functions as an accessory protein to aid DNA replication, elongation factor *efp* and the inhibitor of DNA replication *yfgE* (also known as *hda*) (**Table 23**). Similar to some of the other stresses included in this study, *rfa* and *rfb*

genes had significantly more insertions in the desiccation output library compared to the control, and therefore mutations in these genes were predicted to have a beneficial fitness advantage to the cell (**Table 23**).

Gene	Function	logFC	q-value
nuoL	NADH dehydrogenase subunit L	-12.50	3.80E-11
nuoJ	NADH dehydrogenase subunit J	-10.42	6.74E-05
nuoE	NADH dehydrogenase subunit E	-10.21	0.0001773
STM474_3824	putative cytoplasmic protein	-10.15	0.0002497
atpB	F0F1 ATP synthase subunit A	-10.08	0.0002251
atpD	F0F1 ATP synthase subunit beta	-10.02	0.0003812
STM474_1383	hypothetical protein	-10.00	0.0003812
dnaC	DNA replication protein DnaC	-9.98	0.0002878
efp	Elongation factor P	-9.96	0.0007184
yfgE	DNA replication initiation factor	-9.85	0.0005119
STM474_t2520		10.98	0.0107987
STM474_t3557		8.53	0.0177648
rfaK	putative hexose transferase	5.99	5.45E-66
rfaJ	lipopolysaccharide glucosyltransferase	5.72	1.17E-67
rfbD	dTDP-4-dehydrorhamnose reductase	5.62	2.54E-48
rfaL	O-antigen ligase	5.60	3.49E-65
rfbN	rhamnosyl transferase	5.53	1.49E-47
rfbP	undecaprenol-phosphate galactosephosphotransferase/O-antigen transferase	5.48	1.37E-57
STM474_2816	hypothetical protein	5.28	2.06E-11
rfaH	transcriptional activator RfaH	5.25	1.18E-24

Table 23. Top 20 genes involved in desiccation stress for *S*. Typhimurium strain ST4/74. The associated logFC and q-value (adjusted p-value) is denoted for each gene. Genes with a positive logFC have more insertions in the gene during stress compared to the control, indicating that a loss of the gene would be beneficial for survival during desiccation, whereas genes with a negative logFC have fewer insertions in the stress compared to the control, and therefore loss of the gene would be detrimental to survival during desiccation.

4.3.12 Only three essential genes were identified during heat inactivation at 60°C

There were only three genes predicted to be essential for survival during heat inactivation at 60°C in *S*. Typhimurium strain ST4/74, and these genes were *rnhA*, *clpB* and *tolC* (**Table 24**). Almost all the genes with insertions resulting in a significant q-value (< 0.05) compared to the control had a positive logFC. The genes with the greatest positive logFC included the *rfa* and *rfb* genes, *dnaJ*, *galE*, *ihfA*, *pgm*, *ygcB* and *aroK*. Mutations in the genes with a positive logFC were predicted to provide a beneficial outcome to the cell (**Table 24**).

Gene	Function	logFC	q-value
rnhA	ribonuclease H	-9.04	0.032252
clpB	protein disaggregation chaperone	-5.69	0.007828
tolC	outer membrane channel	-2.67	0.049235
dnaJ	chaperone protein DnaJ	5.38	1.36E-06
rfaK	putative hexose transferase	5.28	1.27E-20
rfbP	undecaprenol-phosphate galactosephosphotransferase/O-antigen transferase	5.18	6.86E-20
rfaJ	lipopolysaccharide glucosyltransferase	5.12	1.40E-20
rfbN	rhamnosyl transferase	4.98	6.90E-20
rfbD	dTDP-4-dehydrorhamnose reductase	4.96	2.58E-20
rfaI	lipopolysaccharide-alpha-1, 3-D-galactosyltransferase	4.76	6.86E-20
galE	UDP-galactose-4-epimerase	4.75	1.10E-14
rfaH	transcriptional activator RfaH	4.73	1.10E-14
STM474_p216	parA	4.66	1.87E-05
rfaG	glucosyltransferase I	4.58	1.70E-14
rfaL	O-antigen ligase	4.56	4.22E-18
ihfA	integration host factor subunit alpha	4.52	0.003336
rfbC	dTDP-4,deoxyrhamnose 3,5 epimerase	4.44	3.49E-17
pgm	phosphoglucomutase	4.20	5.09E-12
yqcB	tRNA pseudouridine synthase C	3.76	0.027358
aroK	shikimate kinase I	3.33	0.014495

Table 24. **Top 20 genes involved in heat inactivation stress for *S*. Typhimurium strain ST4/74**. The associated logFC and q-value (adjusted p-value) is denoted for each gene. Genes with a positive logFC have more insertions in the gene during stress compared to the control, indicating that a loss of the gene would be beneficial for survival during heat inactivation, whereas genes with a negative logFC have fewer insertions in the stress compared to the control, and therefore a loss of the gene would be detrimental to survival during heat inactivation.

4.3.13 *gpmA* and *trmE* are essential cold-stress response genes

The top three genes predicted to be essential for survival during 5-week refrigerated storage were *gpmA*, *trmE* and *gidA* (**Table 25**). Other genes important for survival during 5-week refrigerated storage included, *frdD* (subunit of the fumarate reductase complex), *yhbH* (also known at *hpf*, hibernating promoting factor) and *xerC* (member of the xer site-specific recombination system). The *rfa* and *rfb* genes had the greatest positive logFC compared to the control, and therefore mutations in these genes were likely to be beneficial to the cell during 5-week refrigerated storage (**Table 25**).

Gene	Function	logFC	q-value
gpmA	phosphoglyceromutase	-12.04	0.017032
trmE	tRNA modification GTPase TrmE	-11.97	0.018575
gidA	glucose-inhibited division protein A	-11.95	0.018893
frdD	fumarate reductase subunit D	-11.85	0.023263
yhbH	putative sigma (54) modulation protein	-11.59	0.040215
xerC	site-specific tyrosine recombinase XerC	-11.55	0.043265
proC	pyrroline-5-carboxylate reductase	-11.51	0.043265
eda	keto-hydroxyglutarate-aldolase/keto-deoxy- phosphogluconate aldolase	-11.51	0.043265
yciL	23S rRNA pseudouridylate synthase B	-11.45	0.046834
proP	proline/glycine betaine transporter	-5.58	0.000204
rfaK	putative hexose transferase	5.94	3.10E-64
rfaI	lipopolysaccharide-alpha-1, 3-D-galactosyltransferase	5.89	2.56E-68
rfaL	O-antigen ligase	5.78	1.94E-58
rfaJ	lipopolysaccharide glucosyltransferase	5.70	3.06E-62
rfbP	undecaprenol-phosphate galactosephosphotransferase/O-antigen transferase	5.36	6.68E-54
rfbD	dTDP-4-dehydrorhamnose reductase	5.34	3.76E-55
rfbN	rhamnosyl transferase	5.30	1.39E-50
rfbC	dTDP-4,deoxyrhamnose 3,5 epimerase	4.96	9.24E-47
rfaH	transcriptional activator RfaH	4.90	3.33E-34
galE	UDP-galactose-4-epimerase	4.79	6.55E-41

Table 25. **Top 20 genes involved in 5-week cold-storage stress for *S*. Typhimurium strain ST4/74**. The associated logFC and q-value (adjusted p-value) is denoted for each gene. Genes with a positive logFC have more insertions in the gene during stress compared to the control, indicating that a loss of the gene would be beneficial for survival during 5-week refrigerated storage, whereas genes with a negative logFC have fewer insertions in the stress compared to the control, and therefore a loss of the gene would be detrimental to survival during 5-week refrigerated storage.

4.3.14 *yacC* and *proC* are essential for 8mM Acetic Acid stress survival

Genes essential for survival in 8mM acetic acid in *S*. Typhimurium strain ST4/74 included an uncharacterised protein encoded by *yacC*, amongst other genes such as, *proC*, *ptsO* and *cysE* involved in L-proline biosynthesis, nitrogen metabolism, and cysteine biosynthesis, respectively (**Table 26**). The amino acid sequence of STM474_2746 was identical to *recT*, the Rac prophage in *E*. *coli*, and appears to be essential for survival in acetic acid. *rfbU* is predicted to be an essential gene for survival, whereas other *rfb* genes like *rfbP* and *rfbN* appear to be non-essential in acetic acid, and mutations in these genes may provide the cell with a fitness advantage (**Table 26**).

Gene	Function	logFC	q-value
yacC	Uncharacterised protein yacC	-11.59	0.028773
proC	pyrroline-5-carboxylate reductase	-11.30	0.027616
ptsO	phosphohistidinoprotein-hexose phosphotransferase component of N-regulated PTS system (Npr)	-11.18	0.028773
cysE	serine acetyltransferase	-10.99	0.028773
STM474_2746	gifsy-1 prophage protein	-10.90	0.028773
xerD	site-specific tyrosine recombinase XerD	-10.81	0.030644
nuoH	NADH dehydrogenase subunit H	-10.75	0.030644
ivbL	IlvB leader peptide	-10.69	0.030644
rfbU	mannosyl transferase	-10.65	0.030644
flgM	anti-sigma28 factor FlgM	-10.65	0.030644
rfab	Lipopolysaccharide 1,6-galactosyltransferase	7.39	0.00334
rfaK	putative hexose transferase	6.92	0.005795
rfaJ	lipopolysaccharide glucosyltransferase	6.81	0.005795
rfaH	transcriptional activator RfaH	6.59	0.014443
STM474_0168	putative restriction endonuclease	6.01	0.0255
rfbP	undecaprenol-phosphate galactosephosphotransferase/O-antigen transferase	6.00	0.023239
STM474_p1116	putative phospholipase D	5.98	0.030644
rfbN	rhamnosyl transferase	5.98	0.023239
STM474_1315	hypothetical protein	5.91	0.041426
yfiK	neutral amino-acid efflux protein	5.72	0.030644

Table 26. Top 20 genes involved in 8mM acetic acid stress for *S*. Typhimurium strain ST4/74. The associated logFC and q-value (adjusted p-value) is denoted for each gene. Genes with a positive logFC have more insertions in the gene during stress compared to the control, indicating that a loss of the gene would be beneficial for survival in 8mM acetic acid, whereas genes with a negative logFC have fewer insertions in the stress compared to the control, and therefore loss of the gene would be detrimental to survival in 8mM acetic acid.

4.3.15 No genes are conditionally essential in all six food chain related stress conditions

The essential genes in each stress condition with a logFC < -2 and a q-value of < 0.05 from the tradis_comparison.R output were compared to one another to identify novel genes required for survival in each stress condition, and also those genes required in multiple stresses (**Figure 42**). There were 83 novel genes identified as involved in 14mM citric acid stress survival, including *dnaK* (logFC -10), *imp* (logFC -9.04308), *fruR* (logFC -2.43757) and *aceE* (logFC -9.68789). Comparing the genes required for survival during 24-hour desiccation and the other stress conditions revealed 56 novel genes, some of which were *murD* (logFC -8.15224), *pcnB* (logFC -2.39014), *thiL* (logFC -6.31602) and *xseB* (logFC -8.13341) (**Figure 42**) There were a similar number of unique genes required for 6% NaCl survival as there was for

desiccation survival, at 55 and 56, respectively. Novel genes required for survival in 6% NaCl included, *mraW* (logFC -3.89070), *ftsI* (logFC -8.13559), *yacF* (logFC -3.12259) and *coaE* (logFC -9.58502). There were 36 genes required for survival in 8mM acetic acid that did not appear in any of the essential gene lists for the other stresses featured in this study (**Figure 42**).

Genes required for acetic acid survival that were not required in any other stresses included *apaH* (logFC -10.55010), *yacC* (logFC -11.59164), *rnhA* (logFC -8.46158), and *hha* (logFC -9.96289). Six novel genes were identified as essential during 5-week refrigerated storage and these were *gpmA* (logFC -12.03706), *yciL* (logFC -11.44869), *eda* (logFC -11.50623), *yhbH* (logFC -11.58775), *trmE* (logFC -11.97306) and *frdD* (logFC -11.85439). Only the *clpB* gene was uniquely essential for survival during heat inactivation at 60°C (logFC -5.69277). There were 17 genes identified as essential during both culture in 6% NaCl and 24-hour desiccation, including *mukB* (logFC -8.43958), *prc* (logFC -6.46913) and *ruvB* (logFC -8.54455). Three genes were predicted to be essential in both acid stresses, *hscA* (logFC -8.02172), *rpoS* (logFC -10.6804), and *gppA* (logFC -3.47922). The one gene that was involved in the most stresses, including acetic acid, NaCl, desiccation and citric acid stress, was STM474_4025 (**Figure 42**). A table detailing the genes shared between multiple conditions is detailed in **Supplementary Table 9**.

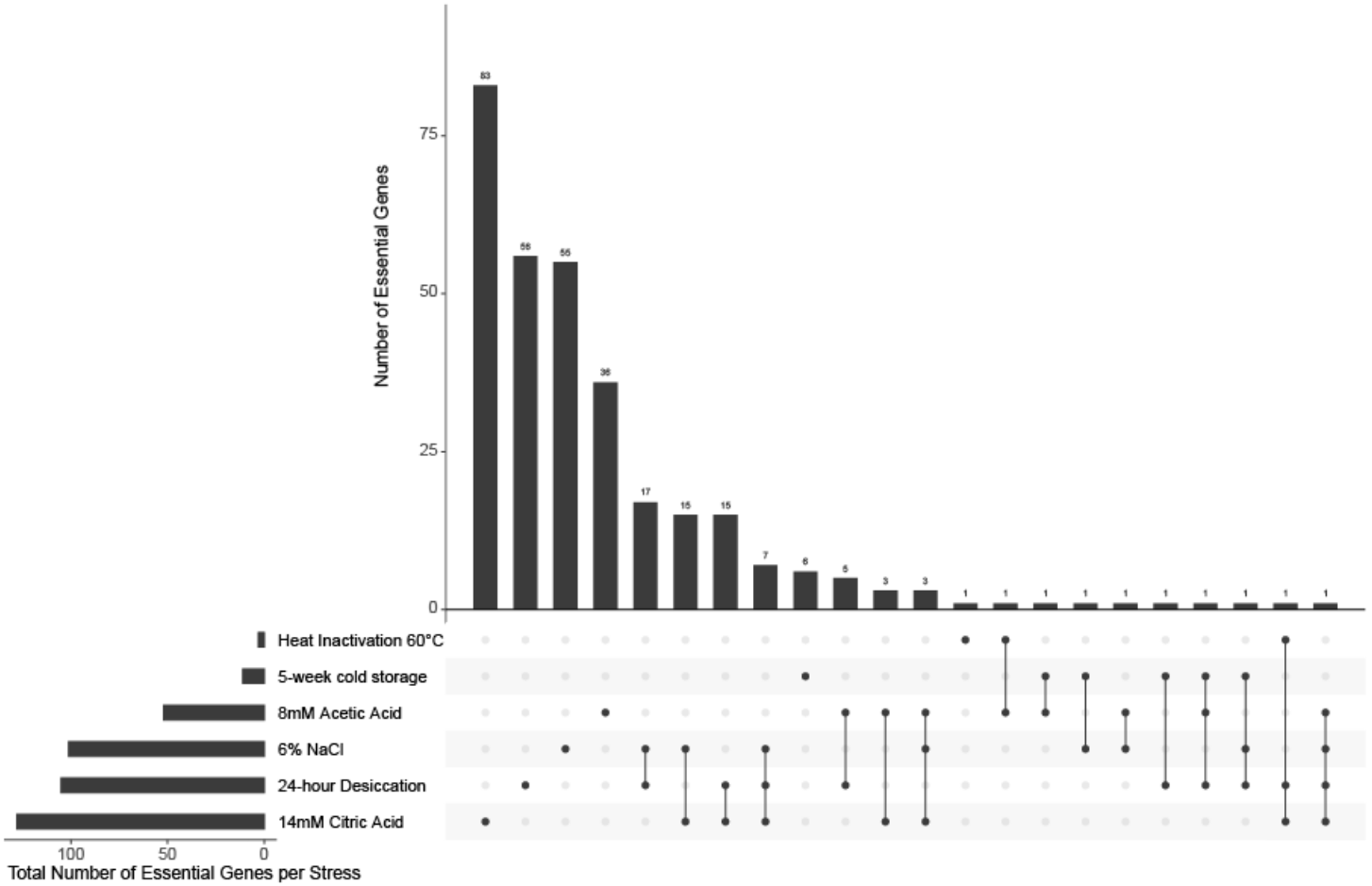


Figure 42. Distribution and abundance of essential genes shared between stress conditions in *S*. Typhimurium strain ST4/74. UpSetR plot showing the number of genes in *S*. Typhimurium strain ST4/74 with a logFC < -2 and a q-value < 0.05, that are shared between stress conditions and the number of genes unique to each stress condition.

4.6 Discussion

Adaptation to various food chain related stresses is important for *Salmonella* survival, however the molecular mechanisms behind food chain related stress tolerance remains largely unknown. In the present study, TraDIS was used to determine the genes essential for survival during heat inactivation at 60°C, 24-hour desiccation, 5-week refrigerated storage, 8mM acetic acid, 14mM citric acid and 6% NaCl stress in *S.* Typhimurium strain ST4/74. During osmotic stress, there were 101 genes with a logFC < -2 and a q-value < 0.05. These genes were therefore predicted to be essential for survival in 6% NaCl. The genes with the smallest logFC and most likely required for NaCl stress survival were *proP*, *dam*, *idcA*, *xerC* and an unannotated reverse transcriptase, STM474_4025. A previous study identified 26 genes required for fitness in 3% NaCl stress (Mandal, 2016). A protein-protein network analysis of the essential genes in 3% NaCl categorised them into three clusters: SPI-3 (*mgtBC*, *misL*, *cigR*, *slsA*, *fidL* and *marT*), two-component system (*dcuBRS*) and sodium ion transport (*yihPO*) (Mandal, 2016). The genes denoted as essential during 3% NaCl stress are all present in the output library during 6% NaCl in the current study, however the logFC values are not significantly different than the control, and therefore not involved in stress survival during 6% NaCl. For example, *mgtB* had a logFC of 0.28 and a q-value of 0.90 and *fidL* had a logFC of -0.24 and a q-value of 0.93. The differences in the number of genes required for NaCl survival and the specific genes identified may be due to the percentage of NaCl used (6% vs 3%), the *S*. Typhimurium strain used (ST4/74 vs 14028s) and the method used to assign essentiality from Tn-Seq data. In another study, the magnesium transporters, *mgtC* and *mgtB,* demonstrated a five-fold increase in mRNA levels during 0.3M NaCl exposure (Lee and Groisman, 2012).

Interruption of *proP* had the greatest negative effect on culture in 6% NaCl stress, with a logFC of -14.29. ProP is an osmoprotectant transporter which protects the cell from dehydration during hyperosmotic stress and has been shown to be essential for survival during desiccation (Finn *et al.*, 2013b). Another gene predicted to be essential in 6% NaCl stress was *dam* (logFC -13.26). DNA methylation, by the *dam* gene, results in the addition of a methyl group ($-CH_3$) to nucleotides in deoxyribonucleic acid, most commonly adenine in prokaryotes (Chatti and Landoulsi, 2008). Inactivation of the *dam* gene has been shown to negatively impact virulence in *S*. *enterica* by affecting the stability of the membrane envelope and increasing sensitivity to bile, however it is currently not clear how *dam* might affect control of osmotic stress (García-Del Portillo *et al*., 1999; Prieto *et al*., 2004).

A logFC of -11.94 was reported for the *xerC* gene during 6% NaCl stress and this gene is involved in xer site-specific recombination (Blakely *et al.,* 1993). Most recently, a Tn-Seq library in *S*. Typhimurium strain ATCC14028 was constructed using the *Himar1* Mariner transposon system and resulted in ~70,000 unique insertions at TA-dinucleotide sites. The transposon mutant library was used to identify genes essential for survival in 17 stress conditions, including three stress conditions relevant to food processing (Wang *et al.*, 2022). In the study, cells were subjected to LB broth containing 4% NaCl (w/v) and grown for 4 hours at 37°C. Post-stress analysis revealed 122 genes essential for survival in 4% NaCl, which is a similar number to the 101 genes essential for 6% NaCl stress survival in the current study. There were just 11 genes predicted to be essential in both the current study during 6% NaCl stress in *S*. Typhimurium strain ST4/74 and in the previous study according to gene annotation. The 11 shared genes and their associated logFC in the present study included *rfbX* (-9.11), *infB* (-6.94), *recB* (-5.66), *dam* (-13.26), *hemC* (-8.62), *xseA* (-2.60), *fur* (-9.13), *corA* (-3.31), *recA* (-10.51), *ispG* (-8.12) and *ftsK* (-7.48). The large number of unique essential genes in each dataset may be due to the different concentrations of NaCl used, but also due to the growth phase of the cells as cells grown to stationary phase were used in the current study. The genes identified as essential in NaCl stress may also be strain specific.

Overall, there were 105 genes predicted to be essential during 24-hour desiccation for *S*. Typhimurium strain ST4/74 in the current study, with a logFC < -2 and a q-value < 0.05. The top three genes required for desiccation survival were *nuoL*, *nuoJ* and *nuoE*, encoding components of the NADH dehydrogenase I system located in the inner membrane. NADH dehydrogenase is a key component of the respiratory chain and can be oxidised (Archer and Elliott, 1995). A similar study, which utilised Tn-seq to identify genes required for desiccation survival in *S*. Typhimurium, found that 37 conditionally essential genes and 24 domain essential genes were required for survival. Amongst them were the *atpCDGAHFEBI* genes, which form part of the F0F1 ATP synthase complex (Mandal and Kwon, 2017). In the present study, *atpB*, *atpD*, *atpG* and *atpC* were also found to be essential for desiccation survival with a significant (q < 0.05) logFC of -10.08, -10.02, -9.24 and -8.88, respectively. As previously mentioned, *xerC,* was predicted to be essential during 6% NaCl stress (logFC = -11.94, q = 0.0000198) and also during desiccation stress (logFC -5.06, q = 0.003) in the current study. This gene has previously been identified as an essential gene in *S*. Typhimurium strain 14028s for desiccation survival (logFC -7.81) (Mandal and Kwon, 2017). XerC and XerD are site specific recombinases which function to ensure the separation of bacterial chromosomes during cell division and replication (Farrokhi *et al.*, 2019). Frequent homologous

recombination events lead to the formation of chromosome dimers in ~15% of cells, causing DNA damage which results in twin filaments (Steiner and Kuempel, 1998). Chromosome dimers can be resolved so that daughter cells retain a complete copy of the genome by XerCD at the *dif*-site (deletion-induced filamentation) located at the terminal end of the chromosome in prokaryotes (Lesterlin *et al.*, 2004). The mechanisms by which *xerC* aid survival during desiccation and NaCl stress remain unknown, however it is clear that *xerC* is important in preventing DNA damage.

The DNA-methyltransferase, *dam*, was found to be essential during desiccation stress in both the current study and in the Tn-seq study in *S*. Typhimurium strain 14028s, with a logFC of -5.27 and -4.20 in each study, respectively (Mandal and Kwon, 2017). Other genes essential for desiccation survival in both strains and the associated logFC in the present study include *lepA* (-3.99), *glmS* (-9.11), *hfq* (-4.82) and *ssaI* (-8.49). *lepA* (also known as EF4) functions during translation as an elongation factor and has been shown to accelerate protein synthesis during certain stresses, but not during optimum growth conditions (Pech *et al.*, 2011). In this study, protein synthesis was increased by 5-fold in the presence of high concentrations of Mg^{2+} (14mM) and by 2-fold at a lower temperature of 20°C and moderate salt conditions (Pech *et al.*, 2011). The initial growth rate of *E. coli ΔlepA* cells was rapidly reduced, but then stabilised after 10-20 generations to wild type levels, in the presence of increased osmotic pressure (Pech *et al.*, 2011). This *ΔlepA* phenotype corresponds with established bacterial osmoregulatory mechanisms, whereby an initial increase in magnesium and potassium ions by 3-7x more than normal levels, results in the synthesis of second phase osmolytes, such as disaccharide trehalose, which in turn reduces the concentration of intracellular solutes to pre-hyperosmotic levels (Csonka and Hanson, 1991). During lower than optimum temperatures and moderate salt concentrations, *lepA* accelerates the synthesis of polyphenylalanine and the increased stability of mRNA secondary structures results in ribosomal stalling, further requiring *lepA* (or EF4) (Pech *et al.*, 2011). Additionally, *lepA* was considered to be essential for the survival and revival of *S*. Typhi strain BRD948 for 6 hours in water (Kingsley *et al.*, 2018). In *E.coli*, *glmS* functions as a L-glutamine D-fructose-6-phosphate amidotransferase enzyme and is involved in amino-sugar containing macromolecule biosynthesis, thus having a beneficial effect in cell envelope stress response (Zhou *et al.*, 2009). Similarly, *glmS* was required for survival during oxidative stress in *S.* Typhimurium strain 14028s (Mandal *et al.*, 2021).

RpoS is a central stress response regulon and enables cells to survive adverse conditions. Specifically, the *rpoS* gene encodes an alternative sigma factor (RpoS) and facilitates the

expression of 30 or more genes during stationary-phase growth, in nutrient limiting conditions such as oxidative stress, acid stress and DNA damage (Loewen and Hengge-Aronis, 1994). RpoS has also been shown to regulate virulence in *Salmonella*, by controlling the expression of the plasmid encoded *spvR* and *spvABCD* genes (Kowarz *et al.,* 1994). However, during the current study the transposon libraries after stress exposure and the control were grown to stationary phase prior to sequencing, therefore stationary phase genes were not expected to be identified as conditionally essential as their presence in the control dataset should have resulted in an insignificant logFC in the stress dataset output. RpoS is regulated by the transcription factor LrhA, which is dependent on the small RNA chaperone, Hfq (Peterson *et al.*, 2006). The *hfq* gene is essential during desiccation survival, as shown in the current study and earlier studies (Mandal and Kwon, 2017). *Salmonella* mutants in the *hfq* gene exhibited reduced survival during storage in pistachios, a low-moisture food product, indicating a role for RpoS, LrhA and Hfq during low-moisture/desiccation stress *(Jayeola et al.*, 2020).

Interestingly, the classic osmolarity response regulators for NaCl and desiccation, EnvZ and OmpR, did not appear to be essential for survival of *S*. Typhimurium strain ST4/74 during these conditions according to their logFC values. The logFC values obtained in the current study were 1.69 (q = 0.00169) and 1.21 (q = 0.12018) during 6% NaCl stress, and 1.96 (q = 9.78×10^{-10}) and 2.21 (q = 2.91×10^{-8}) during desiccation, respectively. One hypothesis for these genes not appearing essential during NaCl and desiccation stress in the current study could be due to the presence of salt in the control media. To verify this, the control library would need to be grown in LB broth without the addition of salt.

Another gene required for desiccation survival in both *S*. Typhimurium strain ST4/74 and strain 14028s was *ssaI*, a subunit of the type 3 secretion system apparatus (T3SS) encoded by *Salmonella* pathogenicity island (SPI) type 2 (Takaya *et al.,* 2019). The *ssa* genes have been identified as virulence genes in many strains of *Salmonella* and *ssaI* was one of the most highly expressed small open reading frames (sORFs) during early-stage host infection (Seribelli *et al*., 2020; Venturini *et al*., 2020). Furthermore, *ssaI* is thought to be specifically involved in interaction with the host after invasion and within the *Salmonella* containing vacuole, as evidenced by the ability of T3SS-2 to suppress inflammasome activation in human macrophages, resulting in *S.* Typhimurium survival (Schultz *et al.*, 2021). Genes that were not required for desiccation survival in *S*. Typhimurium strain ST4/74, but were essential in strain 14028s include; *wecE*, *pagO*, *guaA*, *tpiA*, *pnp*, *miaA*, *rpoN*, *xerD*, *yaeL*, *pal*, *rfbU*, *wzxE*, *dnaKJ*, *glnD*, *dsbC*, *nhaA*, *fepCGD*, *phoU*, *pstB*, *corA*, *ygbF*, *gogB*, *yiiQ*, *ssaH*, *ssaJ* and *envF* (Mandal

and Kwon, 2017). Identification of other mutations in T3SS apparatus (*ssaH* and *ssaJ*) suggest these genes may have an alternate function, in flagella apparatus perhaps, although more research is needed to confirm this. Desiccation for 4 hours in a biosafety cabinet was one of the stresses included in the 17-stress condition study in *S*. Typhimurium strain 14028s by Wang *et al.* (2022). There were 287 essential genes required for desiccation survival compared to 105 genes in the present study, and 40 shared genes between the two studies. The shared genes and their associated logFC in the current study were *fusA* (-8.35), *visC* (-2.37), *sucC* (-3.24), *nuoG* (-5.51), *atpC* (-8.88), *yigC* (-9.24), *sdhD* (-5.31), *orn* (-9.29), *tolC* (-2.66), *atpB* (-10.08), *gidA* (-3.53), *rfaF* (-9.20), *atpE* (-8.19), *atpD* (-10.02), *fre* (-4.53), *ftsE* (-6.90), *efp* (-9.96), *nuoJ* (-10.42), *xerC* (-5.06), *ptsI* (-3.23), *nuoL* (-12.50), *yfgE* (-9.85), *pdxH* (-8.67), *nifU* (-9.33), *dam* (-5.27), *thiL* (-6.32), *mtlD* (-2.06), *fur* (-9.23), *gshB* (-2.53), *nuoH* (-5.02), *surA* (-5.03), *tolA* (-5.84), *fis* (-7.86), *crp* (-7.33), *ruvB* (-8.65), *pfs* (-9.28), *lepA* (-3.99), *atpG* (-9.24), *rpoE* (-9.76) and *nuoE* (-10.21). Many of the shared genes in both studies were the genes with the smallest logFC in the current study, suggesting that they are the genes most likely to be responsible for survival during desiccation stress. There were 65 unique genes required for desiccation survival in the current study compared to the study by Wang *et al*. (2022), which may be due to the *S*. Typhimurium strain used or due to the growth phase of the cells (4 hours vs 24 hours). Due to the differences in number of unique genes shared in the three studies exploring desiccation survival using Tn-Seq based methods, response to desiccation stress seems to be strain specific, however there is some overlap in genes suggesting there may be a core set of desiccation survival genes too.

Transposon directed insertion site sequencing of *S.* Typhimurium strain ST4/74 after exposure to organic acids, revealed that there were 128 and 52 genes essential for survival in 14mM citric acid and 8mM acetic acid, respectively. There were three genes predicted to be essential for survival in both stresses: *hscA*, *rpoS*, and *gppA*. The top genes identified as essential for survival in citric acid stress during the current study include *envZ*, *ompR*, *gshA*, and the two-component system, *phoPQ*. A number of regulons are well established as being involved in acid tolerance in *Salmonella enterica*, including RpoS, Fur, PhoPQ and OmpR/EnvZ. *phoPQ* is a two-component signalling system present in a number of bacteria, including *Salmonella, E.coli* and *Shigella* (Miller *et al.*, 1989). PhoQ senses reduced extracellular concentrations of magnesium and calcium and activates PhoP, via autophosphorylation of PhoQ, resulting in the transcriptional regulation of a subset of genes encoding outer membrane proteins (Gunn *et al.*, 1996). There are approximately 40 proteins which make up the PhoPQ regulon, including *prgH*, *fliC*, *pmrAB*, *mgtA*, *mgtCB*, *phoN*, *pcgL*,

and *pagP*. *S*. Typhimurium utilises PhoQ sensing and activation to regulate lipopolysaccharide biosynthesis, and disruption of the cell structure leads to increased resistance to cationic antimicrobial peptides (CAMP) (Dalebroux and Miller, 2014). *phoP* was one of the first genes to be associated with acid tolerance, and a *ΔphoP* mutant was shown to negatively affect the ability of *S*. Typhimurium to adapt to low pH (Foster and Hall, 1990). OmpR/EnvZ is a two-component system associated with osmolarity. EnvZ senses changes in osmolarity and autophosphorylates, transferring the phosphate to OmpR, which in turn binds to DNA and activates transcription of target genes (Foster *et al.,* 1994). Previous studies have shown that *ompR* is induced during low pH and is central to acid tolerance during stationary phase (Bang *et al.*, 2000).

The glutathione biosynthesis gene, *gshA*, was required for survival during citric acid stress in the current study, and although *gshA* hasn't been implicated in S*almonella* acid stress tolerance before, there have been studies showing its importance in the protection of *Lactococcus lactis* against lactic acid stress (Zhang *et al.*, 2007). Fewer genes were predicted to be essential for survival during 8mM acetic acid stress compared to citric acid stress in *S*. Typhimurium strain ST4/74, which may be due to the mild acetic acid concentration chosen for TraDIS experiments. A different subset of genes was required for survival in 8mM acetic acid stress, and the top genes predicted to be essential included *yacC*, *proC*, *ptsO*, *sysE*, *xerD* and *nuoH*. A putative lipoprotein, *yacC,* had the greatest negative logFC (-11.59) compared to the control during acetic acid stress, and this gene has been previously described as a novel macrophage fitness gene in *S.* Enteritidis strain D7796 (Fong *et al.*, 2022). A Tn-Seq study in *S.* Derby strain 14T identified 35 genes as essential for survival during acid stress, including PhoPQ, which has been recognised as an acid resistance regulon in the present study during citric acid stress, but also previously in *E. coli* and *S.* Typhimurium (Xu *et al*., 2020; Núñez-Hernández *et al*., 2013). Most recently, gene essentiality during acid stress was established in *S*. Typhimurium strain 14028s by challenging cells to 3-hour exposure in EG medium at pH 3.3, which contains 2g/L citric acid (Wang *et al.*, 2022). There were 299 genes identified as essential during acid stress in the study by Wang *et al.* (2022). A comparison of the essential genes found in the current study and the previous study revealed 16 shared genes between the two studies, and 111 unique to *S*. Typhimurium strain ST4/74. The 16 shared genes and the associated logFC from the current study were *yciS* (-9.76), *rfbI* (-2.21), *yajC* (-9.29), *pare* (-9.19), *pgm* (-11.52), *ftsK* (-7.58), *sdhC* (-9.04), *rfaG* (-3.88), *yigP* (-10.72), *yfgE* (-9.81), *infB* (-9.61), *dnaK* (-10.00), *xseA* (-2.21), *sucB* (-10.19), *pfs* (-9.25) and *imp* (-9.04).

Sequencing the transposon mutant library post exposure to heat inactivation at 60°C, identified 53 genes involved in survival during this stress condition with a q-value of less than 0.05. However, only three of these genes had a logFC of less than -2, and therefore were classed as essential genes during heat stress. The three essential genes established during heating *S*. Typhimurium strain ST4/74 at 60°C for 30 seconds were *rnhA, clpB* and *tolC*, with logFC values of -9.04, -5.69 and -2.67, respectively. Ribonuclease H (*rnhA*) is an endonuclease that cleaves RNA in RNA-DNA hybrid molecules, producing mono- and oligoribonucleotides (Keller and Crouch, 1972). In *E. col* strain MIC2067, a *rnhA* and *rnhB* double mutant showed a temperature-sensitive growth phenotype (Itaya *et al.*, 1999). An RNA-sequencing experiment in *S*. Typhimurium ATCC14028 identified 209 genes that were upregulated after exposure to a low-water activity environment (a_w= 0.11), including *rnhA*. Upregulated genes in RNA-sequencing experiments are most likely required for adaptation to a particular stress condition, and since *rnhA* had a logFC of 4.11 it was deemed necessary for adaptation to low water activity environments in the study (Maserati *et al.*, 2017). ClpB is a member of the heat-shock protein 100 family (Hsp100), which functions as a molecular chaperone to aid protein stabilisation and refolding during stress (Neckers and Tatu, 2008). The role of ClpB in bacterial stress response and virulence is well established and protects cells of a number of bacterial species against a range of environmental stressors including heat, acidic pH, osmotic stress, oxidative stress, ethanol and nutrient starvation (reviewed in Alam *et al*. (2021).

In a previous study, heat stress was conducted at 45°C for 6 hours and sequencing analysis identified 307 essential genes. The top genes with the largest negative logFC were *dnaK*, *dnaJ*, *ribF*, *apaH*, *murD and ftsA* (Wang *et al.*, 2022). Only *rnhA* was predicted to be essential during heat inactivation at 60°C for 30 seconds in the current study and during heating at 45°C for 6 hours in the study by Wang *et al*. (2022). The outer membrane protein, TolC, functions as a multidrug efflux pump and the loss of *tolC* has previously been shown to increase sensitivity to bile salts, detergents, antibiotics, and organic solvents in *Salmonella* (Horiyama *et al.*, 2010). Additionally, TolC has been recognised as essential for survival in egg white and deletion of the *tolC* gene resulted in reduced survival in the matrix during a recent study investigating survival of *S.* Enteritidis in egg white (Raspoet *et al.*, 2019).

The ability of *S*. Typhimurium strain ST4/74 to adapt to cold stress was established by incubating *Salmonella* cells at 4°C for 5 weeks, to mimic the shelf life of the vegetarian food product used throughout the study. Only 11 genes were deemed essential for survival in this stress condition in the current study, which may be due to the lack of cell death observed

during 5-week refrigerated storage and the ability of cells to persist in the cold stress environment as discussed in chapter 2. In a previous study, cold stress was investigated by incubating cells for 2-days at 4°C in an air bath (Wang *et al.*, 2022). This study identified 146 genes essential for cold stress survival, which is much more than acknowledged in the current study. Only one gene, *gidA,* was predicted to be essential in both studies and the associated logFC in the present study was -11.95. Glucose inhibited division protein A, *gidA*, is involved in *Salmonella* virulence and is modulated under different environmental conditions (Rehl *et al.*, 2013). The variation in essential genes observed between the two studies suggests that there may be different mechanisms involved in overcoming short-term and long term cold stress. Another study exploring the genetic mechanisms underlying the pathogenicity of *Salmonella* exposed to cold-stress at 5°C for 48 hours in cultured intestinal epithelial cells categorised differentially expressed genes during the stress into three main categories; T3SS associated, plasmid associated and prophage associated (Shah *et al.*, 2014). Genes induced during cold stress belonged to the *ssa* and *sse* operons, amongst others (Shah *et al.*, 2014). None of the essential genes identified during the current study appeared to be differentially expressed during exposure to cold stress in epithelial cells. This is most likely due to the difference in matrix used to adapt cells to a cold environment, as in the current study the *Salmonella* cells were incubated at 4°C within a vegetarian food matrix, whereas in the previous study they were inoculated into intestinal epithelial cells.

No genes were found to be essential for survival in all six food chain related stress conditions in the current study, and only one gene was identified as being involved in four of the stresses. The gene essential for survival in 6% NaCl, desiccation, 8mM acetic acid and 14mM citric acid stress was a putative reverse transcriptase, annotated as STM474_4025. A blastp of the amino acid sequence of STM474_4025 showed it was 100% identical to a retron St85 family RNA-directed DNA polymerase (also known as a reverse transcriptase). Retrons are bacterial genetic elements comprising of non-coding RNA (ncRNA) and a reverse transcriptase. The broad function of retrons was recently revealed as an anti-phage defence mechanism, thirty years post discovery (Millman *et al.*, 2020). There were 12 genes identified as essential in all 17 stress conditions in the study by Wang *et al*. (2022), which were *dnaK*, *recB*, *recC*, *rnhA*, *pdxH*, *dcd*, *xseA*, *bamD*, *yheM*, *gidA*, *orn* and an unannotated gene, STM14_4641. A few of the genes identified as essential in all stress conditions in the previous study were also predicted as essential during the current study during citric acid stress (*dnaK* and *xseA*), salt stress (*recB* and *xseA*), heat inactivation (*rnhA*), acetic acid stress (*rnhA* and *xseA*), desiccation (*pdxH*, *gidA* and *orn*) and during 5-week refrigerated storage

(*gidA*). Genes essential for survival during 6% NaCl stress, 14mM citric acid stress and desiccation stress in the current study were *nuoJ*, *engD*, *recG*, *efp*, *ycar*, *fur* and *dam*. There were three overlapping essential genes for 6% NaCl, 14mM citric acid and 8mM acetic acid stress, which were *recA*, *xseA* and *ftsK*. *xerC* was predicted to be essential during 6% NaCl stress, desiccation and 5-week refrigerated storage in the current study, whilst *tolC* was predicted to be essential for citric acid stress survival, desiccation and during heat inactivation. The ribonuclease reductase enzyme gene, *rnr*, was essential for survival in desiccation and 6% NaCl stress, and this gene has previously been identified as an essential gene during desiccation in *Lactobacillus paracasei* (Palud *et al.*, 2020). *proP* was identified as an essential gene in both salt and cold stress in the current study, whereas *zur*, a zinc uptake transcriptional repressor was only involved in salt stress. Two genes, *rfaI* and *rfaB* were detrimental for survival in all stresses except 14mM citric acid.

4.7 Conclusion

The use of a transposon directed insertion site sequencing (TraDIS) method enabled essential genes in six food chain related stress conditions to be identified in *S*. Typhimurium strain ST4/74. The most essential genes were identified during exposure to 14mM acetic acid, whereas the fewest number of essential genes were found to be associated with heat inactivation at 60°C. Unsurprisingly, there was no overlap between genes predicted to be essential during all six stress conditions included in the study, but this may be due to the stringent cut-off for gene essentiality chosen for the current experiment (logFC < -2). Several essential genes identified during the current study have been previously shown to be involved in food chain related stress, including *proP* during desiccation and osmotic stress, and *phoPQ* during organic acid stress. However, there were also novel genes identified during exposure to each stress condition, indicating response to stress and the mechanisms behind stress resistance is strain specific. To confirm that TraDIS can be a useful tool for identifying essential genes during specific stress exposure, gene doctoring will be used to generate single-gene knockouts in *S*. Typhimurium strain ST4/74 of some of the key essential genes identified during the current study. This will be discussed in chapter five.

Chapter Five

The link between food chain related stress tolerance in *Salmonella enterica* and genotype

5.1 Introduction

Next generation sequencing technologies have improved over the past few decades and has resulted in in-depth analysis of bacterial genomes. All microorganisms can be categorised by genotype depending on the presence of sequence diversity arising from insertions and deletions and sequence polymorphisms. In chapter two, the variation in response to food chain related stress was evaluated for diverse *Salmonella* strains and strains exhibiting increased tolerance or sensitivity to stress were identified. Strains of *S*. Typhimurium have greater known genetic diversity compared to other serovars, including Newport and Enteritidis, and strains of *S*. Typhimurium showed phenotypic variation in response to stress in the current study (Hu *et al.*, 2021). Evolution of the phylogroup of *S*. Typhimurium and its monophasic variants is distinct in comparison to *S*. Heidelberg and *S.* Enteritidis, which could explain the different tolerances to stress observed. The adaptation of *S*. Typhimurium within different ecological niches is driven by genomic variation contributing to the diversity observed within the serovar, which results in greatly adapted and virulent strains (Tanner and Kingsley, 2018). Sequence variation associated with niche adaptation may explain variations in stress tolerance, therefore it is important to explore the genomic disparities between strains, which may aid the identification of highly tolerant strains to common food preservation techniques in industry.

Epidemics are often initiated by novel genotypes that enter a new environmental or host niche. Molecular genotyping is now being used as an efficient method for epidemiological surveillance of foodborne outbreaks (Cliff *et al.*, 2020). Current detection methods for identifying *Salmonella* contamination in food products include the use of PCR and isothermal-based methods, which rely on target genes. Some of the target genes used include *invA* and *ttrRSBCA*, located in the chromosome of *Salmonella s*trains (Afroj *et al*., 2017; Dmitric *et al*., 2018). Despite the importance of these gene-based detection methods in identifying and preventing foodborne epidemics, mutations in these target genes may occur, highlighting the requirement for more robust detection methods. The techniques discussed in this chapter may provide a useful advancement for *Salmonella* detection in food products by identifying genes which are associated with increased tolerance to food chain related stress. Improvements in whole genome sequencing, increased surveillance, and the continual development of bioinformatic tools may resolve this need for more efficient detection methods (Hu *et al.,* 2021).

Sequence variation can result in functional divergence of proteins and therefore a bioinformatic tool, termed Deltabitscore (DBS), was used here to identify whether conditionally essential genes in *S*. Typhimurium strain ST4/74 were associated with an increased tolerance to stress in other *Salmonella* strains. Deltabitscore is a profile-based method for identifying functional divergence in genes derived from a common ancestor in bacterial species. It uses a profile Hidden Markov Model (HMM) based approach for probable alignment of multiple sequences and simultaneously captures information on the expected frequency of point mutations. Bitscore is a measure of how well a protein sequence fits a HMM for a protein family. Sequence divergence may result in changes in residues that are highly conserved within a protein family resulting in increase or decrease in bitscore depending on whether the change results in a better or worse fit to the HMM, respectively. This information is then used to calculate the delta-bit score, which quantifies the difference in fit to the HMM model for pairs of orthologous protein sequences (Wheeler *et al.,* 2016). DBS is therefore a convenient metric to predict the impact of mutations on function and sequence variation can result in functional divergence of proteins.

The presence of genes in a population can fluctuate over time due to horizontal gene transfer (HGT), resulting in a difference in the presence and absence of genes. Since the divergence of *Salmonella* from *E.coli* 100-140 million years ago, 3Mb of novel DNA has been estimated to have been lost or gained from each species (Vernikos *et al.*, 2007). The pangenome can be described as the complete genetic sequence of a species, including the core genome (genes present in all strains of a species) and accessory genome (genes unique to each strain) (Tettelin *et al.*, 2005). Strains can acquire genes, via HGT, from other organisms which can affect stress response and virulence (Medini *et al.*, 2005). Understanding the pangenome can enhance knowledge on bacterial selection and evolution. During this chapter, bioinformatic tools were used to evaluate the presence and absence of genes which were associated with increased stress tolerance. Roary is a tool which constructs the pangenome from bacterial species sequence data and identifies the core and accessory genomes, using the computing power of a desktop computer (Page *et al.*, 2015). ARIBA, which stands for Antimicrobial Resistance (AMR) Identification By Assembly, is an alignment based tool which identifies AMR genes from short read sequence data. ARIBA can also be used for multi-locus sequence typing (MLST) and can identify the presence/absence of genes in each database (Hunt *et al.,* 2017). In addition, nucleotide sequence polymorphisms resulted in sequence divergence of approximately 20%. The strains used in this study share a more recent common ancestor and exhibit no more than 2% sequence divergence.

In the current study, genome variation was explored using a combination of laboratory and computational techniques. Single-gene knockout mutants of conditionally essential genes were generated in *S*. Typhimurium strain ST4/74 and the survival of these mutants in food chain related stresses was assessed to verify conditionally essential genes identified using TraDIS. The pangenome, DBS analysis and knockout mutants were used to determine whether genome variation can explain food chain related stress tolerance in *Salmonella* strains. Genetic markers for food chain related stress tolerance and sensitivity were inferred using computational methods to evaluate the presence or absence of conditionally essential genes in *Salmonella* strains. Ultimately, understanding the molecular mechanisms behind stress tolerance in *Salmonella* can aid surveillance and outbreak detection, and ultimately improve food safety.

5.1.1 Aims

1. Assess the phenotype of *S*. Typhimurium strains in which candidate conditionally essential genes required for survival or growth in food chain related stress, including heat inactivation, long term refrigerated storage, desiccation, organic acids and salt have been deleted by targeted allelic exchange
2. Identify candidate genetic markers for food chain related stress tolerance and sensitivity in *Salmonella* using a range of bioinformatics techniques

5.2 Methods

5.2.1 Primer design for Golden Gate Cloning

Primer sequences with homology to the junction of the desired site of recombination in the chromosome of *S.* Typhimurium strain ST4/74 (forward and reverse) were designed for each gene knock-out target using SnapGene (version 5.3.2) (**Table 27**). The homologous recombination (HR) region was identified for each gene by selecting a 400bp region located 22bp upstream from the start of the target gene (denoted HR1) and reverse primers were designed by selecting a 400bp region, 22bp downstream from the end of the target gene (denoted HR2). The 22bp upstream and downstream of the HR1 and HR2 regions were selected and designated as the forward and reverse primers, respectively. Primer tails were added to the 5' end of HR1 and HR2 forward and reverse primers, which included the BsaI recognition cutting site and the necessary nucleotide overhang to combine nucleotide fragments in the correct order (**Table 28**).

Primer	Sequence	bp	GC	Tm
rfab_HR1_F	GGGCTACTGAGACCAATACTCCCCTTACATGCAATATCTGCA	42	48%	60°C
rfab_HR1_R	GGGGGTCTCGCTCCATTTTGAAGAAGAAGTCATTCAACAGAC TTTAGATTATAACT	56	39%	60°C
rfab_HR2_F	GGGCGCTTGAGACCACCTTAATCTAAAGACTTTAGATATTAA ATAGCAAAAATAGTAATATAACCC	66	33%	60°C
rfab_HR2_R	GGGGGTCTCGTCGTGATTTTGCAAAAAGAATCGGGGTTGATT CA	44	48%	60°C
zur_HR1_F	GGGCTACTGAGACCTATAAAACCGAAGGCCGAATTCCTGC	40	53%	61°C
zur_HR1_R	GGGGGTCTCGCTCCGGCAGTAGAGAGGGGCACGCCC	36	75%	68°C
zur_HR2_F	GGGCGCTTGAGACCTGAAAGTCTAATCCGTTACTTTACCACG AG	44	50%	60°C
zur_HR2_R	GGGGGTCTCGTCGTTAGCCCATCTTATTATCAGGTGAAGTAA TTCC	46	48%	60°C
proP_HR1_F	GGGCTACTGAGACCGGCAGATCGCCGAAAAAGGCCGCAATT A	42	57%	60°C
proP_HR1_R	GGGGGTCTCGCTCCAGCCCTGTAGCGCTATATACGG	36	64%	61°C
proP_HR2_F	GGGCGCTTGAGACCGCTTAATCTCTCGCGGGCATACT	37	59%	60°C
proP_HR2_R	GGGGGTCTCGTCGTAACCCATATCACTATCCACATTAGCGC	41	54%	61°C
rnr_HR1_F	GGGCTACTGAGACCAATCTATATGGCGTCGTTACCAGATGG	41	51%	61°C
rnr_HR1_R	GGGGGTCTCGCTCCATCTCTAATGAAGTTTACTTCACTCCACC A	44	50%	60°C
rnr_HR2_F	GGGCGCTTGAGACCAGAAGAGGGTCAATTATCTGTAGGCCG	41	56%	61°C
rnr_HR2_R	GGGGGTCTCGTCGTGCGCCAAGATTATGCGGATCGG	36	64%	63°C
dam_HR1_F	GGGCTACTGAGACCCACATCCAGTTTGTCCATGACG	36	56%	60°C
dam_HR1_R	GGGGGTCTCGCTCCTGAAACAGTATTTGATTGCCCCCTCAA	41	54%	60°C
dam_HR2_F	GGGCGCTTGAGACCCCTTCTCCGGCTGTGGAGAAAG	36	64%	61°C
dam_HR2_R	GGGGGTCTCGTCGTCATTGCAGCTCAGTAGTTCTTCAAATTA CGAC	46	50%	61°C

Table 27. Primers used for generation of single-gene knockout mutants. Homologous recombination forward and reverse primers for each single-gene knockout target in *S*. Typhimurium strain ST4/74 needed to amplify the 432bp gene region in preparation for Golden Gate Assembly.

Primer	Tail Sequence
HR1 Forward	GGGCTACTGAGACC
HR1 Reverse	GGGGGTCTCGCTCC
HR2 Forward	GGGCGCTTGAGACC
HR2 Reverse	GGGGGTCTCGTCGT

Table 28. Sequence of nucleotide tail added to each primer. A nucleotide tail was added to each homologous recombination forward and reverse primer for each gene knockout target. Nucleotides in red indicate the BsaI recognition cutting site.

5.2.2 Transformation of pACBSCE into *S*. Typhimurium strain ST4/74

Electrocompetent cells of *S.* Typhimurium strain ST4/74 were prepared by inoculating a 50mL conical flask containing 2x YT broth with cells grown for ~18 hours overnight in LB broth (1:1000 dilution). Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, 03690) was added to the flask to a final concentration of 70µM. The inoculated 2x YT broth was incubated at 37°C with shaking at 200rpm until the OD600nm reached between 0.20 and 0.25. The contents of the flask were pelleted by centrifugation at 3000rpm for 10 minutes at 4°C, and the supernatant discarded. The resulting pellet was washed three times with ice cold 10% glycerol, spinning down using a centrifuge at 3000rpm for 10 minutes each time. After the final wash, the bacterial pellet was resuspended in 500µL 10% glycerol. A 2µL aliquot of pACBSCE helper plasmid (Lee *et al.*, 2009) was added to 50µL electrocompetent cells and electroporation was conducted at 2.4V using a MicroPulser electroporator (Bio-rad) with 2mm electroporation cuvettes (EP-102) (Cell Projects).

Three individual electroporation's were conducted for *S.* Typhimurium strain ST4/74 and a pUC19 positive control was included, alongside a plasmid DNA-free negative control. Immediately after electroporation, 1mL LB broth (warmed to 37°C) was added to each electroporation cuvette and the contents transferred to a sterile 1.5mL microcentrifuge tube. *S.* Typhimurium strain ST4/74 transformants were recovered for 2 hours at 37°C in a shaking incubator (200rpm), pooled together and 100µL was plated on LB agar containing chloramphenicol (25µg/mL). The pUC19 positive control was plated on LB agar containing carbenicillin (50µg/mL) and plates were incubated overnight at 37°C. Colonies harbouring the pACBSCE helper plasmid were sub-cultured into 5mL LB broth containing chloramphenicol (25µg/mL) and incubated at 37°C with 200rpm shaking. Glycerol stocks of *S.* Typhimurium strain ST4/74 with pACBSCE were prepared by combining 500µL of culture grown to stationary phase overnight (approximately 18 hours) with an equal volume of 50% glycerol and were stored at -20°C.

5.2.3 Amplification of Homologous Regions by PCR

Homologous regions for each gene target were amplified by polymerase chain reaction (PCR) using the primers listed in **Table 27,** with template DNA from *S.* Typhimurium strain ST4/74. A 50µL PCR reaction consisted of 25µL of Q5 High-Fidelity 2X Master Mix (New England

Biolabs (NEB), M0492), 2.5µL of each primer pair (10µM) (**Table 27**) and 20µL of double distilled and deionised water (Sigma-Aldrich, W4502). For template DNA, a single colony of *S*. Typhimurium strain ST4/74 grown overnight on LB agar at 37°C was picked and resuspended in the PCR reaction. A Veriti thermocycler (Fisher Scientific, 4375786) was used for the PCR reaction and the protocol is described in **Figure 43**. Amplified PCR product was analysed by 1% Agarose gel electrophoresis at 120V for 30 minutes. Bands were stained with Midori green direct DNA stain (Geneflow, MG05) and visualised under blue light. After confirmation that the correct region of each gene had been amplified, the PCR product was purified using the QIAquick PCR purification kit (Qiagen, 28104) following the manufacturer's instructions and the final product was eluted in 30µL elusion buffer (EB). The concentration of purified DNA was quantified using a Qubit 3.0 fluorometer (Invitrogen, Q33216) and the associated Qubit dsDNA Broad Range Assay Kit (Invitrogen, Q32850).

98°C	3 minutes
98°C	15 seconds
58°C	15 seconds
72°C	30 seconds
72°C	7 minutes
16°C	∞

x 35 cycles

Figure 43. Thermocycling conditions for amplification of 432bp homologous region in each target gene in *S*. Typhimurium strain ST4/74.

5.2.4 Assembly of DNA molecules using Golden Gate Reaction

The pDOC-GG plasmid to be used in the golden gate reaction for gene doctoring methodology is described in Thomson *et al*. (2020). The golden gate reaction was assembled using the reagents listed in **Table 29**. The reaction was incubated using a Veriti thermocycler (Fisher Scientific, 4375786) overnight using the protocol denoted in **Figure 44**.

Reagent	Amount
Ligase Buffer (NEB, M0202S)	1µL
BSA (NEB,B9000S)	2µL
pDOC-GG	10 fmol
Tetracycline cassette	10 fmol
HR1 DNA	20 fmol
HR2 DNA	20 fmol
T4 DNA Ligase (NEB, M0202S)	0.5µL
BsaI-HFV2 (NEB, R3733S)	0.5µL
Deionised Water	χµL
Total	10µL

Table 29. Golden Gate Assembly reagents. The exact quantities of reagents used in the current study for each gene target can be found in **supplementary table 10**.

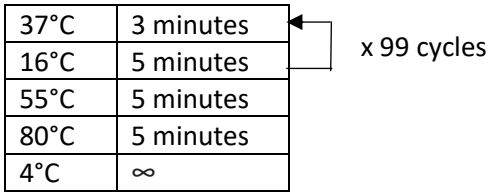


Figure 44. Thermocycling conditions for Golden Gate reactions.

5.2.5 Transformation of pDOC-GG vectors into chemically competent *E. coli*

A 2µL aliquot of the golden gate assembly for each gene target was added to 50µL chemically competent *E. coli* DH5α (NEB, C2987I). The cells were heat shocked by leaving on ice for 2 minutes, heating at 42°C for 2 minutes and then leaving on ice again for 2 minutes. The heat shocked cells were resuspended in 1mL LB broth and incubated for 1 hour at 37°C with the shaking incubator set to 200rpm. The recovered cells were pelleted by centrifugal force at 13,300rpm for 4 minutes and the supernatant was discarded. The resulting pellet was resuspended in 100µL PBS and spread plated onto LB agar containing tetracycline (10µg/mL), kanamycin (50µg/mL), 1mM Isopropyl β-D-thiogalactoside (IPTG) (Sigma-Aldrich, 16758) and 20µg/mL X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside). Wild type colonies were differentiated from transformants by a blue centre. Successful transformants were picked and checked using colony PCR.

5.2.6 Colony PCR for pDOC-GG constructs

To verify the correct pDOC-GG constructs were generated, a 10µL PCR reaction was set up containing 5µL GoTaq G2 Green Polymerase Master Mix (Promega, M7822), 0.5µL of pDOC-K forward and reverse primers (**Table 30**), and 4µL deionised water. Template DNA was added to the PCR reaction tubes by picking four colonies from the p-DOC-GG vector transformants in *E. coli* DH5α and dipping them into the PCR tubes. The 'picked' colony was then streaked onto LB agar containing both tetracycline (10µg/mL) and kanamycin (50µg/mL) and incubated overnight at 37°C. A positive PCR control was included which contained pDOC-GG-*nirD* plasmid DNA, which had previously been confirmed by sanger sequencing, and the negative control contained the 200bp pDOC-GG plasmid backbone as the template DNA. A Veriti thermocycler was used for the PCR reaction and the protocol is described in **Figure 45**. Colony PCR products that were the correct size were sub-cultured from the streak plates into fresh LB broth containing tetracycline (10µg/mL) and kanamycin (50µg/mL) and incubated overnight at 37°C. Plasmids were extracted from the overnight cultures using a QIAprep Spin Miniprep kit, following the manufactures instructions, and eluted in a final volume of 30µL EB. Plasmid preparations were sent for sanger sequencing using Eurofins Genomics.

Primer	Sequence
pDOC-K-fwd	CATGATTACGCCAAGCTCTAG
pDOC-K-rev	GGGTTTTCCCAGTCACGACGT

Table 30. **pDOC-K primers used for colony PCR of pDOC-GG vectors**.

98°C	3 minutes
98°C	15 seconds
53°C	15 seconds
72°C	2 minutes 30 seconds
72°C	7 minutes
16°C	∞

x 25 cycles

Figure 45. **Thermocycling conditions for colony PCR of pDOC-GG vectors in *E. coli* DH5α.**

5.2.7 Transforming pDOC-GG gene doctoring plasmids into *S.* Typhimurium strain ST4/74 with pACBSCE

Electrocompetent cells of *S.* Typhimurium strain ST4/74 containing the pACBSCE helper plasmid were prepared using the method detailed in section 5.2.2, however, *S.* Typhimurium strain ST4/74 with pACBSCE was prepared overnight in LB broth containing chloramphenicol (25µg/mL) prior to inoculating into 2x YT broth. Transformants of *S.* Typhimurium strain ST4/74 containing pACBSCE and pDOC-GG gene doctoring vectors were plated onto LB agar containing chloramphenicol (25µg/mL), kanamycin (50µg/mL) and tetracycline (10µg/mL), and incubated overnight at 37°C. The positive control (pUC19) was plated onto LB agar containing carbenicillin (25µg/mL) and chloramphenicol (25µg/mL).

5.2.8 Lambda Red Recombination

pDOC-GG transformants in *S.* Typhimurium strain ST4/74 of each gene knockout target that had successfully grown on the selective antibiotic plates were resuspended in 500µL LB broth containing chloramphenicol (25µg/mL) and tetracycline (10µg/mL) and incubated for 3 hours at 37°C in a shaking incubator set to 200rpm. Cells were pelleted by centrifugation at 13,300rpm for 4 minutes and the resulting bacterial pellet resuspended in an equal volume of 10% LB broth. The cells were washed 3x with 10% LB broth, and the final pellet resuspended in 10% LB broth and 0.3% arabinose (Sigma, A3256). The cell suspension was incubated for 2 hours at 30°C and 200rpm, and a 100µL aliquot was plated onto LB agar containing tetracycline (10µg/mL), 1mM IPTG and 5% sucrose (Sigma, S0389). Plates were incubated overnight at 37°C. Resulting colonies were streaked onto three separate LB plates containing kanamycin (50µg/mL), chloramphenicol (25µg/mL) and tetracycline (10µg/mL) and incubated overnight at 37°C. Colonies able to grow on tetracycline plates, but not kanamycin or chloramphenicol, were sent for whole genome sequencing to confirm the successful deletion of the target gene and insertion of the tetracycline cassette.

5.2.9 DNA extraction from *S.* Typhimurium strain ST4/74 mutants

S. Typhimurium strain ST4/74 single-gene knock out mutants (*rnr*, *zur*, *rfaB*, *proP* and *dam*) were grown for 18 hours in LB broth containing tetracycline (10µg/mL). A 100µL aliquot of

culture grown to stationary phase was deposited directly into a Maxwell RSC cultured cells DNA kit cassette (Promega, AS1620) and loaded into a Maxwell RSC 48 Instrument (Promega, AS8500). DNA was extracted in duplicate for each gene knockout, following the manufactures standard protocol for DNA extraction from bacterial cells. The DNA concentration was quantified using a Qubit 3.0 fluorometer and the Qubit dsDNA BR Assay Kit. DNA was diluted to 3-7ng/µL with elusion buffer and sent for whole genome sequencing, conducted by the QIB sequencing team, using an Illumina NextSeq500 instrument.

5.2.10 Whole genome sequencing of *S.* Typhimurium strains with single-gene knockout

Genomic DNA extracted from *S*. Typhimurium strain ST4/74 single-gene knockout mutants was normalised to 3-7ng/µL with elusion buffer. Library preparation and sequencing was conducted by the QIB sequencing team, as follows. A 0.5µL of TB1 Tagment DNA Buffer was mixed with 0.5µL BLT (bead linked transposome), Tagment DNA Enzyme (Illumina, 20018704) and 4µL water in a master mix and 5µL of this mixture was added to a 96-well plate on ice. 2µL of normalised DNA (10ng total) was pipette mixed with 5µL of the tagmentation mix and heated to 55°C for 15 minutes in a thermocycler (Veriti). A PCR master mix was prepared using 10µL KAPA 2G Fast Hot Start Ready Mix (Merck, KK5601) and 2µL water (per sample) and 12µL of this was added to each well of a 96-well plate. 1µL of each P7 and P5 Nextera XT Index Kit v2 index primers (Illumina, FC-131-2001-2004) were added to each well. Finally, 7µL of the tagmentation mix was added and mixed. The PCR was run at 72°C for 3 minutes, 95°C for 1 minute, followed by 14 cycles of 95°C for 10 seconds, 55°C for 20 seconds and 72°C for 3 minutes. Libraries were quantified using the QuantiFluor dsDNA System (Promega, E2670) and run on a GloMax Discover Microplate Reader. Libraries were pooled following quantification in equal quantities. The final pool was double-SPRI size selected between 0.5 and 0.7X bead volumes using Illumina DNA Prep, (M) Tagmentation (96 Samples, IPB, 20060059).

The final pool was quantified on a Qubit 3.0 fluorometer and run on a D5000 ScreenTape (Agilent, 5067-5579) using the Agilent Tapestation 4200 to calculate the final library pool molarity. The pool was run at a final concentration of 1.5pM on an Illumina Nextseq500 instrument using a Mid Output Flowcell (NSQ 500 Mid Output KT v2 (300 CYS) Illumina, FC-404-2003) following the Illumina recommended denaturation and loading recommendations, which included a 1% PhiX spik (PhiX Control v3 Illumina, FC-110-3001).

Data was uploaded to Basespace (www.basespace.illumina.com) and the raw data was converted to 8 fastq files for each sample. FastQC (version 0.11.9) was used to check the quality of the sequencing fastq reads. BLAST (version 2.10.0) was used to confirm the deletion of the gene target by aligning the fasta sequence of the gene to the single-gene knockout fasta files. If the gene couldn't be found in the single-gene knockout fasta file, then the knockout was deemed to be successful.

5.2.11 Heat inactivation of *S.* Typhimurium strain ST4/74 single-gene knockout mutants

Overnight cultures of *S.* Typhimurium strain ST4/74 (wild type), ST4/74Δ*dam*::tetR, ST4/74Δ*rnr*::tetR, ST4/74Δ*zur*::tetR, ST4/74Δ*rfaB*::tetR and ST4/74Δ*proP*::tetR were prepared in 5mL LB broth for the wild type control and LB broth containing tetracycline (10µg/mL) for the mutants. Broths were incubated at 37°C with shaking at 200rpm for approximately 18 hours. Cells were collected by centrifugation at 13,000rpm for 4 minutes at room temperature and the supernatant was discarded. The pellet was resuspended in PBS and the optical density of each culture was adjusted to approximately 2.5 x 10^9 CFU/mL, using PBS. The frozen vegetarian food product was thawed and 750mg was deposited into the centre of each thermal cell (provide by Nestlé). A 50µL aliquot of each culture at approximately 2.5 x 10^9 CFU/mL was inoculated into each thermal cell containing the vegetarian food product, in duplicate. Thermal cells were left to incubate for 1 hour at 4°C. Thermal cells were heated in a water bath at 60°C for 30 seconds and immediately plunged into an iced water bath to rapidly cool. One thermal cell was attached to a TC-08 datalogger (Pico Technology) to monitor the temperature of the samples for the duration of the experiment. Control thermal cells were not heated but were treated the same for the rest of the experiment. Food samples (heated and control) were deposited directly into 5mL LB broth bottles, mixed using a vortex and then serially diluted (1:10) in a 96-well plate with PBS. A 5µL aliquot of each dilution was spotted onto a square LB agar plate, in triplicate. Plates were incubated at 30°C overnight and surviving colonies enumerated.

5.2.12 Desiccation of *S.* Typhimurium strain ST4/74 single-gene knockout mutants

Overnight cultures of *S.* Typhimurium strain ST4/74 (wild type) and the mutant strains were prepared in 5mL LB broth and LB broth containing tetracycline (10µg/mL), respectively.

Broths were incubated at 37°C with shaking at 200rpm for approximately 18 hours. Cells were pelleted by centrifugation at 13,000rpm for 4 minutes at room temperature and the supernatant was discarded. The pellet was resuspended in PBS and the optical density of each culture was adjusted to approximately 2.5 x 10^9 CFU/mL, using PBS. Five wells of a 96-well plate were filled with 50µL of each mutant strain and the wild type control. Plates were left to desiccate in a safety cabinet for 24 hours and the temperature and humidity was monitored using a thermohygrometer placed inside the safety cabinet. For a control, 50µL of each culture was deposited into a 96-well plate (five wells for each culture) and mixed with 150µL PBS before being serially diluted (1:10) with PBS. A 5µL aliquot of each dilution from the control plate was spotted, in triplicate, onto a square LB agar plate and incubated overnight at 30°C. After 24 hours, desiccated cells were resuspended in 200µL PBS and serially diluted (1:10) with PBS, and spot plated as before. Surviving colonies were enumerated and the log ratio survival calculated.

5.2.13 Long term refrigerated storage of *S.* Typhimurium strain ST4/74 single-gene knockout mutants

Overnight cultures of *S.* Typhimurium strain ST4/74 (wild type) and the mutant strains were prepared in 5mL LB broth and LB broth containing tetracycline (10µg/mL), respectively. Broths were incubated at 37°C with shaking at 200rpm for approximately 18 hours. Cells were pelleted by centrifugation at 13,000rpm for 4 minutes at room temperature and the supernatant was discarded. The pellet was resuspended in PBS and the optical density of each culture was adjusted to approximately 2.5 x 10^9 CFU/mL, using PBS. Wells of a 24-well tissue culture plate were filled with 750mg thawed vegetarian food product and each well was inoculated with 50µL of each mutant and wild type at approximately 2.5 x 10^9 CFU/mL (four wells per strain). Plates were left to incubate for 5 weeks in a cold-room at 4°C. Control plates were prepared in the same way, but the inoculated vegetarian food product was immediately deposited into individual 5mL LB broth bottles and serially diluted (1:10) with PBS. 5µL of each dilution was spot plated onto a square LB agar plate, in triplicate, and incubated overnight at 30°C. Control colonies were enumerated the following day. After 5 weeks, inoculated food samples were deposited into individual 5mL LB broth bottles and serially diluted (1:10) with PBS. Serial dilutions were spot plated as before and incubated overnight at 30°C. Surviving colonies were enumerated, and the log ratio survival calculated.

5.2.14 Growth of *S*. Typhimurium strain ST4/74 single-gene knock out mutants in 6% NaCl

Overnight cultures of *S*. Typhimurium strain ST4/74 wild type and mutant strains were prepared in 5mL LB broth (for the wild type strain) and LB broth containing tetracycline (10µg/mL) (for the mutant strains) and were incubated at 37°C in a shaking incubator at 200rpm. A 6% concentration of NaCl (Sigma, S9888) solution was prepared in LB broth (w/v). A 5µL aliquot of overnight culture of both ST4/74 wild type and mutant strains were deposited into 5mL 6% NaCl solution. For a control, 5µL of each overnight culture was deposited into 5mL LB broth (no NaCl). 200µL aliquots of each culture in either 6% NaCl or LB broth were dispensed into wells of a CELLSTAR U-Bottom 96-Well plate (Greiner, M0812), in triplicate. Growth was measured at OD600nm using a FLUOstar Omega Microplate Reader (BMG Labtech) at 37°C, with constant shaking. Measurements were taken every 5 minutes for 24 hours.

5.2.15 Growth of *S*. Typhimurium strain ST4/74 single-gene knock out mutants in 14mM Citric Acid

Overnight cultures of *S*. Typhimurium strain ST4/74 and mutant strains were prepared in 5mL LB broth and 5mL LB broth containing tetracycline (10µg/mL), respectively. Cultures were incubated at 37°C with shaking set to 200rpm for approximately 18 hours. A 30mM stock solution of citric acid (Sigma Aldrich, 251275) was prepared in 250mL LB broth and sterilised using a Minisart polyethersulfone (PES) filter (0.22µM) (Sartorius, 16532K) and syringe. A 14mM working stock of citric acid was prepared from the 30mM stock solution. 5mL of citric acid and LB broth (no citric acid) were dispensed into 15mL centrifuge tubes (Corning, CLS430055), in duplicate for each strain. A 1mL aliquot of overnight culture in stationary phase (18-hour growth) of *S*. Typhimurium strain ST4/74 and mutant strains were added to 4mL LB broth adjusted to pH 5.8 with 30mM citric acid and incubated for 30 minutes at 37°C with shaking set to 200rpm. Cultures were then adjusted to approximately 5 x 10^8 CFU/mL using LB broth, and 5µL of each strain was inoculated into the tubes containing 14mM citric acid and LB broth. 200µL of inoculated citric acid with each strain and the associated controls in LB broth were dispensed into a U-Bottom 96-well plate, in triplicate wells. The citric acid was measured using a benchtop pH meter (Mettler Toldeo). Absorbance at an optical density

of 600nm was measured for 24 hours at 37°C using a FLUOstar Omega Microplate Reader, with pre-measurement shaking.

5.2.16 Growth of *S.* Typhimurium strain ST4/74 single-gene knock out mutants in 8mM Acetic Acid

Overnight cultures of *S.* Typhimurium strain ST4/74 wild type and mutant strains were prepared in 5mL LB broth (for the wild type strain) and LB broth containing tetracycline (10µg/mL) (for the mutant strains) and were incubated at 37°C in a shaking incubator at 200rpm for 18 hours. A 60mM stock solution of acetic acid (SLS, 33209) was prepared by combining 345µL acetic acid with LB broth to a final volume of 100mL and sterilised using a 0.22µM PES filter and syringe. The 60mM acetic acid stock solution was used to prepare a working stock of 8mM acetic acid. A 5mL aliquot of 8mM acetic acid was transferred to a 15mL centrifuge tube, in duplicate. Overnight cultures of *S.* Typhimurium strain ST4/74 and mutants were pre-adapted to pH 5.8 using 60mM acetic acid, and incubated for 30 minutes at 37°C, with shaking at 200rpm. The centrifuge tubes containing 5mL acetic acid were inoculated individually with 5µL ST4/74 and the mutant strains. A 200µL aliquot of each inoculated acetic acid solution with ST4/74 and the mutant strains, and the non-inoculated controls were dispensed into a U-Bottom 96-well plate, in triplicate wells. The pH of acetic acid was measured using a benchtop pH meter (Mettler Toldeo) and growth was measured at OD600nm for 24-hours at 37°C using a FLUOstar Omega Microplate Reader (BMG Labtech), with pre-measurement shaking.

5.2.17 Statistical analysis on mutant stress survival data

A one-way ANOVA was conducted on the log ratio survival data during heat inactivation, desiccation and 5-week refrigerated storage in Graphpad prism (version 8.0.2). Fisher's least significance difference test was included and the mean log ratio survival for each mutant during stress was compared to the *S.* Typhimurium strain ST4/74 wild type control.

5.2.18 Determination of presence and identification of potential functional divergence in conditionally essential genes in diverse *Salmonella enterica* strains

To investigate the presence or functional divergence of proteins encoded by conditionally essential genes identified using TraDIS, genome sequence assemblies from Illumina short-read sequences of 14 *Salmonella* strains were constructed using SPAdes (version 3.5.0) (Bankevich *et al.*, 2012). Gene models were constructed using PROKKA (version 1.11) (Seemann, 2014) to identify open reading frames and predicted protein sequences. Polymorphisms in the predicted protein sequences in this model that may result in changes in function were predicted by pairwise comparison to the Pfam HMM each with reference to *S. bongori* strain N268-08 using deltabitscore (version 1.0) (Wheeler *et al*., 2016). This method has been used previously to identify hypothetically disrupted coding sequences (HDCS) that are potential pseudogenes, and polymorphisms that potentially result in loss of function due to changes in key residues in functional domains.

To determine the presence or absence of genes that were conditionally essential in *S*. Typhimurium strain ST4/74 the accessory genome of the 14 *Salmonella s*trains were determined, and Roary (version 3.13.0) (Page *et al.*, 2015) was run on the assembled sequences without splitting orthologs to generate a file containing gene presence and absence data in all strains. ARIBA (version 1.0) (Hunt *et al.*, 2017) was used as an alternative method to roary to identify the presence and absence of conditionally essential genes identified in *S*. Typhimurium strain ST4/74 in the other *Salmonella* strains. Custom python3 scripts were written by Gaetan Thilliez to filter out the gene presence and absence data from the roary and ARIBA output for each strain for the conditionally essential genes in *S.* Typhimurium strain ST4/74 in each stress condition and to determine whether the conditionally essential genes identified during each stress were predicted to have lost their function in the other *Salmonella* strains. The individual plots generated from each pipeline were amalgamated in R, alongside the phylogenetic tree generated using RaxML previously and the log ratio survival or difference in AUC for each mutant during food chain related stress.

5.3 Results

5.3.1 Rationale for selection of single-gene knockout candidates of conditionally essential genes in *S*. Typhimurium strain ST4/74

To investigate whether single-gene knock out strains exhibit stress tolerance phenotypes predicted from the TraDIS analysis of transposon insertion mutant library, single-gene knockout mutants of five candidate conditionally essential genes (*rfaB*, *dam*, *rnr*, *zur* and *proP*) were constructed by allelic exchange using the gene doctoring method in *S.* Typhimurium strain ST4/74. The single-gene knockout mutants were sequenced to verify that the target gene had been successfully replaced with a tetracycline resistance cassette. *proP* and *dam* were selected because they had the greatest negative logFC value in the transposon library comparison before and after exposure to stress and these genes were considered to be the most conditionally essential genes required for survival. The *proP*, *dam*, *zur* and *rnr* genes were also selected because they were essential for survival in multiple stress conditions according to their logFC values. *proP* was also included because it had previously been implicated as an essential gene during desiccation stress response in the literature. *rfaB* was chosen to evaluate the effect of a positive logFC on phenotype. The phenotype of each single-gene knockout mutant generated using the gene doctoring method was tested for each stress condition, regardless of whether the gene was essential in that condition or not. This was to test whether there was a phenotypic difference between genes with a logFC greater than 2 or less than -2.

The associated insertion site plots for each single-gene knockout mutant in *S*. Typhimurium strain ST4/74 in each stress condition compared to the control were visualised using Artemis software (version 17.0.2) to verify the logFC values generated from the Bio-Tradis pipeline output (**Figure 46**). If there were significantly ($q < 0.05$) fewer Tn5 insertions in the stress library compared to the control, then this gene was considered conditionally essential for survival in food chain related stress. No insertions were detected in the *dam* gene following culture in 6% NaCl stress or heat inactivation, which was consistent with the logFC values generated for this gene in these stresses (**Figure 46**). There were insertions present in the plot during 8mM acetic acid stress, however there were still fewer insertions than present in the control library and as the logFC value was not less than -2, this gene was not considered essential for acetic acid stress survival (**Figure 46**). The *proP* gene had few insertions in the plots for 6% NaCl, 8mM acetic acid, desiccation, heat inactivation and 5-week refrigerated

storage, compared to the control. There were no insertions in the *rnr* gene during heat inactivation, and very few insertions during 6% NaCl stress, 8mM acetic acid, 5-week refrigerated storage and desiccation. Insertions in the *rnr* gene during 14mM citric acid stress were situated towards the 3' and 5' ends of the gene (**Figure 46**). The *zur* gene had no insertions following culture in 6% NaCl stress, whereas there were insertions in all other stresses. The *rfaB* gene was the only gene included in the study which had a positive logFC, and the high number of insertions in each stress and the control was also evident when looking at the insertion plots (**Figure 46**).

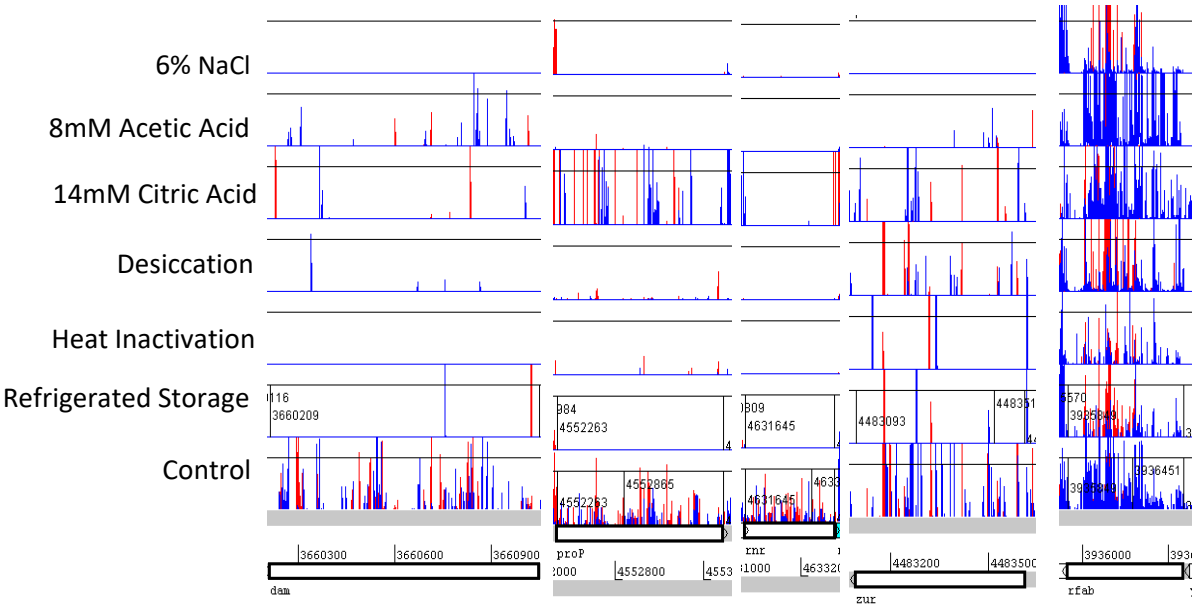


Figure 46. **Insertion site plots for the single-gene knockout candidates in *S*. Typhimurium strain ST4/74**. Plots were visualised in Artemis (version 17.0.2) and stress conditions included 6% NaCl, 8mM acetic acid, and 14mM citric acid stress, 24-hour desiccation, heat inactivation at 60°C for 30 seconds and 5-week refrigerated storage. The red lines denote the insertions are on the forward strand and the blue lines represent insertions on the reverse strand.The height of the bars represent the number of insertions.

The mutant strains were subjected to the same stress conditions used when generating the transposon mutant library in chapter four. The *proP* and *dam* mutants had the greatest negative logFC during 6% NaCl stress at -14.29 and -13.26, respectively (**Table 31**). The *rnr* and *zur* mutants both had negative logFC values during 6% NaCl stress, indicating that there were fewer insertions in the stress compared to the control. However, the *rfaB* mutant had a positive logFC during 6% NaCl stress of 4.24, meaning there were more insertions in the stress condition compared to the control. During 14mM citric acid stress, the *dam* mutant

had the greatest negative logFC out of all the single-gene knockout candidates (**Table 31**). *S.* Typhimurium strain ST4/74Δ*rfaB* had a negative logFC in 14mM citric acid stress compared to the control, whereas this mutant had a positive logFC for all other stresses. However, as the logFC value reported for this mutant was not < -2, it was not deemed a significant difference and is unlikely to be phenotypically different from the wild type strain (**Table 31**).

Only the *dam* mutant had a logFC <-2 and a q-value < 0.05 during 14mM citric acid stress, which was considered significantly different to the control. The *rnr* and *dam* mutants had the greatest negative logFC during 24-hour desiccation and heat inactivation, and both were less than -2. The *proP*, *zur* and *rfaB* mutants were not significantly different from the wild type strain during heat inactivation (**Table 31**). Only ST4/74Δ*proP* had a significant logFC less than -2 during 5-week refrigerated storage out of all the single-knockout gene candidates. The *rnr* mutant had the greatest negative logFC during acetic acid stress, which was significant. The *proP*, *zur* and *dam* mutants also had negative logFC values, however these were not less than -2, and therefore not deemed essential for survival during acetic acid stress even though they had a q-value of less than 0.05 (**Table 31**).

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Gene	6% NaCl	14mM CA	Desiccation	Heat Inactivation	Cold Storage	8mM AA
proP	-14.29	-0.21	-0.31	-0.90	-5.58	-0.37
rfaB	4.24	-1.07	3.37	1.98	2.43	7.39
zur	-4.57	-0.20	-0.31	-0.05	-0.34	-1.56
dam	-13.26	-2.13	-5.27	-2.73	-1.58	-0.77
rnr	-3.35	-1.05	-5.20	-4.81	-1.37	-4.85

Table 31. **Stress response (logFC) for each single gene knock out candidate**. LogFC values with a q-value < 0.05 in *S.* Typhimurium strain ST4/74 during six food chain related stresses are denoted (CA= citric acid, AA= acetic acid).

5.3.2 The *dam, rnr, rfaB* and *zur* genes are essential for survival of *S*. Typhimurium strain ST4/74 during desiccation stress

To assess the fitness of the single gene knock out mutants generated in *S*. Typhimurium strain ST4/74 during desiccation, the mutants were air dried for 24 hours and surviving colonies enumerated (**Figure 47**). There was no recovery of *S*. Typhimurium strain ST4/74Δ*dam* on the LB agar plates after desiccation, consistent with the -5.27 logFC observed for the gene during desiccation stress in the transposon library. There was a ~4.9-log reduction in cell viability for the *rnr* mutant after desiccation, which was significantly different to the wild type control ($p < 0.001$). The *rnr* mutant had the second greatest negative logFC (-5.2) out of all the strains tested, after *dam*, during desiccation stress. Although ST4/74Δ*rfaB* had a positive logFC (3.37) when compared to the wild type control in the transposon library after desiccation stress, the mutant was unable to tolerate desiccation stress.

The reduction in cell viability for ST4/74Δ*rfaB* was approximately 4-log and was significantly different to the wild type control ($p < 0.001$) (**Figure 47**). The *zur* mutant also exhibited sensitivity to desiccation stress with an average log reduction of 3.8 and a significant p-value of less than 0.001 compared to the wild type. The logFC of the *zur* mutant during desiccation stress of the transposon library was not less than -2, and hence this gene was not considered to be essential for desiccation survival in *S*. Typhimurium strain ST4/74, however there was a significant phenotypic difference in response to desiccation observed between the mutant and the wild type. The *proP* mutant showed a 3.23-log reduction in cell viability after 24-hour desiccation, which was insignificant to the control. This strain was not considered to be essential for desiccation according to its logFC value of -0.31. The wild type strain observed a ~3-log reduction in cell viability during desiccation (**Figure 47**).

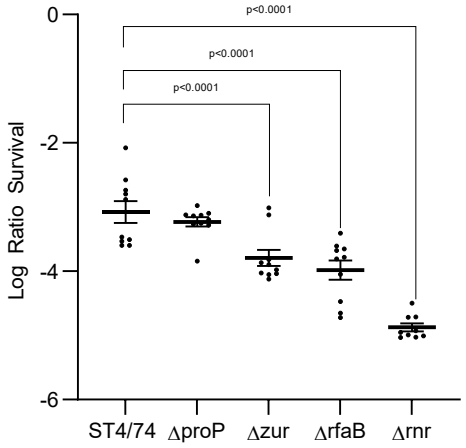

Figure 47. **Effect of 24-hour desiccation on isogenic mutants in *S*. Typhimurium strain ST4/74**. The log ratio survival of single-gene knock out mutants in *S*. Typhimurium strain ST4/74 compared to the wild type strain was assessed during 24-hour desiccation at 21°C and 39% relative humidity. There was no recovery of ST4/74Δ*dam*.

5.3.3 The *rnr* gene is essential for survival of *S*. Typhimurium strain ST4/74 during heat stress

The single-gene knock out mutants generated in *S*. Typhimurium strain ST4/74 were subjected to heat inactivation at 60°C for 30 seconds and compared to the survivability of the wild type, to evaluate the phenotype of each mutant during the stress condition (**Figure 48**). Although there was a lot of variation observed amongst replicates for each mutant, ST4/74Δ*rnr* was significantly different to the wild type (p = 0.0292) and exhibited a ~2.9-log reduction in cell viability (**Figure 48**). The log-reduction in cell viability observed in the *rnr* mutant coincides with the logFC (-4.81) value obtained for this gene during heat inactivation of the transposon library (**Table 31**). Due to the amount of variation observed between replicates, none of the other mutants were significantly different to the wild type. The log reduction observed by the *dam*, *zur* and *proP* mutants were 2.7, 1.9 and 1.2, respectively, and their associated logFC values were -2.7, 0.05 and 0.09, respectively (**Figure 48**) (**Table 31)**. Although the logFC for the *rfaB* mutant was 1.98, and therefore the mutant should survive better than the wild type, the mutant was in fact sensitive to heat inactivation and resulted in a 1.5-log reduction in cell viability (**Figure 48**) (**Table 31**).

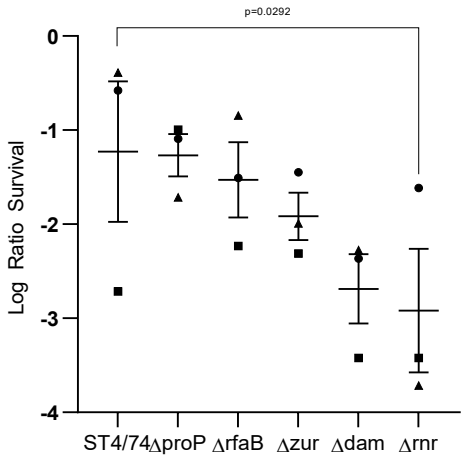

Figure 48. Effect of heat inactivation on isogenic mutants in *S*. Typhimurium strain ST4/74. The log ratio survival of single-gene knock out mutants in *S*. Typhimurium strain ST4/74 compared to the wild type strain was assessed during heat inactivation at 60°C for 30 seconds.

5.3.4 The *rnr* gene is essential for survival of *S*. Typhimurium strain ST4/74 during cold-storage stress

To assess whether the genes knocked out in *S*. Typhimurium strain ST4/74 are essential for survival during extended cold storage, the mutants were subjected to incubation at 4°C in the vegetarian food product for 5 weeks, and surviving colonies were enumerated (**Figure 49**). The *rnr* mutant had the greatest log reduction in cell survival, at ~0.8-log, and was significantly different to the wild type ($p < 0.0001$), however this mutant did not have the greatest negative logFC out of all the mutant candidate genes in the transposon library comparison and the logFC reported for *rnr* was -1.37 (**Table 31**). *S*. Typhimurium strain ST4/74Δ*dam* exhibited a ~0.6-log reduction in cell viability after 5-week incubation at 5°C, and the *dam* gene in the transposon library had a -1.58 logFC compared to the control during long term cold storage (**Figure 49**) (**Table 31**). The *zur* mutant phenotype was also significantly different to the wild type control ($p = 0.019$) and exhibited a ~0.45-log reduction in cell viability. The *zur* gene had a logFC of -0.34 compared to the control (**Figure 49**) (**Table 31**). Surprisingly, *proP* had the greatest negative logFC out of all mutants included in the study at -5.58 (**Table 31**), however there was no significant phenotypic difference between this mutant and the wild type during 5-week refrigerated storage and only a ~0.19-log reduction in cell viability was observed for this strain (**Figure 49**). *S*. Typhimurium strain ST4/74Δ*rfaB* had a positive logFC of 2.43 compared to the control during 5-week refrigerated storage (**Table 31**), however this mutant was affected by extended exposure to refrigerated

temperatures and suffered a 0.45-log reduction in cell viability (**Figure 49**). The wild type strain only reduced in cell viability by 0.16-log during the 5-week experiment, which is comparable to what was observed previously for survival of *S*. Typhimurium strain ST4/74 during cold storage in chapter 2. Overall, the viability of *rnr*, *dam, rfaB* and *zur* mutants were all significantly reduced compared to the wild type strain, and hence these genes are likely to be essential for long term refrigerated storage.

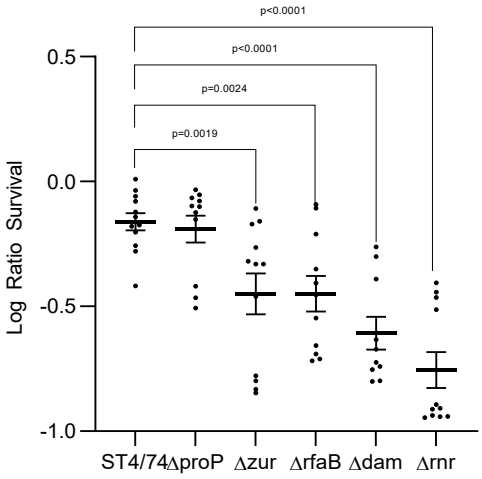

Figure 49. Effect of long term refrigerated storage on isogenic mutants in *S*. Typhimurium strain ST4/74. The log ratio survival of single-gene knock out mutants in *S*. Typhimurium strain ST4/74 compared to the wild type strain was assessed during 5-week refrigerated storage at 4°C.

5.3.5 The *dam* gene is essential for survival of *S*. Typhimurium strain ST4/74 during NaCl stress

To establish whether the mutant gene candidates identified during TraDIS experiments in *S*. Typhimurium strain ST4/74 were essential for survival in salt stress, the mutants were grown in LB broth supplemented with 6% NaCl for 24 hours, alongside a wild type control, and the optical density at 600nm was measured (**Figure 50**). The mutant strains had a similar growth phenotype to the wild type in LB broth (no NaCl) and detectable growth was initiated after ~3 hours incubation for the wild type and mutant strains, however the *dam* mutant had a slightly longer lag phase than the other mutant strains and growth in this strain was first detectable after 5 hours incubation (**Figure 50A**). Detectable growth was initiated after ~8 hours for the wild type strain in LB broth supplemented with 6% NaCl, however there was a slightly extended lag phase observed for the mutant strains and log-phase was initiated after

~11 hours incubation at 37°C (**Figure 50B**). The *dam* mutant had the greatest lag-phase out of all the strains included in this study, and began growing after ~22 hours of incubation at 37°C in LB broth supplemented with 6% NaCl, however, the growth phenotype of this strain in LB broth (no salt) was similar to the wild type (**Figure 50)**

The logFC value for the *dam* gene in the transposon library after exposure to 6% NaCl was -13.28 compared to the control, consistent with the lack of growth observed for the defined mutant (**Table 31**). However, the mutant with the greatest negative logFC during 6% NaCl exposure was *proP* (-14.29), but the growth phenotype for this strain was similar to the *rnr* and *zur* mutants, which only had a logFC of -3.35 and -4.57 in the transposon library, respectively (**Figure 50**) (**Table 31**). The only gene candidate included in the study which had a positive logFC during 6% NaCl stress in the TraDIS experiments was *rfaB* (logFC = 4.24), however, this strain exhibited reduced growth in LB broth supplemented with 6% NaCl, and reached a lower OD600nm than the other mutant strains included in the study (**Figure 50**) (**Table 31**).

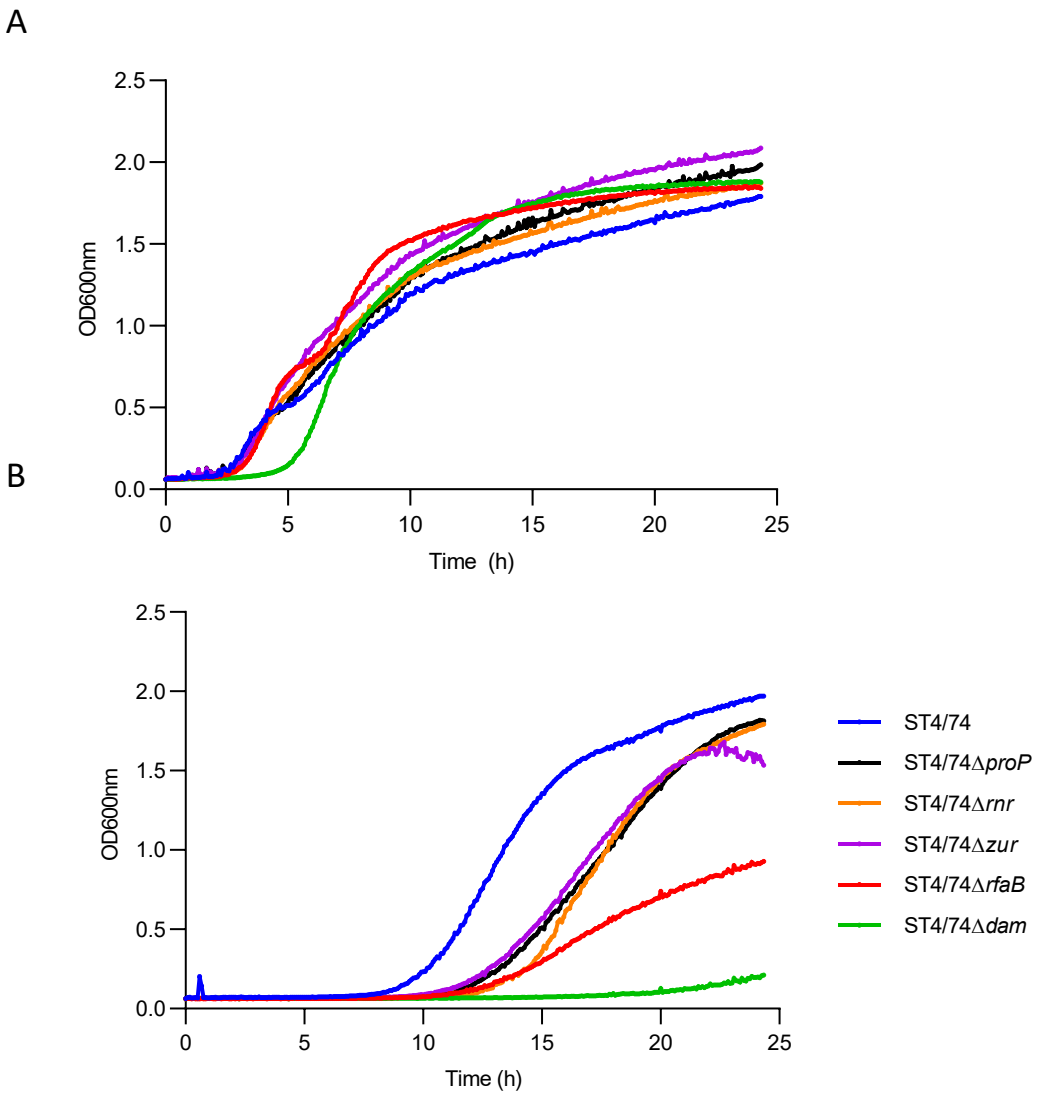

Figure 50. **Effect of NaCl on growth of isogenic mutants in *S.* Typhimurium strain ST4/74.** Growth of *S.* Typhimurium strain ST4/74 single gene knock out mutants measured as OD600nm in (A) LB broth and (B) LB broth supplemented with 6% NaCl, for 24 hours at 37°C. The data represents the mean of three biological replicates.

The maximum OD600nm and maximum generation time, in hours, during growth of each mutant strain and the wild type in LB broth containing 6% NaCl was calculated using Growthcurver R package (**Figure 51**). The maximum optical density reached for the *S.* Typhimurium ST4/74 wild type strain was greater in 6% NaCl compared to the LB broth control at 1.8 and 1.4, respectively (**Figure 51A**). The ST4/74 wild type strain had a shorter generation time in LB broth containing 6% NaCl, which was calculated at 1 hour 8 minutes, whereas the generation time in LB broth was 1 hour 45 minutes. The *proP* and *rnr* mutants also reached a greater OD600nm and exhibited a shorter generation time in 6% NaCl

compared to LB broth, although not significantly different to the wild type strain. The maximum OD600nm achieved for ST4/74Δ*zur* was similar in both media, however the generation time was quicker in 6% NaCl for this mutant (not significant) (**Figure 51)**. ST4/74Δ*dam* only reached an OD600nm of approximately 0.35 in 6% NaCl, which was significantly different to the wild type strain ($p < 0.0001$). The growth phenotype of this strain in LB broth was not significantly different to the other mutant strains and wild type, reaching an OD600nm of 1.7. ST4/74Δ*rfaB* reached an OD600nm of ~0.84 in 6% NaCl, which was significantly different ($p < 0.0001$) than the wild type strain (**Figure 51**).The generation time for ST4/74Δ*dam* in 6% NaCl was ~2 hours, which was longer than in LB broth, however, the *dam* mutant did not reach stationary phase during the 24-hour experiment and the generation time for the *dam* mutant was significantly different than the wild type in 6% NaCl ($p = 0.0009$) and LB broth ($p = 0.0116$). The generation time for the *rfaB* mutant in 6% NaCl and LB broth was similar, at approximately 1 hour and 19 minutes and 1 hour and 8 minutes, respectively, however the generation time of ST4/74Δ*rfaB* in LB broth was significantly different ($p = 0.0186$) to the wild type strain (**Figure 51B**).

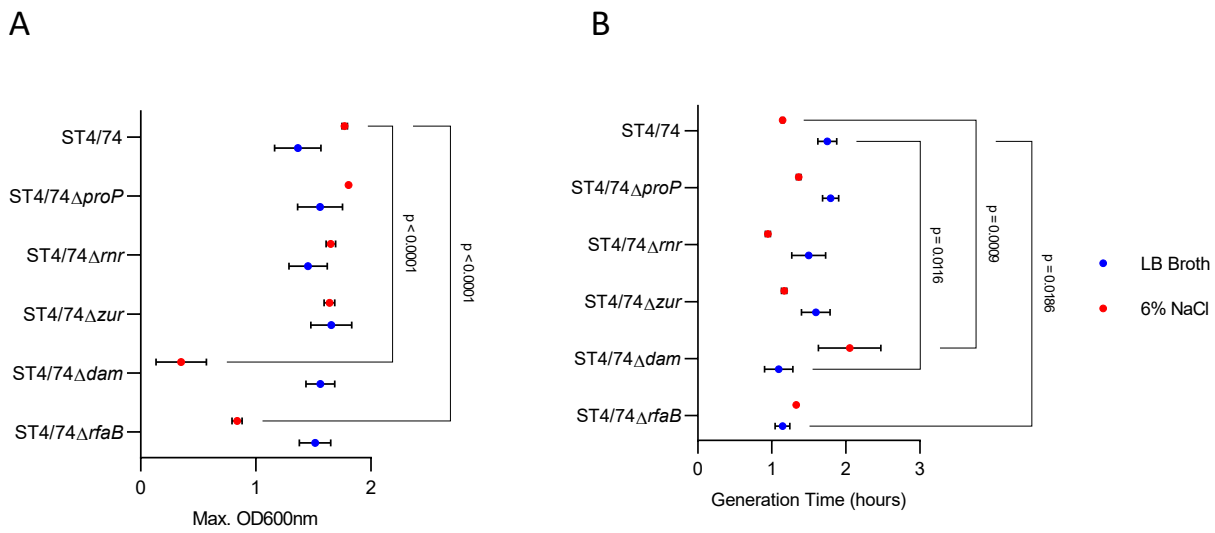

Figure 51. Effect of NaCl on growth rate of isogenic mutants. The (A) maximum OD600nm and (B) maximum generation time (hours) of *S*. Typhimurium strain ST4/74 wild type and mutants in LB broth and LB broth supplemented with 6% NaCl was assessed over a 24-hour period. The output contains the results from the Growthcurver package in R on the mean(±SEM) data of 3 biological replicates for each strain.

5.3.6 The *dam* and *rfaB* genes are essential for survival of *S.* Typhimurium strain ST4/74 during citric acid stress

To determine the genes essential for survival in citric acid stress, the survivability of *S*. Typhimurium strain ST4/74 single-gene knockout mutants were assessed in LB broth supplemented with 14mM citric acid by measuring growth at 37°C (**Figure 52**). All mutant strains and the wild type behaved similarly in LB broth, except ST4/74Δ*dam*, which exhibited a longer lag-phase of ~5 hours compared to the other strains (**Figure 52A**). In the ST4/74 wild type strain, growth was initiated after ~9 hours incubation with 14mM citric acid. The *proP* and *zur* mutants began growing after ~12 hours incubation in citric acid, and the *rnr* mutant was initiated after approximately 13 hours of incubation. The *dam* and *rfaB* mutants exhibited a similar growth phenotype and only started growing after ~18 hours incubation with citric acid (**Figure 52B**)

The growth curve phenotypes for each mutant corresponds with the logFC values obtained from the transposon mutant library. The *proP*, *zur* and *rnr* mutants all reached a similar OD600nm to the wild type strain and had logFC values of -0.21, -0.20 and -1.05, respectively, which were not considered to be significantly different from the control. The *rfaB* gene had a logFC of -1.07 compared to the control and was not considered to be an essential gene for citric acid survival in *S.* Typhimurium strain ST4/74. However, it is evident by looking at the growth curves that deleting this gene has a deleterious effect on growth in LB broth supplemented with 14mM citric acid, as the maximum OD600nm reached was only ~0.3 (**Figure 52B**). The *dam* gene had the greatest negative logFC out of all the mutants included in this study (-2.13) and was greatly affected by the presence of citric acid, as evidenced by the minimal growth observed during the 24-hour experiment (**Figure 52B**).

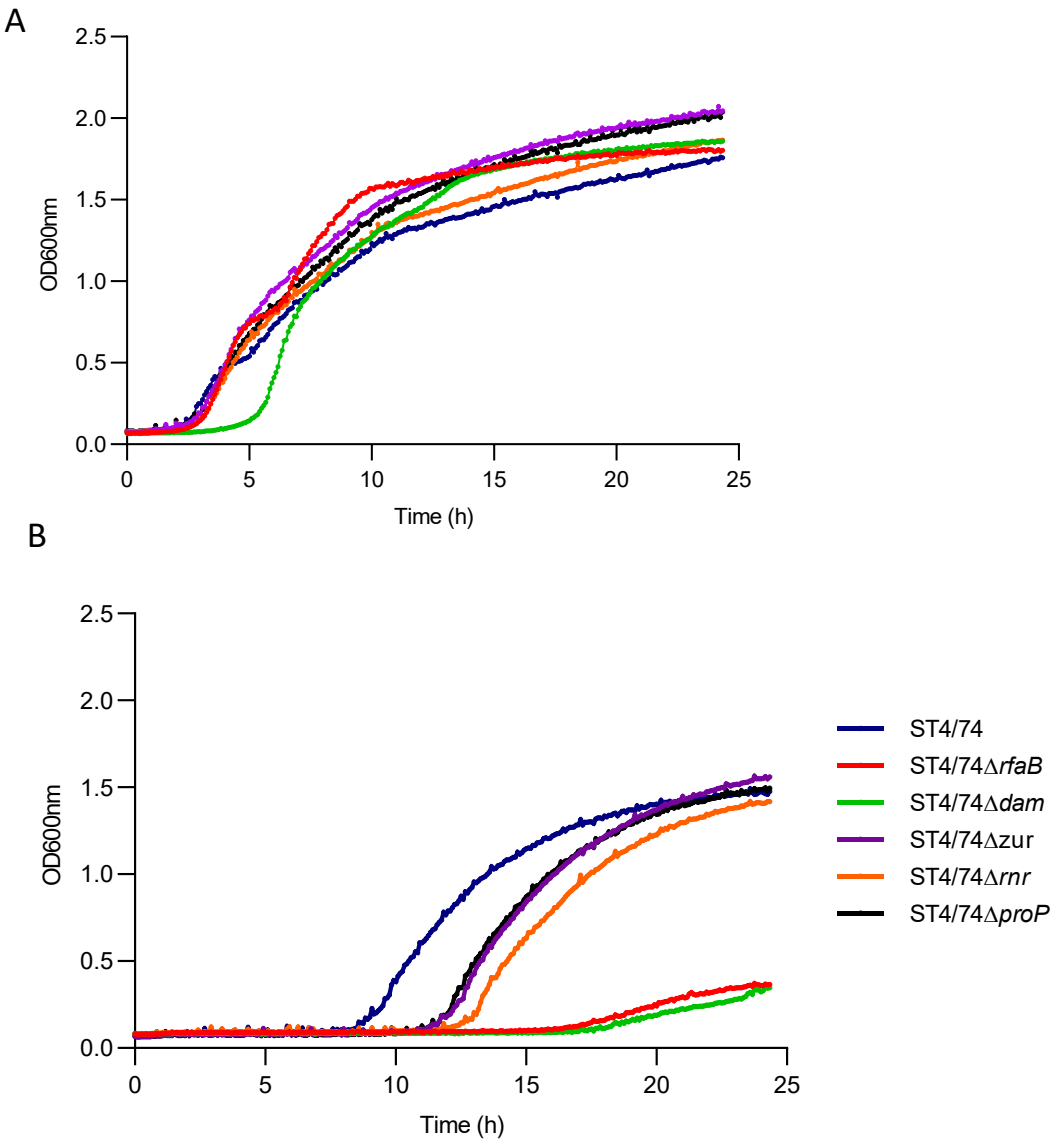

Figure 52. **Effect of citric acid on growth of isogenic mutants in *S*. Typhimurium strain ST4/74.** The growth of *S*. Typhimurium strain ST4/74 single gene knock out mutants and wild type strain was measured as OD600nm in (A) LB broth and (B) LB broth supplemented with 14mM citric acid, for 24 hours at 37°C. The data represents the mean of three biological replicates.

The growth curve kinetics of *S*. Typhimurium strain ST4/74 mutants were evaluated using the Growthcurver package in R to determine maximum OD600nm and generation time during citric acid stress (**Figure 53A**). The maximum OD600nm for the wild type strain was similar in LB broth and LB broth supplemented with 14mM citric acid (**Figure 53A**). The maximum OD600nm for all other strains was greater in LB broth compared to LB broth supplemented with 14mM citric acid. ST4/74Δ*dam* and ST4/74Δ*rfaB* had the greatest difference in

OD600nm between the two growth media, and the OD600nm for these mutants in 14mM citric acid were significantly different than the wild type at 0.49 ($p = 0.0003$) and 0.41 ($p < 0.0001$), respectively (**Figure 53A**). *S*. Typhimurium strain ST4/74Δ*rfaB* had the longest generation time out of all the mutants included in the study, which was significantly different than the wild type strain ($p = 0.0240$). The generation time in 14mM citric acid for Δ*proP*, Δ*rnr* and Δ*zur* were similar to the wild type, and the generation time for Δ*dam* was the quickest reported, however this strain failed to reach stationary phase within 24 hours (**Figure 53B**).

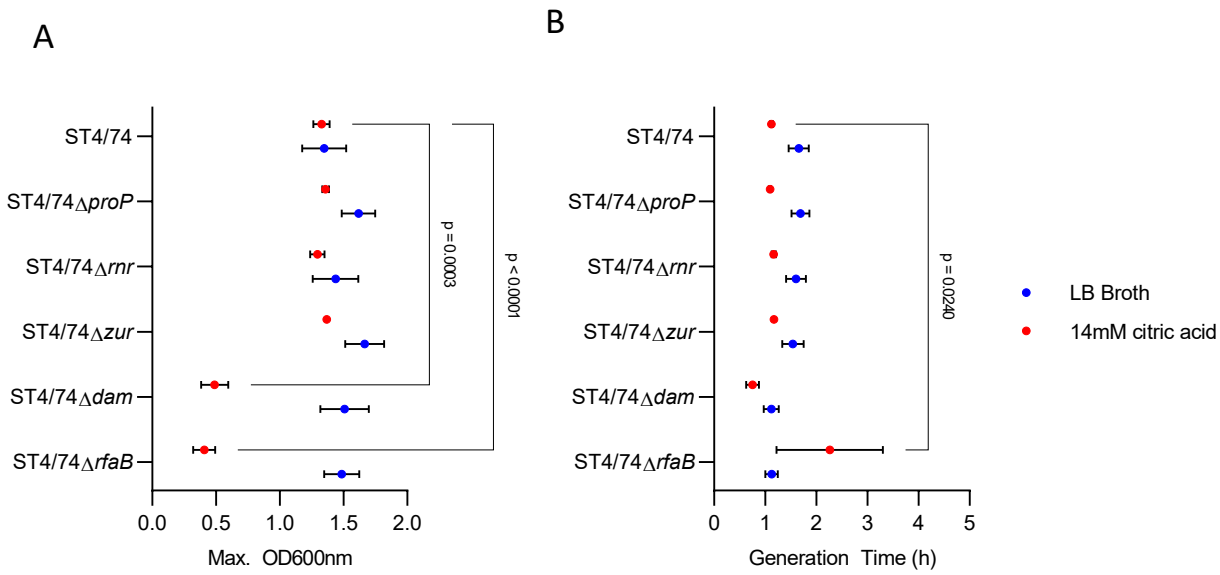

Figure 53. Effect of citric acid on the growth rate of isogenic mutants in *S*. Typhimurium strain ST4/74. The (A) maximum OD600nm, and (B) maximum generation time (hours) of *S*. Typhimurium strain ST4/74 wild type and mutants in LB broth and LB broth supplemented with 14mM citric acid was assessed over a 24-hour period. The output contains the results from the Growthcurver package in R on the mean(±SEM) data of 3 biological replicates for each strain.

5.3.7 None of the single-gene knockout candidates in *S*. Typhimurium strain ST4/74 were essential for survival during acetic acid stress

Single-gene knockout mutants of the genes identified as essential in acetic acid stress from TraDIS data in *S*. Typhimurium strain ST4/74 were subjected to 8mM acetic acid, and the OD600nm of each mutant was monitored to determine whether the genes identified as conditionally essential had a phenotype effect on the strain during acetic acid stress. The

wild type and mutant strains had similar asymptotic shaped growth curves in LB broth (**Figure 54**). The lag-phase for ST4/74Δ*dam* was longer than the other strains in LB broth and growth was initiated after approximately 5 hours (**Figure 54A**). The mutants and wild type strain exhibited an increased exponential growth phase in 8mM acetic compared to LB broth. All strains had a slightly longer lag-phase in LB broth supplemented with 8mM acetic acid (**Figure 54B).** The logFC values obtained from TraDIS data correspond with the phenotype observed for each mutant in 8mM acetic acid. ST4/74Δ*rfaB* had a positive logFC of 7.39, and this strain reached a greater maximum OD600nm than the wild type strain. The Δ*proP*, Δ*dam* and Δ*zur* strains had negative logFC values in the TraDIS library during acetic acid stress, however the values were not less than -2, and were not deemed significant. The growth profile for these strains was similar to the wild type, which agrees with the TraDIS logFC data. *S*. Typhimurium strain ST4/74Δ*rnr* had a logFC of -4.85, however this strain did not show reduced growth during exposure to 8mM acetic acid (**Figure 54B**).

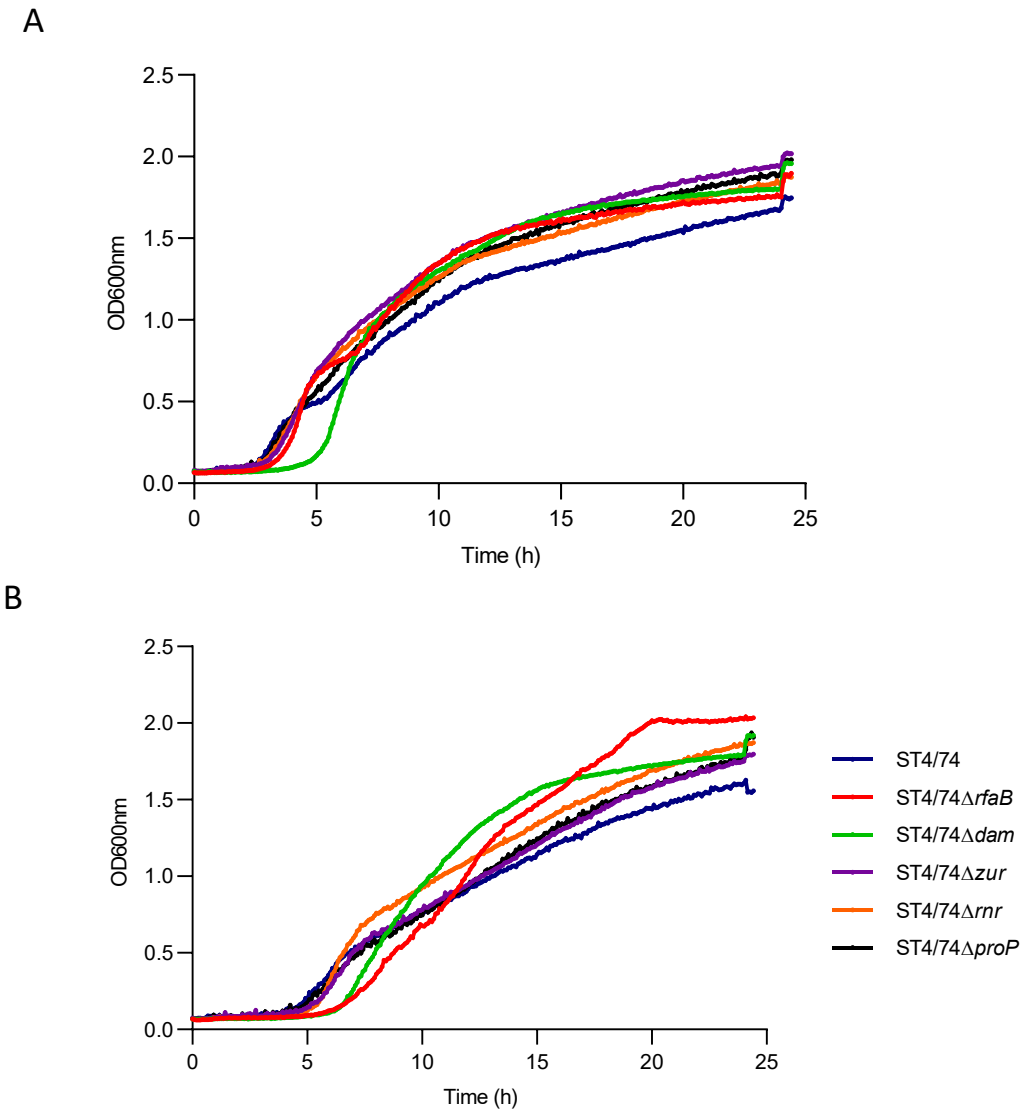

Figure 54. **Effect of acetic acid on growth of isogenic mutants in *S*. Typhimurium strain ST4/74.** The growth of *S*. Typhimurium strain ST4/74 single-gene knock out mutants and wild type strain was measured as OD600nm in (A) LB broth and (B) LB broth supplemented with 8mM acetic acid, for 24 hours at 37°C. The data represents the mean of three biological replicates.

An R package, called Growthcurver, was used to quantify the generation time and maximum OD600nm for *S*. Typhimurium strain ST4/74 wild type and mutant strains during growth in 8mM acetic acid (**Figure 55**).The maximum OD600nm reached for ST4/74, ST4/74Δ*proP*, and ST4/74Δ*dam*, was approximately the same in both types of media, however a greater OD600nm was observed during 8mM acetic acid growth for the *rfaB* mutant and was significantly different ($p < 0.0001$) to the wild type (**Figure 55A**). The *proP* mutant maximum OD600nm during growth in LB broth was significantly different ($p = 0.0163$) to the control. ST4/74Δ*rnr* reached an OD600nm of 1.7 in 8mM acetic acid, which was significantly different

to the wild type (p = 0.0274) and reached an OD600nm 1.61 in LB broth. The *zur* mutant reached a greater maximum OD600nm in LB broth compared to 8mM acetic acid, at 1.7 and 1.6, respectively, and the OD600nm reached in LB both was significantly different to the wild type strain (p = 0.0065) (**Figure 55A)**. The generation time for all strains was longer in LB broth supplemented with 8mM acetic acid compared to the LB broth control (**Figure 55B**) ST4/74Δ*dam* and ST4/74Δ*rfaB* had significantly quicker generation times compared to the wild type control during growth in 8mM acetic acid (p < 0.0001) and LB broth (p < 0.0001 and p = 0.010, respectively) (**Figure 55B**).

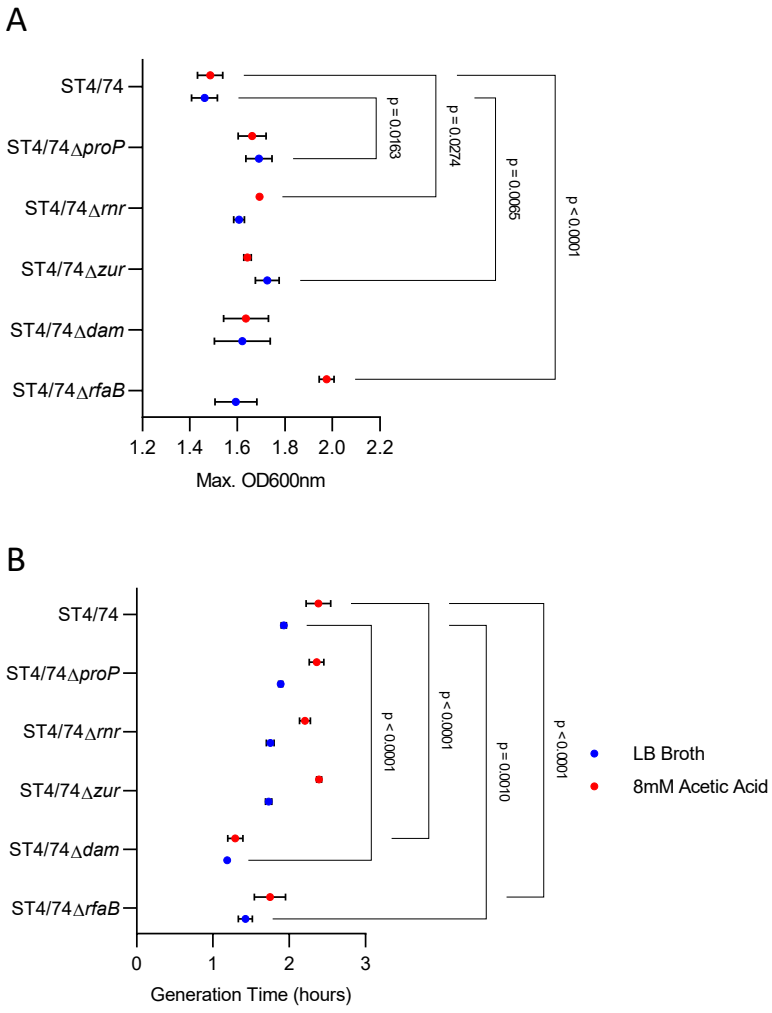

Figure 55. **Effect of acetic acid on the growth rate of isogenic mutants in *S*. Typhimurium strain ST4/74.** The (A) maximum OD600nm, and (B) maximum generation time (hours) of *S*. Typhimurium strain ST4/74 wild type and mutants in LB broth and LB broth supplemented with 8mM acetic acid was assessed over a 24-hour period. The output contains the results from the Growthcurver package in R on the mean(±SEM) data of 3 biological replicates for each strain.

5.3.8 ST4/74Δ*dam* and ST4/74Δ*rfaB* exhibited reduced growth in 6% NaCl and 14mM citric acid

To determine whether the growth curves obtained for the mutant strains of *S*. Typhimurium ST4/74 in each stress condition were significantly different to the wild type, the difference in area under the curve (AUC) was calculated from the mean growth curve data of each replicate in LB broth and during stress (**Figure 56**). A two-way ANOVA, with an uncorrected Fisher's Least Significant Difference (LSD) test, was conducted in Graphpad Prism (version 9.4.0) to establish significance. All of the mutant strains were significantly different to the wild type in 6% NaCl and 14mM citric acid ($p < 0.05$). None of the mutant strains of ST4/74 had a significant difference in AUC compared to the wild type during 8mM acetic acid stress (**Figure 56**). ST4/74Δ*dam* had the greatest difference in AUC in 6% NaCl also the largest negative logFC (-13.26) during 6% NaCl stress compared to LB broth, and ST4/74Δ*rfaB* had the greatest difference in AUC during 14mM citric acid growth and a significant ($q < 0.05$) logFC value of -1.07 (**Figure 56**). The strain with the greatest difference in AUC during acetic acid stress was ST4/74Δ*rnr*, although not phenotypically significantly different than the wild type strain, the logFC value for this mutant was -4.35 in acetic acid. ST4/74Δ*rnr* had the smallest difference in AUC during 6% NaCl and 14mM citric acid stress (**Figure 56**).

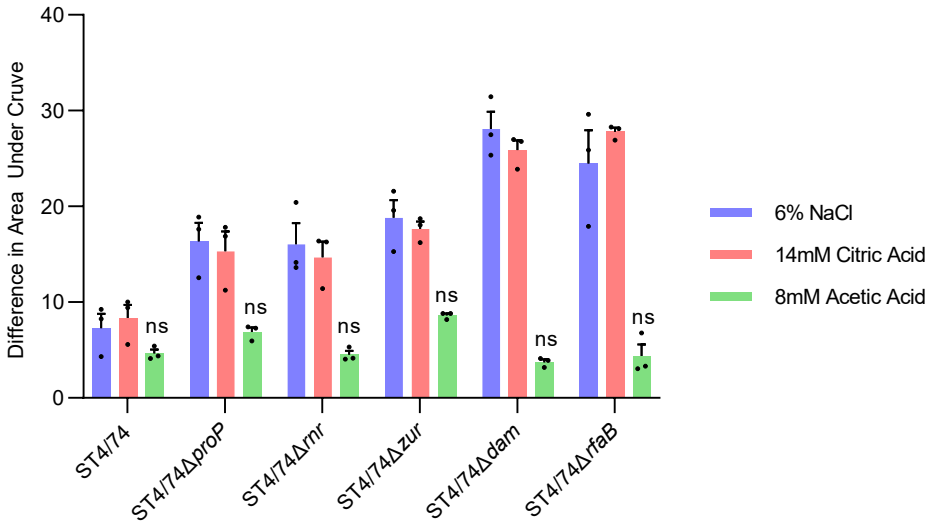

Figure 56. Area under the curve for each isogenic mutant during stress. The difference in area under the curve (AUC) for *S*. Typhimurium strain ST4/74 single-gene knockout mutants grown in LB broth compared to LB broth supplemented with 6% NaCl, 12mM acetic acid or 8mM citric acid. Bars represent the mean of three biological replicates (±SEM). All difference in AUC values were significantly different to the wild type strain ($p < 0.05$, two-way ANOVA test) except the ones denoted with ns (not significant).

5.3.9 The *dam* and *rfaB* genes are essential for survival during food chain related stress in *S.* Typhimurium strain ST4/74

To summarise the phenotypic data obtained for each single-gene knockout mutant of *S.* Typhimurium strain ST4/74 in food chain related stress, a heat map was generated in R (version 4.0.2) to display the log ratio survival and maximum OD600nm reached of each strain (**Figure 57**). The *dam* mutant had a large reduction in cell survival during desiccation and heat inactivation compared to the other mutants and was unable to reach as high an OD600nm as some of the other mutants during 14mM citric acid and 6% NaCl stress (**Figure 57**). ST4/74Δ*rfaB* also exhibited a reduced growth phenotype in 14mM citric acid, 6% NaCl stress and a higher reduction in log ratio survival than some other mutants during desiccation and heat inactivation. Overall, mutants had the greatest log reduction in cell viability during desiccation than during 5-week refrigerated storage and heat inactivation. A lower maximum OD600nm was achieved by mutants during citric acid exposure, than in 6% NaCl and 8mM acetic acid (**Figure 57**).

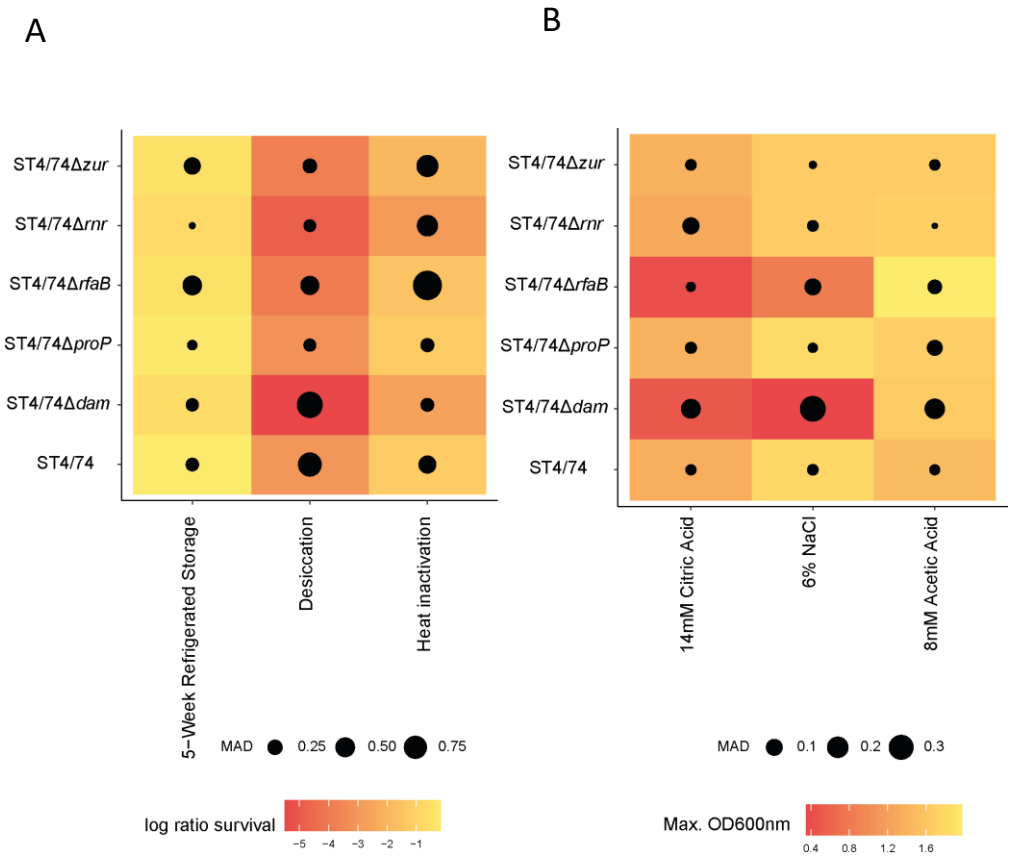

Figure 57. Heatmap summary of *S.* Typhimurium strain ST4/74 mutant phenotypes during food chain related stress. (A) Mean log ratio survival of ST4/74 mutants during 5-week refrigerated storage, desiccation, and heat inactivation and (B) Maximum OD600nm of ST4/74 mutants during growth at 37°C in LB broth supplemented with 14mM citric acid, 6% NaCl and 8mM acetic acid. Black circles represent the mean absolute deviation (MAD).

5.3.10 Presence and absence of conditionally essential genes in other *Salmonella* strains

To establish whether phenotypic variation in response to food chain related stresses in *Salmonella enterica* strains can be explained due to genomic differences, the presence, absence, and functional divergence of conditionally essential genes from TraDIS experiments were identified in the set of 14 *Salmonella* strains used throughout this study. Roary was used to construct the pangenome of the *Salmonella* strains and the presence and absence of conditionally essential genes for survival of *S.* Typhimurium strain ST4/74 were identified. Another method, called ARIBA, was used to verify the presence and absence of conditionally essential genes identified using roary. Deltabitscore was used to identify genes which may be non-functional. Roary and ARIBA were used to evaluate the potential role of the accessory genome in stress variation, whilst DBS identified sequence polymorphisms in the core or accessory genome. The gene presence and absence data was plotted alongside the log ratio survival data for heat inactivation, desiccation and 5-week refrigerated storage, and against the difference in AUC data for 6% NaCl, 8mM acetic acid and 14mM citric acid to assess the phenotype of each strain during stress. A maximum likelihood phylogenetic tree was also included to identify phylogenetic signals for stress response. Only genes absent in both Roary and ARIBA analysis were evaluated during this study.

5.3.10.1 Lack of evidence for phenotypic variability in survival during refrigerated storage of *Salmonella enterica* strains due to polymorphisms in conditionally essential genes in *S*. Typhimurium strain ST4/74

For 5-week refrigerated storage, all of the genes absent from the strains included in this study had a positive logFC value in *S*. Typhimurium strain ST4/74 and none of the genes were considered to be essential for survival. There was no correlation between strains with an increased resistance or sensitivity to 5-week refrigerated storage and phylogenetic relatedness (**Figure 58**). The strain with the greatest reduction in cell viability over the 5-week period was *S.* Gallinarum strain 287/91. The only gene absent in this Gallinarum strain compared to ST4/74 was the *rfbI* gene, which is involved in lipopolysaccharide biosynthesis and had a positive logFC of 1.42 and a q-value of < 0.05. This gene was also absent in all strains except the Typhimurium strains, so does not explain the increased sensitivity to cold stress observed by *S*. Gallinarum strain 287/91 (**Figure 58**).

An O-antigen transferase, *rfbP* (5.36 logFC, q < 0.05) was predicted to be non-functional in *S*. Newport strain SL254, although this Newport strain did not exhibit increased tolerance to extended storage at refrigerated temperature (**Figure 58**). The *rfaK* gene, involved in LPS biosynthesis (5.94 logFC, q < 0.05) was predicted to have lost its function in *S*. Infantis strain S1326/28 and both Newport strains (**Figure 58**). *S*. Kentucky strain SL479 and S. Newport strains SL254 and SGSC4157 were predicted to have a potentially non-functional *rfaL* (5.78 logFC, q < 0.05) gene according to deltabitscore and this gene was predicted to be absent in *S*. Kedougou strain B37 Col9 and *S*. Infantis strain S1326/28 by both roary and ARIBA (**Figure 58**).

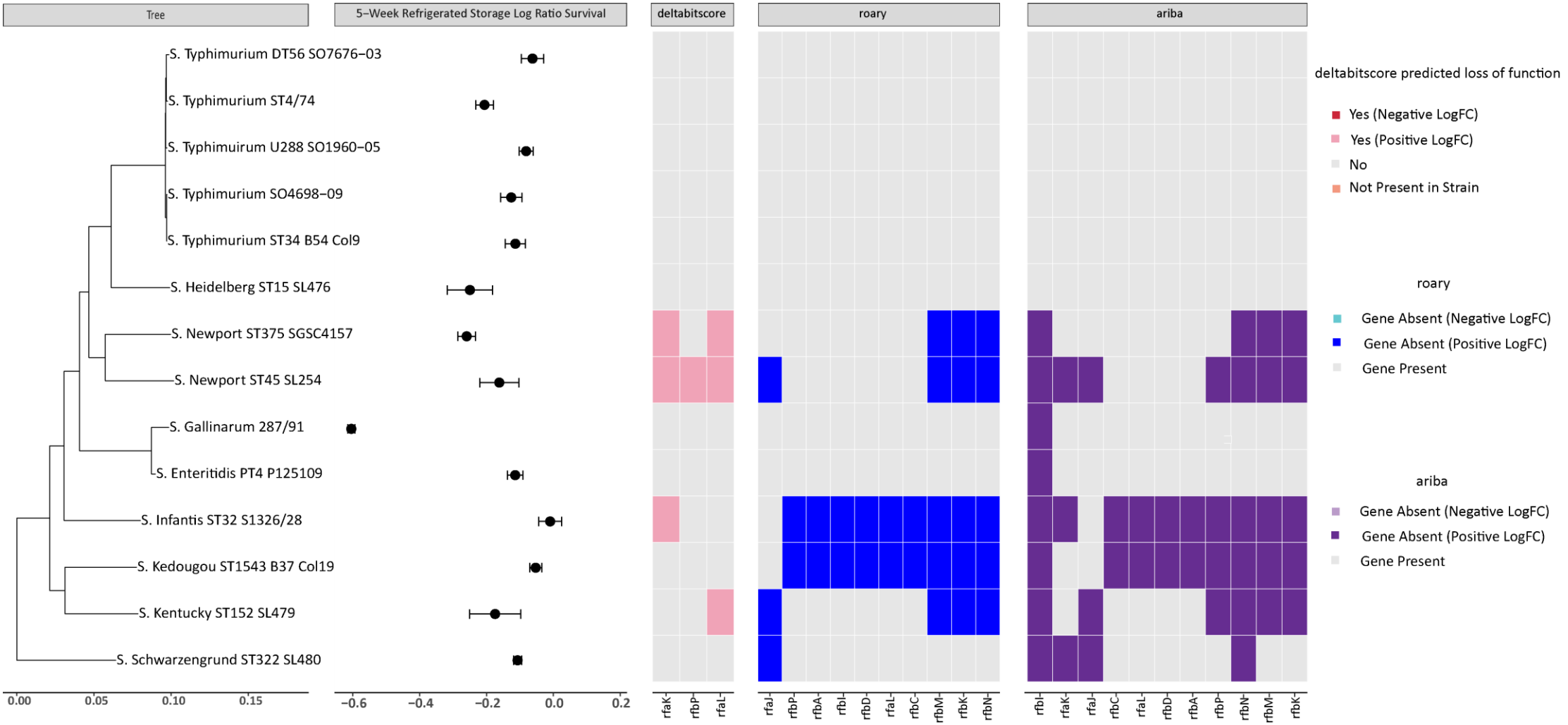

Figure 58. Prediction of conditionally essential genes in other *Salmonella* strains during 5-week refrigerated storage. The presence, absence, and loss of function of conditionally essential genes in *S*. Typhimurium strain ST4/74 during 5-week refrigerated storage for other *Salmonella* strains, alongside a maximum likelihood phylogenetic tree generated using RaxML and log ratio survival data.

5.3.10.2 Loss of function in *visC* may contribute to sensitivity to desiccation in *S.* Kentucky strain SL479

Response to desiccation was not related to phylogeny in the strains included in the current study (**Figure 59**). *S*. Typhimurium strain SO1960-05 and *S.* Kentucky strain SL479 had the biggest log reduction in cell viability compared to other strains (**Figure 59**). *S*. Kentucky strain SL479 was predicted to have a diverged *visC* (*ubiI*) gene (-2.37 logFC), which is involved in ubiquinone biosynthesis, that was not predicted to be non-functional or absent in any other strains (**Figure 59**). There were no genes exclusively absent or non-functional in *S*. Typhimurium SO1960-05 which could explain the increased sensitivity to desiccation observed. Additionally, there were no genes predicted to be non-functional in both strains showing an increased sensitivity to desiccation. The two monophasic *S*. Typhimurium strains had an increased tolerance to desiccation, but there were no genes exclusively absent in these strains that had a positive logFC from the TraDIS comparison data, which could explain the increased tolerance. *S*. Heidelberg strain SL476 exhibited a ~2-log reduction in cell viability during desiccation stress and this strain was predicted to have a non-functional *hfq* gene (-4.82 logFC), which functions as an RNA-binding protein. Deltabitscore predicted *orn* (-9.29 logFC) to be non-functional in *S*. Gallinarum strain 287/91 and *orn* functions as an oligoribonuclease (**Figure 59**).

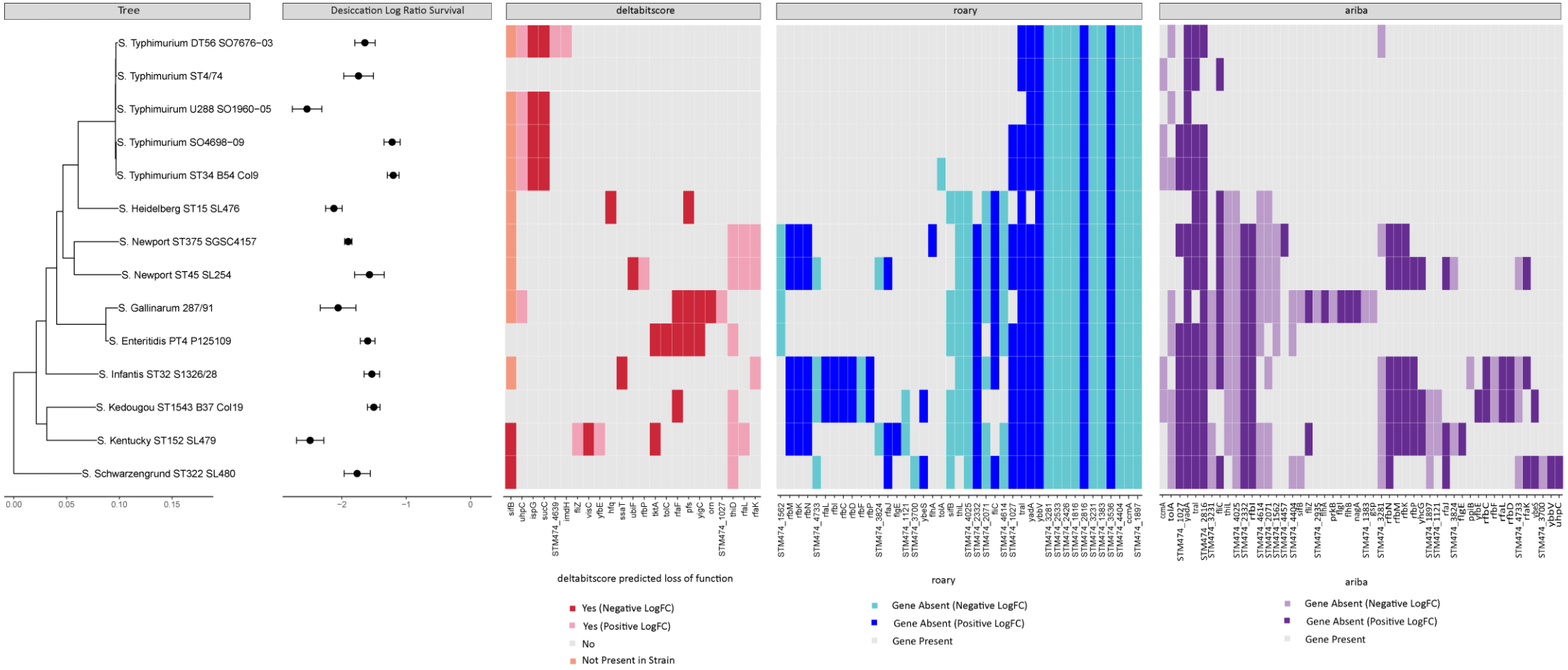

Figure 59. **Prediction of conditionally essential genes in other *Salmonella* strains during 24-hour desiccation.** The presence, absence, and loss of function of conditionally essential genes in *S*. Typhimurium strain ST4/74 during 24-hour desiccation for other *Salmonella* strains, alongside a maximum likelihood phylogenetic tree generated using RaxML and log ratio survival data.

5.3.10.3 Lack of evidence for phenotypic variability during heat stress of *Salmonella enterica* strains due to polymorphisms in conditionally essential genes in *S.* Typhimurium strain ST4/74

There was no correlation between heat resistance and phylogenetic relatedness in the 14 *Salmonella* strains included in this study (**Figure 60**). *S*. Gallinarum strain 287/91 had the greatest log reduction in cell viability at 60°C out of all the strains tested. There were no genes identified as essential for survival during heating at 60°C for 30 seconds in S. Typhimurium strain ST4/74 that were absent from the genome of *S*. Gallinarum strain 287/91 that could explain the sensitivity observed to heat stress. *S*. Kedougou strain B37 Col19 and *S*. Infantis strain S1326/28 were absent in multiple genes which had a positive logFC in S. Typhimurium strain ST4/74 and could explain the increased resistance to heat observed by these two strains (**Figure 60**). *S*. Enteritidis strain P125109 resulted in a ~0.75-log reduction in cell viability during heating at 60°C for 30 seconds, and this strain was predicted to have a non-functional *tolC* gene, which encodes the TolC protein that functions as an outer membrane efflux pump. From the TraDIS comparison data, *tolC* had a logFC of -2.67 and a significant q-value of < 0.05 in *S*. Typhimurium strain ST4/74 during heat inactivation at 60°C for 30 seconds. (**Figure 60**).

The O-antigen transferase, *rfbP* had a positive logFC in *S*. Typhimurium strain ST4/74 of 5.18 (q < 0.05) and was predicted to be non-functional in *S*. Newport strain SL254, but was absent from the genome of *S*. Kentucky strain SL479, *S*. Kedougou strain B37 Col19, *S*. Infantis strain S1326/28, and *S*. Newport strain SL254 in both roary and ARIBA (**Figure 60**). The O-antigen ligase, *rfaL* (4.56 logFC, q < 0.05) was predicted to be degraded in *S*. Kentucky strain SL479 and *S*. Newport strains SL254 and SGSC4157 in the deltabitscore analysis and this gene was absent in *S*. Kedougou strain B37 Col19 and S. Infantis strain S1326/28 in both roary and ARIBA analyses. Deltabitscore predicted *rfaK* (5.28 logFC, q < 0.05) to be non-functional in *S.* Infantis strain S1326/28 and *S*. Newport strains SL254 and SGSC4157 (**Figure 60**). The *yafB* gene (2.44 logFC, q < 0.05) was absent from all strains except *S*. Newport strain SGSC4157 and *S*. Heidelberg strain SL476 and this gene is methylglyoxal reductase involved in methylglyoxal degradation and ketogluconate metabolism (**Figure 60**).

The *traG,* a conjugal transfer mating pair stabilisation protein (2.86 logFC, q < 0.05) was missing in all strains except *S*. Typhimurium strain SO1960-05 in both roary and ARIBA analyses (**Figure 60**). The flagellin gene, *fliC* (2.10 logFC, q < 0.05) was absent in *S*. Schwarzengrund strain SL480, *S*. Infantis strain S1326/28, *S*. Enteritidis strain P125109, *S*.

Gallinarum strain 287/91, *S*. Newport strains SL254 and SGSC4157, and *S*. Heidelberg strain SL476 (**Figure 60**). The lipopolysaccharide transferase, *rfaJ* (5.12, q < 0.05) was absent in *S*. Schwarzengrund strain SL480, *S*. Kentucky strain SL479 and *S*. Newport strain SL254. The absence of multiple LPS biosynthesis genes, *rfbD* (4.96 logFC, q < 0.05), *rfbC* (4.44 logFC, q < 0.05) and *rfaL* (4.56 logFC, q < 0.05), occured in *S*. Infantis strain S1326/28 and *S*. Kedougou strain B37 Col19 (**Figure 60**). Although there was genomic variation observed amongst the *Salmonella* strains included in this study, there were no genes in particular that could explain the increased tolerance or sensitivity to heat stress observed.

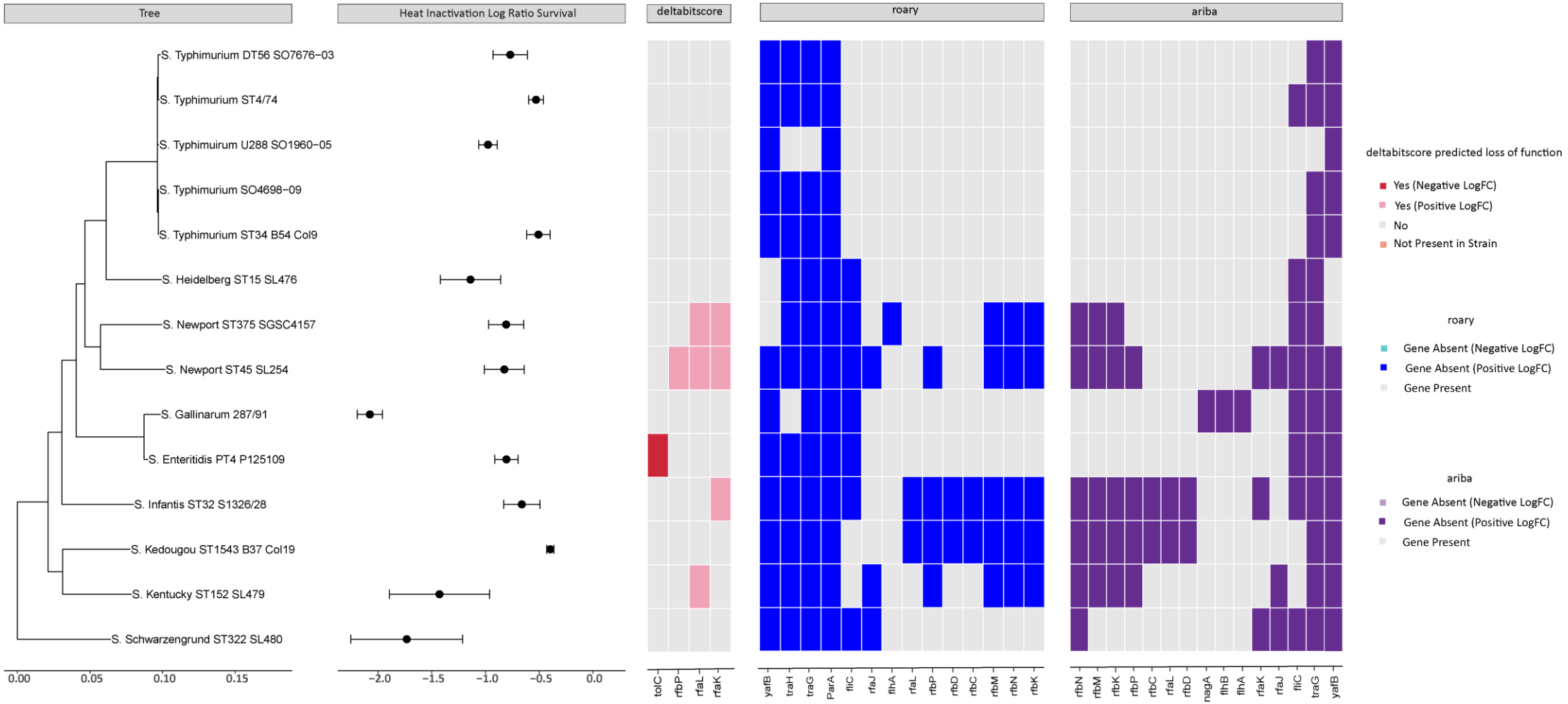

Figure 60. Prediction of conditionally essential genes in other *Salmonella* strains during heat inactivation at 60°C. The presence, absence, and loss of function of conditionally essential genes in *S*. Typhimurium strain ST4/74 during heat inactivation at 60°C for 30 seconds for other *Salmonella* strains, alongside a maximum likelihood phylogenetic tree generated using RaxML and log ratio survival data.

5.3.10.4 There is a lack of evidence for phenotypic variability during NaCl stress of *Salmonella enterica* strains due to polymorphisms in conditionally essential genes in *S*. Typhimurium strain ST4/74

S. Typhimurium strain SO1960-05 had the smallest different in area under curve (AUC) during 6% NaCl stress, whereas *S.* Gallinarum strain 287/91 exhibited the greatest difference in AUC. Two genes were predicted to have lost their function in *S.* Gallinarum strain 287/91, *flgA* and *rhtB*, according to the deltabitcore analysis. Both of these genes had positive logFC values indicating that loss of these genes would be beneficial for survival during NaCl stress in *S*. Typhimurium strain ST4/74 (**Figure 61**). *S*. Enteritidis strain P125109 had a predicted loss of function in the *ppia* gene (-3.33, $q < 0.05$), however this would explain this strains' increased resistance to NaCl. *S*. Schwarzengrund strain SL480 also had a large difference in AUC between growth in 6% NaCl and LB broth, and a predicted loss of function in *bamB* (-6.91 logFC, $q < 0.05$) and *infB* (-6.56 logFC, $q < 0.05$) genes according to the deltabitscore analysis. *S.* Schwarzengrund strain SL480 and *S*. Gallinarum strain 287/91 were both absent in STM474_2992 (-8.26 logFC, $q < 0.05$), and both strains did not survive well in NaCl (**Figure 61**). *S*. Schwarzengrund strain SL480 was missing the hypothetical protein, STM474_3700 gene (-1.31, $q < 0.05$), and this gene was 99.6% similar to an IcIR family transcriptional regulator in *Salmonella enterica*.

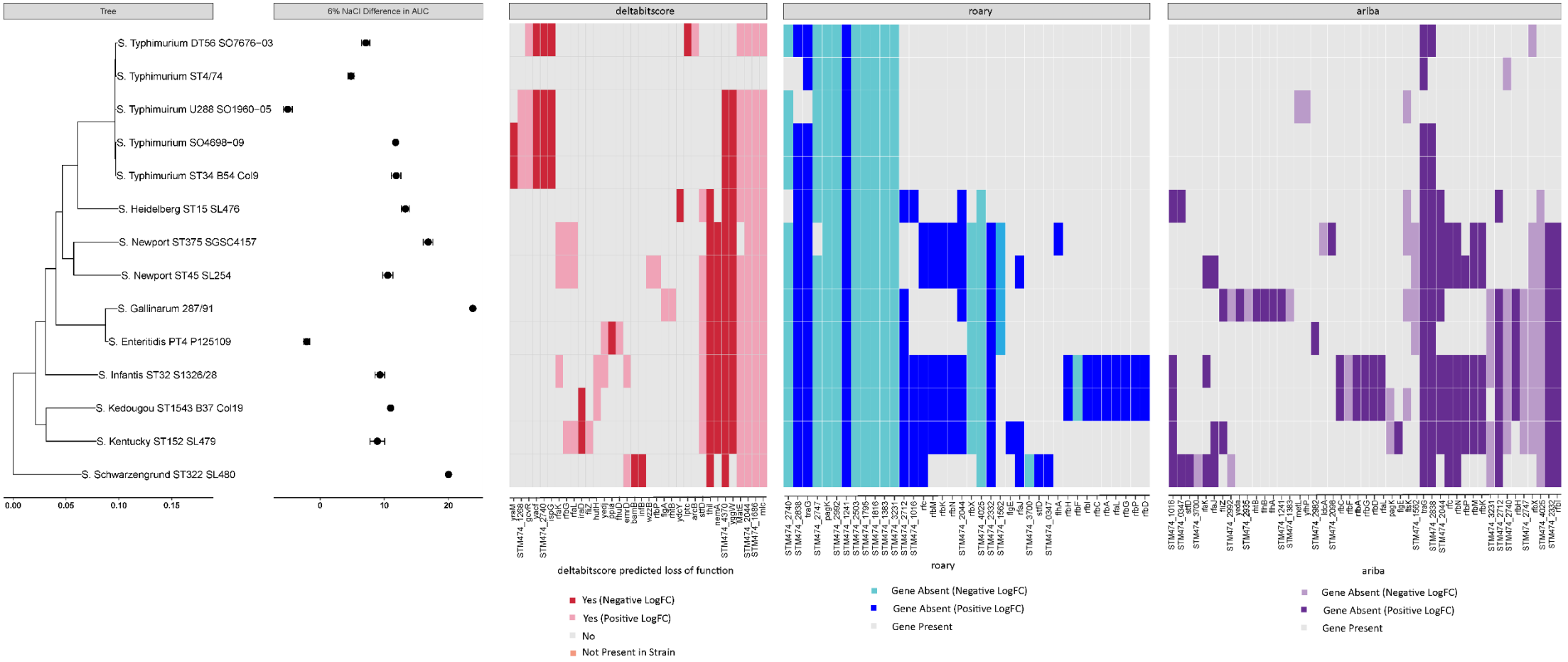

Figure 61. Prediction of conditionally essential genes in other *Salmonella* strains during salt stress. The presence, absence, and loss of function of conditionally essential genes in *S*. Typhimurium strain ST4/74 during 6% NaCl for other *Salmonella* strains, alongside a maximum likelihood phylogenetic tree generated using RaxML and area under the curve (AUC) data.

5.3.10.5 Loss of function of hypothetical protein STM474_3044 may increase sensitivity to acetic acid stress in *S*. Gallinarum strain 287/91

There was no correlation between sensitivity to acetic acid, as evidenced by a greater AUC, and phylogenetic relatedness (**Figure 62**). *S*. Gallinarum strain 287/91 exhibited the greatest difference in AUC between growth in acetic acid and LB broth. Deltabitscore predicted a loss of function in the hypothetical protein, STM474_3044, in the Gallinarum strain, and this gene was absent from *S*. Schwarzengrund strain SL480 and *S.* Newport strain SGSC4157. A blastp of the amino acid sequence of STM474_3044 indicated a 100% similarity with a putative ABC-type transporter in another *Salmonella* serovar. *S*. Typhimurium strain SO7676-03 and *S.* Gallinarum strain 287/91 were both reported to have a non-functional *yfiK* gene (5.72 logFC, $q < 0.05$), which is an amino acid efflux protein (**Figure 62**).

S. Typhimurium strain SO7676-03 was also predicted to have a non-functional *yacC* gene (putative lipoprotein), which had a negative logFC of -11.59 ($q < 0.05$) and was the gene with the greatest logFC recorded during acetic acid stress in the mutant library. Deltabitscore analysis also revealed loss of function in STM474_1002, Gifsy-1 prophage protein, for *S*. Typhimurium strain SO7676-03 (-8.12 logFC, $q < 0.05$) (**Figure 62**). A putative hexose transferase, *rfaK* (6.92 logFC, $q < 0.05$), which is involved in LPS biosynthesis, was predicted to be non-functional by deltabitscore in *S*. Infantis strain S1326/28 and *S*. Newport strains SL254 and SGSC4157. Another lipopolysaccharide biosynthesis gene, *rfaL* (5.45 logFC, $q < 0.05$) was predicted to have lost its function in *S.* Kentucky strain SL479, *S.* Newport strains SL254 and SGSC4157. The *iscA* gene (-10.32 logFC, $q < 0.05$), which encodes for an iron-sulfur assembly protein was predicted to be non-functional in *S*. Newport strain SL254 using deltabitscore (**Figure 62**). The gene cluster, *rfbACD*, involved in O-antigen biosynthesis, were missing from the genome in *S*. Kedougou strain B37 Col19 and *S*. Infantis strain S1326/28 (**Figure 62**).

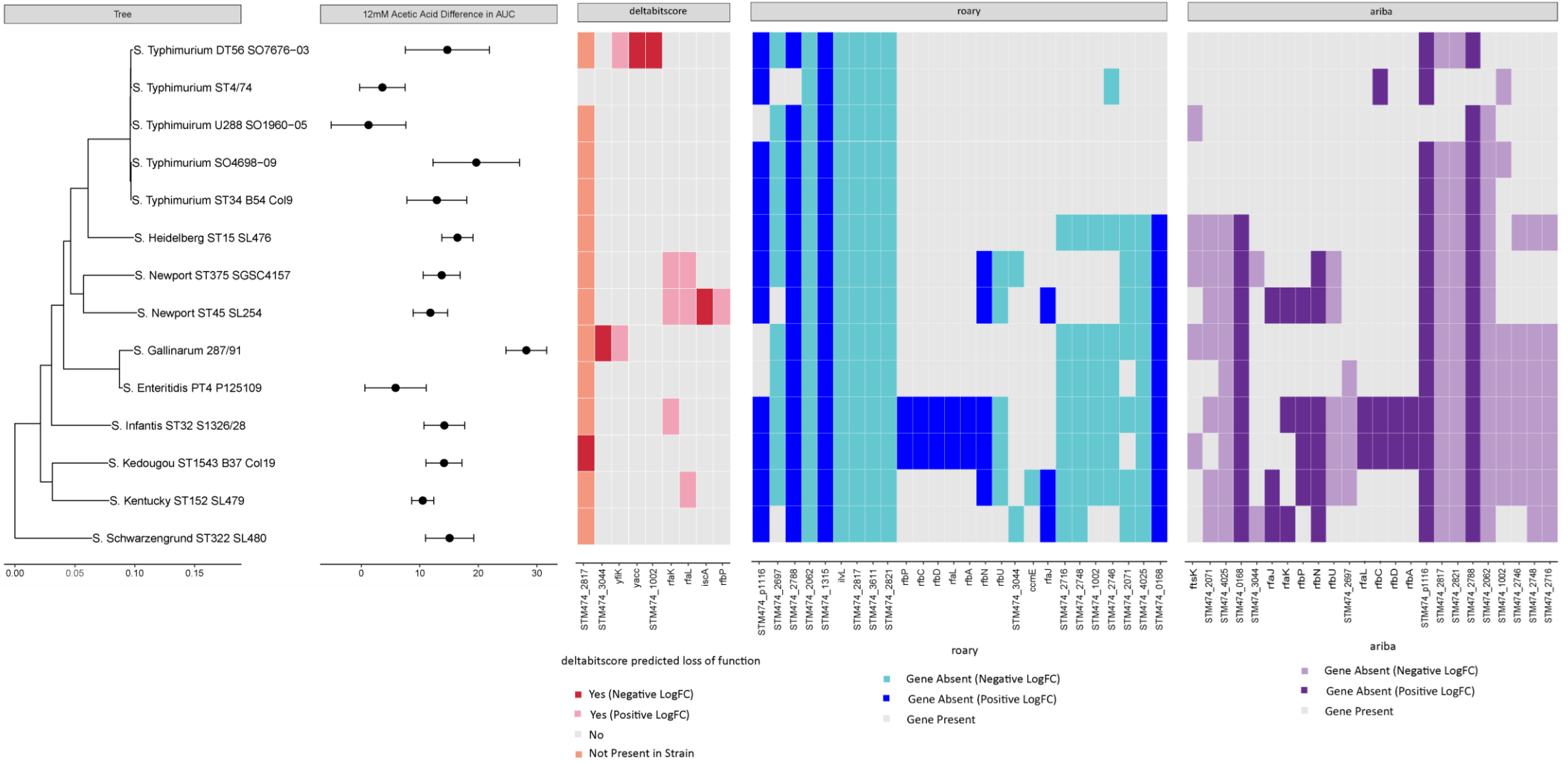

Figure 62. Prediction of conditionally essential genes in other *Salmonella* strains during acetic acid stress. The presence, absence, and loss of function of conditionally essential genes in *S*. Typhimurium strain ST4/74 during 8mM acetic acid stress for other *Salmonella* strains, alongside a maximum likelihood phylogenetic tree generated using RaxML and area under the curve (AUC) data in 12mM acetic acid.

5.4.10.6 Loss of function of *acrB* may increase sensitivity to citric acid stress in *S.* Gallinarum strain 287/91

Response to citric acid stress was strain specific and there was no correlation between resistance of sensitivity and genetic relatedness amongst strains. *S*. Gallinarum strain 287/91 had the greatest difference in AUC during growth in citric acid compared to LB broth and deltabitscore analysis revealed a non-functional gene, *acrB*, which had a logFC -2.15 and functions as an acridine efflux pump (**Figure 63**). Two monophasic Typhimurium strains, SO4698-09 and B54 Col9, had a large difference in AUC during growth in LB broth supplemented with citric acid compared to growth in LB broth with no citric acid. Both of these monophasic strains had a predicted loss of function in *sbmA*, *gshA* and *fepG*, which had logFC values of -7.00, -13.13 and -9.87, respectively (**Figure 63**).

Deltabitscore predicted *S*. Typhimurium SO1960-05 to have a non-functional or missing *endph* gene (1.12 logFC, $q < 0.05$), which could explain why this strain seems to grow better in citric acid than LB broth. *S*. Enteritidis strain P125109 was resistant to 14mM citric acid stress and was missing the STM474_2982 hypothetical protein, which had a positive logFC, indicating loss of this gene is beneficial for survival during stress (**Figure 63**). The unannotated hypothetical protein, STM474_3688 (1.42 logFC, $q < 0.05$), was absent from the genome in *S*. Schwarzengrund strain SL480, *S*. Infantis strain S1326/28, *S*. Newport strain SL254 (**Figure 63**).

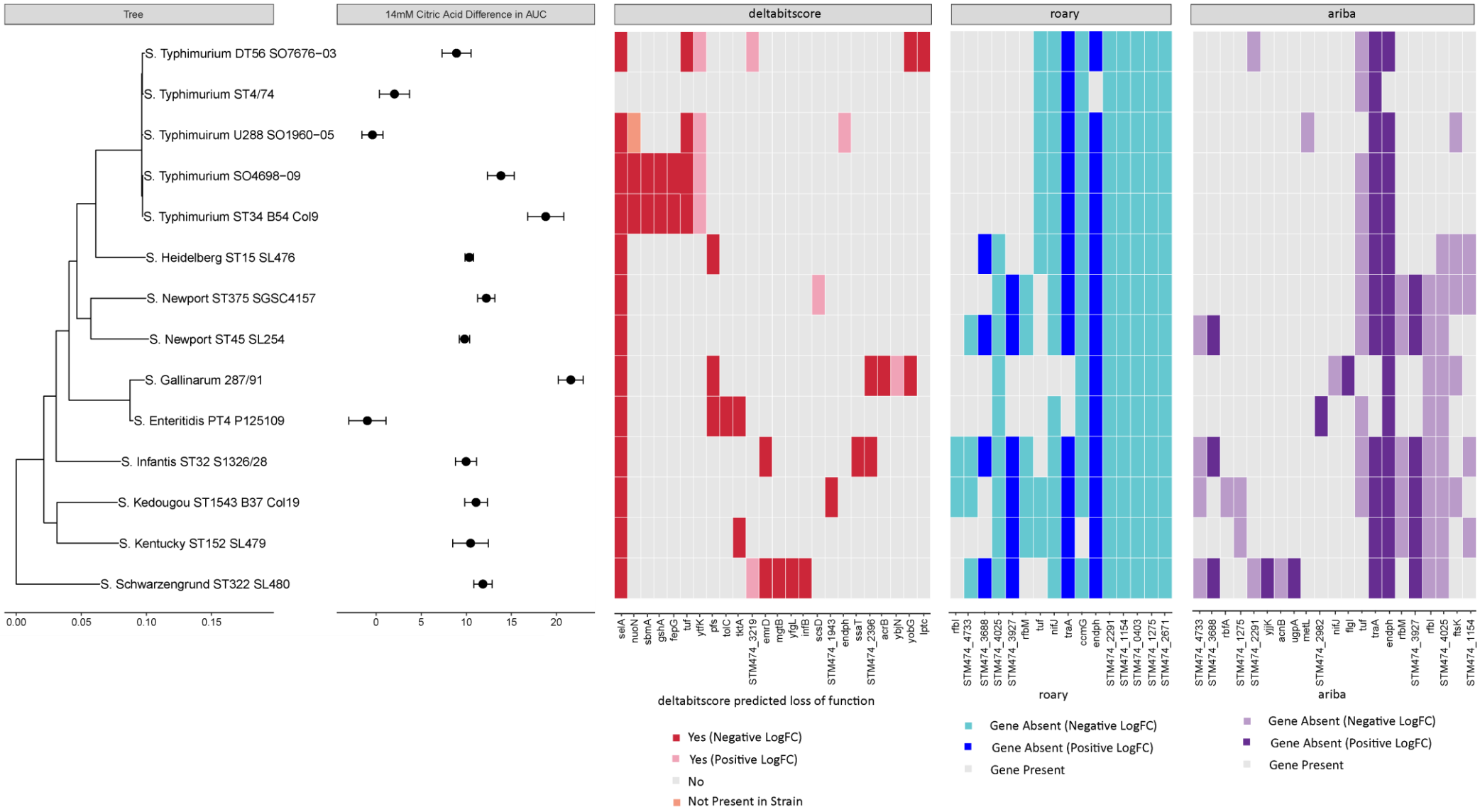

Figure 63. Prediction of conditionally essential genes in other *Salmonella* strains during citric acid stress. The presence, absence, and loss of function of conditionally essential genes in *S.* Typhimurium strain ST4/74 during 14mM citric acid stress for other *Salmonella* strains, alongside a maximum likelihood phylogenetic tree generated using RaxML and area under the curve (AUC) data.

5.4 Discussion

Diverse *Salmonella* strains differ in their risk to food safety likely, at least in part, due to the variability tolerance to food chain related stresses, such as heat and desiccation. However, the molecular mechanisms behind food chain related stress tolerance in *Salmonella enterica* remains largely unknown. In previous chapters, the genes essential for survival during heat inactivation at 60°C, 24-hour desiccation and 5-week refrigerated storage, and during growth in 6% NaCl, 8mM acetic acid and 14mM citric acid were identified using transposon directed insertion site sequencing (TraDIS) in *S*. Typhimurium strain ST4/74. In this chapter, single-gene knockout mutants were constructed using gene doctoring in *S*. Typhimurium strain ST4/74 to verify the computational results from TraDIS experiments, which determined essential genes in each stress condition by comparing the transposon insertions in each gene before and after stress exposure. The genes chosen to be deleted from *S*. Typhimurium strain ST4/74 were *rfaB, dam, proP, zur* and *rnr,* which were shown to be essential, in varying degrees, for survival in the food chain related stresses included in this study, except for *rfaB* which was predicted to be beneficial to the host strain when absent in some stresses.

Phenotypic evaluation of the mutant strains during desiccation indicated that the *dam*, *rnr*, *rfaB*, and *zur* genes were all essential for survival in *S*. Typhimurium strain ST4/74, due to the reduction in cell viability for each mutant. Deletion of the *dam* gene in *S*. Typhimurium strain ST4/74 resulted in a total loss of cells following 24-hour desiccation and removal of the *dam* gene had a negative effect on growth in 6% NaCl. A study looking at the effects of *dam* mutants on *S.* Typhimurium survival in hydrogen peroxide indicated that the *dam* gene fulfils a protective role during oxidative stress, and loss of this gene decreased motility in *Salmonella* cells (Chatti *et al.*, 2012). *dam* mutants have been shown to modify gene expression in *Salmonella* and reduced gene expression in *Salmonella* pathogenicity Island-1 (SPI-1) diminishes epithelial cell invasion and results in a reduction of HilD, the main SGI-1 activator (Balbontín *et al*., 2006).

The *dam* and *rfaB* genes were identified as essential for survival of *S*. Typhimurium strain ST4/74 during citric acid stress, and both mutants had a significant difference in area under the curve in the growth media compared to the wild type. A study has shown that *dam* mutants exhibit a growth defect during prolonged stationary phase (Westphal *et al.*, 2016). Mutations in the *rfa* locus result in a shortened lipopolysaccharide core, which form a deep-rough phenotype (Nikaido and Vaara, 1985). This phenotype increases the cells' sensitivity

to certain hydrophobic antibiotics, anionic detergents and affects motility and biofilm formation (Pagnout *et al.*, 2019). None of the single-gene knockout candidates in *S*. Typhimurium strain ST4/74 were essential for survival during acetic acid stress when tested phenotypically. This may be due to the concentration of acetic acid chosen for the TraDIS experiment. The concentration was most likely too weak to have a deleterious effect on survival, and therefore not many genes were identified as essential for survival as there were not many cells killed by the stress treatment.

Surprisingly, the reduction in cell viability observed for ST4/74Δ*rfaB* during 24-hour desiccation was also significantly different to the wild type strain, even though this gene had a positive logFC in the TraDIS comparison during desiccation stress. A positive logFC indicates that mutations in the gene have a beneficial effect on survival of the strain during stress, therefore the *rfaB* mutant should have survived better than the wild type strain, however this was not the case. The *rfaB* gene (also known as *waaB*) is responsible for adding a galactose group to a glucose group in the lipopolysaccharide of the outer membrane (Pradel *et al.*, 1992). A study investigating the involvement of outer membrane polysaccharides during desiccation stress in *Salmonella* found that lipopolysaccharide mutants, *waaL* (or *rfaL*), *wbaP* (or *rbaP*), *waaC* (or *rfaC*), *waaP* (or *rfaP*), *waaK* (or *rfaK*) and *wzx* were significantly ($p \leq 0.01$) more sensitive to desiccation than *S*. Typhimurium strain SJW1103 (wild type) (Garmiri *et al*., 2008). Reduced desiccation tolerance had previously been linked to mutations in lipopolysaccharide core forming genes resulting in a weakened outer membrane, however, this previous study seems to suggest that sensitivity to desiccation arises from mutations in genes resulting in the loss of O-antigen (or O-polysaccharide) (Irvin *et al*., 1975; Garmiri *et al*., 2008).

The *zur* gene, a zinc uptake transcriptional repressor, was essential for survival of *S*. Typhimurium strain ST4/74 during desiccation, and this mutant resulted in a 3.8-log reduction in cell survival compared to the wild type during 24-hour desiccation. Previously, *zur* was found to be negatively selected for in two studies aimed at understanding *Salmonella* survival in low-moisture foods (pistachios and almonds) (Jayeola *et al*., 2020; Li *et al*., 2020).

The Ribonuclease R (*rnr*) gene synthesises deoxyribonucleotides (dNTPs) from ribonucleotides (NTPs) and provides the precursors required for the synthesis and repair of DNA (Eriksson *et al.*, 1997). This gene was essential for survival of *S*. Typhimurium strain ST4/74 during heat stress, resulting in a ~2.9-log reduction in cell viability. RNA-sequencing data has shown that mutations in exoribonuclease genes impair cell motility by effecting

flagellum assembly in *E. coli* (Pobre and Arraiano, 2015). This phenomenon has been described before in a different species, *Aeromonas hydrophila*, whereby deletion of *rnr* resulted in reduced motility (Erova *et al.*, 2008). *S*. Typhimurium strain ST4/74Δ*rnr* was also an essential gene during long term refrigerated storage at 4°C and during desiccation in the current study, and this gene has previously been identified as a cold-shock protein. Deletion of this gene in *E. coli* strain MG1693 caused a small colony phenotype when grown at 10°C for 3 hours (Cairrão *et al.*, 2003).

During desiccation, ST4/74Δ*proP* did not have a significantly different reduction in log ratio survival compared to the wild type strain, which is surprising as this gene has previously been implicated as essential for survival in desiccated conditions (Finn *et al.*, 2013a). ProP is part of a two-component system, therefore the lack of difference in phenotype observed for this mutant compared to the wild type indicates there is no single-gene effect. The phenotypic variation observed between strains of the same serovar and strains of different serovars indicates that serovars have distinct tolerance to stress, but strains of the same serovar can still vary in their response. More strains would need to be included in the study to evaluate whether stress tolerance is strain dependent. Due to the variability observed, the genetic determinants required for stress tolerance may be found in the accessory genome, rather than the core genome. To evaluate this hypothesis, the pangenome of fourteen *Salmonella* strains was constructed using roary, and the presence and absence of conditionally essential genes in *S*. Typhimurium strain ST4/74 were identified. The increased availability of whole genome sequences of bacterial strains has led to a better understanding of the pangenome, which has enabled the potential role of the accessory genome in stress response to be evaluated during the current study (Page *et al*., 2015). There was no correlation between phylogeny and the phenotypic variability observed in *Salmonella* strains during food chain related stress indicating that variability emerges multiple times throughout the population structure of *S. enterica*.

Deltabitscore was used to determine the functional divergence of the proteome of diverse *Salmonella* isolates using a Hidden Markov Model (HMM), to hypothesise whether loss of function in genes predicted to be essential during TraDIS experiments could explain the sensitivity or resistance to food chain related stress observed in diverse *Salmonella* strains. Furthermore, the absence of conditionally essential genes identified in *S.* Typhimurium strain ST4/74 were identified using roary in other *Salmonella* strains. During desiccation, *S.* Kentucky strain SL479 was most sensitive to desiccation and deltabitscore analysis revealed that this strain had a diverged *visC* gene compared to *S.* Typhimurium strain ST4/74. The *visC*

(or *ubiI*) gene functions as a C5 hydroxylase during aerobic ubiquinone (coenzyme Q) biosynthesis, located downstream of the C1-hydroxylation gene *ubiH* in the same operon (Hajj Chehade *et al.*, 2013). A previous study showed that mutations in *visC* impaired biofilm formation in *E*. *coli* strain UT189, and this was identified during a transposon insertion site sequencing screen to identify genes involved in biofilm formation (Hadjifrangiskou *et al.*, 2012). UT189Δ*ubiI* exhibited decreased motility and pilus-facilitated adherence compared to the wild type strain, in another study by the same group (Floyd *et al.*, 2016).

There were no conditionally essential genes for heat inactivation in *S*. Typhimurium strain ST4/74 identified as hypothetically non-coding or absent in the genomes of *S.* Gallinarum strain 287/91 and *S*. Schwarzengrund strain SL480 which could explain their increased sensitivity to heat stress. The only essential gene predicted to be non-functional in any of the *Salmonella* strains tested phenotypically during heat stress was *tolC* in *S*. Enteritidis P125109. TolC is a member of the outer membrane efflux protein family and transports toxic molecules across the cell membrane (Benz *et al.*, 1993). TolC functions within three types of transport systems, including ATP-binding cassette type (ABC), resistance nodulation division type (RND) and major facilitator superfamily (MF) (Zgurskaya *et al.*, 2011). The AcrAB-TolC belongs to the RND-type transport system and provides *Salmonella* with innate resistance against antibiotics, biocides, dyes and detergents (Buckley *et al.*, 2006). Additionally, TolC was found to be required for cell adhesion and infiltration of epithelial cells and macrophages in *Salmonella* (Webber *et al.*, 2009*)*.

S. Gallinarum strain 287/91 exhibited the greatest sensitivity growth in 6% NaCl. *proP* and *proU* have already been identified as osmoresponsive genes involved in the survival of *Salmonella* during desiccation and NaCl stress (Zhou *et al.*, 2011). However, during the current study removal of the *proP* gene did not affect growth of *S*. Typhimurium strain ST4/74 in 6% NaCl as much as originally hypothesised, as other gene knockouts had a greater difference in area under the curve. This indicates that sensitivity to salt stress is unlikely to be due to a single-gene and is more likely the effect of the interaction between the genes identified as essential during salt stress which results in the lack of growth in salt. Also, mutants in a transposon library are exposed to competition from other mutants during stress selection, whereas during phenotypic experiments of single-gene knockouts, the entire gene is removed, and survival is assessed independently. Furthermore, transposon insertions can occur at any point in the gene, so the mutation causing lack of growth during TraDIS experiments may not have the same effect when the whole gene is removed.

Another hypothetical protein, STM474_3044, was predicted to be non-functional in *S*. Gallinarum strain 287/91 in the deltabitscore analysis and disruptions in this gene may be responsible for the sensitivity to acetic acid stress observed in this strain. STM474_3044 shares 100% sequence identify with DUF1778 in *Salmonella enterica* according to a blastp search. The DUF1778 protein family consists of antitoxins associated with Gcn-5-like *N*-acetyltransferase (GNAT) toxins belonging to a type II toxin-antitoxin system (Cheverton *et al.*, 2016). Bacterial toxin-antitoxin systems (TAS) typically consist of two components occurring in the same operon, which was the case with the STM474_3044 and STM474_3045 operon in *S*. Typhimurium strain ST4/74. The toxin inhibits cellular processes, such as DNA replication or mRNA translation, whereas the antitoxin component inhibits the mechanism of the toxin during certain conditions (Bikmetov *et al.*, 2022). Type II toxin-antitoxin systems function through direct protein-protein interactions. There have been 14 type II toxin-antitoxin systems predicted computationally in *S*. Typhimurium to date (including De la Cruz *et al.* (2013) and Lobato-Márquez *et al.* (2015)). In a study by Cheverton *et al*. (2016), a GNAT-related toxin extended lag phase when overexpressed by inhibiting protein synthesis in *Salmonella*. An extended lag-phase was also observed in the current study in *S*. Gallinarum strain 287/91 when incubated with 12mM acetic acid, which could be the result of a GNAT-related toxin-antitoxin system. Although more research into the function and structure of the hypothetical protein STM474_3044 would need to be performed in order to determine whether it is a component of a type II toxin-antitoxin system.

During citric acid stress, *S*. Gallinarum strain 287/91 growth was negatively affected during culture with citric acid. Deltabitscore analysis revealed a predicted loss of function in the *acrB* gene and ARIBA predicted the absence of *nifJ* in this strain. These genetic differences may increase sensitivity to citric acid stress in *S*. Gallinarum strain 287/91. AcrB is the pump protein in the AcrAB-TolC efflux system. In *S.* Typhimurium strain SL2344, loss of virulence in AcrB mutants was due to loss of efflux activity, rather than the absence of the membrane spanning integral protein (Wang-Kan *et al.*, 2017). In an early study, mutations in *acrB* increased the susceptibility of *E*. coli to a range of small inhibitor molecules, including cephalothin and cephaloridine (Ma *et al.*, 1995). TolC has previously been shown to be responsible for maintaining and improving organic solvent tolerance (e.g. *n*-Hexane and decane) in *E*. *coli* strain K-12, which is regulated by *marA*, *robA* and *soxS* stress response genes (Aono *et al.*, 1998). Interestingly, *tolC* was predicted to only be non-functional in *S*. Enteritidis strain P125109, however this strain grew similar to the wild type strain during 14mM citric acid stress. This strengthens the argument made earlier that sensitivity to food

chain related stress is not caused by a single-gene effect and is more likely a collective effect from many genes identified as essential during transposon directed insertion site sequencing of *S*. Typhimurium strain ST4/74 during exposure to stress.

The loss of function or absence of genes predicted to be essential during food chain related stress in *S.* Typhimurium strain ST4/74 were used to infer the molecular mechanism behind stress tolerance in a number of *Salmonella enterica* strains using computational methods deltabitscore, roary and ARIBA. The disparity between genes identified as absent in the roary and ARIBA analyses may be due to the methods utilised by each pipeline to call gene presence or absence. Roary relies on the annotation provided with each strain sequence, and hence there may be some differences in annotation depending on the annotation pipeline chosen. During the current study, PROKKA was used for genome annotations (Seemann, 2014). The reference strain used in roary and ARIBA was directly downloaded from NCBI, whereas the *S.* Typhimurium strain ST4/74 sequence used in the maximum likelihood phylogenetic tree was from the laboratory strain culture which was sequenced in-house using short-read sequencing. The laboratory strain may have more mutations than present in the NCBI sequence due to its frequency of use in the laboratory. Additionally, the methods used to annotate each sequence may be different and the same gene may have been given different names depending on the programme used. The default cut-off parameters for sequence similarity were used, which were 95% and 90% for roary and ARIBA, respectively. The difference in the sequence similarity threshold used may explain the variances in gene presence or absence identified by each bioinformatic tool. Overall, the bioinformatic tools used to predict gene presence or absence can influence the result, which is one of the major challenges in comparative genomic studies, so care must be taken to choose the most appropriate method for each study.

5.5 Conclusion

In most cases, the phenotype observed for the single-gene knock out mutants in *S*. Typhimurium strain ST4/74 were consistent with the transposon mutant library screen in food chain related stresses. However, this was not the case with *rfaB*, as theoretically removal of this gene should have had a fitness advantage to the cell, however in practice deletion of this gene impeded survival during stress. The pangenome analysis revealed genomic differences in some of the conditionally essential genes identified in *S*. Typhimurium

strain ST4/74 which may contribute to the food chain related stress tolerance and sensitivity observed with some of the strains of *Salmonella* included in this study. However, response to stress is unlikely due a single-gene effect, but rather by a collection of conditionally essential genes.

Chapter 6

General Discussion and Future Research

During this study, the phenotypic variability of 14 *Salmonella* strains of different serovars were subjected to food chain related stress and the survival of each strain was assessed. The stresses applied to the *Salmonella* strains were heat inactivation at 60°C for 30 seconds, 24-hour desiccation, 5-week refrigerated storage, 6% NaCl, 14mM citric acid and 12mM acetic acid, and are all common food preservation techniques implemented by the food manufacturing industry to prevent microbial contamination of food products. Heat inactivation and 5-week refrigerated storage assays were conducted in a vegetarian food product, to determine whether food matrix affects survival during temperature stress, as it has previously been proposed in the literature that the presence of fat may provide bacterial cells with protection from heat. During the present study, there was no correlation between phylogenetic relatedness and response to stress, and closely related strains did not express the same survivability in each stress. This suggests a high degree of variability even within closely related phylogroups i.e Typhimurium. *S.* Gallinarum strain 287/91 was sensitive to many of the stresses encountered during this study, however showed an increased resistance to the addition of salt. The reasoning behind the different response to stress exhibited by the *S*. Gallinarum strain is unknown, however it would be interesting to determine why *S*. Gallinarum can adapt to a high salt environment but cannot withstand desiccation.

From this study it is evident that response to stress is strain specific, rather than serovar specific, and variation occurs between strains of the same serovar. This is unsurprising due to the amount of genetic variability observed within the *Salmonella* strains used in the current study and from previous studies which showed that strains within serovars have different adaptations to host species driven by evolution. It would be useful for food manufacturers to be able to predict the phenotype of a particular strain of interest using a machine learning-based model. The conditionally essential genes predicted from TraDIS data would be used in conjunction with information regarding genotypic diversity to predict the phenotype of strains, however more strains would need to be included for this to be implemented. Some strains showed increased resistance during food chain related stress, such as *S*. Infantis strain S1326/28, *S*. Enteritidis strain P125109 and the monophasic *S*. Typhimurium strains and as a result these strains should be used in food challenge tests during manufacturing. Other strains may be more resistant to food chain related stress, so a similar study to the current including more strains of the same serovar and different serovars should be conducted in the future.

A highly saturated transposon mutant library was generated in *S*. Typhimurium strain ST4/74 using TraDIS and was used to identify genes essential for survival in laboratory growth conditions and during food chain related stresses. Comparisons of the genes identified during the current study and those previously published revealed a large number of novel conditionally essential genes, which suggests that the genes responsible for stress survival are strain dependent. Some of the stress conditions selected to use for TraDIS experiments were not optimal as only a few genes were predicted to be essential in certain stresses i.e heat inactivation. The limited number of genes identified during heat inactivation could be due to the mild heat treatment applied to the strain, and very few genes would need to be selected for to survive in this condition. It would be interesting to subject the TraDIS library to multiple time and temperature combinations to identify the genes consistently predicted to be essential in each condition, and therefore most likely to be involved in heat stress. This technique could also be used to narrow down the essential genes identified in citric acid, desiccation, NaCl and acetic acid stress so that only genes predicted to be essential in each concentration for organic acid and salt stress, or in each time point for desiccation, would be deemed truly essential.

No genes were predicted to be essential in all six stress conditions, which suggests that the genes involved in stress response are specific to each condition. Several essential genes identified during the current study have been previously shown to be involved in food chain related stress, including *proP* during desiccation and osmotic stress, and *phoPQ* during organic acid stress. However, the majority of genes were novel and hadn't been described previously in the literature. It would be interesting to conduct a roary comparison analysis with the essential genes identified from TraDIS experiments in other *Salmonella* strains in the same stresses and the current study to identify overlapping genes by comparing CDS of genes, rather than relying on gene annotation, as this can differ depending on the annotation software used. Single-gene knockout mutants were constructed in *S*. Typhimurium strain ST4/74 to determine whether the genes predicted to be essential in food chain related stress conditions using TraDIS exhibited decreased survival when exposed to stress. The *dam* and *rfaB* genes were essential for survival in all stress conditions when assessed phenotypically, although more work is required to understand the mechanisms by which these genes aid survival in food chain related stresses.

Several bioinformatic tools were used to elucidate the phenotypic variability observed in *Salmonella* strains, by identifying the presence, absence, or loss of function of conditionally essential genes identified in *S*. Typhimurium strain ST4/74. There were no genes identified

as absent or non-functional in strains exhibiting the same phenotype, which indicates that it would be unwise to infer the phenotype of *Salmonella* strains from genotype of one model strain by using bioinformatic tools, such as roary, ARIBA and deltabitscore, due to the large amount of genetic variability between strains. A Genome wide association study (GWAS) could be used to identify conditionally essential genes that are absent or exhibit a high DBS in stress sensitive strains, which would require sensitivity data on many more strains. The current study highlights the disadvantage of using laboratory strains as model organisms in food challenge tests because there is a multitude of phenotypic and genotypic variation amongst strains.

Overall, this study suggested that consideration of *Salmonella* genotype has the potential to improve risk assessments in the food manufacturing industry, due to the inherent phenotypic variability observed in response to food chain related stresses. The conditionally essential genes identified during this study have the potential to be used as biomarkers to identify *Salmonella* strains which exhibit an increased resistance to the methods typically used to inhibit microbial contamination of food products. However, the transposon mutant library generated in the current study would need to be subjected to more concentrations and durations of each stress to deduce the genes identified as essential in all concentrations, and therefore the most likely stress resistance genes. Furthermore, it would be interesting to establish the molecular mechanisms behind stress sensitivity in host adapted strains, such as *S*. Gallinarum strain 287/91 and *S*. Typhimurium strain SO1960-05, which could be established by constructing mutant libraries in these strains specifically to assess essential genes. Ultimately, the research generated throughout this study contributes to the development and enhancement of knowledge-based intervention strategies that can be utilised during food manufacturing to improve food safety.

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Supplementary Tables

Supplementary Table 1. Essential genes required for growth in LB broth at 37°C in *S*. Typhimurium strain ST4/74.

Locus Tag	Gene Name	Start Position	End Position	Strand	Read Count	Insertion Index	Gene Length (bp)	Insertion Count	Function
STM474_0013	*dnaK*	11593	13509	1	146	0.004173187	1917	8	molecular chaperone DnaK
STM474_0034	*STM474_0034*	34376	34816	-1	19	0.009070295	441	4	putative transcriptional regulator
STM474_0046	*rpsT*	52280	52543	-1	0	0	264	0	30S ribosomal protein S20
STM474_0048	*ribF*	52872	53810	1	0	0	939	0	bifunctional riboflavin kinase/FMN adenylyltransferase
STM474_0049	*ileS*	53819	56689	1	0	0	2871	0	isoleucyl-tRNA synthetase
STM474_0050	*lspA*	56689	57189	1	97	0.005988024	501	3	lipoprotein signal peptidase
STM474_0052	*ispH*	57796	58746	1	35	0.002103049	951	2	4-hydroxy-3-methylbut-2-enyl diphosphate reductase
STM474_0058	*STM474_0058*	64862	66637	-1	214	0.004504505	1776	8	pyruvate carboxylase subunit B
STM474_0067	*dapB*	74020	74841	1	50	0.003649635	822	3	dihydrodipicolinate reductase
STM474_0091	*folA*	100842	101321	1	0	0	480	0	dihydrofolate reductase
STM474_0097	*imp*	105813	108173	-1	62	0.002117747	2361	5	LPS-assembly protein
STM474_0127	*ftsL*	141348	141713	1	146	0.010928962	366	4	cell division protein FtsL
STM474_0128	*ftsI*	141729	143495	1	35	0.000565931	1767	1	penicillin-binding protein 3 precursor
STM474_0129	*murE*	143482	144969	1	0	0	1488	0	UDP-N-acetylmuramoylalanyl-D-glutamate--2-6-diaminopimelate ligase
STM474_0130	*murF*	144966	146324	1	5	0.002207506	1359	3	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D- alanine ligase
STM474_0131	*mraY*	146318	147400	1	0	0	1083	0	phospho-N-acetylmuramoyl-pentapeptide- transferase
STM474_0132	*murD*	147403	148719	1	18	0.000759301	1317	1	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase
STM474_0133	*ftsW*	148719	149963	1	51	0.002409639	1245	3	cell division protein FtsW
STM474_0134	*murG*	149960	151027	1	22	0.001872659	1068	2	N-acetylglucosaminyl transferase
STM474_0135	*murC*	151146	152621	1	11	0.001355014	1476	2	UDP-N-acetylmuramate--L-alanine ligase
STM474_0137	*ftsQ*	153536	154366	1	46	0.007220217	831	6	cell division protein FtsQ
STM474_0138	*ftsA*	154363	155625	1	0	0	1263	0	cell division protein FtsA
STM474_0139	*ftsZ*	155686	156837	1	0	0	1152	0	cell division protein FtsZ
STM474_0140	*lpxc*	156938	157855	1	29	0.004357298	918	4	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase

STM474_0142	*secA*	158693	161398	1	271	0.002956393	2706	8	preprotein translocase SecA subunit
STM474_0147	*coaE*	163211	163831	-1	110	0.009661836	621	6	dephospho-CoA kinase
STM474_0160	*aceE*	176244	178907	1	131	0.003753754	2664	10	pyruvate dehydrogenase subunit E1
STM474_0161	*aceF*	178922	180811	1	6	0.000529101	1890	1	dihydrolipoyllysine-residue acetyltransferase
STM474_0162	*lpdA*	181008	182435	1	0	0	1428	0	dihydrolipoamide dehydrogenase
STM474_0180	*yadF*	201406	202068	-1	13	0.004524887	663	3	carbonate dehydratase
STM474_0211	*hemL*	237207	238487	-1	1	0.00078064	1281	1	glutamate-1-semialdehyde aminotransferase
STM474_0213	*yadR*	240115	240504	1	0	0	390	0	iron-sulfur cluster insertion protein ErpA
STM474_0216	*pfs*	242058	242756	-1	126	0.007153076	699	5	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase
STM474_0222	*dapD*	249285	250109	-1	65	0.007272727	825	6	2-3-4-5-tetrahydropyridine-2-carboxylate N-succinyltransferase
STM474_0224	*map*	253048	253842	-1	20	0.003773585	795	3	methionine aminopeptidase
STM474_0225	*rpsB*	254293	255018	1	4	0.00137741	726	1	30S ribosomal protein S2
STM474_0226	*tsf*	255276	256127	1	15	0.002347418	852	2	elongation factor Ts
STM474_0227	*pyrH*	256272	256997	1	0	0	726	0	uridylate kinase
STM474_0228	*frr*	257144	257701	1	4	0.001792115	558	1	ribosome recycling factor
STM474_0229	*dxr*	257842	259038	1	0	0	1197	0	1-deoxy-D-xylulose 5-phosphate reductoisomerase
STM474_0230	*uppS*	259351	260109	1	0	0	759	0	undecaprenyl pyrophosphate synthase
STM474_0231	*cdsA*	260122	260979	1	0	0	858	0	CDP-diglyceride synthase
STM474_0233	*bamA*	262375	264789	1	0	0	2415	0	outer membrane protein assembly factor BamA
STM474_0235	*lpxd*	265401	266426	1	0	0	1026	0	UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase
STM474_0236	*fabZ*	266514	266987	1	92	0.004219409	474	2	(3R)-hydroxymyristoyl-ACP dehydratase
STM474_0237	*lpxA*	266991	267779	1	0	0	789	0	UDP-N-acetylglucosamine acyltransferase
STM474_0238	*lpxB*	267779	268927	1	62	0.003481288	1149	4	lipid-A-disaccharide synthase
STM474_0240	*dnaE*	269544	273026	1	7	0.000287109	3483	1	DNA polymerase III subunit alpha
STM474_0241	*accA*	273039	273998	1	0	0	960	0	acetyl-CoA carboxylase carboxyltransferase subunit alpha
STM474_0245	*tilS*	278666	279958	1	134	0.007733952	1293	10	tRNA(Ile)-lysidine synthetase
STM474_0251	*proS*	282458	284176	-1	5	0.001163467	1719	2	prolyl-tRNA synthetase
STM474_r0259	*STM474_r0259*	289177	290706	1	243	0.010457516	1530	16	
STM474_t0261	*STM474_t0261*	290967	291042	1	0	0	76	0	

STM474_r0262	*STM474_r0262*	291227	294315	1	63	0.001618647	3089	5	
STM474_r0264	*STM474_r0264*	294514	294628	1	0	0	115	0	
STM474_t0265	*STM474_t0265*	294819	294895	1	0	0	77	0	
STM474_0275	*dnaQ*	303384	304124	1	2	0.001349528	741	1	DNA polymerase III epsilon subunit
STM474_0306	*STM474_0306*	338316	338753	1	2	0.002283105	438	1	putative cytoplasmic protein
STM474_0312	*STM474_0312*	341562	341681	1	0	0	120	0	hypothetical protein
STM474_0325	*gmhA*	354741	355337	1	34	0.010050251	597	6	phosphoheptose isomerase
STM474_0387	*hemB*	423164	424138	-1	58	0.001025641	975	1	delta-aminolevulinic acid dehydratase
STM474_0426	*secD*	461817	463664	1	24	0.001623377	1848	3	preprotein translocase subunit SecD
STM474_0427	*secF*	463630	464646	1	29	0.004916421	1017	5	preprotein translocase subunit SecF
STM474_0436	*ribd*	469330	470433	1	63	0.003623188	1104	4	Riboflavin biosynthesis protein ribD
STM474_0437	*ribH*	470522	470992	1	0	0	471	0	6-7-dimethyl-8-ribityllumazine synthase
STM474_0438	*nusB*	471013	471432	1	30	0.007142857	420	3	transcription antitermination protein NusB
STM474_0439	*thiL*	471511	472488	1	36	0.00408998	978	4	thiamine monophosphate kinase
STM474_0442	*dxs*	474116	475978	-1	65	0.002147075	1863	4	1-deoxy-D-xylulose-5-phosphate synthase
STM474_0443	*ispA*	476002	476901	-1	0	0	900	0	geranyltranstransferase
STM474_0469	*clpP*	502579	503319	1	14	0.001349528	741	1	ATP-dependent Clp protease proteolytic subunit
STM474_0470	*clpX*	503571	504842	1	38	0.001572327	1272	2	ATP-dependent protease ATP-binding subunit
STM474_0471	*lon*	505028	507382	1	1	0.000424628	2355	1	DNA-binding ATP-dependent protease La
STM474_0504	*dnaX*	540395	542323	1	5	0.000518403	1929	1	DNA polymerase III tau subunit
STM474_0508	*adk*	545529	546173	1	0	0	645	0	adenylate kinase
STM474_0510	*hemH*	546402	547364	1	25	0.001038422	963	1	ferrochelatase
STM474_0542	*ybbV*	581893	582147	1	1	0.003921569	255	1	putative cytoplasmic protein
STM474_0556	*lpxH*	597231	597953	-1	7	0.001383126	723	1	UDP-2-3-diacylglucosamine hydrolase
STM474_0557	*ppiB*	597956	598468	-1	47	0.009746589	513	5	peptidyl-prolyl cis-trans isomerase B
STM474_0558	*cysS*	598623	600008	1	0	0	1386	0	cysteinyl tRNA synthetase
STM474_0563	*folD*	602190	603056	-1	12	0.002306805	867	2	bifunctional 5-10-methylene-tetrahydrofolate dehydrogenase/ cyclohydrolase
STM474_0610	*fepC*	650273	651067	-1	84	0.010062893	795	8	iron-enterobactin transporter ATP-binding protein
STM474_0612	*fepD*	652053	653060	-1	31	0.006944444	1008	7	iron-enterobactin transporter membrane protein
STM474_0654	*lipA*	694350	695315	-1	2	0.002070393	966	2	lipoyl synthase
STM474_0660	*mrdB*	700318	701430	-1	47	0.002695418	1113	3	cell wall shape-determining protein

STM474_0661	*mrdA*	701433	703334	-1	2	0.000525762	1902	1	penicillin-binding protein 2
STM474_0666	*nadD*	706256	706897	-1	109	0.003115265	642	2	nicotinic acid mononucleotide adenylyltransferase
STM474_0667	*holA*	706899	707930	-1	32	0.004844961	1032	5	DNA polymerase III subunit delta
STM474_0668	*rlpB*	707930	708520	-1	0	0	591	0	LPS-assembly lipoprotein RplB
STM474_0669	*leuS*	708535	711360	-1	138	0.004246285	2826	12	leucyl-tRNA synthetase
STM474_0687	*lnt*	729583	731121	-1	61	0.003248863	1539	5	apolipoprotein N-acyltransferase
STM474_0692	*ubiF*	735460	736641	1	9	0.000846024	1182	1	2-octaprenyl-3-methyl-6-methoxy-1-4-benzoquinol hydroxylase
STM474_t0694	*STM474_t0694*	737419	737493	-1	13	0.013333333	75	1	
STM474_t0697	*STM474_t0697*	737750	737824	-1	0	0	75	0	
STM474_t0698	*STM474_t0698*	737860	737934	-1	0	0	75	0	
STM474_t0699	*STM474_t0699*	737958	738042	-1	0	0	85	0	
STM474_t0700	*STM474_t0700*	738051	738127	-1	0	0	77	0	
STM474_0707	*glnS*	746832	748499	1	172	0.003597122	1668	6	glutaminyl-tRNA synthetase
STM474_0743	*STM474_0743*	784576	785472	1	140	0.012263099	897	11	putative glycosyl transferase
STM474_0744	*STM474_0744*	785485	786618	1	24	0.001763668	1134	2	galactosyltransferase
STM474_0747	*STM474_0747*	788325	790103	1	45	0.005059022	1779	9	putative glycosyltransferase
STM474_0748	*STM474_0748*	790296	791129	1	0	0	834	0	putative glycosyltransferase
STM474_0759	*sucA*	801156	803957	1	62	0.002855103	2802	8	alpha-ketoglutarate decarboxylase
STM474_0760	*sucB*	803972	805180	1	127	0.005789909	1209	7	dihydrolipoamide succinyltransferase
STM474_0765	*cydA*	808940	810508	1	75	0.003186743	1569	5	cytochrome d terminal oxidase polypeptide subunit I
STM474_0766	*cydB*	810524	811663	1	21	0.002631579	1140	3	cytochrome d ubiquinol oxidase subunit II
STM474_0767	*ybgT*	811678	811791	1	0	0	114	0	Cyd operon protein YbgT
STM474_0771	*tolR*	813535	813963	1	10	0.006993007	429	3	colicin uptake protein TolR
STM474_0772	*tolA*	814028	815251	1	61	0.003267974	1224	4	cell envelope integrity inner membrane protein TolA
STM474_0773	*tolB*	815372	816667	1	104	0.006944444	1296	9	translocation protein TolB
STM474_t0776	*STM474_t0776*	818189	818264	1	8	0.013157895	76	1	
STM474_t0778	*STM474_t0778*	818475	818550	1	16	0.013157895	76	1	
STM474_0792	*STM474_0792*	829385	831160	1	32	0.001689189	1776	3	pyruvate carboxylase subunit B
STM474_0880	*STM474_0880*	925442	925957	1	18	0.011627907	516	6	putative cytoplasmic protein
STM474_0881	*STM474_0881*	925875	926729	1	41	0.004678363	855	4	putative electron transfer protein beta subunit
STM474_0933	*tnpA_2*	981703	982161	1	0	0	459	0	transposase for IS200

STM474_0940	*infA*	986884	987102	-1	0	0	219	0	translation initiation factor IF-1
STM474_0943	*cydC*	988163	989884	-1	59	0.005226481	1722	9	cysteine/glutathione ABC transporter membrane/ATP-binding component
STM474_0944	*cydD*	989885	991651	-1	39	0.001697793	1767	3	cysteine/glutathione ABC transporter membrane/ATP-binding component
STM474_0947	*ftsK*	993908	997993	1	228	0.004650024	4086	19	DNA translocase FtsK
STM474_0948	*lolA*	998133	998747	1	38	0.001626016	615	1	outer-membrane lipoprotein carrier protein
STM474_0950	*serS*	1000359	1001651	1	0	0	1293	0	seryl-tRNA synthetase
STM474_0966	*cmk*	1021561	1022244	1	14	0.001461988	684	1	cytidylate kinase
STM474_0967	*rpsA*	1022358	1024031	1	28	0.002389486	1674	4	30S ribosomal protein S1
STM474_0970	*msbA*	1027002	1028750	1	1	0.000571755	1749	1	lipid transporter ATP-binding/permease protein
STM474_0971	*lpxK*	1028747	1029724	1	42	0.001022495	978	1	tetraacyldisaccharide 4'-kinase
STM474_0974	*kdsB*	1031231	1031977	1	39	0.001338688	747	1	3-deoxy-manno-octulosonate cytidylyltransferase
STM474_0978	*mukF*	1034769	1036091	1	0	0	1323	0	condesin subunit F
STM474_0979	*mukE*	1036045	1036776	1	86	0.005464481	732	4	condesin subunit E
STM474_0980	*mukB*	1036776	1041242	1	34	0.000223864	4467	1	cell division protein MukB
STM474_0986	*STM474_0986*	1047729	1047881	1	0	0	153	0	hypothetical protein
STM474_0987	*asnC*	1048046	1049446	-1	0	0	1401	0	asparaginyl-tRNA synthetase
STM474_0994	*recT*	1056703	1057860	-1	279	0.01208981	1158	14	Gifsy-2 prophage RecT
STM474_1000	*STM474_1000*	1061617	1062027	-1	0	0	411	0	regulatory protein
STM474_1001	*STM474_1001*	1061983	1062096	1	0	0	114	0	hypothetical protein
STM474_1018	*STM474_1018*	1071545	1071793	1	14	0.004016064	249	1	Gifsy-2 prophage protein
STM474_1059	*fabA*	1114230	1114748	-1	0	0	519	0	3-hydroxydecanoyl-(acyl carrier protein) dehydratase
STM474_1076	*STM474_1076*	1130174	1130503	-1	0	0	330	0	sulfur transfer protein TusE
STM474_t1078	*STM474_t1078*	1131644	1131731	1	0	0	88	0	
STM474_1125	*STM474_1125*	1176989	1177681	1	75	0.01010101	693	7	putative outer membrane protein
STM474_1150	*htrB*	1198757	1199737	-1	4	0.001019368	981	1	lipid A biosynthesis lauroyl acyltransferase
STM474_1166	*mviN*	1211105	1212679	1	37	0.002539683	1575	4	putative virulence protein
STM474_1181	*rne*	1224496	1227699	-1	230	0.008426966	3204	27	ribonuclease E
STM474_1190	*fabH*	1233454	1234407	1	27	0.004192872	954	4	3-oxoacyl-(acyl carrier protein) synthase III
STM474_1191	*fabD*	1234423	1235352	1	42	0.001075269	930	1	acyl carrier protein S-malonyltransferase
STM474_1192	*fabg*	1235365	1236099	1	0	0	735	0	3-oxoacyl-[acyl-carrier-protein] reductase

STM474_1193	*acpP*	1236255	1236491	1	0	0	237	0	Acyl carrier protein
STM474_1197	*tmk*	1239766	1240407	1	14	0.001557632	642	1	thymidylate kinase
STM474_1198	*holB*	1240404	1241408	1	60	0.005970149	1005	6	DNA polymerase III subunit delta'
STM474_1214	*lolC*	1258821	1260020	1	0	0	1200	0	outer membrane-specific lipoprotein transporter subunit LolC
STM474_1215	*lolD*	1260013	1260714	1	32	0.001424501	702	1	lipoprotein transporter ATP-binding subunit
STM474_1216	*lolE*	1260714	1261958	1	11	0.000803213	1245	1	outer membrane-specific lipoprotein transporter subunit LolE
STM474_1225	*tnpA_2*	1270999	1271457	1	0	0	459	0	transposase for IS200
STM474_1230	*purB*	1276340	1277710	-1	21	0.001458789	1371	2	adenylosuccinate lyase
STM474_1232	*mnma*	1278442	1279593	-1	41	0.004340278	1152	5	tRNA-specific 2-thiouridylase mnmA
STM474_1236	*icdA*	1281238	1282488	1	17	0.001598721	1251	2	isocitrate dehydrogenase
STM474_1243	*STM474_1243*	1288341	1288518	1	0	0	178	0	pseudogene
STM474_1294	*gapA*	1325546	1326550	-1	0	0	1005	0	glyceraldehyde-3-phosphate dehydrogenase
STM474_1314	*nadE*	1346972	1347799	-1	14	0.004830918	828	4	NAD synthetase
STM474_1337	*thrS*	1369055	1370983	1	0	0	1929	0	threonyl-tRNA synthetase
STM474_1338	*infC*	1370987	1371529	1	0	0	543	0	translation initiation factor IF-3
STM474_1339	*rpmI*	1371610	1371822	1	0	0	213	0	50S ribosomal protein L35
STM474_1340	*rplT*	1371873	1372229	1	0	0	357	0	50S ribosomal protein L20
STM474_1341	*pheS*	1372519	1373514	1	0	0	996	0	phenylalanyl-tRNA synthetase subunit alpha
STM474_1342	*pheT*	1373530	1375917	1	7	0.000837521	2388	2	phenylalanyl-tRNA synthetase subunit beta
STM474_1413	*ssaH*	1446582	1446872	1	11	0.003436426	291	1	secretion system apparatus SsaH
STM474_1414	*ssaI*	1446884	1447132	1	29	0.004016064	249	1	type III secretion system apparatus protein
STM474_1427	*ssaT*	1456507	1457286	1	52	0.01025641	780	8	type III secretion system apparatus protein
STM474_t1430	*STM474_t1430*	1458586	1458662	-1	15	0.012987013	77	1	
STM474_1432	*ribE*	1460404	1461045	1	89	0.003115265	642	2	riboflavin synthase subunit alpha
STM474_1455	*pdxH*	1479556	1480212	1	66	0.01065449	657	7	pyridoxamine 5'-phosphate oxidase
STM474_1456	*tyrS*	1480327	1481613	1	0	0	1287	0	tyrosyl-tRNA synthetase
STM474_1614	*sifB*	1649022	1649984	1	35	0.00623053	963	6	secreted effector protein
STM474_1642	*STM474_1642*	1677004	1677720	-1	0	0	717	0	putative inner membrane protein
STM474_1714	*fabI*	1748989	1749777	1	24	0.001267427	789	1	enoyl-(acyl carrier protein) reductase
STM474_1723	*STM474_1723*	1758368	1759537	-1	108	0.001709402	1170	2	tetratricopeptide repeat protein

STM474_1726	*ribA*	1760962	1761552	1	0	0	591	0	GTP cyclohydrolase II
STM474_1731	*topA*	1766645	1769242	-1	0	0	2598	0	DNA topoisomerase I
STM474_1768	*hns*	1804136	1804549	1	0	0	414	0	global DNA-binding transcriptional dual regulator H-NS
STM474_1770	*hnr*	1805792	1806805	-1	28	0.004930966	1014	5	response regulator of RpoS
STM474_1789	*kdsA*	1827260	1828114	-1	0	0	855	0	2-dehydro-3-deoxyphosphooctonate aldolase
STM474_1793	*prfA*	1830184	1831266	-1	37	0.001846722	1083	2	peptide chain release factor 1
STM474_1794	*hemA*	1831307	1832563	-1	17	0.00318218	1257	4	glutamyl-tRNA reductase
STM474_1797	*ipk*	1833497	1834348	1	0	0	852	0	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase
STM474_1798	*STM474_1798*	1834345	1834515	-1	0	0	171	0	hypothetical protein
STM474_1799	*prsA*	1834596	1835561	1	0	0	966	0	ribose-phosphate pyrophosphokinase
STM474_1839	*tnpA_2*	1872138	1872596	-1	0	0	459	0	transposase for IS200
STM474_1842	*yeaz*	1875330	1876025	-1	47	0.004310345	696	3	putative M22 peptidase yeaZ
STM474_1921	*msbb*	1941560	1942531	-1	50	0.00308642	972	3	Lipid A biosynthesis (KDO)2-(lauroyl)-lipid IVA acyltransferase
STM474_1926	*ruvB*	1946622	1947632	-1	65	0.005934718	1011	6	Holliday junction DNA helicase RuvB
STM474_1934	*aspS*	1952678	1954450	-1	24	0.001128032	1773	2	aspartyl-tRNA synthetase
STM474_1942	*argS*	1960164	1961897	1	0	0	1734	0	arginyl-tRNA synthetase
STM474_t1975	*STM474_t1975*	1993028	1993101	-1	0	0	74	0	
STM474_t1976	*STM474_t1976*	1993154	1993229	-1	0	0	76	0	
STM474_1977	*pgsA*	1993381	1993929	-1	18	0.001821494	549	1	phosphatidylglycerophosphate synthetase
STM474_1988	*tnpA_2*	2003303	2003761	-1	0	0	459	0	transposase for IS200
STM474_2072	*STM474_2072*	2072244	2072939	1	0	0	696	0	regulatory protein
STM474_2172	*rfbV*	2165334	2166335	-1	56	0.005988024	1002	6	abequosyltransferase
STM474_2173	*rfbX*	2166340	2167632	-1	111	0.007733952	1293	10	putative O-antigen transporter
STM474_2175	*rfbH*	2168641	2169954	-1	92	0.00913242	1314	12	CDP-6-deoxy-D-xylo-4-hexulose-3-dehydrase
STM474_2176	*rfbG*	2169981	2171060	-1	115	0.013888889	1080	15	CDP glucose 4-6-dehydratase
STM474_2177	*rfbF*	2171065	2171838	-1	47	0.006459948	774	5	glucose-1-phosphate cytidylyltransferase
STM474_2206	*dcd*	2204602	2205183	-1	33	0.003436426	582	2	deoxycytidine triphosphate deaminase
STM474_2242	*metG*	2247374	2249488	1	136	0.000945626	2115	2	methionyl tRNA synthetase
STM474_2282	*folE*	2288160	2288828	-1	0	0	669	0	GTP cyclohydrolase I
STM474_2317	*rplY*	2324149	2324433	1	0	0	285	0	50S ribosomal protein L25
STM474_2344	*ccmG*	2346149	2346706	-1	16	0.003584229	558	2	heme lyase disulfide oxidoreductase

STM474_2345	*ccmF*	2346703	2348634	-1	0	0	1932	0	cytochrome c-type biogenesis protein CcmF
STM474_2347	*ccmD*	2349107	2349319	-1	0	0	213	0	heme exporter protein C
STM474_2348	*ccmC*	2349316	2350062	-1	0	0	747	0	heme exporter protein
STM474_2349	*ccmB*	2350105	2350764	-1	0	0	660	0	heme exporter protein
STM474_2350	*ccmA*	2350761	2351384	-1	151	0.004807692	624	3	cytochrome c biogenesis protein CcmA
STM474_2368	*gyrA*	2371427	2374063	-1	115	0.000758438	2637	2	DNA gyrase A subunit
STM474_2372	*ubiG*	2378032	2378760	1	74	0.006858711	729	5	3-demethylubiquinone-9 3-methyltransferase
STM474_2373	*nrdA*	2379116	2381401	1	29	0.000437445	2286	1	ribonucleotide-diphosphate reductase subunit alpha
STM474_2374	*nrdB*	2381514	2382644	1	0	0	1131	0	ribonucleotide-diphosphate reductase subunit beta
STM474_2454	*ubiX*	2464298	2464867	-1	77	0.010526316	570	6	3-octaprenyl-4-hydroxybenzoate carboxy-lyase
STM474_2464	*folC*	2475070	2476338	-1	110	0.003152088	1269	4	tetrahydrofolate synthase/dihydrofolate synthase
STM474_2465	*accD*	2476406	2477320	-1	2	0.001092896	915	1	acetyl-CoA carboxylase subunit beta
STM474_2516	*gltX*	2527879	2529294	-1	10	0.002118644	1416	3	glutamyl-tRNA synthetase
STM474_t2520	*STM474_t2520*	2529871	2529946	1	0	0	76	0	
STM474_2529	*ligA*	2536717	2538732	-1	14	0.001488095	2016	3	NAD-dependent DNA ligase LigA
STM474_2530	*zipA*	2538804	2539841	-1	36	0.002890173	1038	3	cell division protein ZipA
STM474_2536	*crr*	2544376	2544885	1	1	0.001960784	510	1	glucose-specific PTS system component
STM474_2574	*tnpA_2*	2577047	2577505	-1	0	0	459	0	transposase for IS200
STM474_2586	*dapE*	2595410	2596537	1	100	0.007978723	1128	9	succinyl-diaminopimelate desuccinylase
STM474_2592	*dapA*	2601759	2602637	-1	39	0.002275313	879	2	dihydrodipicolinate synthase
STM474_2611	*guaA*	2620521	2622098	-1	66	0.008238276	1578	13	GMP synthase
STM474_2621	*engA*	2649173	2650645	-1	0	0	1473	0	GTP-binding protein EngA
STM474_2624	*hisS*	2652587	2653861	-1	17	0.002352941	1275	3	histidyl-tRNA synthetase
STM474_2625	*ispG*	2653972	2655090	-1	35	0.00357462	1119	4	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase
STM474_2647	*nifS*	2679253	2680467	-1	85	0.004938272	1215	6	cysteine desulfurase
STM474_2650	*suhB*	2682152	2682955	1	38	0.003731343	804	3	inositol monophosphatase
STM474_2659	*glyA*	2691398	2692651	-1	40	0.003987241	1254	5	serine hydroxymethyltransferase
STM474_2682	*acpS*	2719779	2720159	-1	0	0	381	0	4'-phosphopantetheinyl transferase
STM474_2683	*pdxJ*	2720159	2720890	-1	108	0.008196721	732	6	pyridoxal phosphate biosynthetic protein PdxJ
STM474_2686	*rnc*	2722544	2723281	-1	17	0.001355014	738	1	ribonuclease III
STM474_2687	*lepB*	2723498	2724472	-1	79	0.009230769	975	9	signal peptidase I
STM474_2690	*STM474_2690*	2728390	2728518	-1	7	0.007751938	129	1	hypothetical protein

STM474_2722	*STM474_2722*	2757552	2757800	-1	2	0.004016064	249	1	Gifsy-1 prophage protein
STM474_2740	*STM474_2740*	2767785	2768195	-1	46	0.00973236	411	4	Gifsy-1 prophage cI
STM474_2741	*STM474_2741*	2768447	2768833	1	14	0.005167959	387	2	putative regulator
STM474_2765	*pssA*	2793068	2794423	1	0	0	1356	0	phosphatidylserine synthase
STM474_r2769	*STM474_r2769*	2796856	2796970	-1	0	0	115	0	
STM474_r2770	*STM474_r2770*	2797170	2800175	-1	249	0.0083167	3006	25	
STM474_t2771	*STM474_t2771*	2800371	2800446	-1	2	0.013157895	76	1	
STM474_r2772	*STM474_r2772*	2800537	2802066	-1	102	0.003921569	1530	6	
STM474_2776	*bamD*	2807072	2807809	1	1	0.001355014	738	1	outer membrane protein assembly complex subunit BamD
STM474_2787	*STM474_2787*	2816803	2817798	-1	160	0.011044177	996	11	CI repressor
STM474_2797	*rplS*	2826061	2826408	-1	0	0	348	0	50S ribosomal protein L19
STM474_2798	*trmd*	2826449	2827216	-1	0	0	768	0	tRNA (guanine-N(1)-)-methyltransferase
STM474_2799	*rimM*	2827261	2827812	-1	0	0	552	0	16S rRNA processing protein RimM
STM474_2800	*rpsP*	2827828	2828076	-1	13	0.004016064	249	1	30S ribosomal protein S16
STM474_2801	*ffh*	2828390	2829751	-1	42	0.003671072	1362	5	signal recognition particle protein
STM474_2805	*grpE*	2832775	2833365	-1	32	0.008460237	591	5	co-chaperone GrpE
STM474_2806	*ppnK*	2833488	2834366	1	0	0	879	0	inorganic polyphosphate/ATP-NAD kinase
STM474_2847	*STM474_2847*	2877245	2878222	-1	6	0.00204499	978	2	putative competence protein
STM474_2861	*STM474_2861*	2886863	2887495	1	0	0	633	0	phage repressor protein cI
STM474_2896	*STM474_2896*	2924046	2925920	1	220	0.009066667	1875	17	putative inner membrane protein
STM474_2897	*STM474_2897*	2926180	2927502	-1	147	0.013605442	1323	18	putative inner membrane protein
STM474_2923	*STM474_2923*	2960278	2960391	1	0	0	114	0	hypothetical protein
STM474_t2962	*STM474_t2962*	2993051	2993127	-1	0	0	77	0	
STM474_t2963	*STM474_t2963*	2993131	2993223	-1	0	0	93	0	
STM474_2964	*csrA*	2993527	2993712	-1	0	0	186	0	carbon storage regulator
STM474_2965	*alaS*	2993947	2996577	-1	1	0.000380084	2631	1	alanyl-tRNA synthetase
STM474_3069	*ispF*	3092827	3093306	-1	18	0.002083333	480	1	2-C-methyl-D-erythritol 2-4-cyclodiphosphate synthase
STM474_3070	*ispD*	3093306	3094016	-1	43	0.004219409	711	3	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase
STM474_3086	*STM474_3086*	3108814	3108948	-1	0	0	135	0	hypothetical protein
STM474_3096	*eno*	3118827	3120125	-1	78	0.006928406	1299	9	phosphopyruvate hydratase
STM474_3097	*pyrG*	3120208	3121845	-1	16	0.004884005	1638	8	CTP synthetase
STM474_3120	*STM474_3120*	3147933	3148100	-1	0	0	168	0	hypothetical protein

STM474_3140	*recB*	3167570	3171115	-1	258	0.004512126	3546	16	exonuclease V subunit beta
STM474_3142	*recC*	3174174	3177545	-1	62	0.002965599	3372	10	exodeoxyribonuclease V gamma subunit
STM474_3147	*thyA*	3179479	3180273	-1	0	0	795	0	thymidylate synthase
STM474_3148	*lgt*	3180280	3181155	-1	7	0.001141553	876	1	prolipoprotein diacylglyceryl transferase
STM474_3187	*lysS*	3219939	3221456	-1	4	0.000658762	1518	1	lysyl-tRNA synthetase
STM474_3188	*prfB*	3221466	3222542	-1	0	0	1077	0	peptide chain release factor 2
STM474_3195	*ygfZ*	3227638	3228618	1	35	0.002038736	981	2	putative global regulator
STM474_3204	*ubiH*	3239185	3240363	-1	136	0.005089059	1179	6	2-octaprenyl-6-methoxyphenyl hydroxylase
STM474_3210	*rpiA*	3245497	3246156	-1	12	0.001515152	660	1	ribose-5-phosphate isomerase A
STM474_3215	*fba*	3250231	3251310	-1	128	0.013888889	1080	15	fructose-bisphosphate aldolase
STM474_3216	*pgk*	3251412	3252575	-1	2	0.000859107	1164	1	phosphoglycerate kinase
STM474_3238	*metK*	3272383	3273549	1	3	0.000856898	1167	1	S-adenosylmethionine synthetase
STM474_3245	*yqgF*	3279368	3279784	1	8	0.004796163	417	2	Holliday junction resolvase-like protein
STM474_3326	*plsC*	3356657	3357394	-1	34	0.005420054	738	4	1-acyl-glycerol-3-phosphate acyltransferase
STM474_3327	*parC*	3357651	3359933	-1	191	0.007884363	2283	18	DNA topoisomerase IV subunit A
STM474_3335	*parE*	3364666	3366558	-1	75	0.00264131	1893	5	DNA topoisomerase IV subunit B
STM474_3349	*ribB*	3378556	3379209	-1	126	0.010703364	654	7	3-4-dihydroxy-2-butanone 4-phosphate synthase
STM474_3354	*rfaE*	3383264	3384697	-1	48	0.008368201	1434	12	bifunctional heptose 7-phosphate kinase/heptose 1-phosphate adenyltransferase
STM474_3358	*cca*	3389926	3391167	1	125	0.004830918	1242	6	multifunctional tRNA nucleotidyl transferase
STM474_3360	*folB*	3392191	3392553	-1	7	0.002754821	363	1	bifunctional dihydroneopterin aldolase/dihydroneopterin triphosphate 2'-epimerase
STM474_3362	*gcp*	3393519	3394532	-1	12	0.00295858	1014	3	O-sialoglycoprotein endopeptidase
STM474_3363	*rpsU*	3394760	3394975	1	0	0	216	0	30S ribosomal protein S21
STM474_3364	*dnaG*	3395211	3396956	1	1	0.000572738	1746	1	DNA primase
STM474_3365	*rpoD*	3396971	3398953	1	30	0.001008573	1983	2	RNA polymerase sigma factor RpoD
STM474_t3367	*STM474_t3367*	3399709	3399784	1	0	0	76	0	
STM474_3444	*infB*	3474315	3476993	-1	121	0.003732736	2679	10	initiation factor IF2-alpha
STM474_3445	*nusA*	3477018	3478520	-1	285	0.012641384	1503	19	transcription elongation factor NusA
STM474_3446	*STM474_3446*	3478548	3479006	-1	21	0.004357298	459	2	Ribosome maturation factor rimP
STM474_3452	*glmM*	3483112	3484449	-1	46	0.002242152	1338	3	phosphoglucosamine mutase
STM474_3454	*tnpA_2*	3485437	3485895	-1	0	0	459	0	transposase for IS200

STM474_3455	*hflB*	3486106	3488040	-1	144	0.007235142	1935	14	ATP-dependent metalloprotease
STM474_3456	*rrmJ*	3488144	3488770	-1	26	0.006379585	627	4	23S rRNA methyltransferase J
STM474_3461	*obgE*	3491635	3492807	-1	185	0.011935209	1173	14	GTPase ObgE
STM474_3463	*rpmA*	3493918	3494175	-1	0	0	258	0	50S ribosomal protein L27
STM474_3464	*rplU*	3494195	3494530	-1	11	0.00297619	336	1	50S ribosomal protein L21
STM474_3467	*murA*	3496312	3497571	-1	28	0.003968254	1260	5	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
STM474_3478	*yhbN*	3504472	3505047	1	41	0.003472222	576	2	lipopolysaccharide transport periplasmic protein LptA
STM474_3479	*yhbG*	3505054	3505779	1	86	0.002754821	726	2	putative ABC transporter ATP-binding protein YhbG
STM474_3504	*rpsI*	3533823	3534215	-1	0	0	393	0	30S ribosomal protein S9
STM474_3505	*rplM*	3534231	3534659	-1	5	0.002331002	429	1	50S ribosomal subunit protein L13
STM474_3533	*mreD*	3563328	3563819	-1	39	0.008130081	492	4	rod shape-determining protein MreD
STM474_3534	*mreC*	3563819	3564871	-1	23	0.002849003	1053	3	cell wall structural complex MreBCD transmembrane component MreC
STM474_3535	*mreB*	3564936	3566054	-1	22	0.004468275	1119	5	rod shape-determining protein mreB
STM474_3541	*STM474_3541*	3571515	3571985	1	0	0	471	0	acetyl-CoA carboxylase biotin carboxyl carrier protein subunit
STM474_3542	*STM474_3542*	3571996	3573345	1	0	0	1350	0	acetyl-CoA carboxylase biotin carboxylase subunit
STM474_t3557	*STM474_t3557*	3588202	3588277	-1	0	0	76	0	
STM474_r3558	*STM474_r3558*	3588291	3588405	-1	7	0.008695652	115	1	
STM474_r3559	*STM474_r3559*	3588604	3591591	-1	0	0	2988	0	
STM474_t3561	*STM474_t3561*	3591787	3591862	-1	0	0	76	0	
STM474_r3562	*STM474_r3562*	3591953	3593482	-1	0	0	1530	0	
STM474_3567	*yrdC*	3595571	3596143	-1	90	0.013961606	573	8	putative ribosome maturation factor
STM474_3571	*def*	3598418	3598927	1	49	0.005882353	510	3	peptide deformylase
STM474_3572	*fmt*	3598943	3599890	1	52	0.002109705	948	2	methionyl-tRNA formyltransferase
STM474_3579	*rplQ*	3604359	3604742	-1	0	0	384	0	50S ribosomal protein L17
STM474_3580	*rpoA*	3604783	3605772	-1	80	0.007070707	990	7	DNA-directed RNA polymerase subunit alpha
STM474_3581	*rpsD*	3605798	3606418	-1	3	0.001610306	621	1	30S ribosomal protein S4
STM474_3582	*rpsK*	3606452	3606841	-1	0	0	390	0	ribosomal protein S11
STM474_3583	*rpsM*	3606858	3607214	-1	0	0	357	0	30S ribosomal protein S13
STM474_3584	*rpmJ*	3607361	3607477	-1	1	0.008547009	117	1	50S ribosomal protein L36
STM474_3585	*secY*	3607509	3608840	-1	0	0	1332	0	preprotein translocase SecY subunit

STM474_3586	*rplO*	3608848	3609282	-1	0	0	435	0	50S ribosomal protein L15
STM474_3587	*rpmD*	3609286	3609465	-1	0	0	180	0	50S ribosomal protein L30
STM474_3588	*rpsE*	3609469	3609972	-1	0	0	504	0	30S ribosomal protein S5
STM474_3589	*rplR*	3609987	3610340	-1	0	0	354	0	50S ribosomal protein L18
STM474_3590	*rplF*	3610350	3610883	-1	0	0	534	0	50S ribosomal protein L6
STM474_3591	*rpsH*	3610896	3611288	-1	0	0	393	0	30S ribosomal protein S8
STM474_3592	*rpsN*	3611322	3611627	-1	0	0	306	0	30S ribosomal protein S14
STM474_3593	*rplE*	3611642	3612181	-1	0	0	540	0	50S ribosomal protein L5
STM474_3594	*rplX*	3612196	3612510	-1	0	0	315	0	50S ribosomal protein L24
STM474_3595	*rplN*	3612521	3612892	-1	0	0	372	0	50S ribosomal protein L14
STM474_3596	*rpsQ*	3613056	3613310	-1	21	0.003921569	255	1	30S ribosomal protein S17
STM474_3597	*rpmC*	3613310	3613501	-1	0	0	192	0	50S ribosomal protein L29
STM474_3598	*rplP*	3613501	3613911	-1	0	0	411	0	50S ribosomal protein L16
STM474_3599	*rpsC*	3613924	3614625	-1	0	0	702	0	30S ribosomal protein S3
STM474_3600	*rplV*	3614643	3614975	-1	0	0	333	0	50S ribosomal protein L22
STM474_3601	*rpsS*	3614990	3615268	-1	0	0	279	0	30S ribosomal protein S19
STM474_3602	*rplB*	3615285	3616106	-1	0	0	822	0	50S ribosomal protein L2
STM474_3603	*rplW*	3616124	3616426	-1	0	0	303	0	50S ribosomal protein L23
STM474_3604	*rplD*	3616423	3617028	-1	21	0.001650165	606	1	50S ribosomal protein L4
STM474_3605	*rplC*	3617039	3617668	-1	0	0	630	0	50S ribosomal protein L3
STM474_3606	*rpsJ*	3617701	3618012	-1	0	0	312	0	30S ribosomal protein S10
STM474_3612	*fusA*	3621040	3623154	-1	33	0.001891253	2115	4	elongation factor G
STM474_3613	*rpsG*	3623251	3623721	-1	13	0.004246285	471	2	30S ribosomal protein S7
STM474_3614	*rpsL*	3623817	3624191	-1	7	0.002666667	375	1	30S ribosomal subunit protein S12
STM474_3616	*yheM*	3624612	3624968	-1	36	0.008403361	357	3	sulfur relay protein TusC
STM474_3617	*yheN*	3624968	3625354	-1	68	0.012919897	387	5	sulfur transfer complex subunit TusD
STM474_3646	*tnpA_2*	3656679	3657137	-1	0	0	459	0	transposase for IS200
STM474_3662	*yrfF*	3671706	3673838	1	382	0.007501172	2133	16	putative inner membrane protein
STM474_3706	*asd*	3729951	3731126	-1	236	0.008503401	1176	10	aspartate-semialdehyde dehydrogenase
STM474_3735	*rpoH*	3757250	3758104	-1	14	0.001169591	855	1	RNA polymerase factor sigma-32
STM474_3736	*ftsX*	3758350	3759405	-1	120	0.006628788	1056	7	cell division protein FtsX
STM474_3738	*ftsY*	3760069	3761544	-1	23	0.001355014	1476	2	cell division protein FtsY

STM474_3827	*glyS*	3861086	3863155	-1	0	0	2070	0	glycyl-tRNA synthetase subunit beta
STM474_3828	*glyQ*	3863165	3864076	-1	7	0.002192982	912	2	glycyl-tRNA synthetase subunit alpha
STM474_3883	*rfaD*	3926631	3927563	1	69	0.004287245	933	4	ADP-L-glycero-D-mannoheptose-6-epimerase
STM474_3884	*rfaF*	3927566	3928612	1	71	0.006685769	1047	7	lipopolysaccharide heptosyltransferase II
STM474_3885	*rfaC*	3928612	3929565	1	41	0.007337526	954	7	ADP-heptose:LPS heptosyl transferase I
STM474_3897	*kdtA*	3940653	3941930	1	8	0.001564945	1278	2	3-deoxy-D-manno-octulosonic-acid transferase
STM474_3898	*coaD*	3941939	3942418	1	0	0	480	0	phosphopantetheine adenylyltransferase
STM474_3900	*rpmG*	3943353	3943520	-1	6	0.005952381	168	1	50S ribosomal protein L33
STM474_3901	*rpmB*	3943541	3943777	-1	0	0	237	0	50S ribosomal protein L28
STM474_3903	*dfp*	3944833	3946056	1	21	0.005718954	1224	7	flavoprotein
STM474_3904	*dut*	3946034	3946492	1	0	0	459	0	deoxyuridine 5'-triphosphate nucleotidohydrolase
STM474_3914	*gmk*	3954413	3955036	1	55	0.008012821	624	5	guanylate kinase
STM474_3991	*ccmF*	4035229	4037160	-1	0	0	1932	0	cytochrome c-type biogenesis protein CcmF
STM474_3993	*ccmD*	4037633	4037845	-1	0	0	213	0	heme exporter protein C
STM474_3994	*ccmC*	4037842	4038588	-1	0	0	747	0	heme exporter protein
STM474_3995	*ccmB*	4038631	4039290	-1	0	0	660	0	heme exporter protein
STM474_4013	*gyrB*	4060170	4062584	-1	26	0.000828157	2415	2	DNA gyrase subunit B
STM474_4015	*dnaN*	4063834	4064934	-1	36	0.000908265	1101	1	DNA polymerase III subunit beta
STM474_4016	*dnaA*	4064939	4066351	-1	0	0	1413	0	chromosomal replication initiator protein DnaA
STM474_4017	*rpmH*	4067000	4067140	1	0	0	141	0	50S ribosomal protein L34
STM474_4018	*rnpA*	4067157	4067516	1	65	0.013888889	360	5	ribonuclease P
STM474_4020	*STM474_4020*	4067740	4069386	1	94	0.003642987	1647	6	inner membrane protein OxaA
STM474_4040	*glmS*	4090347	4092176	-1	88	0.003278689	1830	6	D-fructose-6-phosphate amidotransferase
STM474_4041	*glmU*	4092365	4093735	-1	37	0.000729395	1371	1	bifunctional N-acetylglucosamine-1-phosphate uridyltransferase/acetyltransferase
STM474_4044	*atpD*	4095423	4096805	-1	202	0.011569053	1383	16	F0F1 ATP synthase subunit beta
STM474_4046	*atpA*	4097746	4099287	-1	124	0.009079118	1542	14	F0F1 ATP synthase subunit alpha
STM474_4047	*atpH*	4099300	4099833	-1	33	0.011235955	534	6	F0F1 ATP synthase subunit delta
STM474_4048	*atpF*	4099848	4100318	-1	25	0.010615711	471	5	F0F1 ATP synthase subunit B
STM474_r4068	*STM474_r4068*	4121447	4122976	1	0	0	1530	0	
STM474_t4069	*STM474_t4069*	4123067	4123142	1	0	0	76	0	
STM474_r4070	*STM474_r4070*	4123338	4126325	1	0	0	2988	0	

STM474_r4072	*STM474_r4072*	4126524	4126638	1	0	0	115	0	
STM474_t4074	*STM474_t4074*	4126914	4126989	1	0	0	76	0	
STM474_4090	*rep*	4141210	4143333	1	141	0.004237288	2124	9	ATP-dependent DNA helicase Rep
STM474_4094	*rhoL*	4146947	4147042	1	0	0	96	0	pseudogene
STM474_4095	*rho*	4147058	4148389	1	71	0.003753754	1332	5	transcription termination factor Rho
STM474_4106	*wzyE*	4158664	4160022	1	62	0.003679176	1359	5	putative common antigen polymerase
STM474_t4109	*STM474_t4109*	4162454	4162530	1	0	0	77	0	
STM474_t4110	*STM474_t4110*	4162585	4162660	1	15	0.013157895	76	1	
STM474_t4111	*STM474_t4111*	4162681	4162767	1	0	0	87	0	
STM474_t4112	*STM474_t4112*	4162810	4162886	1	0	0	77	0	
STM474_4115	*hemD*	4165858	4166598	-1	149	0.010796221	741	8	uroporphyrinogen-III synthase
STM474_4116	*hemC*	4166595	4167551	-1	86	0.007314525	957	7	porphobilinogen deaminase
STM474_4124	*dapF*	4173805	4174632	1	76	0.009661836	828	8	diaminopimelate epimerase
STM474_4149	*ubiE*	4198837	4199592	1	13	0.002645503	756	2	ubiquinone/menaquinone biosynthesis methyltransferase
STM474_4151	*ubiB*	4200204	4201844	1	0	0	1641	0	putative ubiquinone biosynthesis protein UbiB
STM474_4157	*yigC*	4205144	4206622	1	72	0.006085193	1479	9	3-octaprenyl-4-hydroxybenzoate decarboxylase
STM474_4165	*hemG*	4216682	4217227	1	4	0.001831502	546	1	protoporphyrinogen oxidase
STM474_r4166	*STM474_r4166*	4217612	4219141	1	0	0	1530	0	
STM474_t4167	*STM474_t4167*	4219216	4219292	1	0	0	77	0	
STM474_t4168	*STM474_t4168*	4219404	4219479	1	0	0	76	0	
STM474_r4169	*STM474_r4169*	4219662	4222649	1	0	0	2988	0	
STM474_r4171	*STM474_r4171*	4222745	4222859	1	0	0	115	0	
STM474_4178	*yjcD*	4227570	4230356	1	45	0.001435235	2787	4	putative xanthine/uracil permeases family protein
STM474_4179	*ysxC*	4230691	4231323	-1	118	0.009478673	633	6	ribosome biogenesis GTP-binding protein YsxC
STM474_4232	*STM474_4232*	4283841	4283966	1	0	0	126	0	hypothetical protein
STM474_4278	*priA*	4324800	4326998	-1	27	0.000909504	2199	2	primosome assembly protein PriA
STM474_4314	*murI*	4371338	4372189	1	22	0.004694836	852	4	glutamate racemase
STM474_r4315	*STM474_r4315*	4372576	4374105	1	0	0	1530	0	
STM474_t4316	*STM474_t4316*	4374180	4374256	1	0	0	77	0	
STM474_t4317	*STM474_t4317*	4374368	4374443	1	0	0	76	0	
STM474_r4318	*STM474_r4318*	4374626	4377613	1	0	0	2988	0	
STM474_r4320	*STM474_r4320*	4377709	4377823	1	0	0	115	0	

STM474_4321	*murB*	4378004	4379032	1	168	0.009718173	1029	10	UDP-N-acetylenolpyruvoylglucosamine reductase
STM474_4322	*birA*	4379029	4379991	1	75	0.008307373	963	8	biotin--protein ligase
STM474_4323	*coaA*	4380026	4380982	-1	134	0.007314525	957	7	pantothenate kinase
STM474_t4326	*STM474_t4326*	4381378	4381453	1	3	0.013157895	76	1	
STM474_t4327	*STM474_t4327*	4381462	4381546	1	1	0.011764706	85	1	
STM474_t4329	*STM474_t4329*	4381663	4381737	1	0	0	75	0	
STM474_4332	*secE*	4383349	4383732	1	0	0	384	0	preprotein translocase subunit SecE
STM474_4333	*nusG*	4383734	4384279	1	19	0.003663004	546	2	transcription antitermination protein NusG
STM474_4334	*rplK*	4384437	4384865	1	27	0.002331002	429	1	50S ribosomal protein L11
STM474_4335	*rplA*	4384869	4385573	1	211	0.004255319	705	3	50S ribosomal protein L1
STM474_4336	*rplJ*	4385993	4386490	1	0	0	498	0	50S ribosomal protein L10
STM474_4337	*rplL*	4386557	4386922	1	0	0	366	0	50S ribosomal protein L7/L12
STM474_4338	*rpoB*	4387240	4391268	1	16	0.000496401	4029	2	DNA-directed RNA polymerase subunit beta
STM474_4339	*rpoC*	4391345	4395568	1	370	0.002367424	4224	10	DNA-directed RNA polymerase subunit beta'
STM474_4353	*hemE*	4405477	4406541	1	198	0.005633803	1065	6	uroporphyrinogen decarboxylase
STM474_r4364	*STM474_r4364*	4416016	4417545	1	0	0	1530	0	
STM474_t4365	*STM474_t4365*	4417636	4417711	1	0	0	76	0	
STM474_r4366	*STM474_r4366*	4417905	4420892	1	0	0	2988	0	
STM474_4388	*STM474_4388*	4440133	4440423	-1	0	0	291	0	putative inner membrane protein
STM474_4428	*ubiA*	4476502	4477374	1	92	0.006872852	873	6	4-hydroxybenzoate octaprenyltransferase
STM474_4429	*plsB*	4477473	4479893	-1	119	0.00165221	2421	4	glycerol-3-phosphate acyltransferase
STM474_4431	*lexA*	4480541	4481149	1	14	0.003284072	609	2	LexA repressor
STM474_4440	*dnaB*	4487884	4489299	1	33	0.000706215	1416	1	replicative DNA helicase
STM474_4450	*ssb*	4497579	4498109	1	0	0	531	0	single-strand DNA-binding protein
STM474_4510	*STM474_4510*	4581463	4582338	-1	75	0.007990868	876	7	AraC family transcription regulator
STM474_4526	*groES*	4595356	4595649	1	0	0	294	0	co-chaperonin GroES
STM474_4527	*groEL*	4595693	4597339	1	0	0	1647	0	chaperonin GroEL
STM474_4545	*psd*	4613160	4614128	-1	78	0.004127967	969	4	phosphatidylserine decarboxylase
STM474_4546	*STM474_4546*	4614220	4615296	-1	39	0.002785515	1077	3	ribosome-associated GTPase
STM474_4547	*orn*	4615367	4615924	1	120	0.012544803	558	7	oligoribonuclease
STM474_4554	*yjeE*	4620468	4620929	1	17	0.004329004	462	2	putative ATPase
STM474_4588	*rpsF*	4651095	4651490	1	0	0	396	0	30S ribosomal protein S6

STM474_4589	*priB*	4651497	4651811	1	0	0	315	0	primosomal replication protein N
STM474_4590	*rpsR*	4651816	4652043	1	43	0.00877193	228	2	30S ribosomal protein S18
STM474_4611	*ppa*	4675323	4675853	-1	60	0.007532957	531	4	inorganic pyrophosphatase
STM474_4616	*STM474_4616*	4681690	4681806	1	1	0.008547009	117	1	hypothetical protein
STM474_4627	*STM474_4627*	4695124	4695237	-1	0	0	114	0	hypothetical protein
STM474_4673	*valS*	4737597	4740452	-1	72	0.0017507	2856	5	valyl-tRNA synthetase
STM474_4677	*yjgP*	4742938	4744038	1	60	0.00181653	1101	2	putative permease
STM474_4678	*yjgQ*	4744038	4745120	1	173	0.005540166	1083	6	putative permease
STM474_4747	*dnaC*	4823376	4824113	-1	137	0.013550136	738	10	DNA replication protein DnaC
STM474_4748	*dnaI*	4824116	4824655	-1	0	0	540	0	primosomal protein DnaI
STM474_p1063	*tlpA*	53055	54170	-1	0	0	1116	0	alpha-helical coiled coil protein
STM474_p1064	*STM474_p1064*	54429	54917	1	0	0	489	0	putative cytoplasmic protein
STM474_p1065	*STM474_p1065*	54444	54923	-1	0	0	480	0	hypothetical protein
STM474_p1066	*STM474_p1066*	55572	56312	1	0	0	741	0	putative carbonic anhydrase
STM474_p1067	*rlgA*	56447	57079	1	0	0	633	0	RlgA
STM474_p1077	*spvB*	63874	65649	1	255	0.005630631	1776	10	virulence protein
STM474_p1078	*spvC*	65930	66655	1	215	0.012396694	726	9	SpvC
STM474_p1096	*STM474_p1096*	75427	75723	1	0	0	297	0	putative cytoplasmic protein
STM474_p213	*ydfA*	11610	11870	-1	0	0	261	0	hypothetical protein
STM474_p214	*STM474_p214*	12400	12852	1	0	0	453	0	hypothetical protein
STM474_p256	*traS*	53206	53394	-1	0	0	189	0	TraS protein
STM474_p257	*traR*	53458	53862	-1	0	0	405	0	TraR protein
STM474_p258	*traQ*	53913	54440	-1	0	0	528	0	TraQ protein
STM474_p259	*traP*	54440	55144	-1	0	0	705	0	TraPprotein
STM474_p260	*traO*	55144	56433	-1	0	0	1290	0	TraO protein
STM474_p263	*traL*	58119	58466	-1	0	0	348	0	TraL protein
STM474_p267	*traI*	64339	65157	-1	0	0	819	0	TraI protein
STM474_p268	*traH*	65154	65612	-1	0	0	459	0	TraH protein
STM474_p269	*traG*	66007	66591	-1	27	0.001709402	585	1	TraG protein

Supplementary Table 2. Essential genes shared between *S*. Typhimurium strain ST4/74 and SL3261 and unique to each strain, based on gene orthology.

Strain	Essential Genes
ST4/74 + SL3261	*rplL, ssb, murG, parC, murI, hemH, rplT, uppS, glyQ, dnaG, ftsZ, adk, kdsA, CBW16804.1, rplF, rho, ileS, dnaE, rpoB, lolA, rpsM, ppa, mrdB, ftsX, imp, msbb, asd, ygfZ, cydB, glmU, rplE, rpoH, rplK, eno, lepB, rplB, dapE, rpsF, ubiE, rpoA, ispG, folE, rnc, rplQ, CBW17655.1, lgt, rimM, cmk, rpsI, prfA, mraY, mrdA, prfB, frr, yjeQ, mukF, engA, yjeE, hemG, murC, bamA, htrB, rpoC, CBW16927.1, rpmB, infB, murA, fabA, yhbG, dnaI, accA, priA, serS, pssA, birA, gyrA, ftsQ, dnaN, prsA, rpsQ, lpxK, plsB, parE, rne, accB, ssaT, fabD, rfbF, coaD, metG, dut, ribB, rplW, dxs, rlpB, lolC, ftsI, hemA, thyA, cca, hemC, holA, yeaz, ADX18510.1, ubiG, hnr, folD, grpE, acpP, gmk, psd, rpsH, rpsC, lspA, murB, secF, rpsE, bamD, mnmA, murD, rplY, metK, ribd, glnS, ADX15779.1, cydD, yadR, yrdC, lolD, yadF, CBW20220.1, asnCa, infC, fabH, ftsK, ADX16461.1, cysS, rplR, yidC, ffh, ubiF, lipA, holB, cydC, tsf, coaA, glmM, secD, kdtA, nadD, dnaB, ftsW, pheS, gcp, aspS, rtsA, groEL, ADX17746.1, mukE, ispF, trmd, proS, csrA, ribA, nusB, tyrS, ftsY, ispD, glmS, rpmD, rfbV, suhB, ADX16592.1, yqgF, yhbN, fabZ, ssaI, rpoD, rps16, yjgP, folA, lolE, dapD, acpS, pyrG, fabg, gltX, lexA, lpxB, rplC, rpl19, purB, ftsL, pgsA, engB, alaS, folC, aceF, rfbG, valS, rplD, hemB, rplO, pgk, lpxD, yjgQ, lpxc, dnaA, ubiH, map, rfbX, iscS, CBW17046.1, rpsB, rplM, fmt, thrS, coaE, yigC, hisS, rpsN, rplX, dnaX, mukB, sifB, cdsA, lpxA, nadE, lnt, pfs, lysS, groES, mviN, ubiB, leuS, rplJ, cydA, dxr, gyrB, glyS, ribF, plsC, ADX16465.1, gapA, tilS, fabI, rpsJ, secE, rpsT, zipA, dnaQ, rplP, ispH, CBW16800.1, secA, murF, ppnK, pheT, rpiA, def, ADX18437.1, argS, accD, lpxH, rplV, ipk, ribH, msbA, rpsG, hemE, ADX19172.1, lpdA, crr, fba, rpsA, murE, rpsL, ADX16782.1, rplN, dnaC, rpsD, rpmH, ribE, secY, rpmA, tmk, ligA, thiL, folB, pyrH, rpmC, fusA, rpsS, topA, rplU, ftsA, rpsK, rpmI, dapB, CBW17165.1, infA, dapA, ssaH, wzyE, dfp, kdsB*
ST4/74	*rfaE, nusG, traG, ccmC, tnpA_2, ADX18497.1, tolB, ccmG, traS, tnp, nrdB, traH, ADX17481.1, guaA, tlpA, ftsH, ADX20436.1, ccmC, traH, ccmG, sucA, dcd, clpX, polA, ADX19825.1, mreC, glyA, ccmD, tnpA_2, asnCb, sucB, ADX18378.1, ADX16724.1, atpF, ADX18396.1, ADX20466.1, CBW16157.1, rfaF, ADX18395.1, rplA, yciM, rfbH, dcoA, ccmF1, ispA, hemL, ccmB, ADX18571.1, sciY, pdxJ, traQ, atpA, rfaC, hns, ADX18546.1, ccmD, ccmF, spvC, mreD, ligB, tnpA_1b, ADX20501.1, ADX16045.1, traG, ybbV, ydfA, priB, rpsU, CBW19798.1, tnpA_1a, rpmJ, atpD, hemD, ADX16695.1, clpP, tolR, ppiB, ADX20186.1, ccmA, traR, ADX18346.1, rlgA, traL, yheM, nusA, himD, traL, ADX18762.1, traI, yheN, pdxH, orn, ybgT, ADX20437.1, ADX20435.1, atpH, rnpA, ADX16707.1, obgE, yjcD, fepC, ccmB, recB, nrdA, rfaD, traP, spvB, traQ, gmhA, fepD, rpsR, recT, icdA, ADX18728.1, tolA, traO, traR, mreB, ubiA, traI, igaA, recC, tnpA_1d, traS, rpmG, ADX19078.1, CBW18845.1, tnpA_2, ccmA2, rep, lon, ubiX, ftsJ, ruvB, traP, dapF, ADX20197.1, rpmJa, aceE*
SL3261	*group_584, ADX17031.1, sseJ, ADX17369.1, era, fabB, ADX17571.1, minE, fldA, CBW17298.1, ydgQ, yoeI, CBW17575.1, CBW17659.1, lolB, steC, dprA, pqaA, envF, pagO, ssaB, ispB, ADX17133.1, tyrP, ADX17563.1, group_583, sseA, ADX20084.1, ydcX, rfbJ, ybed, ssaS, CBW18649.1, yfhC, ftsE, yfiG, cysB, pth, pagK, CBW17658.1, yciG, sapD, ADX16502.1, ycaL, ssaJ, ycar, group_582, lipB*

Supplementary Table 3. Genes predicted to be essential for survival during growth in LB broth supplemented with 6% NaCl for *S.* Typhimurium strain ST4/74 (logFC < -2, q-value < 0.05).

Locus Tag	Gene Name	Function	logFC	q-value
STM474_4486	*proP*	proline/glycine betaine transporter	-14.2864	1.82E-29
STM474_3651	*dam*	DNA adenine methylase	-13.258	1.44E-17
STM474_4025	*STM474_4025*	putative reverse transcriptase	-12.3099	1.93E-07
STM474_1821	*ldcA*	L,D-carboxypeptidase A	-11.9385	1.48E-06
STM474_4126	*xerC*	site-specific tyrosine recombinase XerC	-11.5447	1.98E-05
STM474_3679	*yhgI*	putative DNA uptake protein	-11.1424	8.51E-05
STM474_3477	*lptc*	Lipopolysaccharide export system protein lptC	-10.8693	0.000193
STM474_2425	*nuoA*	NADH dehydrogenase subunit A	-10.7939	0.000249
STM474_2967	*recA*	recombinase A	-10.5075	0.000639
STM474_1927	*ruvA*	Holliday junction DNA helicase RuvA	-10.4632	0.000864
STM474_2648	*yfhP*	DNA-binding transcriptional regulator IscR	-10.4021	0.000827
STM474_2417	*nuoJ*	NADH dehydrogenase subunit J	-10.3128	0.00095
STM474_0760	*sucB*	dihydrolipoamide succinyltransferase	-10.123	0.001683
STM474_4531	*efp*	Elongation factor P	-9.85999	0.004662
STM474_0773	*tolB*	translocation protein TolB	-9.8132	0.003464
STM474_3873	*gpsA*	NAD(P)H-dependent glycerol-3-phosphate dehydrogenase	-9.80019	0.003231
STM474_0759	*sucA*	alpha-ketoglutarate decarboxylase	-9.79139	0.005523
STM474_t4330	*STM474_t4330*		-9.78179	0.003464
STM474_0147	*coaE*	dephospho-CoA kinase	-9.58502	0.005209
STM474_0979	*mukE*	condesin subunit E	-9.49353	0.006599
STM474_1723	*STM474_1723*	tetratricopeptide repeat protein	-9.45133	0.00666
STM474_3736	*ftsX*	cell division protein FtsX	-9.35984	0.00787
STM474_1796	*lolB*	outer membrane lipoprotein LolB	-9.35833	0.00787
STM474_0973	*ycar*	UPF0434 protein ycaR	-9.3371	0.00787
STM474_0602	*ybdJ*	putative inner membrane protein	-9.32608	0.009085

STM474_t2960	*STM474_t2960*		-9.30194	0.008796
STM474_2372	*ubiG*	3-demethylubiquinone-9 3-methyltransferase	-9.2771	0.009228
STM474_4045	*atpG*	F0F1 ATP synthase subunit gamma	-9.13357	0.011851
STM474_0714	*fur*	ferric uptake regulator	-9.12608	0.01268
STM474_2173	*rfbX*	putative O-antigen transporter	-9.11242	0.012603
STM474_2586	*dapE*	succinyl-diaminopimelate desuccinylase	-9.0584	0.012686
STM474_2454	*ubiX*	3-octaprenyl-4-hydroxybenzoate carboxy-lyase	-8.93441	0.014673
STM474_0236	*fabZ*	(3R)-hydroxymyristoyl-ACP dehydratase	-8.91153	0.015899
STM474_t0260	*STM474_t0260*		-8.88469	0.015731
STM474_3231	*STM474_3231*	putative racemase	-8.84994	0.017713
STM474_1894	*pagK*	PagK	-8.7465	0.021985
STM474_t1263	*STM474_t1263*		-8.70024	0.021003
STM474_4279	*rpmE*	50S ribosomal protein L31	-8.6853	0.021985
STM474_1562	*STM474_1562*	putative cytoplasmic protein	-8.64575	0.025729
STM474_4116	*hemC*	porphobilinogen deaminase	-8.62716	0.027045
STM474_1926	*ruvB*	Holliday junction DNA helicase RuvB	-8.54455	0.025799
STM474_4322	*birA*	biotin--protein ligase	-8.51101	0.029869
STM474_0980	*mukB*	cell division protein MukB	-8.43958	0.032516
STM474_2177	*rfbF*	glucose-1-phosphate cytidylyltransferase	-8.41213	0.029938
STM474_2503	*STM474_2503*	hypothetical protein	-8.35481	0.036732
STM474_1816	*STM474_1816*	hypothetical protein	-8.34478	0.033695
STM474_2992	*STM474_2992*	hypothetical protein	-8.26492	0.038157
STM474_3612	*fusA*	elongation factor G	-8.24291	0.041646
STM474_2747	*STM474_2747*	putative bacteriophage protein	-8.18896	0.046678
STM474_2740	*STM474_2740*	Gifsy-1 prophage cI	-8.1733	0.04307
STM474_0128	*ftsI*	penicillin-binding protein 3 precursor	-8.13559	0.046527
STM474_2625	*ispG*	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	-8.12238	0.046678
STM474_4049	*atpE*	F0F1 ATP synthase subunit C	-8.08707	0.047333
STM474_0947	*ftsK*	DNA translocase FtsK	-7.48221	0.004779

STM474_0658	*dacA*	D-alanyl-D-alanine carboxypeptidase fraction A	-7.42634	2.10E-05
STM474_0774	*pal*	peptidoglycan-associated lipoprotein precursor	-7.02068	0.014846
STM474_0968	*infB*	integration host factor subunit beta	-6.94157	0.012828
STM474_1383	*STM474_1383*	hypothetical protein	-6.91572	0.015608
STM474_2622	*bamBL*	outer membrane protein assembly complex subunit BamB	-6.90942	0.010882
STM474_3737	*ftsE*	cell division protein FtsE	-6.8096	0.019669
STM474_3444	*infB*	initiation factor IF2-alpha	-6.55836	0.025307
STM474_1795	*STM474_1795*	hypothetical protein	-6.47218	0.033569
STM474_1868	*prc*	tail-specific protease	-6.46913	6.17E-06
STM474_1188	*rpmF*	50S ribosomal protein L32	-6.25376	0.047288
STM474_3191	*xerD*	site-specific tyrosine recombinase XerD	-6.13513	0.015281
STM474_3140	*recB*	exonuclease V subunit beta	-5.66244	0.03312
STM474_4370	*STM474_4370*	acetyltransferase, gnat family	-5.61135	0.039737
STM474_4356	*hupA*	transcriptional regulator HU subunit alpha	-5.0074	0.00666
STM474_4435	*zur*	zinc uptake transcriptional repressor	-4.56706	0.021302
STM474_0445	*thiI*	thiamine biosynthesis protein ThiI	-4.0288	3.07E-05
STM474_4285	*metL*	bifunctional aspartate kinase II/homoserine dehydrogenase II	-4.02247	8.28E-10
STM474_0690	*STM474_0690*	PhoH-like ATP-binding protein	-3.95654	0.019669
STM474_0126	*mraW*	S-adenosyl-methyltransferase MraW	-3.8907	0.015731
STM474_1605	*ydcY*	putative cytoplasmic protein	-3.85442	0.049005
STM474_2535	*ptsI*	phosphoenolpyruvate-protein phosphotransferase	-3.79121	0.027797
STM474_3918	*recG*	ATP-dependent DNA helicase RecG	-3.69813	2.12E-07
STM474_3660	*mrcA*	penicillin-binding protein 1A	-3.5631	6.58E-12
STM474_4785	*radA*	DNA repair protein RadA	-3.55514	0.000146
STM474_2604	*ppk*	polyphosphate kinase	-3.46132	4.30E-10
STM474_1803	*engD*	GTP-binding protein EngD	-3.44698	0.021302
STM474_2811	*smpB*	SsrA-binding protein	-3.44131	0.000303
STM474_3483	*yhbJ*	nucleotide-binding protein yhbJ	-3.36073	0.012603
STM474_4565	*rnr*	ribonuclease R	-3.35415	0.000555

STM474_3639	*ppia*	Peptidyl-prolyl cis-trans isomerase A	-3.33423	2.45E-07
STM474_4129	*corA*	magnesium/nickel/cobalt transporter CorA	-3.30723	0.047288
STM474_3421	*yraM*	putative transglycosylase	-3.18624	2.58E-06
STM474_0146	*yacf*	UPF0289 protein yacF	-3.12259	0.003935
STM474_2470	*flk*	flagella biosynthesis regulator	-2.8966	0.009089
STM474_3878	*yibP*	M23 peptidase domain-containing protein	-2.81147	0.001897
STM474_4128	*uvrD*	DNA-dependent helicase II	-2.76327	1.31E-05
STM474_3279	*iraD*	putative cytoplasmic protein	-2.73281	0.045476
STM474_2613	*xseA*	exodeoxyribonuclease VII large subunit	-2.60393	0.00095
STM474_3337	*icc*	cyclic 3',5'-adenosine monophosphate phosphodiesterase	-2.58545	0.001244
STM474_1727	*acnA*	aconitate hydratase	-2.57113	0.001667
STM474_2756	*srmB*	ATP-dependent RNA helicase SrmB	-2.50466	0.009664
STM474_2949	*proX*	glycine betaine transporter periplasmic subunit	-2.44067	0.009089
STM474_4244	*pfkA*	6-phosphofructokinase	-2.24445	0.012828
STM474_2935	*STM474_2935*	DNA binding protein, nucleoid-associated	-2.20742	0.020718
STM474_1443	*nemA*	N-ethylmaleimide reductase	-2.17362	0.020788
STM474_3509	*degS*	serine endoprotease	-2.08007	0.047333
STM474_0424	*tgt*	queuine tRNA-ribosyltransferase	-2.07551	0.046696

Supplementary Table 4. Genes predicted to be essential for survival during growth in LB broth supplemented with 14mM citric acid for *S.* Typhimurium strain ST4/74 (logFC < -2, q-value < 0.05).

Locus Tag	Gene Name	Function	logFC	q-value
STM474_3668	*envZ*	osmolarity sensor protein	-14.60147816	1.36E-46
STM474_1228	*phoQ*	sensor protein PhoQ	-13.72924998	3.89E-24
STM474_3669	*ompR*	osmolarity response regulator	-13.6962973	1.12E-23
STM474_2956	*gshA*	glutamate--cysteine ligase	-13.12774772	1.57E-12
STM474_4025	*STM474_4025*	putative reverse transcriptase	-12.38149182	4.01E-06
STM474_1229	*phoP*	DNA-binding transcriptional regulator PhoP	-12.24877377	1.03E-05
STM474_4176	*dsbA*	thiol:disulfide interchange protein	-12.22316256	1.27E-05
STM474_0218	*htrA*	serine endoprotease	-11.97564605	3.14E-51
STM474_2467	*truA*	tRNA pseudouridine synthase A	-11.8901239	6.35E-05
STM474_4152	*tatA*	twin arginine translocase protein A	-11.64877987	0.000239824
STM474_0719	*pgm*	phosphoglucomutase	-11.52186488	0.000260662
STM474_0651	*crcB*	camphor resistance protein CrcB	-11.47852774	0.000467344
STM474_1792	*hemK*	N5-glutamine S-adenosyl-L-methionine-dependent methyltransferase	-11.38656437	0.000444
STM474_1863	*yobG*	hypothetical protein	-11.07344879	0.001399477
STM474_3477	*lptc*	Lipopolysaccharide export system protein lptC	-10.94078608	0.001325959
STM474_2622	*bamB*	outer membrane protein assembly complex subunit BamB	-10.88549825	0.002682387
STM474_2425	*nuoA*	NADH dehydrogenase subunit A	-10.86534629	0.001582386
STM474_4150	*yigP*	putative inner membrane protein	-10.71651284	0.002540417
STM474_3064	*rpoS*	RNA polymerase sigma factor RpoS	-10.68044004	0.003381504
STM474_2967	*recA*	recombinase A	-10.57893455	0.003110562
STM474_1927	*ruvA*	Holliday junction DNA helicase RuvA	-10.53475913	0.003951079
STM474_2417	*nuoJ*	NADH dehydrogenase subunit J	-10.38425062	0.004650679
STM474_4241	*cpxR*	DNA-binding transcriptional regulator CpxR	-10.33666148	7.49E-13
STM474_0756	*sdhD*	succinate dehydrogenase cytochrome b556 small membrane subunit	-10.26171374	0.007176649

STM474_3358	*cca*	multifunctional tRNA nucleotidyl transferase/2'3'-cyclic phosphodiesterase/2'nucleotidase/phosphatase	-10.21903771	0.006894412
STM474_0760	*sucB*	dihydrolipoamide succinyltransferase	-10.19451845	0.007005952
STM474_2422	*nuoE*	NADH dehydrogenase subunit E	-10.1742611	0.007170568
STM474_2291	*STM474_2291*	hypothetical protein	-10.13065454	0.009446217
STM474_1702	*pspB*	phage shock protein B	-10.07323675	0.008002566
STM474_1814	*STM474_1814*	putative periplasmic protein	-10.0325225	0.01042434
STM474_1454	*mliC*	lysozyme inhibitor	-10.03104894	0.008512449
STM474_4733	*STM474_4733*	putative cytoplasmic protein	-10.00572761	0.009251909
STM474_0013	*dnaK*	molecular chaperone DnaK	-10.00003397	0.008908555
STM474_0403	*STM474_0403*	hypothetical protein	-9.977891306	0.011374586
STM474_4531	*efp*	Elongation factor P	-9.93120444	0.013331306
STM474_3915	*rpoZ*	DNA-directed RNA polymerase subunit omega	-9.896299767	0.010562026
STM474_0773	*tolB*	translocation protein TolB	-9.884666556	0.011374586
STM474_0611	*fepG*	iron-enterobactin transporter permease	-9.866612432	0.010991715
STM474_2599	*yfgE*	DNA replication initiation factor	-9.812703755	0.012548099
STM474_1724	*yciS*	inner membrane protein yciS	-9.763818496	0.015033328
STM474_2753	*rpoE*	RNA polymerase sigma factor RpoE	-9.728707501	0.013892894
STM474_3990	*ccmG*	heme lyase disulfide oxidoreductase	-9.722845549	0.014316743
STM474_1154	*STM474_1154*	hypothetical protein	-9.722525273	0.014507132
STM474_0160	*aceE*	pyruvate dehydrogenase subunit E1	-9.687892244	0.014564841
STM474_2671	*STM474_2671*	putative periplasmic protein	-9.66391858	0.016441702
STM474_3444	*infB*	initiation factor IF2-alpha	-9.607162896	0.016441702
STM474_3443	*rbfA*	ribosome-binding factor A	-9.53350679	0.019553303
STM474_0973	*ycar*	UPF0434 protein ycaR	-9.408449093	0.021719983
STM474_1276	*STM474_1276*	putative transglycosylase associated protein	-9.316777966	0.025644372
STM474_1858	*STM474_1858*	penicillin-binding protein	-9.306846191	4.75E-18
STM474_2646	*nifU*	scaffold protein	-9.295882442	0.031177112
STM474_0425	*yajC*	preprotein translocase subunit YajC	-9.291394573	0.025644372

STM474_0216	*pfs*	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase	-9.249055628	0.032208833
STM474_0656	*lipB*	lipoyltransferase	-9.204073587	0.032948071
STM474_0714	*fur*	ferric uptake regulator	-9.197474218	0.032948071
STM474_3335	*parE*	DNA topoisomerase IV subunit B	-9.19305981	0.031177112
STM474_4106	*wzyE*	putative common antigen polymerase	-9.176600222	0.032948071
STM474_t4757	*STM474_t4757*		-9.149044952	0.034106821
STM474_t3806	*STM474_t3806*		-9.13896519	0.032948071
STM474_4677	*yjgP*	putative permease	-9.082784052	0.036489875
STM474_0097	*imp*	LPS-assembly protein	-9.043080032	0.037508037
STM474_0755	*sdhC*	succinate dehydrogenase cytochrome b556 large membrane subunit	-9.038113777	0.036975177
STM474_0610	*fepC*	iron-enterobactin transporter ATP-binding protein	-9.027227397	0.037508037
STM474_1275	*STM474_1275*	hypothetical protein	-8.983880562	0.040702257
STM474_0236	*fabZ*	(3R)-hydroxymyristoyl-ACP dehydratase	-8.982836953	0.041987859
STM474_t0260	*STM474_t0260*		-8.956004372	0.041987859
STM474_3243	*gshB*	glutathione synthetase	-8.933323631	1.48E-05
STM474_1427	*ssaT*	type III secretion system apparatus protein	-8.898664634	0.046786105
STM474_4043	*atpC*	F0F1 ATP synthase subunit epsilon	-8.844947588	0.04745179
STM474_0096	*surA*	peptidyl-prolyl cis-trans isomerase SurA	-8.621425714	0.000223249
STM474_2643	*hscA*	chaperone protein HscA	-8.021717383	0.002643016
STM474_t2493	*STM474_t2493*		-7.907287992	0.003078037
STM474_0947	*ftsK*	DNA translocase FtsK	-7.575051989	0.016682685
STM474_3340	*tolC*	outer membrane channel	-7.566094543	1.58E-31
STM474_3874	*secB*	preprotein translocase subunit SecB	-7.530209978	0.018930986
STM474_2423	*nuoC*	bifunctional NADH:ubiquinone oxidoreductase subunit C/D	-7.469515307	0.000666886
STM474_0968	*infB*	integration host factor subunit beta	-7.246447416	0.019916798
STM474_t0339	*STM474_t0339*		-7.19116881	0.022742072
STM474_0391	*sbmA*	transport protein	-7.004166322	1.90E-09
STM474_t4330	*STM474_t4330*		-6.97266962	0.044742806
STM474_1343	*ihfA*	integration host factor subunit alpha	-6.916795332	0.049520055

STM474_4240	*cpxA*	two-component sensor protein	-6.407598606	1.47E-06
STM474_1867	*htpX*	heat shock protein HtpX	-6.317776753	0.014907178
STM474_2808	*STM474_2808*	small membrane protein A	-5.809537527	0.021697036
STM474_3480	*rpoN*	RNA polymerase factor sigma-54	-5.676289862	0.00246266
STM474_4059	*trkD*	potassium transport protein Kup	-5.556069528	5.07E-26
STM474_2413	*nuoN*	NADH dehydrogenase subunit N	-5.392374484	0.049520055
STM474_1803	*engD*	GTP-binding protein EngD	-5.131331677	0.005432237
STM474_4308	*oxyR*	DNA-binding transcriptional regulator OxyR	-5.019374613	2.84E-21
STM474_0199	*mrcB*	penicillin-binding protein 1b	-4.802529023	1.70E-28
STM474_3223	*tktA*	transketolase	-4.721212153	0.003186912
STM474_1943	*STM474_1943*	putative penicillin-binding protein	-4.506632699	8.00E-08
STM474_2420	*nuoG*	NADH dehydrogenase subunit G	-4.488426904	0.024065316
STM474_4158	*fre*	FMN reductase	-3.897053487	4.78E-07
STM474_3895	*rfaG*	glucosyltransferase I	-3.879241202	1.25E-10
STM474_3653	*aroB*	3-dehydroquinate synthase	-3.866461571	0.009868157
STM474_3610	*tuf*	Elongation factor Tu	-3.751439526	0.025135149
STM474_1363	*aroD*	3-dehydroquinate dehydratase	-3.645913556	0.023068056
STM474_4608	*ytfP*	putative cytoplasmic protein	-3.623009952	0.002848224
STM474_3894	*rfaP*	lipopolysaccharide core biosynthetic protein	-3.557928404	5.88E-05
STM474_1146	*mdoH*	glucosyltransferase MdoH	-3.53082494	8.23E-09
STM474_3891	*rfaI*	lipopolysaccharide-alpha-1, 3-D-galactosyltransferase	-3.523030289	2.61E-25
STM474_3673	*feoB*	ferrous iron transport protein B	-3.519105083	2.45E-23
STM474_0496	*acrA*	acridine efflux pump	-3.493030089	1.53E-05
STM474_4091	*gppA*	guanosine pentaphosphate phosphohydrolase	-3.479215559	8.50E-09
STM474_3878	*yibP*	M23 peptidase domain-containing protein	-3.37231918	8.01E-05
STM474_4033	*pstB*	phosphate transporter subunit	-3.231851794	0.000676184
STM474_3509	*degS*	serine endoprotease	-3.210391172	0.000118225
STM474_4266	*fpr*	ferredoxin-NADP reductase	-3.122134377	0.002413957
STM474_0167	*acnB*	bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase	-3.078675099	0.007176649

STM474_2166	*gnd*	6-phosphogluconate dehydrogenase, decarboxylating	-2.873333119	2.60E-08
STM474_0172	*pdxA*	4-hydroxythreonine-4-phosphate dehydrogenase 2	-2.736768238	0.000502392
STM474_0331	*pepD*	aminoacyl-histidine dipeptidase	-2.643586225	2.77E-05
STM474_3672	*feoA*	ferrous iron transport protein A	-2.60914368	0.004167612
STM474_3325	*sufI*	repressor protein for FtsI	-2.550016184	0.000666886
STM474_1663	*nifJ*	putative pyruvate-flavodoxin oxidoreductase	-2.487615067	1.12E-11
STM474_0124	*fruR*	DNA-binding transcriptional regulator FruR	-2.437573819	0.004680888
STM474_0424	*tgt*	queuine tRNA-ribosyltransferase	-2.365285138	0.026739435
STM474_1727	*acnA*	aconitate hydratase	-2.346865439	0.000114599
STM474_1145	*mdoG*	glucans biosynthesis protein G	-2.320908382	0.004226316
STM474_3331	*ygiY*	sensor protein QseC	-2.293194569	0.011863637
STM474_4034	*pstA*	phosphate transporter permease subunit	-2.237838295	0.049520055
STM474_2613	*xseA*	exodeoxyribonuclease VII large subunit	-2.208691822	0.00524185
STM474_2178	*rfbI*	CDP-6-deoxy-delta-3,4-glucoseen reductase	-2.207923219	3.66E-07
STM474_0495	*acrB*	acridine efflux pump	-2.146968671	6.06E-07
STM474_3651	*dam*	DNA adenine methylase	-2.125476277	0.019175174
STM474_4310	*yijC*	DNA-binding transcriptional repressor FabR	-2.08424804	0.002896705
STM474_3918	*recG*	ATP-dependent DNA helicase RecG	-2.025958985	1.84E-05

Supplementary Table 5. Genes predicted to be essential for survival during 24-hour desiccation in *S.* Typhimurium strain ST4/74 (logFC < -2, q-value < 0.05).

Locus Tag	Gene Name	Function	logFC	q-value
STM474_2415	*nuoL*	NADH dehydrogenase subunit L	-12.50115842	3.80E-11
STM474_2417	*nuoJ*	NADH dehydrogenase subunit J	-10.41698534	6.74E-05
STM474_2422	*nuoE*	NADH dehydrogenase subunit E	-10.20713591	0.000177269
STM474_3824	*STM474_3824*	putative cytoplasmic protein	-10.14925211	0.000249669
STM474_4050	*atpB*	F0F1 ATP synthase subunit A	-10.07603318	0.000225132
STM474_4044	*atpD*	F0F1 ATP synthase subunit beta	-10.01714298	0.000381176
STM474_1383	*STM474_1383*	hypothetical protein	-9.996283181	0.000381176
STM474_4747	*dnaC*	DNA replication protein DnaC	-9.984911097	0.000287834
STM474_4531	*efp*	Elongation factor P	-9.963492929	0.00071845
STM474_2599	*yfgE*	DNA replication initiation factor	-9.845211169	0.000511861
STM474_2753	*rpoE*	RNA polymerase sigma factor RpoE	-9.761409652	0.000593947
STM474_4678	*yjgQ*	putative permease	-9.758483333	0.000887899
STM474_0973	*ycar*	UPF0434 protein ycaR	-9.441046486	0.001350444
STM474_2646	*nifU*	scaffold protein	-9.328992108	0.002722351
STM474_4547	*orn*	oligoribonuclease	-9.290247604	0.002496285
STM474_0216	*pfs*	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase	-9.281275858	0.002722351
STM474_4157	*yigC*	3-octaprenyl-4-hydroxybenzoate decarboxylase	-9.240424329	0.002374341
STM474_4045	*atpG*	F0F1 ATP synthase subunit gamma	-9.237902613	0.002557862
STM474_0714	*fur*	ferric uptake regulator	-9.230509271	0.002810607
STM474_3884	*rfaF*	lipopolysaccharide heptosyltransferase II	-9.205395524	0.00251519
STM474_2586	*dapE*	succinyl-diaminopimelate desuccinylase	-9.16210577	0.002722351
STM474_4040	*glmS*	D-fructose-6-phosphate amidotransferase	-9.111409569	0.002722351
STM474_2454	*ubiX*	3-octaprenyl-4-hydroxybenzoate carboxy-lyase	-9.038347361	0.003027664
STM474_3231	*STM474_3231*	putative racemase	-8.954199514	0.00405867
STM474_4043	*atpC*	F0F1 ATP synthase subunit epsilon	-8.87754261	0.004358021
STM474_3572	*fmt*	methionyl-tRNA formyltransferase	-8.786086751	0.005346584

STM474_3281	*STM474_3281*	hypothetical protein	-8.778278462	0.009824294
STM474_1562	*STM474_1562*	putative cytoplasmic protein	-8.749119163	0.007627319
STM474_1455	*pdxH*	pyridoxamine 5'-phosphate oxidase	-8.670535585	0.007250224
STM474_1926	*ruvB*	Holliday junction DNA helicase RuvB	-8.64825676	0.0076237
STM474_2687	*lepB*	signal peptidase I	-8.625077403	0.009824294
STM474_1837	*minE*	cell division topological specificity factor MinE	-8.595713627	0.00822753
STM474_2426	*STM474_2426*	putative cytoplasmic protein	-8.563469329	0.009514988
STM474_0980	*mukB*	cell division protein MukB	-8.543991484	0.010798658
STM474_1897	*STM474_1897*	hypothetical protein	-8.509054931	0.012311909
STM474_1414	*ssaI*	type III secretion system apparatus protein	-8.486225107	0.012506491
STM474_1816	*STM474_1816*	hypothetical protein	-8.448863279	0.010798658
STM474_2530	*zipA*	cell division protein ZipA	-8.380852614	0.012532345
STM474_3612	*fusA*	elongation factor G	-8.347212172	0.013896231
STM474_4611	*ppa*	inorganic pyrophosphatase	-8.272673349	0.016437028
STM474_2533	*STM474_2533*	hypothetical protein	-8.234146825	0.020464749
STM474_4049	*atpE*	F0F1 ATP synthase subunit C	-8.190781226	0.016387402
STM474_0132	*murD*	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase	-8.152237588	0.025044614
STM474_0692	*ubiF*	2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase	-8.146920029	0.040551437
STM474_0444	*xseB*	exodeoxyribonuclease VII small subunit	-8.133410351	0.018375734
STM474_2592	*dapA*	dihydrodipicolinate synthase	-8.130898604	0.017764842
STM474_0557	*ppiB*	peptidyl-prolyl cis-trans isomerase B	-7.966648234	0.026814417
STM474_t3450	*STM474_t3450*		-7.907538235	0.026814417
STM474_3467	*murA*	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	-7.868935309	0.028401018
STM474_3547	*fis*	DNA-binding protein Fis	-7.855317023	2.80E-06
STM474_1614	*sifB*	secreted effector protein	-7.848541833	0.029175863
STM474_3452	*glmM*	phosphoglucosamine mutase	-7.793027388	0.040277217
STM474_4590	*rpsR*	30S ribosomal protein S18	-7.778928903	0.037812563
STM474_4404	*STM474_4404*	hypothetical protein	-7.643173251	0.040604813
STM474_3633	*crp*	cAMP-regulatory protein	-7.325746976	0.00088422

STM474_0718	*seqa*	Protein seqA	-7.059744263	0.00101299
STM474_3737	*ftsE*	cell division protein FtsE	-6.896596508	0.004358021
STM474_0439	*thiL*	thiamine monophosphate kinase	-6.316018672	0.014121063
STM474_1427	*ssaT*	type III secretion system apparatus protein	-6.266050157	0.011467296
STM474_2177	*rfbF*	glucose-1-phosphate cytidylyltransferase	-5.852145632	0.0234833
STM474_0772	*tolA*	cell envelope integrity inner membrane protein TolA	-5.838207398	0.024486514
STM474_1198	*holB*	DNA polymerase III subunit delta'	-5.779732484	0.027481556
STM474_4541	*STM474_4541*	superoxide dismutase	-5.66939665	0.003388265
STM474_2625	*ispG*	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	-5.564435055	0.039541805
STM474_2350	*ccmA*	cytochrome c biogenesis protein CcmA	-5.52633251	0.007669188
STM474_2420	*nuoG*	NADH dehydrogenase subunit G	-5.510038409	0.001562264
STM474_1343	*ihfA*	integration host factor subunit alpha	-5.448984945	0.011907394
STM474_0756	*sdhD*	succinate dehydrogenase cytochrome b556 small membrane subunit	-5.311852268	0.03405146
STM474_3651	*dam*	DNA adenine methylase	-5.270321178	2.16E-08
STM474_4565	*rnr*	ribonuclease R	-5.197971837	0.002722351
STM474_2534	*ptsH*	phosphocarrier protein ptsH	-5.144687583	0.039584471
STM474_2071	*STM474_2071*	hypothetical protein	-5.142140068	0.016437028
STM474_4126	*xerC*	site-specific tyrosine recombinase XerC	-5.058899159	0.002967968
STM474_0096	*surA*	peptidyl-prolyl cis-trans isomerase SurA	-5.028014816	0.0076237
STM474_2419	*nuoH*	NADH dehydrogenase subunit H	-5.021008031	0.036579661
STM474_4733	*STM474_4733*	putative cytoplasmic protein	-4.93426666	0.024611846
STM474_0402	*proC*	pyrroline-5-carboxylate reductase	-4.91124337	0.019150972
STM474_4558	*hfq*	RNA-binding protein Hfq	-4.818654728	0.026814417
STM474_4158	*fre*	FMN reductase	-4.525691525	2.19E-12
STM474_2975	*srlL*	DNA-binding transcriptional repressor SrlR	-4.423640659	9.76E-07
STM474_1803	*engD*	GTP-binding protein EngD	-4.416676724	0.000799209
STM474_1868	*prc*	tail-specific protease	-4.381481219	2.53E-06
STM474_3223	*tktA*	transketolase	-4.170298993	9.49E-05
STM474_1716	*rnb*	exoribonuclease II	-4.114767627	4.59E-06

STM474_2688	*lepA*	GTP-binding protein LepA	-3.988163847	0.000906914
STM474_4614	*STM474_4614*	putative transcriptional regulator	-3.674860613	9.78E-10
STM474_4053	*gidA*	glucose-inhibited division protein A	-3.53080099	0.009824294
STM474_0761	*sucC*	succinyl-CoA synthetase subunit beta	-3.238595626	0.037978568
STM474_2535	*ptsI*	phosphoenolpyruvate-protein phosphotransferase	-3.232994121	0.013465379
STM474_3139	*recD*	exonuclease V subunit alpha	-3.187860288	1.19E-06
STM474_1827	*nhaB*	sodium/proton antiporter	-3.156883084	1.24E-06
STM474_3340	*tolC*	outer membrane channel	-2.663408263	4.57E-08
STM474_0753	*gltA*	type II citrate synthase	-2.6052994	0.000772144
STM474_4025	*STM474_4025*	putative reverse transcriptase	-2.573448594	0.029185664
STM474_3243	*gshB*	glutathione synthetase	-2.532865897	0.013896231
STM474_3918	*recG*	ATP-dependent DNA helicase RecG	-2.488043226	1.24E-06
STM474_3859	*mtlR*	mannitol repressor protein	-2.46735888	0.027232303
STM474_0193	*pcnB*	poly(A) polymerase I	-2.390137052	0.004477484
STM474_3203	*visC*	protein VisC	-2.369470377	0.045145333
STM474_0991	*pncB*	nicotinate phosphoribosyltransferase	-2.206958367	0.001192128
STM474_3690	*glpR*	DNA-binding transcriptional repressor GlpR	-2.145754332	0.004197862
STM474_2442	*STM474_2442*	putative transcriptional regulator	-2.135477162	0.01739489
STM474_3700	*STM474_3700*	putative transcriptional regulator	-2.120257421	5.61E-09
STM474_0862	*ybis*	putative L,D-transpeptidase YbiS	-2.098361155	0.009457115
STM474_3858	*mtlD*	mannitol-1-phosphate 5-dehydrogenase	-2.061246049	0.00071845

Supplementary Table 6. Genes predicted to be essential for survival during heat inactivation at 60°C for 30 seconds for *S.* Typhimurium strain ST4/74 (logFC < -2, q-value < 0.05).

Locus Tag	Gene Name	Function	logFC	q-value
STM474_0274	*rnhA*	ribonuclease H	-9.044145137	0.032252089
STM474_2773	*clpB*	protein disaggregation chaperone	-5.692761425	0.007827574
STM474_3340	*tolC*	outer membrane channel	-2.672705805	0.049234765

Supplementary Table 7. Genes predicted to be essential for survival during 5-week refrigerated storage for *S.* Typhimurium strain ST4/74 (logFC < -2, q-value < 0.05).

Locus Tag	**Gene Name**	**Function**	**logFC**	**q-value**
STM474_0797	*gpmA*	phosphoglyceromutase	-12.03706073	0.017032041
STM474_4021	*trmE*	tRNA modification GTPase TrmE	-11.97306404	0.018575361
STM474_4053	*gidA*	glucose-inhibited division protein A	-11.9507516	0.01889291
STM474_4537	*frdD*	fumarate reductase subunit D	-11.85439024	0.023263189
STM474_3481	*yhbH*	putative sigma(54) modulation protein	-11.58774655	0.04021521
STM474_4126	*xerC*	site-specific tyrosine recombinase XerC	-11.54530559	0.043265058
STM474_0402	*proC*	pyrroline-5-carboxylate reductase	-11.50930236	0.043265058
STM474_1916	*eda*	keto-hydroxyglutarate-aldolase/keto-deoxy- phosphogluconate aldolase	-11.50622572	0.043265058
STM474_1736	*yciL*	23S rRNA pseudouridylate synthase B	-11.44869031	0.046833775
STM474_4486	*proP*	proline/glycine betaine transporter	-5.581831091	0.00020388
STM474_4186	*typA*	GTP-binding protein	-2.402416787	0.012396137

Supplementary Table 8. Genes predicted to be essential for survival during growth in LB broth supplemented with 8mM acetic acid for *S.* Typhimurium strain ST4/74 (logFC < -2, q-value < 0.05).

Locus Tag	Gene Name	Function	logFC	q-value
STM474_0176	*yacc*	Uncharacterized protein yacC	-11.59163888	0.028772932
STM474_0402	*proC*	pyrroline-5-carboxylate reductase	-11.30324231	0.027615519
STM474_3484	*ptsO*	phosphohistidinoprotein-hexose phosphotransferase component of N-regulated PTS system (Npr)	-11.17908717	0.028772932
STM474_3872	*cysE*	serine acetyltransferase	-10.99233061	0.028772932
STM474_2746	*STM474_2746*	gifsy-1 prophage protein	-10.90475419	0.028772932
STM474_3191	*xerD*	site-specific tyrosine recombinase XerD	-10.80977362	0.030643943
STM474_2419	*nuoH*	NADH dehydrogenase subunit H	-10.74886769	0.030643943
STM474_3971	*ivbL*	IlvB leader peptide	-10.68868075	0.030643943
STM474_2171	*rfbU*	mannosyl transferase	-10.64824366	0.030643943
STM474_1168	*flgM*	anti-sigma28 factor FlgM	-10.6454465	0.030643943
STM474_0718	*seqa*	Protein seqA	-10.63656027	0.030643943
STM474_2748	*STM474_2748*	excisionase-like protein	-10.6170735	0.030643943
STM474_2697	*STM474_2697*	hypothetical protein	-10.60203381	0.030643943
STM474_3351	*glgS*	glycogen synthesis protein GlgS	-10.55944814	0.030643943
STM474_0092	*apaH*	diadenosine tetraphosphatase	-10.55010222	0.030823969
STM474_3150	*ygdP*	dinucleoside polyphosphate hydrolase	-10.51327702	0.031595912
STM474_2346	*ccmE*	cytochrome c-type biogenesis protein CcmE	-10.50561191	0.031595912
STM474_1440	*STM474_1440*	glutaredoxin-like protein	-10.49131105	0.031595912
STM474_2071	*STM474_2071*	hypothetical protein	-10.40937545	0.039395589
STM474_3064	*rpoS*	RNA polymerase sigma factor RpoS	-10.40538212	0.039395589
STM474_2645	*iscA*	iron-sulfur cluster assembly protein	-10.31878277	0.039395589
STM474_1933	*nudB*	dATP pyrophosphohydrolase	-10.31317682	0.039395589
STM474_2967	*recA*	recombinase A	-10.30305492	0.039395589
STM474_1732	*yciN*	protein YciN	-10.26193068	0.041426286

STM474_2817	*STM474_2817*	late control-like protein	-10.21588521	0.043850923
STM474_2716	*STM474_2716*	head-to-tail joining-like protein	-10.20778394	0.041426286
STM474_r0259	*STM474_r0259*		-10.20552799	0.041426286
STM474_0947	*ftsK*	DNA translocase FtsK	-10.17543425	0.041426286
STM474_3311	*STM474_3311*	putative inner membrane protein	-10.16619411	0.041426286
STM474_3044	*STM474_3044*	putative ABC-type transporter	-10.1657439	0.041426286
STM474_3611	*STM474_3611*	hypothetical protein	-10.13448822	0.044819438
STM474_1754	*tonB*	transport protein TonB	-10.12774688	0.04512646
STM474_4455	*STM474_4455*	cation efflux pump	-10.12606435	0.042849431
STM474_2062	*STM474_2062*	hypothetical protein	-10.10088486	0.04370353
STM474_4078	*ilvL*	ilvG operon leader peptide	-9.987902008	0.046449945
STM474_2821	*STM474_2821*	P2 gpE-like protein	-9.981560234	0.046449945
STM474_0493	*hha*	hemolysin expression-modulating protein	-9.962886883	0.047445155
STM474_3139	*recD*	exonuclease V subunit alpha	-8.798242085	0.001665603
STM474_4025	*STM474_4025*	putative reverse transcriptase	-8.642308554	0.028385488
STM474_0274	*rnhA*	ribonuclease H	-8.461580689	0.028772932
STM474_4576	*yjfO*	putative lipoprotein	-8.347020792	0.02882017
STM474_1002	*STM474_1002*	Gifsy-1 prophage cI protein	-8.119576916	0.030643943
STM474_2643	*hscA*	chaperone protein HscA	-7.896725658	0.036480131
STM474_1932	*yebC*	protein YebC	-7.144046484	0.045845546
STM474_3143	*ppdC*	Prepilin peptidase dependent protein C	-7.113182238	0.046893769
STM474_3112	*syd*	SecY interacting protein Syd	-6.883896524	0.045845546
STM474_2415	*nuoL*	NADH dehydrogenase subunit L	-6.198891859	0.044830088
STM474_2613	*xseA*	exodeoxyribonuclease VII large subunit	-5.571035772	0.028772932
STM474_3757	*pitA*	putative low-affinity inorganic phosphate transporter	-5.038052955	0.033608098
STM474_4186	*typA*	GTP-binding protein	-4.240365557	0.028772932
STM474_4000	*torC*	trimethylamine N-oxide reductase cytochrome c-like subunit	-4.148268921	0.025500243
STM474_4091	*gppA*	guanosine pentaphosphate phosphohydrolase	-3.697319908	0.030643943

Supplementary Table 9. Genes essential for survival of S. Typhimurium strain ST4/74 in one or more food chain related stresses. Essential genes are denoted with 1 and coloured grey, whereas non-essential genes are denoted with 0 and are uncoloured.

Locus Tag	Gene Name	8mM Acetic Acid	14mM Citric Acid	Heat Inactivation at 60°C	24-hour Desiccation	6% NaCl	5-week Refrigeated Storage
STM474_0013	*dnaK*	1	0	0	1	0	1
STM474_0092	*apaH*	0	0	0	0	0	1
STM474_0096	*surA*	0	0	0	0	0	1
STM474_0097	*imp*	0	0	0	0	0	1
STM474_0124	*fruR*	0	0	0	0	0	1
STM474_0126	*mraW*	0	0	0	0	0	1
STM474_0128	*ftsI*	0	0	0	1	0	1
STM474_0132	*murD*	0	0	0	1	1	1
STM474_0146	*yacf*	1	0	0	0	0	1
STM474_0147	*coaE*	0	0	0	0	1	1
STM474_0160	*aceE*	0	0	0	0	0	1
STM474_0167	*acnB*	0	1	0	0	0	0
STM474_0172	*pdxA*	1	0	0	0	0	0
STM474_0176	*yacc*	0	1	0	1	0	0
STM474_0193	*pcnB*	0	1	0	0	0	0
STM474_0199	*mrcB*	0	1	0	0	0	0
STM474_0216	*pfs*	0	0	0	0	1	0
STM474_0218	*htrA*	0	0	0	0	1	0
STM474_0236	*fabZ*	0	0	0	1	0	0
STM474_0274	*rnhA*	0	0	0	0	1	0
STM474_0331	*pepD*	0	0	0	0	1	0
STM474_0391	*sbmA*	0	1	0	0	0	0

STM474_0402	*proC*	0	1	0	0	0	0
STM474_0403	*STM474_0403*	0	1	0	0	0	0
STM474_0424	*tgt*	1	0	0	0	0	0
STM474_0425	*yajC*	0	0	0	1	0	0
STM474_0439	*thiL*	0	1	0	0	0	0
STM474_0444	*xseB*	0	1	0	1	0	0
STM474_0445	*thiI*	0	1	0	0	0	0
STM474_0493	*hha*	0	1	0	0	1	0
STM474_0495	*acrB*	1	0	1	0	0	0
STM474_0496	*acrA*	0	1	0	0	0	0
STM474_0557	*ppiB*	0	1	0	0	0	0
STM474_0602	*ybdJ*	0	1	0	0	0	0
STM474_0610	*fepC*	0	1	0	0	1	0
STM474_0611	*fepG*	0	1	0	0	0	0
STM474_0651	*crcB*	0	0	0	1	0	0
STM474_0656	*lipB*	0	0	0	1	0	0
STM474_0658	*dacA*	0	0	0	0	1	0
STM474_0690	*STM474_0690*	1	0	0	0	0	0
STM474_0692	*ubiF*	0	1	0	0	0	0
STM474_0714	*fur*	0	1	0	0	0	0
STM474_0718	*seqa*	0	0	0	1	0	0
STM474_0719	*pgm*	0	0	0	0	1	0
STM474_0753	*gltA*	0	1	0	0	0	0
STM474_0755	*sdhC*	0	1	0	0	0	0
STM474_0756	*sdhD*	0	1	0	0	0	0
STM474_0759	*sucA*	0	1	0	0	0	0

STM474_0760	*sucB*	0	0	0	0	1	0
STM474_0761	*sucC*	0	0	0	0	1	0
STM474_0772	*tolA*	0	0	0	1	0	0
STM474_0773	*tolB*	0	1	0	1	1	0
STM474_0774	*pal*	1	0	0	1	0	0
STM474_0797	*gpmA*	0	1	0	0	0	0
STM474_0862	*ybis*	0	0	0	1	0	0
STM474_0947	*ftsK*	0	1	0	0	0	0
STM474_0968	*infB*	0	1	0	1	0	0
STM474_0973	*ycar*	0	0	0	0	1	0
STM474_0979	*mukE*	0	1	0	0	1	0
STM474_0980	*mukB*	0	0	0	1	0	0
STM474_0991	*pncB*	0	0	0	1	0	0
STM474_1002	*STM474_1002*	0	1	0	0	1	0
STM474_1145	*mdoG*	0	0	0	0	1	0
STM474_1146	*mdoH*	0	0	0	1	0	0
STM474_1154	*STM474_1154*	1	1	0	0	1	0
STM474_1168	*flgM*	0	1	0	0	1	0
STM474_1188	*rpmF*	0	1	0	1	1	0
STM474_1198	*holB*	0	0	0	0	1	0
STM474_1228	*phoQ*	0	0	0	1	1	0
STM474_1229	*phoP*	0	0	0	1	0	0
STM474_1275	*STM474_1275*	1	0	0	0	0	0
STM474_1276	*STM474_1276*	0	1	0	0	0	0
STM474_1343	*ihfA*	0	1	0	0	0	0
STM474_1363	*aroD*	0	1	0	0	0	0

STM474_1383	*STM474_1383*	1	0	0	0	0	0
STM474_1414	*ssaI*	0	0	0	0	1	0
STM474_1427	*ssaT*	0	0	0	1	0	0
STM474_1440	*STM474_1440*	0	1	0	0	0	0
STM474_1443	*nemA*	0	1	0	0	0	0
STM474_1454	*mliC*	0	1	0	0	0	0
STM474_1455	*pdxH*	0	1	0	0	0	0
STM474_1562	*STM474_1562*	0	1	0	1	0	0
STM474_1605	*ydcY*	0	1	0	0	0	0
STM474_1614	*sifB*	0	0	0	1	1	0
STM474_1663	*nifJ*	0	0	0	1	0	0
STM474_1702	*pspB*	0	1	0	1	0	0
STM474_1716	*rnb*	1	0	0	0	0	0
STM474_1723	*STM474_1723*	0	0	0	0	1	0
STM474_1724	*yciS*	0	1	0	0	0	0
STM474_1727	*acnA*	0	0	0	1	0	0
STM474_1732	*yciN*	0	0	0	1	1	0
STM474_1736	*yciL*	0	0	0	0	1	0
STM474_1754	*tonB*	0	0	0	1	0	0
STM474_1792	*hemK*	0	1	0	0	0	0
STM474_1795	*STM474_1795*	0	1	0	0	0	0
STM474_1796	*lolB*	0	0	0	1	0	0
STM474_1803	*engD*	0	0	0	0	1	0
STM474_1814	*STM474_1814*	0	1	0	0	0	0
STM474_1816	*STM474_1816*	0	1	0	0	1	0
STM474_1821	*ldcA*	1	0	0	0	0	0

STM474_1827	*nhaB*	1	0	0	0	0	0
STM474_1837	*minE*	0	1	0	0	0	0
STM474_1858	*STM474_1858*	0	0	0	0	1	0
STM474_1863	*yobG*	0	0	0	0	1	0
STM474_1867	*htpX*	0	1	0	1	1	0
STM474_1868	*prc*	0	1	0	0	0	0
STM474_1894	*pagK*	0	0	0	1	1	0
STM474_1897	*STM474_1897*	0	0	0	0	1	0
STM474_1916	*eda*	0	0	0	1	0	0
STM474_1926	*ruvB*	0	0	0	1	0	0
STM474_1927	*ruvA*	0	1	0	0	0	0
STM474_1932	*yebC*	0	1	0	0	0	0
STM474_1933	*nudB*	0	1	0	0	0	0
STM474_1943	*STM474_1943*	0	0	0	1	1	0
STM474_2062	*STM474_2062*	0	0	0	0	1	0
STM474_2071	*STM474_2071*	0	0	0	1	0	0
STM474_2166	*gnd*	0	0	0	1	1	0
STM474_2171	*rfbU*	0	1	0	0	1	0
STM474_2173	*rfbX*	1	0	0	0	0	0
STM474_2177	*rfbF*	1	0	0	0	0	0
STM474_2178	*rfbI*	0	1	0	0	0	0
STM474_2291	*STM474_2291*	1	0	0	0	0	0
STM474_2346	*ccmE*	1	0	0	1	0	0
STM474_2350	*ccmA*	0	1	0	0	0	0
STM474_2372	*ubiG*	1	0	0	0	0	0
STM474_2413	*nuoN*	0	0	0	0	1	0

STM474_2415	*nuoL*	0	0	0	1	1	0
STM474_2417	*nuoJ*	0	1	0	0	0	0
STM474_2419	*nuoH*	0	1	0	0	0	0
STM474_2420	*nuoG*	1	0	0	0	0	0
STM474_2422	*nuoE*	0	0	0	1	0	0
STM474_2423	*nuoC*	0	0	0	0	1	0
STM474_2425	*nuoA*	0	1	0	0	0	0
STM474_2426	*STM474_2426*	1	0	0	1	0	0
STM474_2442	*STM474_2442*	0	1	0	1	1	0
STM474_2454	*ubiX*	1	0	0	1	0	0
STM474_2467	*truA*	0	1	0	1	0	0
STM474_2470	*flk*	0	1	0	1	0	0
STM474_2503	*STM474_2503*	0	1	0	0	0	0
STM474_2530	*zipA*	0	1	0	0	1	0
STM474_2533	*STM474_2533*	0	0	0	1	0	0
STM474_2534	*ptsH*	0	0	0	1	0	0
STM474_2535	*ptsI*	0	0	0	1	1	0
STM474_2586	*dapE*	0	1	0	0	0	0
STM474_2592	*dapA*	0	0	0	0	1	0
STM474_2599	*yfgE*	0	0	0	0	1	0
STM474_2604	*ppk*	0	0	0	1	0	0
STM474_2613	*xseA*	0	0	0	1	0	0
STM474_2622	*bamB*	0	0	0	1	0	0
STM474_2625	*ispG*	0	0	0	1	1	0
STM474_2643	*hscA*	0	0	0	1	1	0
STM474_2645	*iscA*	0	0	0	1	0	0

STM474_2646	*nifU*	0	1	0	1	0	0
STM474_2648	*yfhP*	0	0	0	0	1	0
STM474_2671	*STM474_2671*	1	1	0	0	1	0
STM474_2687	*lepB*	0	1	0	0	1	0
STM474_2688	*lepA*	0	0	0	1	1	0
STM474_2697	*STM474_2697*	1	1	0	0	0	0
STM474_2716	*STM474_2716*	1	0	0	0	0	0
STM474_2740	*STM474_2740*	0	1	0	1	0	0
STM474_2746	*STM474_2746*	0	0	0	0	1	0
STM474_2747	*STM474_2747*	0	1	0	0	0	0
STM474_2748	*STM474_2748*	0	0	0	1	0	0
STM474_2753	*rpoE*	0	0	0	1	0	0
STM474_2756	*srmB*	1	0	0	0	0	0
STM474_2773	*clpB*	1	0	0	0	0	0
STM474_2808	*STM474_2808*	0	0	0	0	1	0
STM474_2811	*smpB*	1	0	0	0	0	0
STM474_2817	*STM474_2817*	0	0	0	0	1	0
STM474_2821	*STM474_2821*	1	0	0	0	0	0
STM474_2935	*STM474_2935*	0	1	0	1	0	0
STM474_2949	*proX*	0	0	0	0	1	0
STM474_2956	*gshA*	0	0	1	0	0	0
STM474_2967	*recA*	0	1	0	0	0	0
STM474_2975	*srlL*	0	0	0	0	1	0
STM474_2992	*STM474_2992*	1	0	0	0	0	0
STM474_3044	*STM474_3044*	1	0	0	0	0	0
STM474_3064	*rpoS*	0	0	0	0	1	0

STM474_3112	*syd*	0	0	0	0	1	0
STM474_3139	*recD*	0	1	0	0	0	0
STM474_3140	*recB*	1	1	0	0	1	0
STM474_3143	*ppdC*	0	0	0	1	0	0
STM474_3150	*ygdP*	0	0	0	0	1	0
STM474_3191	*xerD*	1	0	0	0	0	0
STM474_3203	*visC*	1	1	0	0	0	0
STM474_3223	*tktA*	1	0	0	0	0	0
STM474_3231	*STM474_3231*	1	0	0	1	0	0
STM474_3243	*gshB*	0	0	0	0	1	0
STM474_3279	*iraD*	1	0	0	0	0	0
STM474_3281	*STM474_3281*	1	0	0	0	0	0
STM474_3311	*STM474_3311*	1	0	0	0	1	0
STM474_3325	*sufI*	0	0	0	1	0	0
STM474_3331	*ygiY*	0	1	0	1	0	0
STM474_3335	*parE*	0	0	0	1	1	0
STM474_3337	*icc*	0	1	0	1	0	0
STM474_3340	*tolC*	0	0	0	0	1	0
STM474_3351	*glgS*	0	0	0	1	0	0
STM474_3358	*cca*	1	0	0	0	0	0
STM474_3421	*yraM*	0	1	0	0	0	0
STM474_3443	*rbfA*	0	1	0	0	0	0
STM474_3444	*infB*	0	1	0	0	0	0
STM474_3452	*glmM*	0	0	0	0	1	0
STM474_3467	*murA*	0	1	1	1	0	0
STM474_3477	*lptc*	1	0	0	0	0	0

STM474_3480	*rpoN*	0	1	0	0	0	0
STM474_3481	*yhbH*	0	0	0	0	1	0
STM474_3483	*yhbJ*	0	1	0	0	0	0
STM474_3484	*ptsO*	0	1	0	0	1	0
STM474_3509	*degS*	0	0	0	1	0	0
STM474_3547	*fis*	0	0	0	1	0	0
STM474_3572	*fmt*	0	1	0	0	1	0
STM474_3610	*tuf*	0	1	0	0	0	0
STM474_3611	*STM474_3611*	0	0	0	0	1	0
STM474_3612	*fusA*	1	0	0	0	0	0
STM474_3633	*crp*	0	1	0	0	1	0
STM474_3639	*ppia*	0	0	0	1	0	0
STM474_3651	*dam*	0	0	0	1	0	0
STM474_3653	*aroB*	0	1	0	0	0	0
STM474_3660	*mrcA*	1	0	0	0	0	0
STM474_3668	*envZ*	0	0	0	1	1	0
STM474_3669	*ompR*	0	0	0	1	0	0
STM474_3672	*feoA*	0	0	0	0	1	0
STM474_3673	*feoB*	0	1	0	1	1	0
STM474_3679	*yhgI*	0	1	0	0	0	0
STM474_3690	*glpR*	0	0	0	0	1	0
STM474_3700	*STM474_3700*	0	1	0	0	0	0
STM474_3736	*ftsX*	0	1	0	0	0	0
STM474_3737	*ftsE*	0	1	0	0	0	0
STM474_3757	*pitA*	0	1	0	0	0	0
STM474_3824	*STM474_3824*	0	0	0	0	1	0

STM474_3858	*mtlD*	0	0	0	1	0	0
STM474_3859	*mtlR*	0	0	0	1	0	0
STM474_3872	*cysE*	0	0	0	0	1	0
STM474_3873	*gpsA*	0	0	0	1	1	0
STM474_3874	*secB*	1	0	0	0	0	0
STM474_3878	*yibP*	0	0	0	1	0	0
STM474_3884	*rfaF*	0	0	0	1	0	0
STM474_3891	*rfaI*	0	0	0	1	0	0
STM474_3894	*rfaP*	1	0	0	0	0	0
STM474_3895	*rfaG*	0	0	0	0	1	0
STM474_3915	*rpoZ*	0	1	0	0	0	0
STM474_3918	*recG*	0	1	0	0	1	0
STM474_3971	*ivbL*	0	0	0	1	0	0
STM474_3990	*ccmG*	0	1	0	0	0	0
STM474_4000	*torC*	0	1	0	0	0	0
STM474_4021	*trmE*	0	1	0	0	0	0
STM474_4025	*STM474_4025*	0	1	0	0	0	0
STM474_4033	*pstB*	0	1	0	1	1	0
STM474_4034	*pstA*	1	0	0	0	0	0
STM474_4040	*glmS*	0	1	0	0	0	0
STM474_4043	*atpC*	1	0	0	0	0	0
STM474_4044	*atpD*	1	1	0	1	1	0
STM474_4045	*atpG*	0	1	0	0	0	0
STM474_4049	*atpE*	0	1	0	0	0	0
STM474_4050	*atpB*	0	0	0	1	0	0
STM474_4053	*gidA*	0	1	0	1	0	0

STM474_4059	*trkD*	0	0	0	1	0	0
STM474_4078	*ilvL*	0	0	0	1	1	0
STM474_4091	*gppA*	0	0	0	1	1	0
STM474_4106	*wzyE*	0	0	0	1	0	0
STM474_4116	*hemC*	0	1	0	0	0	0
STM474_4126	*xerC*	1	0	0	0	0	0
STM474_4128	*uvrD*	1	1	0	0	0	0
STM474_4129	*corA*	0	1	0	0	0	0
STM474_4150	*yigP*	0	0	0	0	1	0
STM474_4152	*tatA*	0	0	0	0	1	0
STM474_4157	*yigC*	0	0	0	0	1	0
STM474_4158	*fre*	0	1	0	0	0	0
STM474_4176	*dsbA*	0	1	0	0	0	0
STM474_4186	*typA*	0	0	0	1	0	0
STM474_4240	*cpxA*	0	1	0	1	0	0
STM474_4241	*cpxR*	0	1	0	0	0	0
STM474_4244	*pfkA*	0	1	0	0	0	0
STM474_4266	*fpr*	0	1	0	0	0	0
STM474_4279	*rpmE*	0	0	0	0	1	0
STM474_4285	*metL*	0	1	0	0	0	0
STM474_4308	*oxyR*	0	0	0	0	1	0
STM474_4310	*yijC*	0	0	0	0	1	0
STM474_4322	*birA*	0	1	0	0	0	0
STM474_4356	*hupA*	0	1	0	0	0	0
STM474_4370	*STM474_4370*	0	0	0	0	1	0
STM474_4404	*STM474_4404*	0	0	0	0	1	0

STM474_4435	*zur*	0	0	0	0	1	0
STM474_4455	*STM474_4455*	0	0	0	1	0	0
STM474_4486	*proP*	0	0	0	0	1	0
STM474_4531	*efp*	1	0	0	0	0	0
STM474_4537	*frdD*	0	1	0	1	1	0
STM474_4541	*STM474_4541*	0	0	0	1	0	0
STM474_4547	*orn*	0	0	0	1	0	0
STM474_4558	*hfq*	0	0	0	1	0	0
STM474_4565	*rnr*	0	0	0	1	1	0
STM474_4576	*yjfO*	1	0	0	0	0	0
STM474_4590	*rpsR*	0	0	0	1	0	0
STM474_4608	*ytfP*	0	1	0	0	0	0
STM474_4611	*ppa*	0	0	0	1	0	0
STM474_4614	*STM474_4614*	0	0	0	1	0	0
STM474_4677	*yjgP*	0	1	0	0	0	0
STM474_4678	*yjgQ*	0	0	0	1	0	0
STM474_4733	*STM474_4733*	0	1	0	1	0	0
STM474_4747	*dnaC*	0	0	0	1	0	0
STM474_4785	*radA*	0	0	0	0	1	0
STM474_r0259	*STM474_r0259*	1	0	0	0	0	0
STM474_t0260	*STM474_t0260*	0	1	0	0	1	0
STM474_t0339	*STM474_t0339*	0	1	0	0	0	0
STM474_t1263	*STM474_t1263*	0	0	0	0	1	0
STM474_t2493	*STM474_t2493*	0	1	0	0	0	0
STM474_t2960	*STM474_t2960*	0	0	0	0	1	0
STM474_t3450	*STM474_t3450*	0	0	0	1	0	0

STM474_t3806	*STM474_t3806*	0	1	0	0	0	0
STM474_t4330	*STM474_t4330*	0	1	0	0	1	0
STM474_t4757	*STM474_t4757*	0	1	0	0	0	0

Supplementary Table 10. Reagents required for Golden Gate reaction mix for single-gene knockouts generated in the current study in *S*. Typhimurium strain ST4/74 for (A) *proP* (B) *rnr* (C) *zur* (D) *rfaB* and (E) *dam*.

(A) proP

Volume required 10µL

Plasmid/part name	Part length (bp)	Concentration (ng/µL)		Target amount (fmol)	Volume (µL)
p-DOC-GG	5960	48.4		10	0.8
tet cassette	3671	73.2		10	0.3
proP HR1	432	2.7		20	2.0
proP HR2	432	5.28		20	1.0

GOLDEN GATE REACTION MIX	Volume (µL)
Water	1.9
Ligase buffer	1
BSA	2
DNA FRAGMENTS	4.1
T4 Ligase	0.5
BsaI-HF v2	0.5
	10.0

(B) rnr

Volume required 10µL

Plasmid/part name	Part length (bp)	Concentration (ng/µL)		Target amount (fmol)	Volume (µL)
p-DOC-GG	5960	48.4		10	0.8
tet cassette	3671	73.2		10	0.3
rnr HR1	432	7.48		20	0.7
rnr HR2	432	8.4		20	0.6

GOLDEN GATE REACTION MIX	Volume (µL)
Water	3.6
Ligase buffer	1
BSA	2
DNA FRAGMENTS	2.4
T4 Ligase	0.5
BsaI-HF v2	0.5
	10.0

Volume required 10µL

(C) zur

Plasmid/part name	Part length (bp)	Concentration (ng/µL)	Target amount (fmol)		Volume (µL)
p-DOC-GG	5960	48.4		10	0.8
tet cassette	3671	73.2		10	0.3
zur HR1	432	7.64		20	0.7
zur HR2	253	4.04		20	0.8

GOLDEN GATE REACTION MIX	Volume (µL)
Water	3.5
Ligase buffer	1
BSA	2
DNA FRAGMENTS	2.5
T4 Ligase	0.5
BsaI-HF v2	0.5
	10.0

Volume required 10µL

(D) rfab

Plasmid/part name	Part length (bp)	Concentration (ng/µL)	Target amount (fmol)		Volume (µL)
p-DOC-GG	5960	48.4		10	0.8
tet cassette	3671	73.2		10	0.3
rfab HR1	432	3.64		20	1.5
rfab HR2	432	3.84		20	1.4

GOLDEN GATE REACTION MIX	Volume (µL)
Water	2.1
Ligase buffer	1
BSA	2
DNA FRAGMENTS	3.9
T4 Ligase	0.5
BsaI-HF v2	0.5
	10.0

Volume required 10µL

(E) dam

Plasmid/part name	Part length (bp)	Concentration (ng/µL)		Target amount (fmol)	Volume (µL)
p-DOC-GG	5960	48.4		10	0.8
tet cassette	3671	73.2		10	0.3
dam HR1	432	8.86		20	0.6
dam HR2	432	7.58		20	0.7

GOLDEN GATE REACTION MIX	Volume (µL)
Water	3.6
Ligase buffer	1
BSA	2
DNA FRAGMENTS	2.4
T4 Ligase	0.5
BsaI-HF v2	0.5
	10.0