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Publication Date	2014-07-22
Item record	http://hdl.handle.net/10379/4628

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**Elucidation of the role of the
glutamate decarboxylase system and
the γ -aminobutyric acid shunt
pathway in the stress response of
*Listeria monocytogenes***

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Thesis submitted for a PhD Degree by Research

Conducted at the National University of Ireland, Galway

Research was conducted in: Microbiology

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Submission Date: July 2014

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“When eating a fruit, think of
the person who planted the
tree.” – *Vietnamese proverb*

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Abstract

The foodborne pathogen *Listeria monocytogenes* is the causative agent of listeriosis in humans and other animals. This disease can have a fatality rate of up to 30% in infected patients and leads to spontaneous abortion in pregnant women. The most common source of the bacteria is from contaminated food stuffs that are either consumed raw or are minimally processed. The bacterium has evolved several mechanisms to ensure survival in sometimes harsh environments such as acidified foods and the low pH of the stomach. Among these mechanisms is the glutamate decarboxylase (GAD) system. This is widely considered to allow the bacteria to maintain a favourable intracellular pH in conditions where the extracellular pH has dropped. The GAD system involves the import of extracellular glutamate coupled to the export of γ -aminobutyric acid (GABA). Inside the cell, the glutamate is converted to GABA by GAD which consumes intracellular protons. GABA can then leave the cell in exchange for further glutamate molecules. The glutamate/GABA antiport is carried out by GadT, a membrane protein.

Our work shows that the GAD system has undergone divergent evolution between closely related strains of *L. monocytogenes*. There is a split between the action of extracellular GAD activity (GAD_e) and intracellular GAD activity (GAD_i) in strains 10403S and EGD-e. We demonstrate that among the 3 genes encoding for decarboxylase enzymes, *gadD2*, is closely associated with GAD_e while *gadD3* is central to GAD_i. Overall the regulation of the GAD system does not appear to occur at a transcriptional level in stationary phase cells. Furthermore, we present the first evidence of the GABA shunt pathway in *L. monocytogenes*. This pathway has the potential to metabolise GABA produced by the cell and we identify a link between this pathway and acid resistance. A murine model of study has shown that deletion of up to two GAD genes reduces bacterial load in the spleen and liver indicating a redundancy in the GAD system in *L. monocytogenes* and a potential role in virulence.

List of Abbreviations

Amp	Ampicillin
BHI	Brain Heart Infusion
BSA	Bovine serum albumin
cDNA	Complementary DNA
Cml	Chloramphenicol
dH ₂ O	Distilled water
DM	Defined medium
DMSO	Dimethyl sulfoxide
Erm	Erythromycin
FBS	Fetal bovine serum
<i>g</i>	Force of gravity
GABA	γ-aminobutyric acid
GABA-AT	GABA amino transferase
GAD	Glutamate decarboxylase
Kan	Kanamycin
LB	Luria-bertani
MW	Molecular weight
OD	Optical density
PCR	Polymerase chain reaction
PenStrep	Penicillin streptomycin
PMA	Phorbol myristate acetate
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RT-PCR	Real-time PCR
SAP	Shrimp alkaline phosphatase
SGR	Specific growth rate

SSA	Succinic semialdehyde
SSDH	Succinic semialdehyde dehydrogenase
wt or WT	Wild-type
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1

Introduction

1.1 Biology and taxonomy of *Listeria monocytogenes*

Over the last two decades *Listeria monocytogenes* has emerged to become one of the leading causes of food-borne illness in Europe and the USA (Goulet *et al.*, 2008; Scallan *et al.*, 2011). *L. monocytogenes* was first isolated from rabbits by E.G.D. Murray in 1926 (Murray *et al.*, 1926) with the first human isolate obtained in 1929 (Farber & Peterkin, 1991). It was first named as *Bacterium monocytogenes* by Murray in 1926 and later changed to *Listerella hepatolytica* before assuming its current nomenclature in 1940 by James Hunter Harvey Pirie (Gray & Killinger, 1966). The first reported outbreak of human listeriosis was in New Zealand in 1979 and since then there has been a steady increase in both outbreaks and sporadic cases (Farber & Peterkin, 1991; Goulet *et al.*, 2008).

It is a gram positive rod-shaped bacterium that is catalase positive and oxidase negative (Farber & Peterkin, 1991). It is a non spore-forming bacterium that can grow either aerobically or anaerobically and is motile below 37°C via peritrichous flagella (Gründling *et al.*, 2004; Peel *et al.*, 1988). Although it was first isolated from mammals it is now accepted as being ubiquitous in the environment with isolates characterised from water, vegetation, birds, fish, mammals and many foods and food processing environments (Farber & Peterkin, 1991; Sauders *et al.*, 2012; Vázquez-Boland & Kuhn, 2001). Due to its ubiquity in nature and its intrinsic stress resistance, its elimination from the food chain is particularly difficult, however current EU regulations (EC/1411/2007) state that in foods that supports the growth of *L. monocytogenes*, none should be detected in 25 g of food. The economic burden caused by *L. monocytogenes* in both prevention and treatment is high. In the USA, it is estimated that treatment carries a 94% hospitalisation rate with costs running into the millions annually (Cartwright *et al.*, 2013; Scallan *et al.*, 2011), while the estimated cost to the food industry in the USA to control for *L. monocytogenes* in 2005 was as high as 2.4 billion US dollars a year (Ivanek *et al.*, 2005). The bacterium is often considered as a model organism for the study of pathogenesis and as such much work has been undertaken to understand key aspects of its biology including its genetics and

metabolic capabilities (Cossart & Archambaud, 2009; Eisenreich *et al.*, 2010; Hamon *et al.*, 2006).

L. monocytogenes is a member of the genus *Listeria* spp. which currently comprises 10 different species (den Bakker *et al.*, 2013; Liu, 2013; Figure 1.1). These include *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. grayi*, *L. rocourtiae*, *L. ivanovii*, *L. marthii* and the newly characterised species *L. weihenstephanensis* and *L. fleischmannii*. Among these, only *L. monocytogenes* and *L. ivanovii* are considered to be pathogenic, both possessing the virulence gene cluster (Sauders *et al.*, 2012). Despite *L. seeligeri* displaying properties of haemolysis and possessing a form of the *Listeria* virulence gene cluster, it is considered to be non-pathogenic (Gouin *et al.*, 1994; Hain *et al.*, 2007). The genomes of both *L. monocytogenes* and *L. innocua* were first sequenced in 2001 and compared as representatives of a pathogenic species and non-pathogenic species of *Listeria*. One of the main findings was that the non-pathogenic strain, *L. innocua* lacked most genes that form a virulence cluster in *L. monocytogenes* and also lacked the central virulence regulator PrfA (Glaser *et al.*, 2001). It is generally accepted that ancestral strains of *Listeria* spp. gained the virulence gene cluster and this was more recently lost by *L. innocua* (Vázquez-Boland & Kuhn, 2001). The genus *Listeria* is a member of the *Firmicutes* and is closely related to both the *Lactobacillus* and *Bacillus* families (Bergey's Manual of Systematic Bacteriology).

Within the species *L. monocytogenes*, there is a further classification into thirteen serovars which are divided into 3 lineages. Most cases of listeriosis arise from strains belonging to lineages I and II including serotype 1/2a and 4b (Buchrieser, 2007; Nightingale *et al.*, 2005). Apart from possessing seemingly different capacities to cause disease, some serovars differ on the possession of stress survival genes such as the *gadDIT1* operon, which is associated with acid tolerance (Ryan *et al.*, 2010; Section 1.6.2).

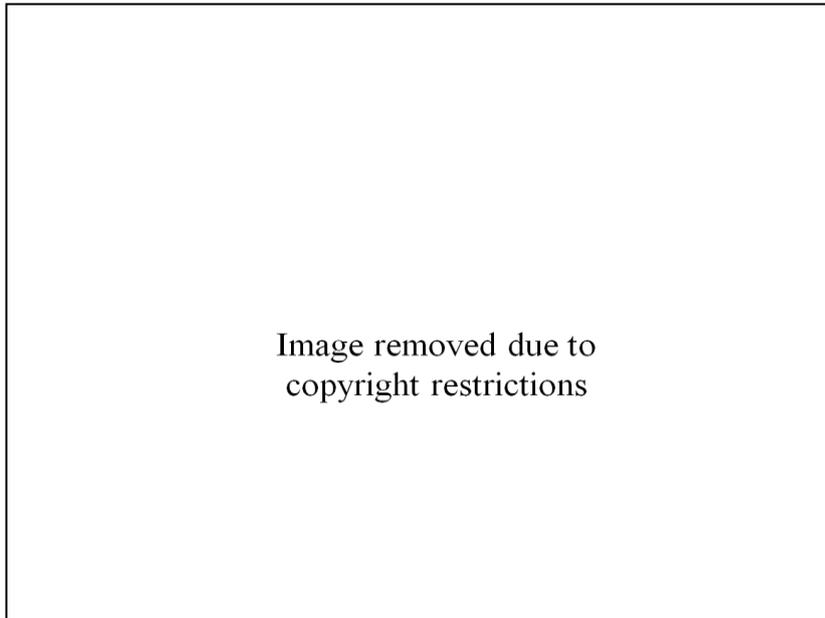


Figure 1.1. Relationship of the nine species of the *Listeria* genus. The relationship is based on comparison of 16S rRNA sequence. Diagram adapted from den Bakker *et al.*, 2013.

1.2 Metabolism

With the sequencing of the genome of EGD-e in 2001 (Glaser *et al.*, 2001), *L. monocytogenes* was shown to possess most of the anabolic and catabolic pathways found in *Bacillus subtilis*, a closely related organism. *Listeria* has a limited variety of carbohydrates from which it can obtain energy including glucose, fructose, glycerol and mannose (Tsai & Hodgson, 2003). ¹³C isotopologue work has uncovered that glucose is metabolised aerobically via the pentose phosphate pathway until α -ketoglutarate (Eisenreich *et al.*, 2006). The next step cannot be achieved however because the tricarboxylic acid cycle is incomplete. *L. monocytogenes* lacks the genes for converting α -ketoglutarate to succinyl co-A, succinyl co-A to succinate and also for converting malate to oxaloacetate (Trivett & Meyer, 1971; Fig. 1.2). Oxaloacetate is thus thought to be derived from the carboxylation of PEP-derived pyruvate by pyruvate carboxylase (Eisenreich *et al.*, 2006). This pathway results in a slowdown of branched-chain

amino acid biosynthesis, which is reflected in the requirement of these (isoleucine, leucine and valine) in minimal media (Hain *et al.*, 2007; Tsai & Hodgson, 2003). Pyruvate carboxylase (PycA) has been shown to be highly important in virulence with *pycA* mutants unable to replicate in mammalian cells and the mutant strain is attenuated in a murine model (Schär *et al.*, 2010). Much knowledge has been attained in recent years as to the specific carbon metabolism profile of intracellular *L. monocytogenes*. The pentose phosphate pathway and not glycolysis is the primary pathway for carbohydrate metabolism and it appears that *L. monocytogenes* switches to utilising phosphorylated glucose and glycerol as carbon sources when inside the host cell (Joseph *et al.*, 2006).

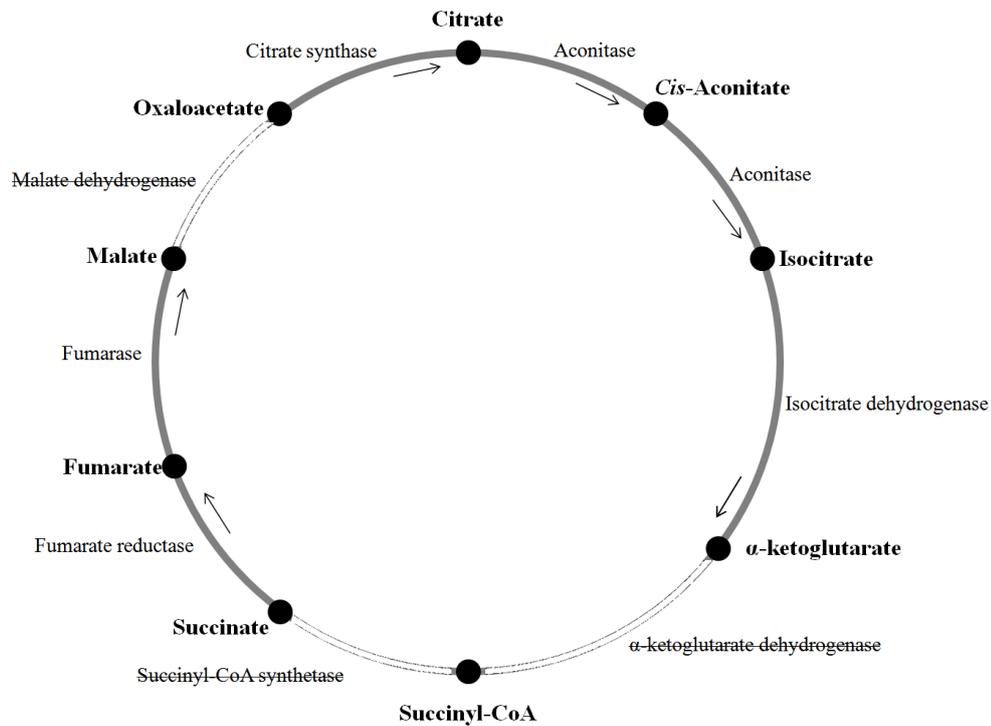


Figure 1.2. The Krebs cycle of *L. monocytogenes*. Simplified diagram of the Krebs cycle showing intermediates of the standard cycle are in bold. The enzymes missing from *L. monocytogenes* are indicated with a strike-through line. All the remaining enzymes are present.

While nitrogen metabolism inside host cells is not fully understood, ethanolamine, ammonium and arginine are expected sources as opposed to glutamine (Joseph *et al.*, 2006; Schauer *et al.*, 2010). Taken together with the knowledge on intracellular carbon sources, it would appear that *Listeria* avoids competition with the host cell for both glucose and glutamine (Hain *et al.*, 2007).

Energy production in *Listeria* is not thought to be normally derived from a respiratory chain despite possessing the aromatic amino acid biosynthesis (*aro*) genes that are responsible for producing the electron acceptor menaquinone and also displaying an NADH oxidase activity (Glaser *et al.*, 2001; Patchett *et al.*, 1991; Stritzker *et al.*, 2004). Due to its split TCA cycle (Trivett & Meyer, 1971) it is expected that reducing agents cannot be efficiently supplied to carry out an electron transport chain (Feld *et al.*, 2012). As the by-products of glucose metabolism, both aerobically and anaerobically, are lactic acid and acetic acid (Pine *et al.*, 1989) it is expected that *L. monocytogenes* derives energy primarily by the generation of a proton motive force (PMF) similar to other lactic acid bacteria (Lungu *et al.*, 2010; Salema *et al.*, 1996).

1.3 Listeriosis - disease and treatment

Although infections are rare, listeriosis carries an estimated mortality of about 30% (Farber & Peterkin, 1991). The disease manifests in two main forms, a gastrointestinal listeriosis which is non-invasive and invasive listeriosis. Non-invasive listeriosis often develops in otherwise healthy adults following the consumption of heavily contaminated food and presents as febrile gastroenteritis (Schlech & Acheson, 2000). Invasive listeriosis often develops as septicaemia or meningoencephalitis in immunocompromised adults, elderly and patients undergoing immunosuppressive therapy (Schlech & Acheson, 2000). *L. monocytogenes* is now the third most common cause of bacterial meningitis in adults in the world (Dzupova *et al.*, 2013). About 10 - 20% of cases of listeriosis in Europe are pregnancy-associated (Allerberger & Wagner, 2010) while similar figures are

found in the USA (Pouillot *et al.*, 2012). Listeriosis can be acquired via the pathogen crossing the placental barrier from the infected mother resulting in spontaneous abortion, still birth or infection of the neonate (Allerberger & Wagner, 2010). This usually occurs in the third trimester of pregnancy when T-cell immunity is low (Allerberger & Wagner, 2010). In recent years there has been an increase in the reported cases of listeriosis in the elderly (>65 years of age) (Goulet & Marchetti, 1996; Goulet *et al.*, 2008). Historically, listeriosis was first identified as a problem in animals (Farber & Peterkin, 1991; Murray *et al.*, 1926) and veterinary cases are still a problem, particularly in ruminants where the organism is contracted from contaminated silage (Schlech & Acheson, 2000).

L. monocytogenes does not have β -lactamase genes and can usually be treated with either penicillin or ampicillin in combination with gentamicin, however penicillin and ampicillin are bacteriostatic and usually require the body's defence mechanisms to clear the infection (Hof, 2003). In general, susceptibility of *L. monocytogenes* to clinically used antibiotics has not changed in the last 35 years despite reports of isolates from food and the environment with varying resistance to antibiotics including tetracycline (Poyart-Salmeron *et al.*, 1992; Walsh *et al.*, 2001). Clindamycin and aminoglycosides are now believed to have little effect while cephalosporins have no effect (Allerberger & Dierich, 1992). The use of cephalosporins to treat bacterial cases of meningitis is problematic in that it is ineffective against *L. monocytogenes*. Therefore it is advised that these third-generation drugs are used in combination with ampicillin to prevent mistreatment (Schlech & Acheson, 2000).

1.4 Pathogenesis - intracellular cycle

L. monocytogenes is an intracellular pathogen. After transit through the stomach of the host, *L. monocytogenes* can pass through the intestinal barrier by invading the Goblet cells and enterocytes of the epithelium along the gut and also into hepatocytes in the liver (Cossart, 2011; Dramsi *et al.*, 1995). Entry is mediated via

an interaction between the host receptors E-cadherin and hepatocyte growth factor (Met) and the *Listerial* surface ligands InlA and InlB, respectively (Bonazzi *et al.*, 2009; Dramsi *et al.*, 1995; Gaillard *et al.*, 1991). Although *L. monocytogenes* possesses several further internalins, it does not appear that these can independently induce entry into non-phagocytic cells (Dramsi *et al.*, 1997). InlA mediated entry does however rely on the support of these internalins (Bergmann *et al.*, 2002). Interaction of InlA with E-cadherin causes a rearrangement of the actin cytoskeleton allowing the bacterium to be engulfed in the cell membrane in a "zipper" fashion (Bonazzi *et al.*, 2009). After internalization, the bacterium is encased in the membrane vacuole (Camejo *et al.*, 2011; Gaillard *et al.*, 1987) from which it escapes through expressing a pore-forming toxin, listeriolysin O (LLO) and two phospholipases (PlcA and PlcB; Vázquez-Boland & Kuhn, 2001). Subsequently, while it is free in the cytosol of the host cell, *L. monocytogenes* replicates and commandeers host cell actin, via ActA, to form an actin tail. This allows the bacterium to propel itself through the cytosol, pushing out through the cell membrane and into neighbouring cells. Now inside a second cell, the bacterium is surrounded by a double membrane from which it escapes once more via LLO and PlcB (Portnoy & Chakraborty, 1992; Vázquez-Boland & Kuhn, 2001; Fig. 1.3).

Additional to the ability to invade epithelial cells, *L. monocytogenes* can also be taken up by and replicate inside professional phagocytes. The ability of *L. monocytogenes* to survive inside macrophages heavily depends upon the source of the cells. Primary macrophages derived from peritoneal tissue kill up to 90% of engulfed *L. monocytogenes* (Portnoy & Schreiber, 1989) while permanent cell lines including murine derived J774 and human lymphocyte THP-1 macrophages are much more acquiescent to survival and growth (Conte *et al.*, 2002; Donaldson *et al.*, 2011). After engulfment in the phagolysosome of the macrophage *L. monocytogenes* can express LLO due to the optimal pH of 5.5 inside the compartment allowing escape into the cytosol where a more neutral pH switches off expression (Glomski *et al.*, 2002). This optimal acid pH inhibits expression of

LLO in the cytosol, preventing cellular perforation and giving the bacteria time to replicate.

A key factor in the survival of *L. monocytogenes* inside host cells, particularly macrophages, is the ability to abate and avoid a host immune response. The addition of IFN- γ to THP-1 macrophages was seen to prevent escape of *L. monocytogenes* from the phagolysosome (Scorneaux *et al.*, 1996). In healthy individuals, the onset of infection is prevented by a Th1 focused immune response involving the action of TNF- α and INF- γ (Abram *et al.*, 2003; Tripp *et al.*, 1993). Successful pregnancy is generally associated with a switch from Th1 immunity to Th2 in order to prevent maternal rejection of the developing foetus (Raghupathy, 2001). This significantly increases the risk of infection from *L. monocytogenes* in pregnant women and the bacteria is well known to be capable of crossing the placental barrier (Farber & Peterkin, 1991; Jacquet *et al.*, 2004). A more recent theory however has been suggested by Bakardjuev *et al.* that *L. monocytogenes* arrives at the placenta in a very low dose and due to favourable conditions, proliferates and crosses back to the mother. As a result abortion is induced in order to clear infection (Bakardjiev *et al.*, 2006). In any case, crossing of the placental barrier is mediated by InlA, which binds to E-cadherin on villous trophoblasts of the placenta directly in contact with the maternal blood (Lecuit *et al.*, 2004).

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Figure 1.3. Electron micrograph images and associated schematic displaying the infectious lifecycle of *L. monocytogenes*. (1) Attachment and entry into non-phagocytic cells mediated by InlA and InlB. (2) Escape from the phagosomal vacuole through excretion of the pore-forming toxin LLO and phospholipases. (3) & (4) Replication and polymerisation of host cell actin to form a comet tail. (5) Propulsion into adjacent cells via the comet tail. (6) Escape from double-membrane vacuole. All pathogenicity factors associated with each step are included beside each micrograph image. The above figure is adapted from Tilney & Portnoy, 1989.

All of the genes that are essential for virulence in *L. monocytogenes* are under the control of the central virulence regulator PrfA (positive regulator factor A; Chakraborty *et al.*, 1992; Milohanic *et al.*, 2003), including *hly*, *actA*, *plcA*, *plcB*, *inlA*, *inlB*, *inlC*. This 27 kDa transcriptional regulator is a member of the Crp/Cap-Fnr family of bacterial transcription factors and is directly responsible for the switch from the flagellar state to intracellular state of *L. monocytogenes* (Scotti *et al.*, 2007). PrfA acts by binding directly to the palindromic 'PrfA box' for up to 12

core genes, however it may also have indirect effects on sets of genes through an undefined interaction with alternative sigma factors (Milohanic *et al.*, 2003). The PrfA binding site is located at position -41 upstream from the promoter sequence to which the RNA polymerase complex initiates transcription. A total of 70 genes were found to be differentially expressed in a strain lacking *prfA* including genes involved in stress response like *lmo0913* (similar to succinic semialdehyde dehydrogenase), *lmo0043* (similar to arginine deiminase) and the *opuC* operon (an ABC transporter). PrfA can be regulated either at a transcriptional level (Schwab *et al.*, 2005), translational level (Johansson *et al.*, 2002; Loh *et al.*, 2009) or at the post-translational level (Eiting *et al.*, 2005; Renzoni *et al.*, 1997). The different methods of transcriptional control have been shown to play roles at different stages during the intracellular life cycle. For instance, transcription initiated from promoters P1_{*prfA*} or P2_{*prfA*} control escape from the vacuole (Freitag & Portnoy, 1994) while transcription from P_{*plcA*} controls cell to cell spread (Camilli *et al.*, 1993). Temperature is a key factor in controlling expression due to the presence of an RNA thermosensor which forms an RNA hairpin preventing translation of the *prfA* transcript. This hairpin melts at temperatures higher than 30°C allowing translation (Johansson *et al.*, 2002). Transcripts from the P1_{*prfA*} are only controlled by this thermosensor. Activity of PrfA has been shown to be affected by addition of charcoal to media (Ripio *et al.*, 1996) and also by the presence of certain sugar sources such as glucose and cellobiose (Milenbachs *et al.*, 1997). *In vivo*, *L. monocytogenes* has been reported to use a non phosphotransferase system (PTS) mechanism of uptake of sugars. One of these, the hexose phosphate transporter, is controlled by PrfA and allows the use of phosphorylated sugars which do not inhibit PrfA activity (Chico-Calero *et al.*, 2002). If an active PTS system were to uptake non-phosphorylated sugars, the PTS permeases would receive phosphates from enzyme II domain A (EIIA). This non dephosphorylated EIIA has been shown to repress PrfA activity (Stoll *et al.*, 2008).

1.5 Response to environmental stress

The ability of *L. monocytogenes* to survive encounters with a wide spectrum of environmental stresses makes it a key concern for the food industry and in disease control environments. The major route of infection is via contaminated foodstuffs and *L. monocytogenes* displays multiple mechanisms for overcoming the main methods used to preserve food and prevent bacterial spoilage. Refrigeration (Walker *et al.*, 1990), curing (Abram *et al.*, 2008a), acidification (Heavin *et al.*, 2009) and high pressure treatment (Karatzas *et al.*, 2003) do not completely eliminate the growth and survival of *L. monocytogenes*. While exposure to low temperature (0 - 7°C) significantly reduces growth rate *L. monocytogenes* can still be recovered from refrigerated food samples within a detectable range (Cole *et al.*, 1990; Ryser *et al.*, 1996). It does appear however that pre-exposure to low temperature reduces the ability of the bacterium to withstand a subsequent acid shock (Ivy *et al.*, 2012). Strains of *L. monocytogenes* however have been shown to survive for extended periods in inorganic acid (Bergholz *et al.*, 2012; Dykes & Moorhead, 2000; Ferreira *et al.*, 2003) and can grow, albeit at much reduced rates in organic acids commonly found in foodstuffs (Heavin *et al.*, 2009).

1.5.1 Sensing and Regulation

In order for *L. monocytogenes* to respond to changes in its environment it must first be able to sense the changes occurring around it. One way the bacterium has evolved to respond to stress involves an elaborate cascade pathway leading to the activation of the alternative sigma factor, σ^B . This sigma factor binds to the inactive RNA polymerase allowing it to recognise specific promoter sequences for a set of genes, thereby commencing their transcription (Borukhov & Nudler, 2003). σ^B in *L. monocytogenes* is homologous to σ^B found in *B. subtilis*, in which it was first discovered (Haldenwang & Losick, 1979). Much of the early work performed to elucidate σ^B function and regulation was carried out in *B. subtilis* and with the sequencing of these two organisms it was found that they share many

of the common genetic features and components of the pathway (Ferreira *et al.*, 2004).

In *L. monocytogenes*, the gene encoding σ^B (*sigB*) is transcribed as part of an eight-gene operon comprising *rsbR*, *rsbS*, *rsbT*, *rsbU*, *rsbV*, *rsbW*, *sigB* and *rsbX* (Ferreira *et al.*, 2004). The transcription of *sigB* is itself controlled by σ^B , however the activity of σ^B is primarily controlled post-translationally. The other *rsb* genes of the operon encode regulators that form part of the σ^B activation pathway and due to their close homology to these proteins in *B. subtilis* the same manner of protein-protein interactions is thought to be followed for *L. monocytogenes* (Ferreira *et al.*, 2004). Whilst the entire operational mechanism of the pathway has yet to be elucidated, it is thought that *L. monocytogenes* senses a stress signal via the stressosome, a large (approx. 2 MDa) multi-subunit complex consisting of RsbR, RsbS and RsbT proteins as well as a homologue of the blue light sensor in *B. subtilis*, YtvA. Various putative paralogues of RsbR exist in *L. monocytogenes*, each predicted to play a role in the stressosome in sensing different stresses (Heavin & O'Byrne, 2012; Losi, 2004; Ondrusch & Kreft, 2011; Pané-Farré *et al.*, 2005). Upon sensing a stress, RsbT is released from the stressosome activating RsbU. This phosphatase dephosphorylates RsbV which in turn sequesters RsbW. In an unstressed condition, RsbW is bound to σ^B , however with its release due to the RsbV-RsbW interaction, σ^B is now free to associate with RNA polymerase and dictate gene transcription (Fig. 1.4).

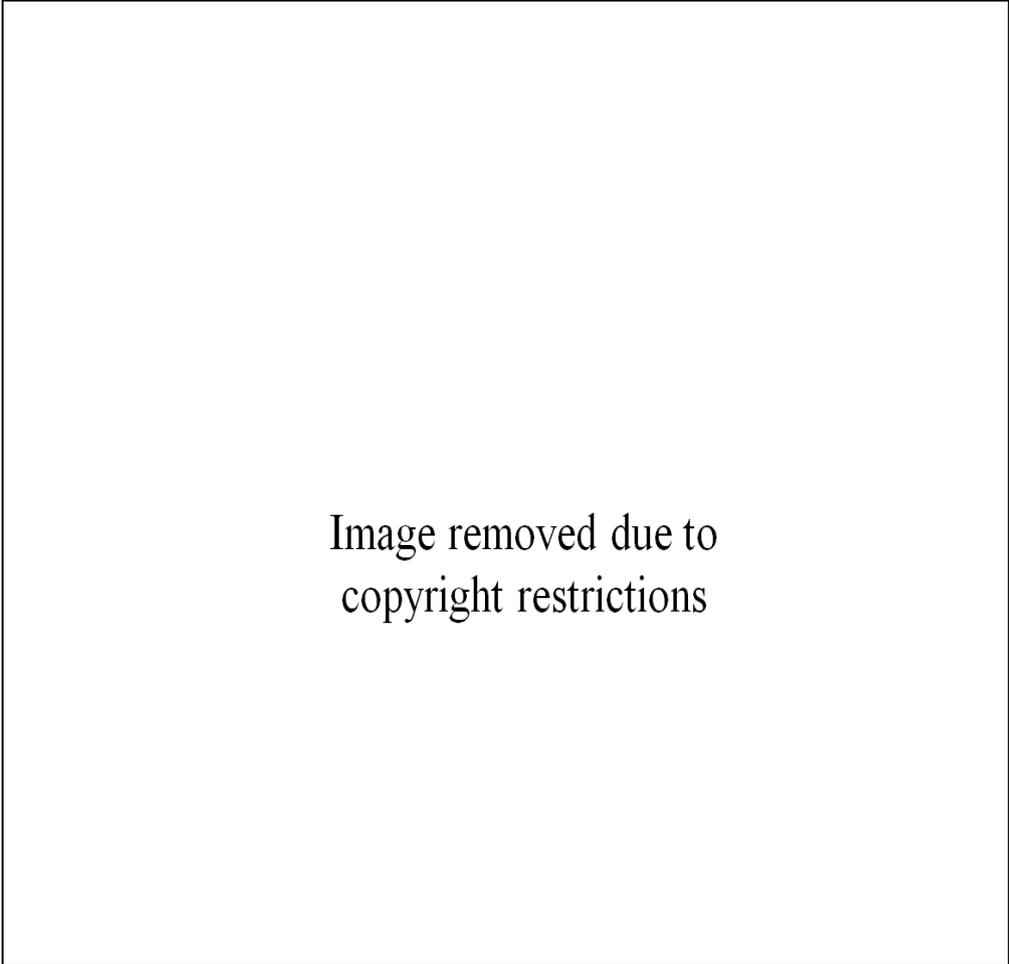


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Figure 1.4. Schematic representation of the σ^B activation pathway modelled from *B. subtilis*. In an unstressed state σ^B is bound to RsbW. During stress the dephosphorylation of RsbV leads to the sequestering of RsbW and the release of σ^B , allowing it to associate with the RNA polymerase. RsbV is dephosphorylated by the phosphatase activity of RsbU, which in turn was activated by the RsbT kinase that was released from the stressosome complex during stress. Figure taken from Hardwick *et al.*, 2007

Mutants lacking a functional *sigB* were found to be sensitive to low pH (Ferreira *et al.*, 2003; Wiedmann *et al.*, 1998), osmotic stress (Abram *et al.*, 2008a), cold stress (Chan *et al.*, 2007), oxidative stress (Becker *et al.*, 1998), high hydrostatic pressure (Wemekamp-Kamphuis *et al.*, 2004a), antibiotics and bacteriocins (Begley *et al.*, 2006). Microarray analysis has shown that σ^B is involved in the

regulation of up to 200 genes (Hain *et al.*, 2008). These genes fall across a broad range of classes and functions however many of them are seen to be involved in mechanisms of stress response. Promoters for σ^B are found upstream of both the *gadT2D2* and *gadD3* operons, which encode for proteins comprising the GAD system that is important for acid tolerance (Kazmierczak *et al.*, 2003). In a σ^B mutant expression of both these operons is abolished in response to low pH (Wemekamp-Kamphuis *et al.*, 2004; Section 1.6.2). σ^B also plays a role in osmotic stress, where its activity is found to be proportional to the level of stress (Utratna *et al.*, 2011). In a σ^B mutant there was no transcription of *opuCA* (a component of the carnitine uptake system involved in osmotic stress) in response to salt treatment (Utratna *et al.*, 2011).

1.5.2 Osmotic Stress

L. monocytogenes has been seen to grow in environments containing up to 2 M NaCl and survive up to 3 M NaCl (Cole *et al.*, 1990). To do this, it accumulates high concentrations of compatible solutes intracellularly to counter the loss of turgor pressure. These compatible solutes include betaine, carnitine, glycine and proline (Bayles & Wilkinson, 2000). OpuC, an ABC transporter encoded by four genes from a single operon has been identified as a key component of osmotic stress survival (Fraser *et al.*, 2000). A mutant lacking the transporter OpuC has been shown to have an impaired ability to grow under hyperosmotic stress due to the inability to uptake carnitine as a compatible solute (Fraser *et al.*, 2000). During osmotic shock, studies have shown large transcriptional changes in the cell that appear to be dependent upon σ^B . A total of 166 genes with predicted σ^B -dependent promoters were identified as a result of exposing *L. monocytogenes* 10403S to osmotic stress (Kazmierczak *et al.*, 2003). Exposing *L. monocytogenes* to osmotic stress also results in a large change in the expression of proteins by the cell with many proteins identified also involved in protecting the cell from cold-shock (Abram *et al.*, 2008b; Duché *et al.*, 2002). In the absence of compatible solutes, the *ctc* gene, encoding Ctc, (similar to the general stress protein of *B.*

subtilis) has been shown to protect *L. monocytogenes* against osmotic shock (Gardan *et al.*, 2003). The function of this remains unknown.

1.5.3 Piezotolerance

The treatment of foods with high hydrostatic pressure has increased since 1998 (Hugas *et al.*, 2002; Rastogi *et al.*, 2007). With this method, food is packaged in special airtight containers and treated with high pressure of up to 600 MPa for short periods of time (Mertens, 1995). Despite the high pressures involved there is evidence that *L. monocytogenes* strains have emerged that are capable of withstanding the treatment (Karatzas *et al.*, 2003). A mutation in the *ctsR* gene showed an increase in expression of the *clp* genes which encode molecular chaperones involved in protein folding (Karatzas *et al.*, 2003). The increase in high pressure resistance was however accompanied by a reduction in virulence.

1.5.4 Tolerance to temperature change

L. monocytogenes is capable of growth at temperatures as low as -0.4°C and isolates have been recovered from meat products exposed to temperatures up to 70°C (Farber & Peterkin, 1991). As refrigeration is a major means of controlling bacterial growth, much work has been undertaken in understanding the adaptation of *L. monocytogenes* to cold temperatures. The bacterium undergoes a large induction of both cold shock and cold acclimation proteins in response to cold stress (Bayles *et al.*, 1996). It appears that *L. monocytogenes* can uptake compatible solutes to serve as cryoprotectants (Bayles & Wilkinson, 2000) and also alters the composition of the cell membrane by increasing the proportion of unsaturated fatty acids (Beales, 2004). The latter allows for a higher membrane fluidity as these fatty acids have a lower melting point. Overall transcriptomic studies have identified 245 genes that are differentially expressed in cultures of *L. monocytogenes* grown at 4°C (Chan *et al.*, 2007). These included genes involved

in both lipid and sugar metabolism and transport and also motility and amino acid metabolism. The alternative sigma factor σ^B also showed increased activity while several genes under the control of PrfA were down regulated (Chan *et al.*, 2007).

The matter of resistance to high temperature became an issue following an outbreak of listeriosis from pasteurised milk in 1983 in the USA (Farber & Peterkin, 1991). It appeared that *L. monocytogenes* was able to survive pasteurisation if first the cells developed a heat shock response after being pre-exposed to sublethal temperatures between 44 and 48°C (Bunning *et al.*, 1990). More recently highly heat-resistant strains, isolated after exposure to 72°C have been identified among strains also resistant to high-hydrostatic pressure (Van Boeijen *et al.*, 2011). *L. monocytogenes* can grow at temperatures up to 45°C (Petran & Zottola, 1989) and work in this area has shown an induction of genes involved in heat shock, DNA repair and SOS response in cells exposed to a heat shock of 48°C (van der Veen *et al.*, 2007). The response of the bacteria appears to be rapid with most transcriptional changes occurring within three minutes post treatment. There is also a concerted effort to repress cell division, with genes involved in cell division and cell wall synthesis down-regulated (van der Veen *et al.*, 2007).

1.5.5 Acid survival

Strains of *L. monocytogenes* have been shown to survive pH as low as pH 2.7 (Cole *et al.*, 1990; Conner *et al.*, 1986; Karatzas *et al.*, 2012). Acid survival is aided by the action of at least four different systems including the arginine deiminase system, an F₀F₁-ATPase system, the glutamate decarboxylase system (Section 1.6) and a mechanism for developing an adaptive acid tolerance response.

1.5.5.1 F_0F_1 -ATPase

The F_0F_1 -ATPase was first examined as a potential acid survival mechanism in *L. monocytogenes* in 2000 (Cotter *et al.*, 2000). Expression of F_0F_1 -ATPase was increased following exposure to a mild-acid stress (Phan-Thanh & Mahouin, 1999). This multi-subunit protein normally operates either aerobically to generate ATP or anaerobically to hydrolyse ATP and generate a proton motive force by excluding H^+ ions from the cell (Nelson & Taiz, 1989). In *Salmonella enterica* it was shown to be involved in tolerance to acid (Foster & Hall, 1991) and is thought to function in removing H^+ ions and maintaining pH homeostasis. Disruption of the ability of the F_0F_1 -ATPase to extrude protons resulted in acid sensitivity in *L. monocytogenes* (Cotter *et al.*, 2000).

1.5.5.2 Adaptive acid tolerance response

When *L. monocytogenes* cells are exposed to a pre-treatment of mild pH (~pH 5.0) for 1 h hour, they are seen to have an improved rate of survival when subsequently treated at a severe pH of 3.0 compared to cells exposed directly to the severe pH (Davis *et al.*, 1996). This phenomenon is called the adaptive acid tolerance response (ATR) and is also seen in *E. coli* (Goodson & Rowbury, 1989) and *Salmonella* Typhimurium (Lee *et al.*, 1994). Organic acids, commonly used to preserve foods have been shown to induce an ATR in *L. monocytogenes* which leads to the idea that their use may increase the risk of infection through consumption of bacteria pre-conditioned for gastric transit. The development of an ATR also leads to a marked increase to thermal and osmotic resistance (O'Driscoll *et al.*, 1996). As mentioned above, the F_0F_1 -ATPase transporter is induced during a mild pH treatment (Phan-Thanh & Mahouin, 1999), and while it is important in the ATR, *L. monocytogenes* can still develop an ATR when the F_0F_1 -ATPase has been disrupted (Cotter *et al.*, 2000).

1.5.5.3 Arginine deiminase system

Genome analysis determined that *L. monocytogenes* possesses three genes encoding the arginine deiminase system (Glaser *et al.*, 2001). This system maintains the pH homeostasis by importing arginine via the ArcD membrane transporter. The arginine is converted to ornithine by an arginine deiminase (ArcA) and catabolic ornithine carbamoyltransferase (ArcB) and the ammonia produced in the process combines with free H⁺ ions in the cytoplasm of the cell to produce ammonium ions (Cunin *et al.*, 1986). This helps to raise the pH in the cytoplasm. Furthermore, ATP is produced in the conversion of arginine and this has the potential to drive the F₀F₁ -ATPase system of acid tolerance as discussed previously (Section 1.5.5.1). Deletion of the genes encoding the arginine deiminase system in *L. monocytogenes* reduced acid survival for these strains and also reduced growth in acidic conditions (Ryan *et al.*, 2009a). The addition of arginine to media was also shown to have a positive effect on acid survival (Ryan *et al.*, 2009b). Regulation of the system is via ArgR, while σ^B and PrfA also play a role in controlling transcription of the systems genes (Ryan *et al.*, 2009a).

1.6 The glutamate decarboxylase system in bacteria and *L. monocytogenes*

1.6.1 General overview

The glutamate decarboxylase (GAD) is well known for its role in regulating neurotransmission in mammals (Porter & Martin, 1984; Sloviter *et al.*, 1996). In fact GAD has been identified across all kingdoms of life including *Animalia*, *Plantae*, and *Prokaryota* (Kezmarsky *et al.*, 2005; Kim *et al.*, 2009). Furthermore, there is a high similarity between glutamate decarboxylases from all kingdoms (Sukhareva & Mamaeva, 2002). The decarboxylation of glutamate has been studied in bacteria since the 1940's (Gale, 1940, 1945) and it is now generally accepted that the main role for the GAD system in bacteria is the maintenance of intracellular pH.

The *E. coli* based model involves the action of a membrane bound antiport protein, generally designated GadC, and a cytosolic pyridoxal 5' phosphate dependent decarboxylase enzyme, GadB/A. Upon a decrease in intracellular pH, GadB/A undergoes a conformational change and moves towards the cytosolic side of the cell membrane (Capitani *et al.*, 2003). The antiporter then imports an extracellular molecule of glutamate in exchange for intracellular GABA. The imported glutamate is decarboxylated by GadB/A to form GABA. Through this decarboxylation reaction, there is a consumption of intracellular protons, leading to an increase in cytosolic pH (Cotter & Hill, 2003; Tsai *et al.*, 2013). Finally the GABA that is formed is exported via the antiporter in exchange for further glutamate and so the process continues (Fig. 1.5 a).

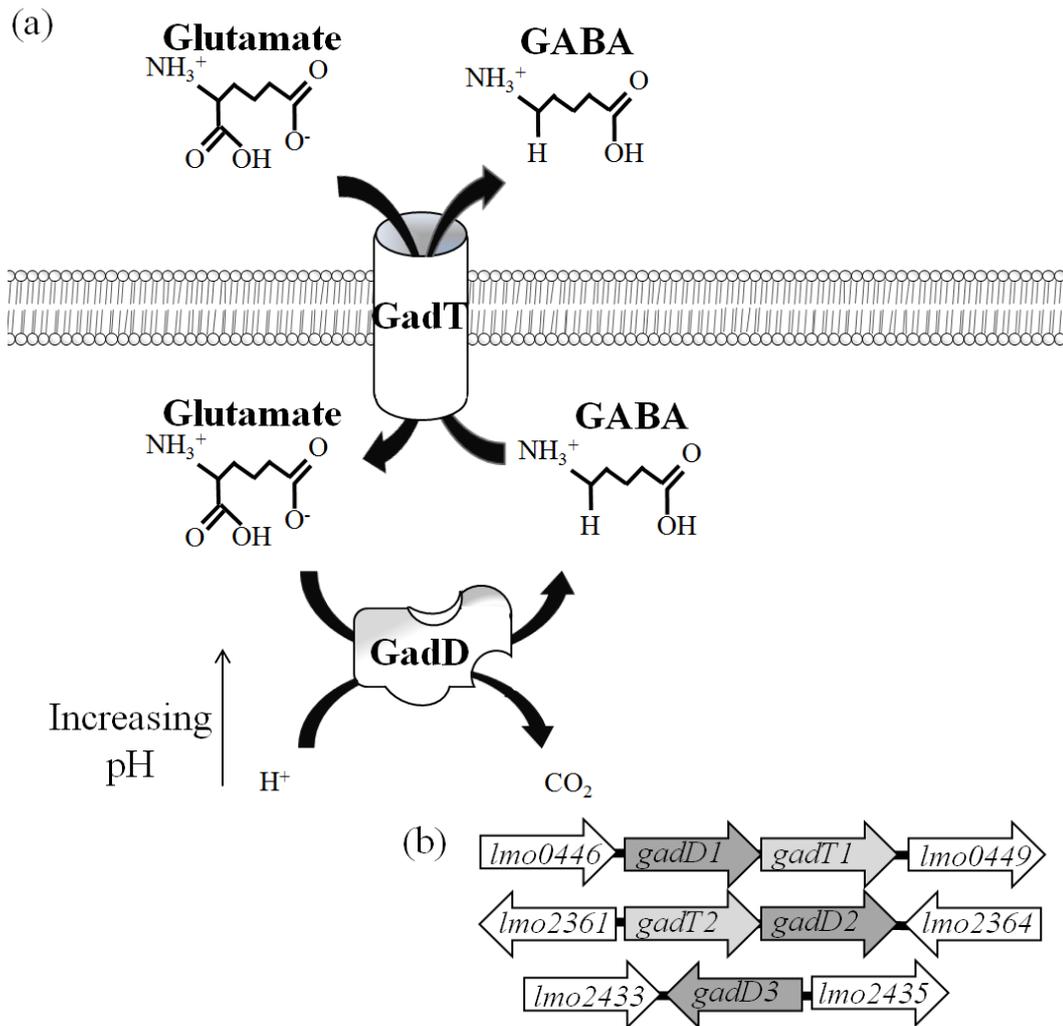


Figure 1.5. (a) The standard model for the action of the GAD system. A membrane bound antiporter carries glutamate into the cell in exchange for GABA. A cytosolic decarboxylase enzyme converts glutamate to GABA, with a consumption of H^+ . **(b)** The genetic loci carrying the GAD genes in *L. monocytogenes* EGD-e.

While the consumption of H^+ ions inside the cell by the action of the decarboxylase is considered beneficial in raising the intracellular pH, Richard & Foster (2004) have argued that this is not the complete story. Following from work by Iyer *et al.*, (2002), where these authors suggest that a net negative charge inside the cell can occur due to an intracellular build up of Cl^- , Richard and Foster describe how a reverse membrane potential can occur. This is due in part to a

build up of positively charged products of both the GAD system (GABA) and also the arginine deiminase system (agmatine). The build up of this net positive internal charge in the cell could actually act to either repel proton intrusion or shut down the cells proton motive force (PMF; Richard & Foster, 2004).

Further publications have suggested that GABA acts as a buffering agent for bacteria exposed to low pH (Lei *et al.*, 2011; Tramonti *et al.*, 2006), however it has been shown that below pH 3.0, glutamate has a more pronounced buffering capacity than GABA (Feehily & Karatzas, 2012). Recent work by Tsai *et al.* (2013) has shown that glutamate is imported into the cell in a deprotonated form while the GABA that is exported is protonated. This confirms the hypothesis that the GAD system acts to reduce the overall net pH inside the cell. Previously, the futile proton hypothesis argued that when considering the pK_a values for both glutamate and GABA, the GAD system would not contribute a net removal of H⁺ (Booth *et al.*, 2002; Feehily & Karatzas, 2012). Under mild pH (>4.5) extracellular glutamate is deprotonated and imported into the cell via GadT. During decarboxylation, an intracellular H⁺ is incorporated to the formed GABA and carried out of the cell. However, under low pH, extracellular glutamate is protonated on the side-chain carboxyl group. Therefore in this condition, glutamate was thought to be imported in a protonated state and once it reaches the milder pH of the cytoplasm, the proton will dissociate and negate any proton consumed in the decarboxylation. However, by performing substrate analysis for the antiporter, it was shown that the antiporter can discriminate between positively, neutrally and negatively charged glutamate based on the overall charge of the molecule (Tsai *et al.*, 2013). The antiporter will only import glutamate with a neutral or negative charge, both of which lack protonation on the side-chain carboxyl group. This work proves that even under low pH, the antiporter will not import protonated forms of glutamate (Tsai *et al.*, 2013).

The genomic architecture of the GAD system among bacteria is quite varied. Despite being considered a major factor in the survival of gastric transit, many

food-borne pathogens do not possess a functional GAD system including *Salmonella* spp. (Foster, 2004) and *Campylobacter* spp. (Feehily & Karatzas, 2012). In the species that do possess a GAD system, the number of genes encoding either antiporters or decarboxylases can vary (De Biase & Pennacchietti, 2012). For example *E. coli* possesses two decarboxylases, GadA and GadB, and one antiporter, GadC (Smith *et al.*, 1992), while *Lactococcus lactis* so far has been shown to possess only one antiporter and one decarboxylase (Su *et al.*, 2011). Interestingly, *Mycobacterium tuberculosis* possesses only a decarboxylase and no antiporter suggesting that the GAD system operates without the expected action of Glutamate/GABA antiport (Cole *et al.*, 1998). Intra-species variation is also common, *Bifidobacterium dentium* is the only member of the *Bifidobacterium* genus that appears to possess a GAD system (Ventura *et al.*, 2009) while among *L. monocytogenes*, serotype 4 strains do not possess the *gadTID1* operon (Cotter *et al.*, 2005).

Regulation of the GAD system has mainly been studied in *E. coli*. Here a complex process involves an interaction between specific GAD regulators, GadE, GadW, GadX and GadY, the general stress response sigma factor RpoS and modulation of intracellular levels of cAMP (Ma *et al.*, 2003). Although the specific molecular mechanisms have not been clearly elucidated, *in vitro* work has shown that RpoS activates transcription of *gadY*, whose sRNA product stabilises *gadX* transcripts. As a result GadX levels increase and along with GadW it binds to the promoters for *gada* and *gadBC* to positively regulate expression of these genes (Sayed *et al.*, 2007; Tramonti *et al.*, 2006). In contrast however, it has been seen that *in vivo* activation of *gadE* by both GadW and GadX occurs. Subsequently, GadE activates transcription of *gada* and *gadBC* (Gong *et al.*, 2004; Ma *et al.*, 2004). Apart from the genetic mechanisms of regulation, it appears that various compounds and conditions regulate the expression of the GAD system in *E. coli*. Both sodium (Richard & Foster, 2007) and polyamines (Chattopadhyay & Tabor, 2013; Jung & Kim, 2003) have been shown to up-regulate the GAD system while limited oxygen also favours an increase in GAD system protein expression (Blankenhorn *et al.*, 1999).

When the crystal structure of the GadB enzyme from *E. coli* was solved (Capitani *et al.*, 2003) it was found that the structure is assembled in a hexameric fashion where dimers of the individual GadB proteins form trimers. The N-terminal ends of each of the subunits hold the structure in place. The structure undergoes some modifications as a result of a drop in pH. Among these includes the re-ordering of residues 452-466 of the C-terminal end of each unit. Under neutral pH these are arranged into the active site (Fig. 1.6 left), behaving like a plug, and at pH of 4.6 they fold out, exposing the active site (Fig. 1.6 right). Further structural modifications allow for the localization of the enzyme to the cell membrane where it can fully carry out its role (Capitani *et al.*, 2003). The overall structures of GAD enzyme can vary depending on the organism and they are arranged according to their specific role (De Biase & Pennacchietti, 2012). The human GAD enzyme is active at neutral pH as a dimer (Fenalti *et al.*, 2007) while that of the plant *Arabidopsis thaliana* is hexameric, shares 39% sequence identity with *E. coli* and possesses a calmodulin binding pocket (Gut *et al.*, 2009).

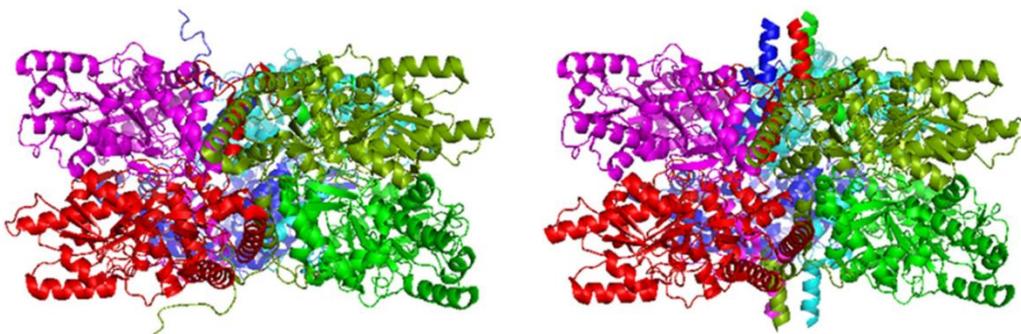


Figure 1.6. Structure of GAD enzyme in *E. coli*. The crystal structure for GadB at neutral pH (left) and acidic pH (right). Each subunit is shown in a different colour displaying the hexameric structure of the enzyme. The N-terminal regions can be seen protruding from the enzyme as a result of acid induced conformational change (right). The figure is taken with permission from De Biase & Pennacchietti, 2012

As the model above suggests (Fig. 1.3), the GAD system is reliant on the import of glutamate coupled to the export of GABA. Recent work particularly in *L. monocytogenes* has moved to separate the system into an extracellular GAD system (GAD_e) and an intracellular GAD system (GAD_i) (Karatzas *et al.*, 2012). This idea is based on the detection of intracellular GABA (GABA_i) accumulation independent of GABA export. GABA_i has been recorded in *E. coli* (Castanie-Cornet *et al.*, 1999), *Shigella flexneri* (Waterman & Small, 2003) and *L. monocytogenes* (Karatzas *et al.*, 2010). It is possible that a bacterium could provide glutamate for the GAD system from its intracellular pool and it has been seen that genes involved in glutamate transport and metabolism are involved in acid resistance and are acid induced (Krastel *et al.*, 2010; Satorhelyi, 2005). Furthermore, if unprotonated glutamate is only used by the antiporter (Tsai *et al.*, 2013), then a functional GAD_i would help to supplement for low availability of unprotonated glutamate in the extracellular environment when the pH is below the pK_a value of the side-chain carboxyl group. Indeed in *M. tuberculosis*, a genome analysis suggests that this bacteria should rely entirely on GAD_i due to a lack of any gene encoding for the glutamate/GABA antiporter (Cole *et al.*, 1998).

1.6.2 The GAD system in *L. monocytogenes*

Glutamate decarboxylase activity was first reported in *L. monocytogenes* in 1981 (Shah *et al.*, 1981). It was shown that the enzyme activity of purified glutamate decarboxylase was similar to that previously seen in *E. coli* whereby one μmol of glutamic acid added to purified enzyme produced similar amounts of CO₂ after 10 min (Najjar & Fisher, 1954; Shah *et al.*, 1981). It wasn't however until 2001 that the role of the GAD system in protecting *L. monocytogenes* from acid stress was examined. Work by Cotter *et. al* (2001) demonstrated that addition of exogenous glutamate to cultures treated with synthetic gastric fluid increased survival compared to cultures without added glutamate. In this work they also identified three genes, one glutamate/GABA antiporter (*gadA*) and two decarboxylases

(*gadB* and *gadC*) thought to encode the GAD system. Deletion of these resulted in an increased sensitivity to acid treatment (Cotter *et al.*, 2001). The GAD system has now been implicated in the survival of *L. monocytogenes* in synthetic gastric fluid, low pH foods, modified atmosphere-packaged foods (Francis *et al.*, 2007), frankfurters (Ryan *et al.*, 2010) and also potentially plays a role in resistance to nisin (Begley *et al.*, 2010). Interestingly it appears that the GAD system does not play a role in protecting *L. monocytogenes* from weak organic acid commonly used in the food industry (Heavin *et al.*, 2009). Deletion of *gadA* and *gadB* together resulted in minor increases in specific growth rate in a defined media acidified with either acetic acid, benzoic acid or sorbic acid compared to wild-type cells (Heavin *et al.*, 2009).

As a result of the sequencing of *L. monocytogenes* strain EGD-e in 2001 (Glaser *et al.*, 2001) it was seen that there are in fact 5 genes encoding the GAD system (Fig. 1.3 b). The previously identified genes *gadA* and *gadB* form an operon and were reclassified as *gadT2* and *gadD2* respectively, while *gadC* was reclassified as *gadD3*. A third operon encoding another pair of GAD genes, *gadD1* and *gadT1* was also identified (Cotter *et al.*, 2005). Unlike the glutamate decarboxylase pairs in *E. coli* which are almost identical at the level of amino acid sequence (Smith *et al.*, 1992), the three decarboxylase genes from *L. monocytogenes*, *gadD1*, *gadD2* and *gadD3* share a maximum 71.1% identity in their protein products (Cotter *et al.*, 2005). Furthermore it appears that *gadDIT1* operon is not present in all strains of *L. monocytogenes* and there is no clear correlation between serotype and possession. While EGD-e possesses the *gadDIT1* genes, F6854 does not, despite both belonging to serotype 1/2a (Cotter *et al.*, 2005; Ryan *et al.*, 2010). However, serotype 4 strains all lack this operon and this appears to correlate with these strains having a reduced capacity to grow at low pH (Cotter *et al.*, 2005; Karatzas *et al.*, 2010). A putative stem-loop structure in the intragenic region between *gadD1* and *gadT1* may prevent these genes operating in concert (Conte *et al.*, 2002). Comparison of two strains, EGD-e with LO28, appears to confirm that despite intra-strain variation in GAD proteins (Fig. 1.7 A), identity between

homologs of another strain is much closer (Fig. 1.7 **B**), indicating common ancestral lineage (Cotter *et al.*, 2005).

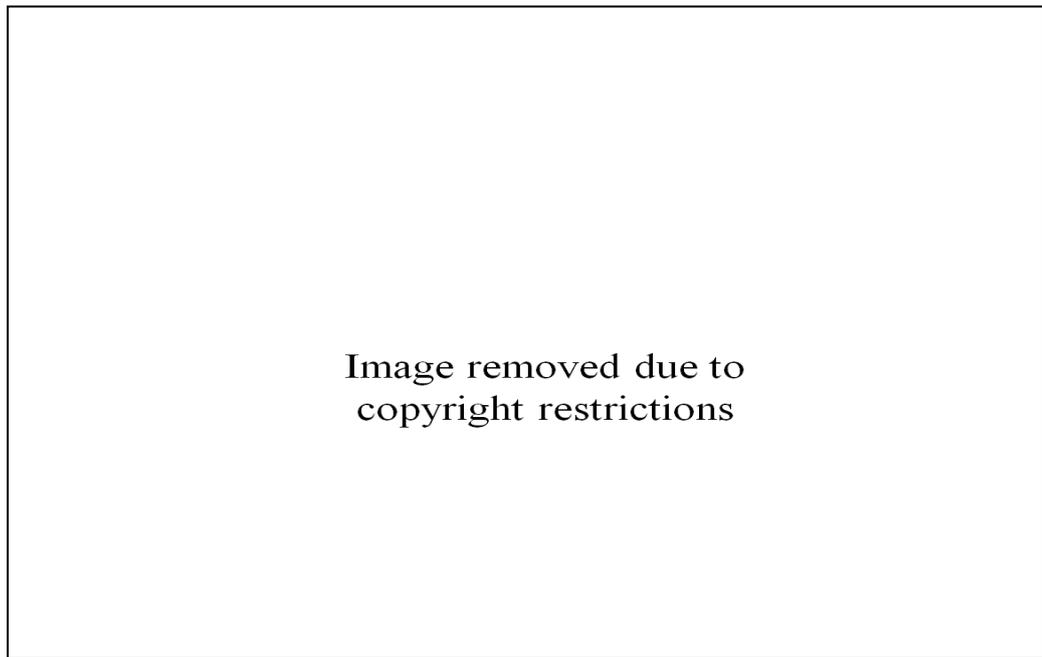


Figure 1.7. Relationship of all *Listerial* gad genes **A.** Percentage identity of the protein products for each of the GAD system genes. Annotations in brackets represent the gene name as listed for EGD-e. **B.** Relationship tree of the three decarboxylase proteins between strains LO28, EGD-e, F2365 and *L. innocua* strain 2463. Figure is taken from Cotter *et al.*, 2005

The *gadT2D2* genes have been shown to play a key role in the survival of *L. monocytogenes* LO28 under conditions of extreme acid stress. Deletion of either *gadT2* or *gadD2* produces strains that exhibit high sensitivity to pH <3.0 (Cotter *et al.*, 2001). In contrast, *gadD1T1* appears to play a role in growth at mild pH (Cotter *et al.*, 2005). This may be explained by the fact that transcription of *gadD1* appears to occur during log phase growth, while transcription of *gadD2* appears to occur upon entry into stationary phase and also in response to acid (Cotter *et al.*, 2001, 2005). As no mutant lacking *gadD3* has been previously constructed and tested at low pH its role is not fully understood. Induction of the

transcription of *gadD3* does occur during acid adaption (Kazmierczak *et al.*, 2003).

The regulation of the GAD system in *L. monocytogenes* has not yet been studied in detail. Unlike *E. coli* there are no genes to encode the regulatory components such as *gadE*, *gadW* *gadX* or *gadY* (Glaser *et al.*, 2001). The system appears to rely on regulation via σ^B (O'Byrne & Karatzas, 2008). Both *gadT2D2* and *gadD3* are down-regulated in a *sigB* mutant and their expression increases upon entry into stationary phase in a σ^B –dependent manner (Abram *et al.*, 2008a; Cotter *et al.*, 2001; Wemekamp-Kamphuis *et al.*, 2004a). Predicted promoters for σ^B are located upstream of both the *gadT2D2* and *gadD3* transcriptional units (Wemekamp-Kamphuis *et al.*, 2004b) however only the promoter preceding *gadD3* has been confirmed (Kazmierczak *et al.*, 2003). Acidification of *L. monocytogenes* 10403S to pH 3.5 increases expression of *gadDIT1* and *gadD3*, despite the reported role for *gadDIT1* playing in mild acid (Cotter *et al.*, 2005; Karatzas *et al.*, 2010). It appears that media type also plays a role in the regulation and the activity of the GAD system. No extracellular GABA (GABA_e) is produced by *L. monocytogenes* 10403S when it is grown and acid-challenged in a defined minimal media (DM; Karatzas *et al.*, 2010). Addition of glutamate to this medium did not induce extracellular GABA production and it appears that *gadT2D2* is not transcribed (Karatzas *et al.*, 2010).

As discussed previously (Section 1.6.1), not all *L. monocytogenes* strains appear capable of conducting the glutamate/GABA antiport of the GAD system (Feehily & Karatzas, 2012). Despite possessing both *gadT1* and *gadT2*, EGD-e does not appear to have a functional glutamate/GABA antiport system as no GABA_e has been detected in this strain in response to acidification (Karatzas *et al.*, 2012). Furthermore, the strain LO28 does not appear to produce GABA_e in Brain Heart Infusion (BHI) media however it does produce GABA_e in Tryptone Soya Broth supplemented with glutamate (Karatzas *et al.*, 2012). Coupled to the evidence that 10403S can only produce intracellular GABA_i and not GABA_e in DM, it appears

that there is a split between an intracellular GAD (GAD_i) system and an extracellular GAD (GAD_e) system in *L. monocytogenes*. As GABA_i is detectable in strains that do not produce GABA_e it appears that an internal source of glutamate is fed into the system (Karatzas *et al.*, 2010, 2012). Furthermore, this behaviour of utilising only the GAD_i seems to be inherently the case in *Mycobacterium tuberculosis* since it possesses GadA but no glutamate/GABA antiporters. As there is no export of GABA via the glutamate/GABA antiporter for GAD_i it has been suggested that in *L. monocytogenes* the GABA could be catabolised via the product of *lmo0913*, a succinic semialdehyde dehydrogenase into succinate (Cotter *et al.*, 2005; Karatzas *et al.*, 2010). This would involve the action of the GABA shunt pathway discussed in the following section.

1.7 The GABA shunt pathway in bacteria

The conversion of glutamate to GABA by glutamate decarboxylase is the first step of the GABA shunt pathway. However, in bacteria where most of the GABA is exported by the glutamate/GABA antiporter, the GABA shunt deals with the steps that follow the generation of GABA. The accumulation of intracellular GABA under acidic conditions suggests that its removal should involve a catabolic pathway. This could be assumed as the glutamate/GABA antiporters are only able to remove quantities of GABA that are equimolar to those of the glutamate they import, leaving the excess in the cell.

The first enzyme in the GABA shunt is a GABA aminotransferase (GABA-AT). This involves the reversible conversion of GABA to succinic semialdehyde (SSA), where the amino group of GABA is transferred to an α -ketoglutarate molecule. The product of this reaction is glutamate (Fig. 1.8). It has been shown that plants can use pyruvate and glyoxylate as alternative amino group acceptors (Bouché & Fromm, 2004; Clark *et al.*, 2009), however bacteria appear to only have α -ketoglutarate-dependent GABA-AT activity, with the exception of some *Rhizobium* spp. which can use both (Prell *et al.*, 2002). The sole reliance on α -

ketoglutarate as the receiving oxo-acid is also the case for GABA-ATs present in mammals (Shelp *et al.*, 1999). The importance of this enzyme in bacteria is often highlighted by the presence of more than one gene encoding a functional GABA-AT. Studies with *E. coli* and *Pseudomonas syringae* have identified the possession of up to three genes encoding functional GABA-ATs (Buell *et al.*, 2003; Kurihara *et al.*, 2010). In *E. coli* it appears that induction of GabT, the primary GABA-AT, occurs at pH 9.0 (Stancik *et al.*, 2002) suggesting that this pathway may be more active at alkaline pH conditions.

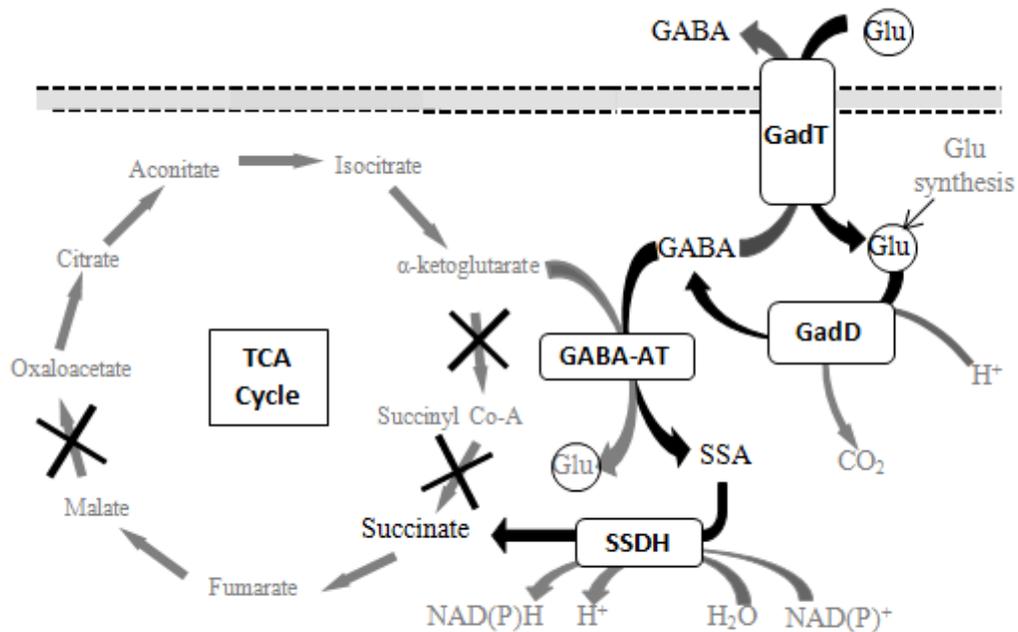


Figure 1.8. Proposed model for the metabolism of GABA_i in *L. monocytogenes*. Glutamate is decarboxylated to GABA_i by GadD. The GABA can either be exported by GadT in exchange for another glutamate or enter the GABA shunt pathway. Here GABA_i donates its amino group to α -ketoglutarate via a transaminase enzyme (GABA-AT), resulting in the formation of succinyl semialdehyde (SSA) and glutamate. The SSA is then oxidised to succinate by a dehydrogenase (SSDH). The incomplete TCA of *L.*

monocytogenes is shown with the missing steps marked with 'X'. The GABA shunt pathway can provide an alternative source of succinate for the bacteria.

The second enzyme in the pathway is a Succinic Semialdehyde Dehydrogenase (SSDH). This irreversible step involves the oxidation of SSA to succinate with the formation of CO₂. Similarly to GABA-AT, bacteria can possess up to three SSDH encoding genes (Buell *et al.*, 2003). Each SSDH can possess different characteristics and co-factor specificities. For example in *E. coli* there are currently two described SSDH enzymes, GabD and YneI; the former is dependent on NADP⁺ and YneI preferentially utilises NAD⁺ (Donnelly & Cooper, 1981b; Fuhrer *et al.*, 2007). YneI can however use NADP⁺, but the activity utilising this cofactor is only 15% of what is achieved using NAD⁺ (Donnelly & Cooper, 1981a). The presence of this varying NAD(P)⁺ dependency has also been well described in several species of *Pseudomonas* (Jakoby & Scott, 1959; Padmanabhan & Tchen, 1969), as well as both NADP⁺ and NAD⁺ dependent SSDH activity shown in *Bacillus thuringiensis* (Zhu *et al.*, 2010). Partially purified SSDH from plants shows an optimal pH of 9 (Shelp *et al.*, 1999). This correlates well with the optimal pH for the GABA-AT if the two enzymes are to work together in a metabolic pathway and suggests that this pathway is not likely to be active under acidic conditions

Metabolism through the GABA shunt is an important route of nitrogen sourcing for bacteria. Arginine, ornithine, agmatine and putrescine are all used as nitrogen sources by *E. coli* (Schneider *et al.*, 2002). Arginine, ornithine and agmatine are first converted to putrescine and the pathway subsequently converts putrescine to GABA which is then catabolised via the GABA shunt. In *E. coli* mutants, despite the absence of either the *gabT* or *gabD* genes, GABA-AT and SSDH activity remains. This activity is induced through the use of putrescine as a sole nitrogen source (Kurihara *et al.*, 2010). Furthermore, *puuE*, a gene forming an operon with other members of the putrescine metabolic pathway was shown to act as a secondary GABA-AT (Fuhrer *et al.*, 2007). It is interesting to note that none of

the other genes in the *puu* gene cluster encode an SSDH, however YneI, which has been predicted as a secondary SSDH (Kurihara *et al.*, 2010), is induced, along with PuuE, by putrescine (Zaboura & Halpern, 1978). Despite this evidence for the role of the GABA shunt in nitrogen metabolism, *E. coli* cannot use GABA as a sole source of nitrogen (Belitsky & Sonenshein, 2002). The reasons for this are unclear, as *E. coli* does possess a transporter capable of transporting GABA. *Bacillus subtilis* has also been shown to utilise GABA as a nitrogen source, however unlike *E. coli*, *B. subtilis* cannot catabolise putrescine or generate GABA through GAD due to the absence of any GAD encoding genes (Dover & Halpern, 1972a). This coincides with the fact that GABA is known to be highly toxic for *B. subtilis* and the high levels of GABA produced by the GAD system could be lethal for this bacterium (Belitsky & Sonenshein, 2002). However, it imports extracellular GABA via a GABA permease *gapP* (Ferson *et al.*, 1996). This pathway can serve as the sole nitrogen source for *B. subtilis*. Prell *et al.*, (2002) suggested that the pathway may be used in nitrogen metabolism for bacteroids, in particular *Rhizobium leguminosarum*. Some atmospheric nitrogen that is fixed to ammonium during its symbiotic relationship with legume nodules is retained by the bacteria. Results show that GabT is specifically induced by the bacteroid in the nodule state, indicating the enzyme may be involved in catabolising the glutamate derived from ammonium.

The regulation of the GABA shunt pathway is a subject on which little is known about. Much of the initial work investigating this pathway was performed in *E. coli*. It was observed that the pathway is regulated by a form of catabolite repression (Dover & Halpern, 1972b). Further work has since localised the genes to a region called the *gab* cluster, which contains the four genes *gabT*, *gabD*, *gabP* and *gabC* (Bartsch *et al.*, 1990; Metzner *et al.*, 1979). Transcriptional control of these genes is complex and involves the *csiD-ygaF-gabDTP* regulon (Fig. 1.9). This operon is reported to be controlled by the three promoters *csiDp*, *gabDp1* and *gabDp2*. The first two promoters are σ^S -dependent while the third is likely to be induced under limited nitrogen availability by Nac which is a σ^{70} -dependent transcriptional activator or by σ^{70} itself (Metzner *et al.*, 2003). Recent work with *B. thuringiensis* (Zhu *et al.*, 2010) identified the role of a sigma factor, σ^{54} , in

controlling the GABA shunt. This regulates a transcriptional activator, GabR, which itself is also auto-regulated. The complete mechanism for regulation has not yet been elucidated in these bacteria however the *gab* cluster in *B. thuringiensis* is a good example of how difficult is to make comparisons and generalisations on GABA shunt regulation, even between species of the same genus. Both *gabD* and *gabT* form a GABA inducible operon in *B. subtilis* (Belitsky & Sonenshein, 2002), however these two genes are individually regulated in *B. thuringiensis* by GabR (Zhu *et al.*, 2010). The involvement of ATP as a non-competitive inhibitor of *Arabidopsis thaliana* SSDH increases the ways by which the GABA shunt in bacteria could be regulated. This implies that the energy status of the cell itself could be involved in the regulation of the pathway (de Carvalho *et al.*, 2011).

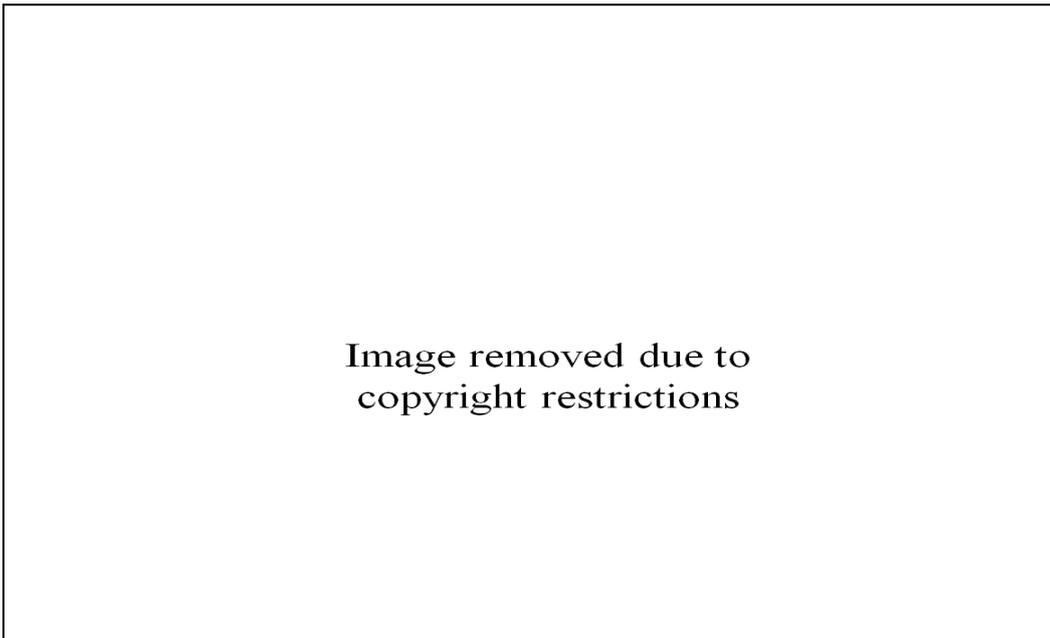


Figure 1.9. Regulation of the GABA shunt encoding genes in *E. coli*. Three promoter upstream of the *gab* operon control transcription of these genes. Carbon starvation and osmotic up-shift induce transcription from the first promoter. Carbon starvation, pH reduction and osmotic up-shift induce transcription from the second promoter while transcription from the third is induced by nitrogen limitation and the activity of $E\sigma^{70}$. The product of *csiR* prevents transcription from the first promoter. Figure is taken from (Metzner *et al.*, 2003).

While much of the focus of the GABA shunt research has been on its role in carbon and nitrogen metabolism, there are grounds to suggest that it plays a much broader role in bacteria and has a significant impact on both survival under environmental stresses and on their ability to cause disease. As discussed earlier, the GABA shunt can operate as an alternative to certain steps of the TCA cycle, providing an alternative pathway for succinate biosynthesis. Many bacteria including *L. monocytogenes*, *M. tuberculosis* and *B. thuringiensis* lack a complete set of genes required for the TCA cycle (Aronson *et al.*, 1975; Glaser *et al.*, 2001; Tian *et al.*, 2005a, b). In fact it has been demonstrated that the SSDHs of *M. tuberculosis* along with an α -ketoglutarate decarboxylase can lead to the generation of succinate in a pathway which is required for normal growth of the bacteria (Tian *et al.*, 2005a). Whilst *M. tuberculosis* does not use the first stage of the GABA shunt, it does indicate that shunt-derived succinate could be beneficial to bacteria. Furthermore, the TCA cycle has been proposed as an important process in the formation of spores in *Bacillus* species (Rutberg & Hoch, 1970), and Aronson *et al.*, (1975) demonstrated that the GABA shunt can successfully complement the loss of α -ketoglutarate dehydrogenase in this process.

As the *gab* gene cluster in *E. coli* is under the control of the stress inducible sigma factor σ^S , it is thought that the GABA shunt may play a role in stress response. The mechanism of its involvement is unclear however, it has been suggested that control of the GABA shunt may affect glutamate levels which are seen to increase in response to osmotic stress (Metzner *et al.*, 2003). This may well be the case as the first step in the pathway generates glutamate. Furthermore, a putative SSDH in *L. monocytogenes*, namely Lmo0913 has been shown to be induced in response to NaCl (Abram *et al.*, 2008b) which would be required to remove the toxic SSA (Fuhrer *et al.*, 2007) accumulated by the generation of glutamate in this manner. The GABA shunt may also play a role in acid tolerance. The antiport independent accumulation of GABA in response to acid by *L. monocytogenes* (Karatzas *et al.*, 2010) would necessitate the catabolism of this metabolite. In fact deletion of

lmo0913 in this bacterium results in an acid sensitive strain (Abram *et al.*, 2008a) highlighting the potential link between acid stress survival and the GABA shunt.

A further, albeit under-studied, role for the pathway has been a possible link between GABA metabolism and virulence. A mutation in GABA-AT has been reported to reduce bacterial virulence in the plant pathogen *P. syringae* (Tian *et al.*, 2005a). Another plant pathogen, *Agrobacterium tumefaciens* uses GABA in damaged plant tissues as a signalling molecule which regulates virulence functions (Chevrot *et al.*, 2006). Levels of GABA increase in wounded plant tissues and they signal the modulation of quorum sensing in the bacterium thereby affecting its virulence on plants. A role for GABA metabolism has been outlined in plant fungal pathogens such as *Fusarium graminearum* and *Cladosporium fulvum* (Carapito *et al.*, 2008; Solomon & Oliver, 2002). These fungi alter the metabolism of the plant-host in order to provide GABA as a nitrogen source. The GABA shunt is involved in the formation of crystals and spores in *Bacillus thuringiensis* (Zhu *et al.*, 2010).

Overall the GABA shunt pathway is a much more important metabolic route than the attention it has received would suggest. Apart from its role in glutamate cycling, it has been shown to play a role in both secondary carbon and nitrogen metabolism of bacteria. This simple two step pathway is quite variable among bacteria, with the number of genes encoding the pathway as well as the regulation of these genes varying greatly. This makes the development of a general model for the GABA shunt in bacteria difficult and necessitates the need for species specific studies. The majority of our knowledge in this area has been attained through work with mammals, typically with an emphasis on novel drug discovery.

1.8 Aims

The project described herein set out to further elucidate the role that the GAD system plays in the survival of *L. monocytogenes* both *in vitro* and *in vivo* and to determine if the GABA shunt pathway is present and active in *L. monocytogenes*.

To achieve these goals deletion mutants were constructed in key genes of both pathways and the phenotypes of these mutants was characterised. As mentioned in section 1.6.2, there is a wide variation in the activity of the GAD system between strains. Therefore, the glutamate decarboxylase genes of two important reference strains, EGD-e and 10403S, were deleted, and a comparison of the roles for these in each strain was undertaken. A complete set of glutamate decarboxylase mutants, $\Delta gadD1$, $\Delta gadD2$ and $\Delta gadD3$ had not been tested for low pH phenotypes in any previous study and this project aimed to achieve this. (Chapters 3 & 4)

Furthermore the extent to which the GAD system aids the survival of *L. monocytogenes* through stomach of a model organism and cause infection had not been tested. Using a murine model, the proposed deletion mutants were tested by orally infecting mice and monitoring dissemination through the host. (Chapter 4)

Finally, as previously reported, there is an accumulation of GABA_i inside *L. monocytogenes* strains as a result of exposure to low pH. Despite the presence of genes encoding a glutamate/GABA antiport system, this GABA does not appear to get removed. In order for GABA to be catabolised, *L. monocytogenes* must possess a functional GABA shunt pathway. Despite genetic evidence for the presence of genes encoding this pathway no direct biochemical evidence had been shown to confirm its existence. Both biochemical and genetic analyses were undertaken to determine if *L. monocytogenes* possesses a functional GABA shunt pathway. (Chapter 5)

Overall, the importance of both GABA production and its subsequent metabolism in relation to acid survival and virulence of *L. monocytogenes* was analysed.

CHAPTER 2

Materials and Methods

2.1 Bacterial strains and plasmids

The bacterial strains used in this study are presented in Table 2.1. Permanent stocks were made by centrifuging 5 ml of overnight culture and resuspending in 3 ml of appropriate media supplemented with DMSO to a concentration of 7% (v/v). One ml aliquots were put in 2 ml cryovials and stored at -80°C. Working cultures of the strains were streaked onto BHI agar plates (*L. monocytogenes*) or LB agar (*E. coli*) and grown overnight at 37°C. Plates were supplemented with selective antibiotics where appropriate and stored at 4°C. For experiments 1 isolated colony was used from a plate for each biological replicate. Plasmids used in the study are listed in Table 2.2.

Table 2.1 Bacterial strains used in this study

Strain	Genotype	Source
<i>L. monocytogenes</i>		
EGD-e	Wild-type	K. Boor (Cornell University)
EGD-e Δ <i>gadD1</i>	Δ <i>lmo0447</i> (Δ <i>gadD1</i>)	This study
EGD-e Δ <i>gadD2</i>	Δ <i>lmo2363</i> (Δ <i>gadD2</i>)	This study
EGD-e Δ <i>gadD3</i>	Δ <i>lmo2434</i> (Δ <i>gadD2</i>)	This study
EGD-e Δ <i>gabD</i>	Δ <i>lmo0913</i> (Δ <i>gabD</i>)	This study
EGD-e Δ <i>lmo0913</i> pKAK0913	<i>gabD</i> complemented with a cloned copy of the full <i>lmsg_02013</i> gene	This study
EGD-e <i>argD</i> ::pLSV101	<i>argD</i> ::pLSV101	K. Schauer
EGD-e Δ <i>gsaB</i>	Δ <i>gsaB</i>	This study
EGDm	<i>inlA</i> residues S192N and Y369S modified	Cormac Gahan (UCC)

EGDm $\Delta gadD1$	<i>inlA</i> residues S192N and Y369S modified; $\Delta gadD1$	This study
EGDm $\Delta gadD2$	<i>inlA</i> residues S192N and Y369S modified; $\Delta gadD2$	This study
EGDm $\Delta gadD3$	<i>inlA</i> residues S192N and Y369S modified; $\Delta gadD3$	This study
EGDm $\Delta gadD1D3$	<i>inlA</i> residues S192N and Y369S modified; $\Delta gadD1$; $\Delta gadD3$	This study
EGDm $\Delta gadD2D3$	<i>inlA</i> residues S192N and Y369S modified; $\Delta gadD2$; $\Delta gadD3$	This study
EGDm $\Delta gabD$	<i>inlA</i> residues S192N and Y369S modified; $\Delta lmo0913$	This study
10403S	Wild-type	K. Boor (Cornell University)
10403S $\Delta gadD1$	$\Delta lmrG_02013$ ($\Delta gadD1$)	This study
10403S $\Delta gadD2$	$\Delta lmrG_02013$ ($\Delta gadD2$)	This study
10403S $\Delta gadD3$	$\Delta lmrG_02013$ ($\Delta gadD3$)	This study
10403S $\Delta gabD$	$\Delta lmrG_02013$ ($\Delta gabD$)	Abram <i>et al.</i> , 2008

E. coli

K-12 BW25113	$\Delta(araD-araB)567$ $\Delta lacZ4787(::rrnB-3)$ λ^{-} rph-1 $\Delta(rhaD-$ $rhaB)568$ $hsdR514$	NBRP (Japan), <i>E. coli</i>
JW2636	BW25113 $\Delta gabD$	NBRP (Japan), <i>E. coli</i>
JW2636 pKAK0913	JW2636 possessing a full copy of <i>lmrG_02013</i> from 10403S cloned into pKSV7	This study
TOP10	F ⁻ <i>mcrA</i> $\Delta(mrr-hsdRMS-mcrBC)$ $\Phi 80 lacZ \Delta M15$ $\Delta lacX74$ <i>recA1</i>	Invitrogen

	<i>araD139</i>	$\Delta(ara-leu)7697$		
	<i>galU</i>			
	<i>galK</i>	<i>rpsL</i>	(Str ^R)	<i>endA1</i>
	<i>nupG</i>			
DH5 α pKSV7	F-80 Φ <i>lacZ</i> Δ M15 $\Delta(lacZYA-$	<i>argF)</i> U169 <i>endA1</i> <i>hsdR17</i>	N. Freitag	(University of Illinois)
	(<i>rk⁻mk⁻</i>) <i>supE44</i> <i>thi-I</i> <i>gyrA96</i>			
	<i>relA1</i> <i>phoA</i> ; pKSV7			

Table 2.2 Plasmids used in this study

Plasmid	Relevant property(ies)	Source
pKSV7	Cm ^r , Amp ^r , temperature sensitive ori, <i>lacZ</i> -MCS	(Smith & Youngman, 1992)
pCR-XL-TOPO	Kan ^r , Amp ^r , <i>lacZ</i> , <i>E. coli</i> cloning vector	Invitrogen
pKSV7:: Δ <i>gadD1</i>	pKSV7 vector carrying <i>gadD1</i> [AD] deletion fragment	This study
pKSV7:: Δ <i>gadD2</i>	pKSV7 vector carrying <i>gadD2</i> [AD] deletion fragment	This study
pKSV7:: Δ <i>gadD3</i>	pKSV7 vector carrying <i>gadD3</i> [AD] deletion fragment	This study
pKSV7:: Δ <i>lmo0913</i>	pKSV7 vector carrying <i>lmo0913</i> [AD] deletion fragment	This study
pKSV7:: Δ <i>gsaB</i>	pKSV7 vector carrying <i>gsaB</i> [AD] deletion fragment	This study
pKSV7:: <i>lmo0913</i>	pKSV7 vector carrying full-copy of <i>lmo0913</i>	Abram

2.2 Culture media

All media where stated were autoclaved at 121°C for 15 min in a Labo autoclave (Sanyo). Filter sterilisation was performed using a 0.22 µm syringe filter (Sartorius) and syringe (BD Plastipack)

2.2.1 Brain Heart Infusion (BHI)

BHI broth was prepared by adding 37 g of BHI broth powder (LabM) per 1000 ml dH₂O. Where 0.5M sucrose was required, 171.15 g sucrose was added to media prior to autoclaving. BHI agar was prepared by adding 49 g BHI agar powder (LabM) per 1000 ml dH₂O.

2.2.2 Luria-bertani (LB)

LB broth was prepared by adding 10 g LB powder (Sigma) per 1000 ml dH₂O. For LB agar, 15 g Agar No. 2 (LabM) was included per 1000 ml.

2.2.3 Defined media (DM)

DM is a defined media for *L. monocytogenes* described by Amezaga *et. al.*, (1995). The media was prepared from stocks described below:

Salt solution (10X)

The salt solution was made by adding 79.9 g of K₂HPO₄ (dipotassium hydrogen orthophosphate), 31 g of NaH₂PO₄·2H₂O (sodium dihydrogen orthophosphate)

and 10 g of NH₄Cl (ammonium chloride) per 1000 ml dH₂O. This was then autoclaved and stored at room temperature.

Magnesium sulphate solution (100X)

The magnesium sulphate solution was made by adding 40 g MgSO₄.7H₂O (Magnesium sulphate) per 1000 ml dH₂O and autoclaved and stored at room temperature.

Ferric citrate (50X)

Ferric citrate solution was made by adding 5 g FeC₆H₅O₇ (Ferric citrate) per 1000 ml dH₂O. This was autoclaved and stored at room temperature.

Amino acids (100X)

An essential amino acid solution was made by adding 10 g L -leucine, L -isoleucine, L -valine and L -methionine and 20 g L -histidine monohydrochloride monohydrate and arginine monohydrochloride per 1000 ml dH₂O. This was filter sterilised and stored at 4°C.

Cysteine and Tryptophan (100X)

The cysteine and tryptophan solution was made by adding 10 g L -cysteine hydrochloride and L -tryptophan per 1000 ml dH₂O. This was filter sterilised and stored at 4°C.

Glutamine (50X)

Glutamine solution was prepared by adding 30 g L-glutamine per 1000 ml dH₂O. This was filter sterilised and stored at 4°C.

Vitamin solution (100X)

To make the vitamin solution, 5 mg α - lipoic acid (D- L 6,8 Thiotic acid) was added per 200 ml 70% ethanol. Four ml of this solution was mixed with 10 mg biotin, 100 mg thiamine, 100 mg riboflavin and 250 ml ethanol (95%). The solution was brought to a final volume of 1000 ml with dH₂O and filter sterilised. The solution was stored at 4°C in the dark.

Trace elements (100X)

A trace element solution was prepared by adding 6.75 g NaOH (Sodium hydroxide) and 13.50 g N(CH₂COOH)₃ (Nitriloacetic acid) per 800 ml dH₂O. This was then slowly added to a 160 ml solution containing 0.55 g of CaCl₂·2H₂O (calcium chloride dihydrate), 0.17 g of ZnCl₂ (zinc chloride), 0.059 g of CuCl₂·2H₂O (cupric chloride dihydrate), 0.06g of CoCl₂·6H₂O (cobaltous chloride 6-hydrate) and 0.060 g Na₂MoO₄·2H₂O (sodium molybdate dihydrate). Finally the solution was brought to a final volume of 1000 ml with dH₂O and filter sterilised. The solution was stored 4°C.

To make a working stock of DM, 810 ml of autoclaved dH₂O was mixed with 100 ml salt solution (10X), 10 ml Magnesium sulphate solution (100X), 20 ml Ferric citrate (50X), 10 ml amino acids solution (100X), 10 ml cysteine and tryptophan solution (100X), 20 ml glutamine solution (50X), 10 ml vitamin solution (100X) and 10 ml trace elements. The pH was adjusted to pH 7.0 with 3 M HCl. The solution was supplemented with glucose to a final concentration of 0.4% (v/v) prior to the growth of any culture.

2.2.4 Media supplements

Depending on the strain or experimental condition, certain supplements were added to the media as required. Stocks of these supplements were made as outlined below:

Antibiotics

A stock solution of ampicillin (Amp) was prepared by dissolving 50 mg ampicillin sodium salts (Sigma) in 1 ml dH₂O. Chloramphenicol (Chl) was prepared by adding 50 mg chloramphenicol (Sigma) per 1 ml ethanol (70%). Erythromycin (Erm) was prepared by adding 50 mg erythromycin (Sigma) per 1 ml ethanol (70%). Kanamycin (Kan) was prepared by adding 20 mg kanamycin salt (Sigma) per 1 ml dH₂O. All antibiotic solutions were filter sterilised and

stored at -20°C . Antibiotics were added to media after sterilisation and once the media had reached 55°C or below.

Glutamate

A stock solution of glutamate (2M) was prepared by adding 338.2 g Monosodium glutamic acid (Sigma) per 1000 ml dH_2O . The solution was filter sterilised and stored at 4°C .

X-gal

A 40 mg ml^{-1} solution of X-gal was prepared by adding 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) to N-dimethylformamide (DMF). The solution was stored at -20°C in the dark.

Lysozyme

A 10 mg ml^{-1} solution of lysozyme was prepared by adding 10 g lysozyme from chicken egg white (Sigma) per 1000 ml dH_2O . This was filter sterilised and stored at -20°C .

2.3 Acid survival challenges

To test the survival of strains during low pH shock, overnight cultures were inoculated at an $\text{OD}_{600\text{nm}}$ of 0.05 into 25 ml broth in 200 ml Erlenmeyer flasks. These were then incubated shaking at 37°C for 18 h until the cells were in stationary phase. A bacterial count was taken by serially diluting $20\ \mu\text{l}$ of the culture in PBS and spot plating $10\ \mu\text{l}$ onto BHI agar. The pH in the remaining culture was subsequently reduced with 2M HCl using a Mettler Toledo SevenEasy pH meter and the culture was returned to 37°C . At specified time-points $20\ \mu\text{l}$ of the culture was serially diluted in PBS and spot plated as before. All plates were allowed to dry and then incubated inverted at 37°C for 24 h. Colony forming units (cfu) were then counted for each time-point and calculated as cfu ml^{-1} .

2.4 Bacterial growth curves

Initial overnight cultures for the strains to be tested were grown in 25 ml of liquid media for 16 h. The OD_{600nm} of each culture was recorded and the culture was diluted to OD_{600nm} 0.02 in sterile medium. Two hundred microlitres of this dilution was added to the well of a 96-well microtiter plate. The plate was incubated at the required temperature in a Tecan Sunrise plate reader and OD_{600nm} readings were recorded by the machine every 30 min. The plate underwent a shaking for 1 min, 30 sec prior to each reading. Data was exported to a Microsoft Excel spreadsheet via Magellan software.

2.5 Anaerobic culture

To measure the ability of strains to grow under anaerobic conditions, media was specifically prepared in order to remove any oxygen. Either 10 g LB broth (Sigma Aldrich), 18.5 g BHI broth (LabM) or 15 g Tryptic Soya Broth (TSB;LabM) plus 1.5 g Yeast Extract (LabM) was added to 15 ml 1% (w/v) cysteine (Sigma Aldrich) and 485 ml H₂O depending on the strain or condition to be tested. For LB, 10 mM L-glutamic acid monosodium salt hydrate (Sigma Aldrich) was also added. The solution was boiled for 15 min and sealed with aluminium foil while left to cool on ice for 15 min. The solution was subsequently flushed with 100% N₂ gas for 15 min and 19.4 ml was aliquoted into 50 ml serum bottles and sealed with a rubber stopper. The head space of each bottle was further flushed with a mix of CO₂ and N₂ gas for 30 s and finally sealed with an aluminium crimp cap. All bottles were sterilised by autoclaving at 121°C for 15 min and after cooling, 600 µl of a 12.5 mg ml⁻¹ filter sterilised sodium sulphide solution was added via needle and syringe. As an indication of oxygen removal every fifth bottle per batch had flakes of rezasurin power (Sigma Aldrich) added. The presence of

oxygen was detected by the presence of a purple/ pink colour in the medium. Bottles were stored at room-temperature until required.

An overnight of culture was inoculated into each bottle via syringe to give a starting OD_{600nm} of 0.02. Cultures were then grown with shaking at 37°C. Growth was measured by placing the entire bottle into a spectrophotometer (Spectronic 20 Genesys) and recording the OD_{600nm}.

2.6 Biofilm assay

An overnight culture was grown at 37°C for 16-18 h. One millilitre of the overnight was centrifuged at 8000 × *g* for 6 min and the pellet was washed once in 1 ml PBS (pH 7.0). The supernatant was discarded and the washed pellet was resuspended in 1 ml PBS. Five microlitres of the washed cells were added to 5 ml of either BHI broth or DM supplemented with glucose to a final concentration of 50 mM and vortexed gently. Two hundred microlitres of this resuspension was transferred to a flat bottomed 96-well tissue culture plate (Sarstedt) with eight technical replicates used. Sterile media was added to each plate as a control. The plate was subsequently incubated statically at 37°C for the required time. After incubation, the OD_{595nm} was recorded using a Tecan Sunrise absorbance reader. The media was carefully removed from all wells using a pipette and each well was washed 3 times with 200 µl PBS. The plate was allowed to dry at room temperature for 45 min and 150 µl of a 1% (w/v) crystal violet solution was added to each well. The plate was incubated at 37°C for 30 min and the crystal violet was removed. The plate was washed 4 times in 200 µl PBS and finally 160 µl 95% ethanol was added. The plate was incubated for a further 30 min at room-temperature and the OD_{595nm} was recorded.

2.7 Metabolite assays

2.7.1 Culture treatment and extraction

Strains were inoculated into 20 ml broth and incubated overnight for 16-18 h at 37°C shaking. Cultures were prepared in duplicate where one was acid treated and one remained untreated. For acid treatment, the pH was adjusted as required using 3 M HCl and the cultures were incubated for a further 1 h at 37°C. Following treatment, 500 µl of culture was removed to retrieve the extracellular metabolite fraction. This was centrifuged at 10,000 × *g* for 10 mins and the supernatant retained. The remaining culture was centrifuged at 10,000 × *g* for 10 min and the supernatant discarded. The pellet was resuspended in either 1 ml or 0.5 ml sterile dH₂O depending on the strain and transferred to a 1 ml microcentrifuge tube. This was then boiled for 10 min and centrifuged at 10,000 × *g* for 10 min. The supernatant was then transferred to a new tube. All samples obtained were frozen at -20°C until needed.

2.7.2 Glutamate

Glutamate was measured from samples using a modified method for the Roche BioPharm L-Glutamic acid enzymatic kit (Cat. no. 10139092035). The method was adjusted to operate in a 300 µl 96-well micro-titre plate as opposed to a 3 ml cuvette. Standards of monosodium glutamate were made in sterile dH₂O with concentrations ranging from 0 – 1.0 mM. One hundred and ninety seven micro litres of master mix (60 µl Solution 1, 20 µl Solution 2, 20 µl Solution 3 and 97 µl dH₂O) was added to each well with 100 µl sample/standard. This was incubated at 25°C for 5 min in a Tecan Sunrise absorbance reader with OD_{500nm} recorded every 1 min. After this pre-incubation, 3 µl of Suspension 4 was added to each well and the plate was further incubated at 25°C as above with OD_{500nm} readings taken

every 1 min for 60 min. The maximum value obtained in the first reaction was subtracted from the maximum value obtained in the second reaction. A standard curve was constructed with the monosodium glutamate standards and the unknown concentrations in samples were obtained by converting the OD_{500nm} value using the equation for the slope of the standard curve.

2.7.3 γ -Aminobutyric acid

The concentration of γ -aminobutyric acid (GABA) was measured using the modified micro-titre plate assay described by O'Byrne *et. al* (2011). Ninety micro litres of master mix containing 80 mM Trizma base (Sigma Aldrich), 750 mM sodium sulphate (BDH), 10 mM dithiothreitol (DTT; MSC), 1.4 mM β -nicotinamide adenine dinucleotide phosphate sodium salt hydrate (NADP; Sigma Aldrich) and 0.3 mg ml⁻¹ Gabase from *Pseudomonas fluorescens* (Sigma Aldrich) was added to each well along with 10 μ l of sample/standard. Standards of γ -aminobutyric acid (Sigma Aldrich) were made in dH₂O to a concentration of 0 – 10 mM for measurement of intracellular GABA, whilst they were made in the growth media if extracellular GABA concentrations were to be measured. Plates were subsequently incubated at 37°C in a Tecan Sunrise absorbance reader and OD_{340nm} readings were taken every 60 s with shaking for 5 s prior to each reading for 3 h.

2.7.4 Succinic semialdehyde

The concentration of succinic semialdehyde (SSA) was measured using the modified micro-titre plate assay described by O'Byrne *et. al* (2011). Ninety micro litres of a master mix containing 80 mM Trizma base (Sigma Aldrich), 750 mM sodium sulphate (BDH), 10 mM dithiothreitol (MSC), 80 mM 2-aminoethyl hydrogen sulfate (Sigma Aldrich), 1.4 mM β -nicotinamide adenine dinucleotide phosphate sodium salt hydrate (Sigma Aldrich) and 0.3 mg ml⁻¹ Gabase from

Pseudomonas fluorescens (Sigma Aldrich) was added to each well along with 10 µl of sample/standard. Standards of Succinic semialdehyde solution (Sigma Aldrich) were made in dH₂O to a concentration of 0 – 10 mM for measurement of intracellular SSA, whilst they were made in the growth media if extracellular SSA concentrations were to be measured. Plates were subsequently incubated at 37°C in a Tecan Sunrise absorbance reader and OD_{340nm} readings were taken every 60 sec after shaking for 5 sec, for 3 h.

2.7.5 Succinate

Succinate was measured from samples using a modified method for the Roche BioPharm Succinic acid enzymatic kit (Cat. no. 10176281035). The method was adjusted to operate in a 300 µl 96-well micro-titre plate as opposed to a 3 ml cuvette. Standards of sodium succinate were made in sterile dH₂O with concentrations ranging from 0 - 1.0 mM. One hundred and ninety eight micro litres of master mix (100 µl Reaction 2, 5 µl Suspension 3 and 93 µl dH₂O) was added to each well with 100 µl sample/standard. This was incubated at 37°C for 5 min in a Tecan Sunrise absorbance reader with OD_{340nm} recorded every 1 min. After this pre-incubation, 2 µl of Suspension 4 was added to each well and the plate was further incubated at 37°C as above with OD_{340nm} readings taken every 1 min for 60 min. The maximum value obtained in the first reaction was subtracted from the maximum value obtained in the second reaction. A standard curve was constructed with the succinate standards and the unknown concentrations in samples were obtained by converting the OD_{340nm} value using the equation for the slope of the standard curve.

2.8 Murine gastric survival

Eleven week old Balb/c mice were used for infection studies. Animals were housed within the biological services unit, University College Cork and fed

2018S Teklad Global 18% Protein Rodent Diet (Harlan). Overnight cultures of bacteria were grown in 200 ml BHI broth at 37°C for 16-18 h. Fifty millilitres of culture was centrifuged at $10000 \times g$ for 10 min and washed once with PBS before being resuspended in 5 ml PBS. Mice were inoculated with bacteria at a concentration of 10^{10} cfu in 200 μ l via direct gastric gavage at 5 mice per strain and housed in groups of 5.

Three days post infection, mice were sacrificed and the liver, spleen, mesenteric lymph nodes (MLN) and intestinal faeces were harvested. Liver, spleen and MLN were homogenised in 5 ml PBS in stomacher bags (Seward). The weight of the faeces was determined and then homogenised in 1 ml PBS in 1.5 ml microcentrifuge tubes. All samples were serially diluted in tenfold steps from neat to 10^{-5} and 30 μ l spots of each dilution were plated in duplicate onto either BHI agar (Liver & Spleen) or LSA (MLN & faeces). Plates were incubated at 37°C for 48 h and colony forming units were counted.

2.9 Macrophage survival assays

Permanent stocks of a human derived THP-1 macrophage cell line were maintained at a concentration of 1×10^6 in 1 ml Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma Aldrich) supplemented with 10 % heat-inactivated foetal bovine serum (FBS; Lonza) and 10% DMSO and stored at -80°C. These were recovered by resuspension in 3 ml RPMI supplemented with 10 % FBS and 100 U ml^{-1} penicillin/streptomycin (PenStrep; Sigma Aldrich) and incubated at 37°C in 5 % CO_2 for 2 days. The culture was then centrifuged at $400 \times g$ for 5 min, the pellet was washed in 5 ml phosphate buffered saline and the centrifugation was repeated. The pellet was then resuspended in 5 ml RPMI with 10% FBS and PenStrep and incubated as above for 5 – 7 days.

For survival assays, the macrophages were centrifuged at $400 \times g$ for 5 min, washed twice in 5 ml PBS and resuspended in RPMI plus 10 % FBS supplemented with 0.16 μ M phorbol myristate acetate (PMA) to a concentration of 1×10^5 cells ml^{-1} . One millilitre was then seeded per well of a 24 well tissue

culture plate and the plate was incubated for a further 24 h at 37°C with 5 % CO₂ to allow the cells to adhere to the surface of the well. After incubation, the media was removed from each well and 1 ml of PBS was added to wash the cells. The plate was shaken gently and the PBS removed.

In parallel, *L. monocytogenes* cultures were grown to stationary phase in BHI. 1 ml of culture was then centrifuged at 10,000 × *g* for 5 min, washed once in 1 ml PBS and resuspended in 1 ml PBS to give an estimated concentration of 1 × 10⁹ cfu ml⁻¹. Bacterial cells were then diluted to 1 × 10⁶ in RPMI and 10% FBS. One millilitre of bacteria was added to each washed well of THP-1 cells to give a multiplicity of infection (MOI) of 10. Plates were incubated at 37°C with 5 % CO₂ for 1 h to allow for invasion.

After the 1 h co-incubation period, the media was removed from the wells and the cells were washed with 1 ml PBS. 1 ml of RPMI and 10 % FBS supplemented with 30 µg ml⁻¹ Gentamicin was added to kill remaining extracellular bacteria. Plates were returned to incubate for 1 h at 37°C with 5 % CO₂.

Bacterial counts were performed by removing the media from each well, washing once in 1 ml PBS and resuspending the cells with 1 ml ice cold sterile dH₂O for 1 min. Serial dilutions were performed and the suspension was spread plated in duplicate onto BHI agar plates. All agar plates were incubated for 24 h at 37°C.

2.10 Enzymatic activity assays

2.10.1 Protein extraction

To prepare crude protein extracts, a culture of bacteria was grown overnight in 200 ml of BHI (*L. monocytogenes*) or LB (*E. coli*) medium at 37°C with continuous shaking. The pellet was retained after centrifugation at 12,000 × *g* for 10 min, followed by two wash steps in wash buffer (10 mM NaCl, 10 mM Na₃O₄P [pH 7.0]). Pellets were then resuspended in 4 ml of a sonication buffer optimized for their downstream application. For GABA-AT, the sonication buffer

comprised 20 mM $\text{Na}_3\text{O}_4\text{P}$, 0.01 mM pyridoxal phosphate, 5 mM mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM dithiothreitol (DTT) (pH 7.0). For SSDH, it comprised 100 mM sodium phosphate, 1 mM DTT, 1 mM PMSF, and 9% glycerol (pH 9.5). Samples were then sonicated on ice by six 30 s pulses at an amplitude of 16 μm , allowing 30 s of rest between each pulse to lyse the cells, followed by centrifugation at $6,000 \times g$ for 10 min and retention of the supernatant. The supernatant was then centrifuged at $15,000 \times g$ for 15 min to remove any remaining cell debris and the supernatant was retained.

2.10.2 Protein concentration determination

Protein concentrations for both assays were normalised to 7 mg ml^{-1} after determining each sample concentration using the Bio-Rad DC protein assay (Bio-Rad) according to the manufacturer's instructions. All protein was first diluted 1:10 in its respective sonication buffer. Standards of bovine serum albumin (BSA; Sigma Aldrich) were used at a concentration of 0 to 1.5 mg ml^{-1} diluted in dH_2O .

2.10.3 GABA-AT assay

One hundred microlitres of protein was added to 1,000 μl reaction mix (85 mM Tris base, 15 mM Tris-HCl, 0 to 100mM GABA, 5 mM α -ketoglutarate, 0.02% BSA) and incubated at 37°C for 1 h. In a 96-well plate, the SSA produced by the reaction was measured as described in section 2.7.4. Any background signal detected from the 0 mM GABA reaction mix was subtracted to highlight the increase in the level of SSA production.

2.10.4 SSDH assay

NADP to a final concentration of 1 mM was added to 2 ml of a normalized protein preparation (7 mg ml⁻¹), and 150 µl of this mix was added to 100 µl of SSA (0 to 1.0 mM) in a 96-well micro-titre plate. Plates were incubated at 37°C in a Tecan Sunrise absorbance reader, with OD_{340nm} readings being recorded every 60 s by Magellan software.

2.11 Molecular techniques - DNA

2.11.1 Polymerase chain reaction (PCR)

PCRs were carried out in a 50 µl volume using a thermocycler (Mastercycler Gradient, Eppendorf) as per the conditions outlined in table 2.3. Template DNA was prepared by resuspending 1 bacterial colony in 500 µl sterile dH₂O and vortexing. A working stock of 10 mM dNTP mix was prepared from 100 mM stock solutions of dTTP, dATP, dGTP and dCTP in sterile dH₂O and stored at -20°C. Primers were ordered from MWG Eurofins and reconstituted with sterile dH₂O to a stock concentration of 100 µM as per manufacturer's instructions. A working stock of primers was diluted to 25 µM in sterile dH₂O and stored at -20°C. For routine screening PCR Taq DNA polymerase from *Thermus aquaticus* (Sigma Aldrich) was used. Each mixture contained 5 µl 10x PCR reaction buffer, 1 µl dNTP mix, 0.5 µl polymerase, 1 µl primer A, 1 µl primer B, 1 µl template DNA and 40.5 µl sterile dH₂O. For PCR requiring high fidelity amplification, Velocity DNA polymerase (Bioline) was used. Here each mixture contained 10 µl 5x HI Fi buffer, 1.5 µl DMSO, 0.5 µl dNTP mix, 1 µl primer A, 1 µl primer B, 1 µl template DNA and 35 µl sterile dH₂O. After amplification DNA was stored at -20°C or used in a downstream application.

Table 2.3 PCR thermocycler conditions

Step	Temperature (°C)	Time (min)
Cell lysis	94	2
Denaturing	94	1
Annealing	54.5	0.5
Elongation	72	<1 - 2 ^a
Final elongation	72	5

^aVaried depending on the fragment size being amplified

Table 2.4 Table of primers

Number	Sequence (5' to 3')	Name (use)	T _m (°C)
COB 611	CGGAATTCCAAGCAAATT ACCAGGTG	<i>lmo0447</i> A <i>gadD1</i>	71.88
COB 612	GAGTAAAGCCAGAGCCAA ACACCGGTACA	<i>lmo0447</i> B <i>gadD1</i>	74.61
COB 613	CGGTGTTTGGCTCTGGCTT TACTCATTAAG	<i>lmo0447</i> C <i>gadD1</i>	72.99
COB 614	CGGAATTCATAACGTAAG ATAGTGCGCC	<i>lmo0447</i> D <i>gadD1</i>	71.46
COB 649	ACAAATACGCCACGCATC	<i>lmo0447</i> Outside A <i>gadD1</i>	62.16
COB 650	GGCAAGAACCATAAGAAT CCAC	<i>lmo0447</i> Outside D <i>gadD1</i>	63.33

COB 615	CGGAATTCGTAGTCATTTA <i>lmo2363 A gadD2</i>	69.69
	TTTAGTCGGC	
COB 616	AAGCCGTGACTATATAAC <i>lmo2363 B gadD2</i>	68.38
	ATGATTTTTTCCTC	
COB 617	TATATAGTCACGGCTTCAC <i>lmo2363 B gadD2</i>	69.84
	ACATTAATAAAAAGGC	
COB 618	CGGAATTCTCGCATATTA <i>lmo2363 D gadD2</i>	68.61
	ATTATTTGACG	
COB 690	CGGAATTCTCTCATATTA <i>lmrg_01479 D 10403S</i>	64.94
	TTATTTGACG <i>gadD2</i>	
COB 651	TCATTCCTAACTGCCATTT <i>lmo2363 Outside A gadD2</i>	62.65
	CC	
COB 695	TCTTTCCTAACCGCAATTT <i>lmrg_01479 Outside A</i>	62.65
	CC <i>10403S gadD2</i>	
COB 652	TGGAATGAGAATAGTGGA <i>lmo2363 Outside D gadD2</i>	63.70
	CGG	
COB 642	CGGAATTCCTTTATAGTGA <i>lmo2434 A gadD3</i>	65.45
	AGACGAC	
COB 643	TTGTCATGATACATAAA <i>lmo2434 B gadD3</i>	68.3
	GCTTCCGAAG	
COB 644	GCTTGTATGTATCATGACA <i>lmo2434 C gadD3</i>	70.08
	AAGAACGCAAC	
COB 622	TAGAATTCATTTTCAGTACG <i>lmo2434 D gadD3</i>	72.86
	CGAGCCATCAC	
COB 674	GAACCTCCTTATAAGTAC <i>lmo2434 Outside A gadD3</i>	51.00
	CATC	

COB 675	GGTGGTTACGGTGCATTC	<i>lmo2434</i> Outside D	<i>gadD3</i>	50.00
COB 354	CGGAATTCGGAGCAGTTT TTGTTAGCC	<i>lmo0913</i> A		73.41
COB 355	TGCTGTTTCTTTAATACTC AAAAATACCACTCC	<i>lmo0913</i> B		68.39
COB 356	AGTATTAAGAAACAGCA ATCCAAGTGAAATTC	<i>lmo0913</i> C		67.78
COB 357	CGGAATTCAAGTGTGCCA TAAGTTGC	<i>lmo0913</i> D		71.41
COB 372	GGTACTATTCATGGGCA G	<i>lmo0913</i> Outside A		56.95
COB 373	CATCCTGTTATCCCTCC	<i>lmo0913</i> Outside D		54.87
COB 717	AAAGAAAGGAAGTGTGGC G	<i>gsaB</i> Outside A		61.78
COB 718	TGCAATGATGGCTCCAAT TAC	<i>gsaB</i> Outside D		64.56
COB395	GTAAAACGACGGCCAG	M13 Forward		56.00
COB396	CAGGAACAGCTATGAC	M13 Reverse		51.00
COB578	CGCTTCGGATATGAGGGT TA	<i>gadD1</i> Forward (RT-PCR)		63.60
COB579	AGTGGATACGCCGGTACT TG	<i>gadD1</i> Reverse (RT-PCR)		63.70
COB584	AATACCTTGCCCATGCAG TC	<i>gadD2</i> Forward (RT-PCR)		63.70
COB585	GGCTTGGAATCTTGGAT	<i>gadD2</i> Reverse (RT-PCR)		63.80

	GA	
COB580	GAAACGCTCGAGAAAAAT <i>gadD3</i> Forward (RT-PCR)	63.60
	GC	
COB581	AGTTTGGTCGTTTTGCCTG <i>gadD3</i> Reverse (RT-PCR)	63.30
	T	
COB629	CATATTCGAAGTGCCATT <i>lmo2230</i> Forward (RT-PCR)	55.3
	GC	
COB630	CTGAACTAGGTGAATAAG <i>lmo2230</i> Forward (RT-PCR)	57.6
	ACAAAC	
COB493	TGGGGAGCAAAACAGGAT 16S Forward (RT-PCR)	63.80
	TAG	
COB494	TAAGGTTCTTCGCGTTGCT 16S Reverse (RT-PCR)	63.60
	T	

2.11.2 Agarose gel electrophoresis-DNA

To visualise DNA samples, a 1 % (w/v) agarose gel was made by adding agarose powder to a 1X TAE buffer (40 mM Tris base, 0.114 % (v/v) glacial acetic acid and 1 mM EDTA; pH 8.0). Gels were stained with SYBR Safe and poured into a casting tray. Cast gels were placed in a gel electrophoresis tank and covered with 1X TAE buffer. DNA samples were mixed with 5 µl of crystal 5X loading buffer (Bioline) and added to a formed gel well. Five microlitres of Hyperladder I was added each side of the DNA samples to give a standard size marker for samples. The DNA sample was separated across the gel by running a current at 100 V for 45 min using a Powerpack Consort E132. DNA was then visualised by excitation of the SYBR safe dye on a UV transilluminator and images were captured using a G:BOX gel imager (Syngene) and GeneSnap software (Syngene).

2.11.3 PCR product purification

PCR products were purified in one of two ways using either a Qiagen PCR purification kit for direct PCR product clean up or a Qiagen Gel extraction kit for isolating products first run on a 1 % agarose gel, to allow for targeted isolation. Elution of DNA was performed with 40 μ l pre-warmed sterile dH₂O. The concentration of purified products was determined either using a NanoDrop (Thermo Scientific) or comparison with 5 μ l of Hyperladder I (Bioline) in an agarose gel. Samples were stored at -20°C.

2.11.4 Plasmid isolation and purification

A strain containing a plasmid was inoculated into media supplemented with the correct selective antibiotic and incubated for 16 h at 37°C. Following incubation 5 ml of the culture was centrifuged at 6,800 $\times g$ for 3 min and the supernatant was discarded. Pellets were frozen for a minimum of 3 h at -80°C and then plasmid DNA was isolated following manufacturer's instructions for the GenElute Plasmid Miniprep Kit (Sigma). Purified plasmid DNA was eluted with 40 μ l pre-warmed sterile dH₂O and stored at -20°C. The concentration of the plasmid DNA was determined by NanoDrop (Thermo Scientific).

2.11.5 Restriction enzyme treatment

All enzymes used were FastDigest enzymes (Thermo Scientific). Target DNA was quantified using agarose gel electrophoresis. For plasmid digestion, 2 μ l (up to 1 μ g) of plasmid was added to 2 μ l 10X FastDigest buffer, 15 μ l nuclease free water and 1 μ l FastDigest enzyme and incubated at 37°C for 3 h. Reactions were subsequently incubated at 65°C for 15 min to inactivate the enzyme. Digestion

was confirmed by agarose gel electrophoresis and the required DNA band was excised and purified. For PCR product digestion, 10 µl (100 – 200 ng) of DNA was added to 2 µl 10X FastDigest buffer, 17 µl nuclease free water and 1 µl FastDigest enzyme and incubated at 37°C for 3 h, followed by 65°C for 15 min to inactivate the enzyme. Purification was carried out using a QIAquick PCR purification kit (Qiagen) as per manufacturer's guidelines. All purified digested samples were stored at -20°C until required.

2.11.6 Phosphatase treatment

In order for ligation of DNA inserts to digested plasmids, phosphate ends were removed using Shrimp Alkaline Phosphatase (SAP; Roche) to prevent the self-religation of the plasmid. One microlitre of SAP was added to the digested plasmid and incubated at 37°C for 30 min, followed by inactivation at 65°C for 15 min.

2.11.7 Ligations

DNA was ligated to plasmid DNA at a molar ratio of 3:1 (insert:plasmid). This ratio was calculated as follows:

$$\text{ng insert} = \frac{\text{ng vector} \times \text{kb insert}}{\text{kb vector}} \times \text{ratio (insert/vector)}$$

Ligations were performed in 20 µl volumes with 10 µl insert, 2 µl 10X T4 Ligase Buffer (Fermentas), 1 µl T4 Ligase (Fermentas), the required volume of vector and made up to 20 µl with nuclease free water. The reaction was incubated at 16°C overnight for 16 h and was either used directly in a transformation reaction or stored at -20°C.

2.12 Molecular techniques - RNA

2.12.1 RNA extraction

An RNeasy RNA isolation kit (Qiagen) was used to isolate total RNA from bacterial samples according to the protocol below. Cultures of bacteria were grown to stationary phase and 1 ml of culture was removed before and after treatment as required. Two millilitres of RNAlater^(R) (Sigma Aldrich) were added and the mixture vortexed and incubated at room-temperature for 5 min. The sample was then centrifuged at 8000 x g for 5 min and the supernatant discarded. Pellets were subsequently stored for up to 6 months at -80°C.

To isolate the total RNA from each sample, the thawed pellet was resuspended in 1 ml 1M Tris solution adjusted to pH 7.4 in DEPC treated water. The suspension was transferred to a 1 ml microcentrifuge tube, centrifuged at 8000 x g for 5 min and the supernatant discarded. The cells were then lysed by microwaving at 540 W for 30 s. The pellet was immediately resuspended in 350 µl RLT buffer, supplemented with β-mercaptoethanol to a final concentration of 10% (v/v), supplied with an RNeasy RNA isolation kit (Qiagen). Following a centrifugation step at 12,000 × g for 2 min, the supernatant was transferred to a separate 1.5 ml microcentrifuge tube containing 350 µl 70% ethanol and mixed by pipetting. The solution was transferred to a filter column and centrifuged at 8000 × g for 15 s. The flow-through was discarded and the column was washed twice with 500 µl of Buffer RPE, once for 15 s and then for 2 min at 8000 × g, discarding the flow through each time. The dry column was finally centrifuged at 8000 × g for 1 min to remove any remaining wash buffer. Elution of the membrane bound RNA was performed by adding 40 µl of RNA secure (Ambion) pre-warmed to 65°C for 10 min and centrifuging at 8000 × g for 1 min into a clean tube. Samples of RNA were stored at -80°C.

2.12.2 Agarose gel electrophoresis-RNA

To confirm the integrity of RNA samples 1 μ l of RNA was added to 5 μ l crystal 5X loading buffer and run on a 1% TBE agarose gel. TBE gels were prepared by adding 1% (w/v) agarose powder to a 0.5X stock of TBE made from 21.6 g l⁻¹ Tris base, 11 g l⁻¹ Boric acid and 1.16 g l⁻¹ EDTA made up in dH₂O. Gels were stained with SYBR safe (Invitrogen) to allow for detection of the sample in the gel. Samples were run at 100 V for 30 min and gels were imaged by excitation of the SYBR safe dye on a UV transilluminator and images were captured using a G:BOX gel imager (Syngene) and GeneSnap software (Syngene). Two distinct bands indicating the 16S and 30S ribosomal RNA and a lack of smearing were indicative of good quality RNA.

2.12.3 DNase treatment

To remove contaminating DNA from the RNA, samples were treated with DNase. Briefly, 15 μ l of RNA was added to 1.9 μ l of 10X Turbo DNase buffer (Ambion) and 0.4 μ l Turbo DNase (Ambion). Following incubation at 37°C for 20 min, a further 0.4 μ l of Turbo DNase was added to each sample and another incubation at 37°C for 30 min was performed. Finally 2 μ l DNase Inactivation Reagent (Ambion) was added and the sample was incubated at room-temperature for 5 min with intermediate mixing. The sample was then centrifuged at 10,000 \times g for 2 min and the supernatant carefully removed and stored in a clean microcentrifuge tube. To confirm removal of DNA a 40-cycle PCR was performed on the DNase treated RNA using *Listerial* 16S primers. The absence of contaminating DNA was also assessed by running 1 μ l of the DNase treated RNA on a TBE gel and checking for a DNA band.

2.12.4 RNA integrity and quantification

The quality and quantity of the RNA post DNase treatment was checked using an Agilent 2100 Bioanalyzer chip. This allowed for the separation of up to 12 samples of RNA in the wells of a chip and quality was determined by 2100 Bioanalyzer software and returned as an RNA integrity number (RIN). This assay followed the RNA 6000 Nano Assay protocol.

2.12.5 Generation of cDNA

RNA was normalised between related samples based on the concentration determined by either the Bioanalyzer or NanoDrop. Fifteen microlitres of RNA was added to 1 μ l 10 mM dNTPs and 1 μ l Random Primers (Invitrogen) and incubated at 65°C for 5 min. The samples were chilled on ice and briefly centrifuged. Four microlitres of 5X First Strand buffer (Invitrogen) was then added along with 2 μ l DTT (Invitrogen) and an incubation at 25°C for 2 min followed. One microlitre of Superscript II (Invitrogen) was then added and the tube was incubated for 10 min at 25°C, 50 min at 42°C and 15 min at 70°C using a thermocycler (Mastercycler Gradient, Eppendorf). Samples were stored at -20°C.

2.12.6 Real-time PCR (RT-PCR)

RT-PCR reactions were carried out in 10 μ l volume in white Lightcycler 480 Multiwell Plates (Roche) using a Lightcycler 480 (Roche) to detect excitation of SYBR green upon binding to double stranded DNA. All cDNA samples were diluted 1:10 in nuclease free H₂O. For each pair of primers, a separate master mix was made as per table 2.5. Samples were incubated for 45 cycles of 15 s at 94°C, 15 s at 56°C and 30 s at 72°C.

Table 2.5 Master mix for RT-PCR

Component	Volume/reaction (μ l)
2X QuantiTect SYBR Green (Qiagen)	5
Primer A (0.5 μ M)	0.5
Primer B (0.5 μ M)	0.5
H ₂ O	1.5

Following completion of the RT-PCR cycle the crossing-point (Cp) value for each sample was obtained using the "Advanced Relative quantification" method (Lightcycler 480; Roche). The Cp value is the point at which fluorescence rises above any background fluorescence and is determined by the amplified gene copy number. Cp values were converted to relative quantification values using the $2^{-\Delta\Delta CT}$ method described previously (Livak 2001). Each sample was normalised against values obtained for the 16S rRNA gene.

2.13 Generation of electrocompetent bacteria

2.13.1 *E. coli* K12 MG1655 and Top10

An overnight culture of *E. coli* was incubated in 20 ml LB broth at 37°C for 16-18 h. This was subsequently diluted 1:100 into 500 ml LB broth and incubated at 37°C for 3-5 h until OD_{600nm} 0.35 - 0.40 was reached. The culture was chilled on ice for 10 min, split into two 250 ml chilled centrifuge pots and centrifuged at 2,500 \times g for 15 min at 4°C. The supernatant was discarded and each pellet was washed 4 times in 25 ml ice-cold 10% (v/v) glycerol (Sigma Aldrich) with centrifuging at 2,500 \times g for 15 min at 4°C. Each pellet was resuspended in 1 ml

ice-cold 10% glycerol and combined into a 50 ml chilled centrifuge tube. The tube was centrifuged at $2500 \times g$ for 15 min at 4°C , supernatant discarded and the pellet resuspended in 500 μl ice-cold 10% glycerol. Finally 50 μl aliquots of the cells were transferred to frozen 1.5 ml microcentrifuge tubes and stored immediately at -80°C .

2.13.2 *L. monocytogenes* EGD-e

The method described by Monk *et. al.*, (2008) was followed for producing electrocompetent transformable EGD-e. An overnight culture was grown in 20 ml BHI broth at 37°C for 16-18 h. This was diluted 1:100 into 500 ml of BHI broth supplemented with 500 mM sucrose (Sigma Aldrich) to result in an $\text{OD}_{600\text{nm}}$ of 0.01 - 0.02. The culture was incubated shaking at 37°C until an $\text{OD}_{600\text{nm}}$ of 0.2 - 0.25 was reached. Ten micrograms per millilitre of ampicillin was then added to the culture and a further incubation at 37°C was carried out for 2 h to allow the cells to double in number. The culture was subsequently chilled on ice for 10 min, split into two 250 ml chilled centrifuge pots and centrifuged at $5,000 \times g$ for 10 min at 4°C . The supernatant was discarded and each pellet washed in 250 ml ice-cold filter sterilised sucrose-glycerol wash buffer (SGWB) composed of 10% (v/v) glycerol and 500 mM sucrose adjusted to pH 7 with 100 mM NaOH. After centrifuging at $5,000 \times g$ for 10 min at 4°C , the supernatant was discarded and each pellet was washed further with 87.5 ml ice-cold SGWB as above. The pellets were finally resuspended in 25 ml ice-cold SGWB, combined into a 250 ml Erlenmeyer flask and $10 \mu\text{g ml}^{-1}$ lysozyme from chicken egg white (Sigma Aldrich) was added. The suspension was incubated at 37°C for 20 min. The cells were immediately centrifuged at $3,000 \times g$ for 10 min at 4°C , washed once in 20 ml ice-cold SGWB and centrifuged at $3,000 \times g$ for 10 min at 4°C . The pellet was finally resuspended in 2.5 ml ice-cold SGWB and aliquoted at 50 μl in 1.5 ml pre-frozen microcentrifuge tubes. The cells were stored at -80°C .

2.13.3 *L. monocytogenes* 10403S

This strain of *L. monocytogenes* is not as competent as EGD-e and so the method varies to get optimal transformation efficiency. An overnight culture was grown in 20 ml BHI broth at 37°C for 16-18 h. This was diluted 1:100 into 500 ml BHI broth supplemented with sucrose to a concentration of 500mM and incubated at 37°C until an OD_{600nm} of 0.2 was obtained. Five micrograms per millilitre of Penicillin G was added and the cells were grown for an additional 2 h. The cells were split into two 250 ml centrifuge pots, centrifuged at 5,000 × g for 10 min at 4°C and the supernatant was discarded. The pellets were washed twice in 450 ml and 225 ml ice-cold electroporation buffer (1 mM HEPES, 500 mM sucrose) and centrifuged at 5,000 × g for 10 min at 4°C. The cells were resuspended in 4 ml ice-cold electroporation buffer, combined into one tube and centrifuged once more at 5,000 × g for 10 min at 4°C. Finally the cells were resuspended in 4 ml ice-cold electroporation buffer and aliquoted at 50 µl into pre-frozen microcentrifuge tubes. The cells were stored at -80°C.

2.14 Transformation of electrocompetent bacteria

Purified plasmid DNA was prepared as described in section 2.11.4. One microlitre of DNA (~100 ng) was added to 50 µl of electrocompetent cells and transferred to a 0.2 cm electroporation cuvette (Ingenio). The cells were then exposed to a charge of 2.1 - 2.5 kV at 200 Ω and 25 µF in a GenePulser (BioRad) at time constants between 4.5 and 4.8 ms. The cells were immediately resuspended in 450 µl of either SOC media (*E. coli*) or BHI broth supplemented with sucrose to a final concentration of 500 mM (*L. monocytogenes*) and allowed to recover at 37°C for 1 h. Fifty microlitre aliquots were subsequently spread plated onto agar plates with the required selective agent. Where blue/white screening was required, 40 µl of X-gal (40 mg ml⁻¹) was spread onto each plate 30 min prior to the bacteria. All plates were incubated at 37°C for 24 - 48 h. Positive transformants were confirmed by PCR with primers to target the plasmid.

2.15 Generation of knockout mutants

To delete regions of targeted genes, a method using Splicing by Overlap Extension (SOEing) PCR, followed by allelic replacement by homologous recombination as described by Horton *et. al.*, (1993) was used. This involves the generation by PCR of a truncated copy of a gene which is swapped into the bacterial chromosome in place of the wild-type gene. A set of 4 primers were designed as shown in Figure 2.1(i), with primers A and B amplifying a region upstream of the target gene and primers C and D amplifying a downstream region. Primers B and C were designed such that they had extended nucleotide regions that were homologous to the other primer. Primers A and D were designed with 5' *EcoRI* restriction enzyme sites.

An initial PCR was performed to generate the flanking fragments [AB] and [CD] using the conditions described in Section 2.11.1 and a high fidelity polymerase (Velocity DNA polymerase; Bionline). The fragments were imaged and subsequently purified from an agarose gel. To splice the two regions together (Fig. 2.1 ii) a second PCR was carried out as follows: 1 μ l [AB], 1 μ l [CD] 0.5 μ l dNTPs, 10 μ l 5X Hi-Fi Reaction Buffer (Bionline), 1 μ l Velocity DNA polymerase (Bionline) and 34.5 μ l H₂O was incubated for 5 cycles of 30 s at 96°C, 30 s at 54.5°C and 30 s at 72°C in a thermocycler. After the fifth cycle 1 μ l each of primers A and D was added and a full PCR cycle program was followed as described in Section 2.11.1. The product of the PCR was imaged by gel electrophoresis and gel purified.

To increase the efficiency of cloning into the shuttle vector, each fragment was first sub-cloned using a TOPO XL PCR Cloning Kit (Invitrogen). Four microlitres of purified DNA fragment [AD] was added to 1 μ l pCR-XL-TOPO vector and incubated at room-temperature for 5 min. After incubation 1 μ l of 6X TOPO Cloning Stop Solution was added, the reaction was gently mixed by pipetting, centrifuged briefly and placed on ice. Transformation of the cloning reaction into

electrocompetent Top10 cells (Fig. 2.1 **iii**) was carried out as described in Section 2.10. Positive transformants were identified by the formation of white colonies on LB agar supplemented with kanamycin ($30 \mu\text{g ml}^{-1}$) and confirmed by PCR using M13 primers, specific for a region on the cloning vector outside of the multi-cloning site (MCS).

An overnight culture of a positive transformant was subsequently grown at 37°C in LB broth supplemented with kanamycin. Five millilitres of this was used to isolate a preparation of concentrated, purified plasmid containing the cloned fragment as per section 2.11.4. The fragment was removed from the plasmid by restriction digestion with *EcoRI* as described in Section 2.11.5 and further purified from the plasmid by running the digestion reaction on an agarose gel and excising the required DNA band.

All knockout mutations were generated using pKSV7 as a temperature sensitive shuttle vector. This vector allows integration of the truncated fragment either upstream or downstream of the target gene by homologous recombination when grown at temperatures above 42°C . To clone the *EcoRI* digested fragment into pKSV7 (Fig. 2.1 **iv**), the plasmid was first purified from a host strain, *E. coli* DH5 α pKSV7, and digested with *EcoRI*. To prevent re-ligation of the plasmid to itself, the plasmid was treated with shrimp alkaline phosphatase (SAP) as described in Section 2.11.6. The purified digested DNA fragment [AD] was subsequently ligated with the SAP treated, digested pKSV7 using T4 ligase (Section 2.11.7) and transformed into *E. coli* Top10 electrocompetent cells (Fig 2.1(v)). Following confirmation of successful transformation with the newly produced pKSV7::[AD], the plasmid was purified from the intermediary *E. coli* host and transformed into *L. monocytogenes* by electroporation (Fig. 2.1(vi); section 2.14).

Successful *Listeria* transformants were confirmed by PCR and streaked onto BHI agar supplemented with chloramphenicol ($10 \mu\text{g ml}^{-1}$) and grown at the non-permissive temperature of 45°C for 24-48 h. Growth was re-streaked 2-3 times until isolated colonies grew, indicative of integration of the plasmid into the *Listerial* chromosome. Integration was confirmed by PCR using a combination of primers specific to the plasmid or chromosome (Fig. 2.2 i). The plasmid can integrate either upstream or downstream of the target gene as shown in Fig. 2.2 i.

Following integration, the plasmid is removed by a second homologous recombination step. This can result in the removal of the wild-type gene and the plasmid or removal of the plasmid and the truncated gene fragment, resulting in reversion to the wild-type genotype (Fig. 2.2 ii). Cultures of the integrant strains were grown in 20 ml BHI broth without antibiotic selection at 30°C and passaged to fresh media every 12 h. At each passage, a sample of culture was serially diluted and spread onto BHI agar and incubated at 37°C for 24 h. Colonies were then replica spotted onto BHI agar with and without chloramphenicol ($10 \mu\text{g ml}^{-1}$) and incubated for a further 36 h at 37°C . Colonies that failed to grow on BHI with chloramphenicol were indicative of lacking the pKSV7 plasmid. Confirmation of a successful mutant was performed by PCR with primers targeting nucleotide sequences outside of the deletion region (Fig. 2.2 ii). Stocks of each mutant strain were prepared as described in Section 2.1 and stored at -80°C .

2.16 Bioinformatics

All nucleotide sequences for *L. monocytogenes* EGD-e were obtained from the Listlist database (genolist.pasteur.fr). Homologous gene sequences for 10403S were obtained using the Basic Local Alignment Search Tool (BLAST; NCBI), aligning the EGD-e sequence against a database of all sequenced *L. monocytogenes* strains.

2.17 Statistical analysis

All data was analysed using either SBSS software (IBM) or GraphPad Prism 5.01 (GraphPad). Data were checked for normality and variance and an appropriate test was chosen to determine significance of results. Data were considered significant where a *P* value of less than 0.05 was obtained.

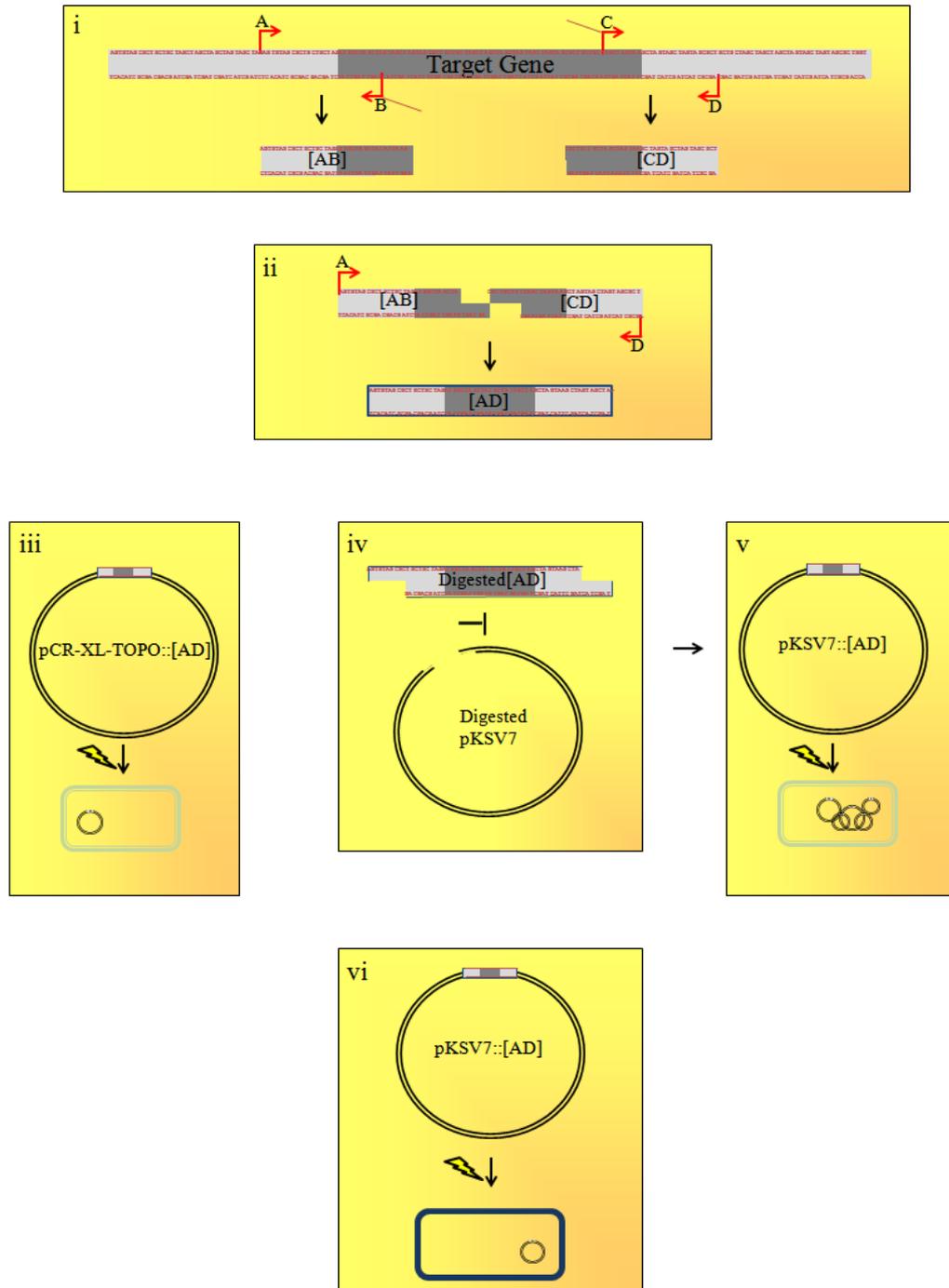


Figure 2.1. Generation of truncated gene fragment by SOEing PCR and cloning into the shuttle vector. (i) Two initial PCR reactions generate two DNA fragments, [AB] & [CD], each side of the region intended to be deleted using primers A & B and primers C & D, respectively. (ii) A second PCR reaction allows for the annealing of the overlapping ends of [AB] fragment with [CD] and the eventual generation of the product [AD]. (iii) The [AD] fragment is sub-cloned into pCR-XL-TOPO and transformed into *E. coli* Top10 cells by electroporation. (iv) After plasmid purification of the pCR-XL-

TOPO::[AD] clone from transformed *E. coli* Top10 cells, the [AD] fragment is digested with restriction enzymes to remove it from the pCR-XL-TOPO vector and eventually ligated together with SAP treated, digested pKSV7 shuttle vector. (v) The ligated DNA is transformed into *E. coli* Top10 cells by electroporation. (vi) Following purification of pKSV7::[AD] from *E. coli* Top10, the plasmid is transformed into *L. monocytogenes* by electroporation.

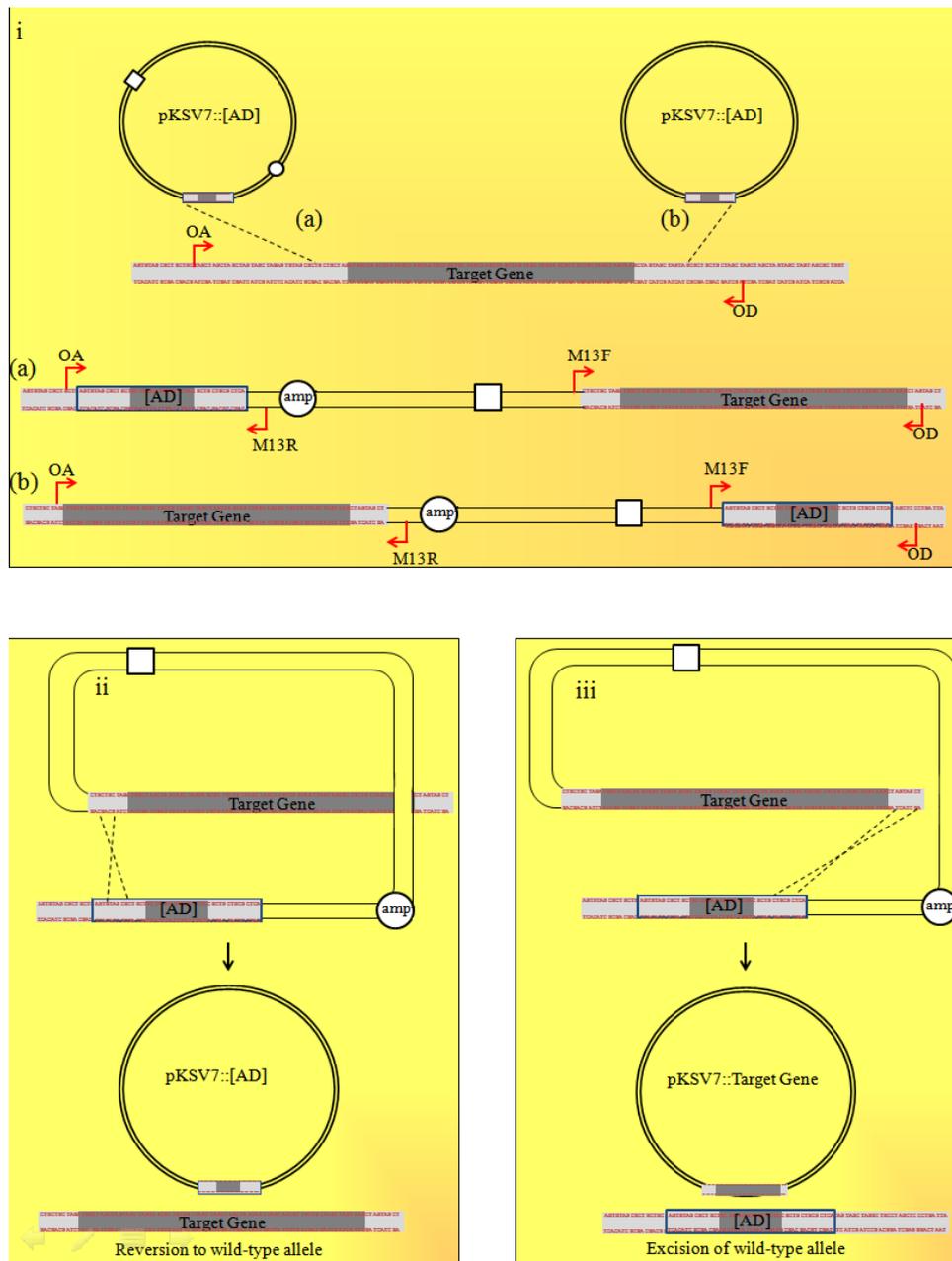


Figure 2.2. Integration and excision of pKSV7 by second recombination. (i) Through growth above 42°C the plasmid may recombine either upstream (a) or downstream (b) of the target gene on the chromosome of *L. monocytogenes*. Primers OA and OD are specific to the chromosome and primers M13F and M13R are specific to pKSV7. These allow for a PCR to differentiate between an upstream or downstream integration. (ii) A second homologous recombination event, during growth at 30°C can result in excision of the plasmid and a reversion to the wild-type genotype. (iii) A different second homologous recombination event through growth at 30°C can result in the wild type gene crossing onto the plasmid resulting in the truncated [AD] fragment remaining in the chromosome.

CHAPTER 3

Deletion of glutamate decarboxylase and GABA shunt genes in *Listeria* *monocytogenes*

3.1 Introduction

Previous work has shown that the *L. monocytogenes* strain EGD-e does not produce extracellular GABA (GABA_e) in response to acid treatment suggesting a defunct transporter system (Karatzas *et al.*, 2012). Therefore, the three glutamate decarboxylase genes, *lmo0447* (*gadD1*), *lmo2363* (*gadD2*), and *lmo2434* (*gadD3*) were selected for deletion in this strain. This would allow a focused study on the importance of the intracellular GAD system (GAD_i). Furthermore, a gene thought to be involved in the breakdown of intracellular GABA (*lmo0913*; Karatzas *et al.*, 2010) was also chosen for deletion, analysis of which is covered in chapter 4. As described below, initial single mutants were generated in EGD-e and phenotypic analysis was carried out to ascertain the role that each gene plays in survival of EGD-e at low pH. In order to compare the function of the GAD system between strains, the same mutations were introduced into a second strain, 10403S. This particularly robust strain is capable of producing both GABA_e and GABA_i (Karatzas *et al.*, 2012). Finally, introduction of the above mutants into a murinised EGD-e strain (EGDm) allowed for a strain that could be subsequently used in a mouse model of infection.

3.2 Generation of Deletion mutants in EGD-e

Mutants of *L. monocytogenes* were constructed via a technique known as splicing by overlap extension (SOEing) PCR (Horton *et al.*, 1993) followed by two homologous recombination events. SOEing PCR removes an internal region of the target gene through the initial generation of two fragments of DNA (Fig 2.1; Chapter 2). The two fragments are joined together in a second PCR reaction and cloned into a suicide plasmid vector. The plasmid is subsequently transformed into competent cells of *L. monocytogenes*. Above 42°C, the plasmid cannot replicate independently and must recombine into regions of the *L. monocytogenes* chromosome that are homologous to the deletion cassette it is carrying. A second recombination event, is selected at 30°C, which results in the plasmid excising

from the chromosome carrying a copy of either the wild-type target gene or the deletion cassette.

3.2.1 Creation of deletion fragments

All nucleotide sequences for the design of mutants in EGD-e were obtained from the published genome for this strain (Glaser *et al.*, 2001; Listilist). Primers A and D included a 5' *Eco*RI restriction site (Section 2.15). Primer B contained a 5' tail between 6-10 bp that was complementary to a corresponding tail on the 5' end of primer C. Primers were designed to produce AB and CD fragments between 300 and 500 bp as shown in Table 3.1. The first PCR successfully generated both AB and CD fragments required for each gene deletion (Fig. 3.1). These fragments were subsequently purified from an agarose gel (Section 2.11.3). A second PCR reaction was performed to join the AB and CD fragments for each gene. This PCR reaction allows for the joining of the complementary tails included from primers B and C by exposing a 1:1 mix of purified AB and CD fragments to 5 cycles of PCR in the absence of primers (Section 2.11). The PCR successfully generated AD fragments for $\Delta gadD1$, $\Delta gadD2$, $\Delta gadD3$ and $\Delta lmo0913$ (Fig. 3.2). The agarose gel image displayed several non specific bands after the PCR reaction. These are probably due to AB or CD fragments annealing incorrectly to each other. All of the bands of correct size were purified after separation on an agarose gel (Section 2.11.3).

Table 3.1. Sizes of designed deletion regions

Gene deleted	AB fragment (bp)	CD fragment (bp)	Deletion cassette (bp)	Deleted region (bp)	Codons remaining
<i>gadD1</i>	459	469	904	1326	21
<i>gadD2</i>	449	421	854	1365	10
<i>gadD3</i>	398	374	752	969	144
<i>lmo0913</i>	343	442	764	1407	20
<i>gsaB</i>	Commercially synthesised		616	1287	4

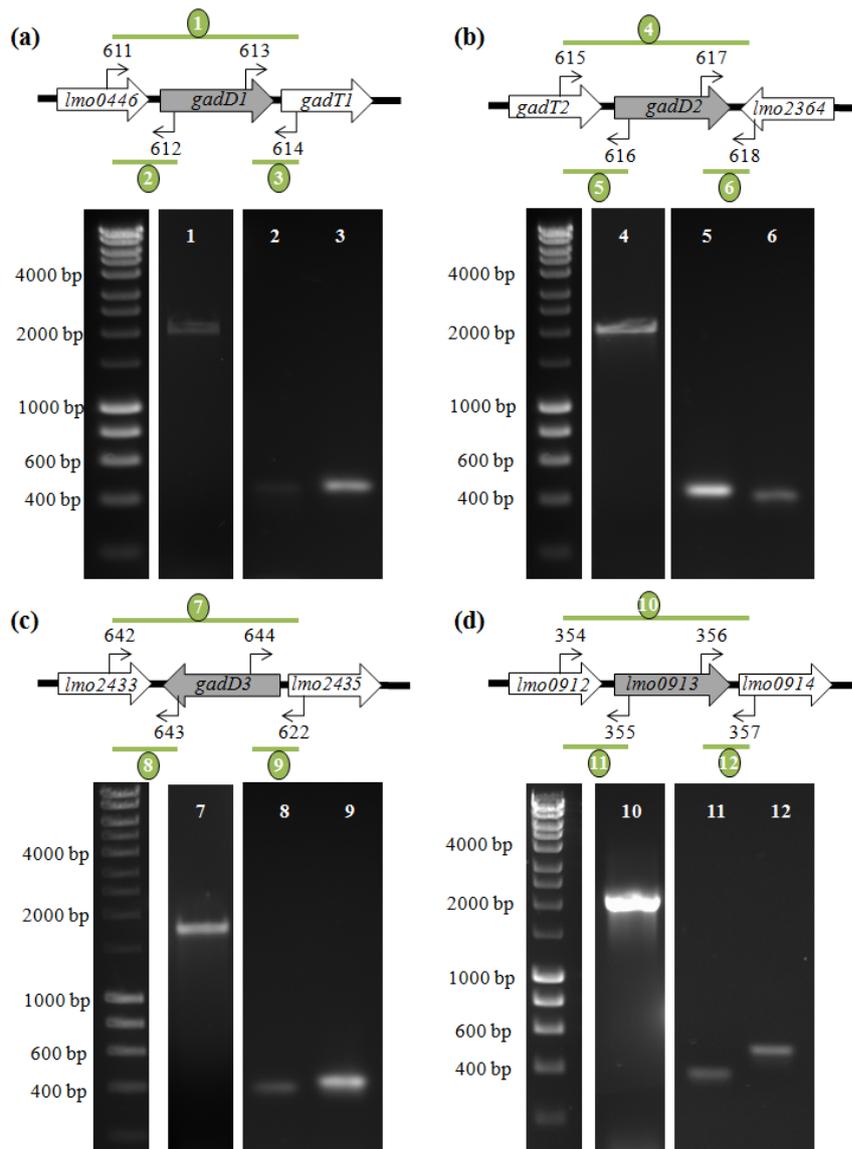


Figure 3.1. Schematics and gel images of the genes to be deleted and SOEing PCR fragments. (a) A full copy of the *gadD1* gene is shown (1) using primers 611/614 along with the [AB] fragment (2) and the [CD] fragment (3) using primers 611/612 and 613/614 respectively. (b) A full copy of *gadD2* gene (4) was generated using primers 615/618 along with the [AB] fragment (5) and the [CD] fragment (6) using primers 615/616 and 617/618 respectively. (c) A full copy of the *gadD3* gene (7) using primers 642/622 along with the [AB] fragment (8) and the [CD] fragment (8) using primers 642/643 and 644/622 respectively. (d) A full copy of the *lmo0913* gene (10) using primers 354/357 along with the [AB] fragment (11) and the [CD] fragment (12) using primers 354/355 and 356/357 respectively.

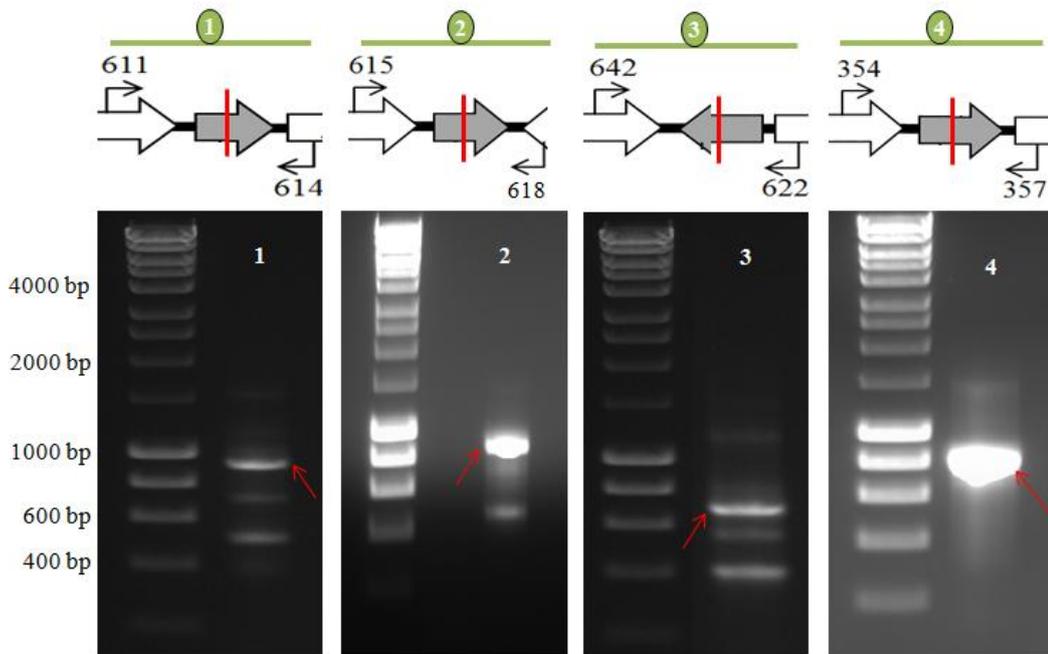


Figure 3.2. SOEing PCR generation of deletion fragments. (1) The deletion cassette (904 bp) for Δ *gadD1* using primers 611/614 is shown. (2) The deletion cassette (854 bp) for Δ *gadD2* using primers 615/618 is shown. (3) The deletion cassette (752 bp) for Δ *gadD3* using primers 642/622 is shown. (4) The deletion cassette (785 bp) for Δ *lmo0913* using primers 354/357 is shown.

3.2.2 Cloning of the deletion cassette into pCR-XL-TOPO and pKSV7

Difficulty experienced in the cloning of each fragment directly into pKSV7 following *EcoRI* digestion led to the use of an intermediary host vector. Here a commercial cloning kit TOPO TA Cloning[®] (Invitrogen) was used (Fig. 3.3 a). This kit allows for rapid, highly efficient cloning of a PCR fragment without the need for digestion or ligation. It utilises the "A" nucleotide overhangs that are left on a PCR product following its generation (Invitrogen, 2012). Following ligation of each AD fragment with the pCR-XL-TOPO vector, the reaction product was transformed into electrocompetent *E. coli* TOP 10 cells (Section 2.14). Due to the presence of a the *lacZ α* gene at the multi-cloning site on the vector, colonies of *E.*

coli that no longer produce a blue colour in the presence of X-gal but can grow on kanamycin are indicative of correct transformants.

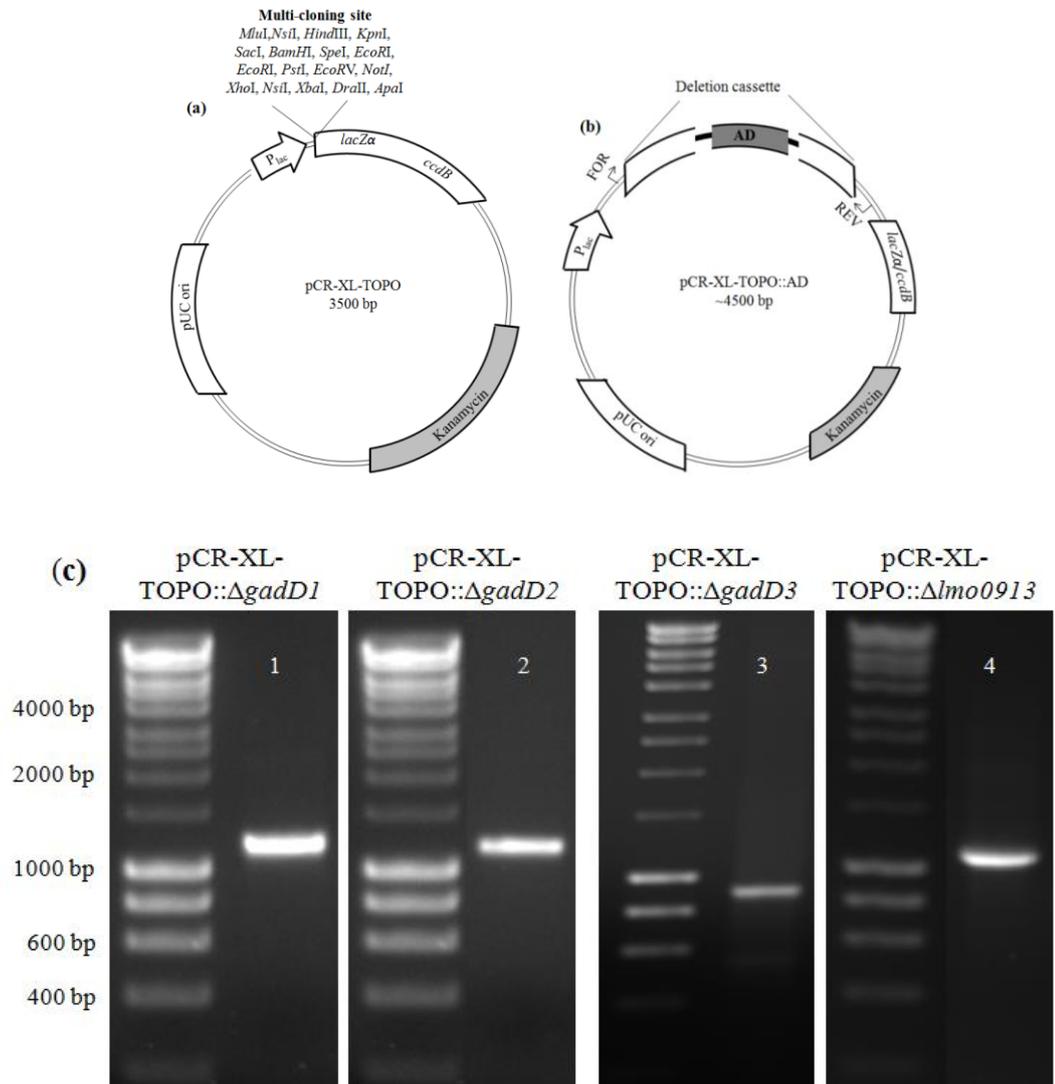


Figure 3.3. Cloning into p-CR-XL-TOPO. (a) Map of plasmid pCR-XL-TOPO indicating the location of the multi-cloning site, *lacZ α* and kanamycin resistance genes is depicted. (b) pCR-XL-TOPO plasmid containing a cloned AD fragment disrupting the *lac* promoter. (c) PCR with M13 FOR/REV primers indicating correct cloned AD product size for (1) pCR-XL-TOPO:: Δ *gadD1* [1174 bp] (2) pCR-XL-TOPO:: Δ *gadD2* [1124 bp] (3) pCR-XL-TOPO:: Δ *gadD3* [940 bp] (4) pCR-XL-TOPO:: Δ *lmo0913* [1055 bp].

Between 150 and 200 white colonies were screened for each deletion construct by using M13 FOR and REV primers in order to ensure that a clone was identified. These primers bind specifically to the plasmid (Fig. 3.3 b). All AD deletion cassettes for $\Delta gadD1$, $\Delta gadD2$, $\Delta gadD3$ and $\Delta lmo0913$ were successfully cloned into pCR-XL-TOPO to generate pCR-XL-TOPO:: $\Delta gadD1$, pCR-XL-TOPO:: $\Delta gadD2$, pCR-XL-TOPO:: $\Delta gadD3$ and pCR-XL-TOPO:: $\Delta lmo0913$, respectively (Fig. 3.3 c).

Following confirmation of a positive pCR-XL-TOPO clone for each gene deletion, a PCR was performed to amplify the deletion cassette using M13 primers and the product of each PCR reaction was purified after gel electrophoresis. The purified product was digested with *EcoRI* and ligated to *EcoRI* digested pKSV7 at a ratio of 3:1 (insert:vector; Section 2.11.7). Following ligation, the mixture was transformed into electrocompetent *E. coli* Top10 cells. Between 250 and 300 colonies were screened by PCR with M13 primers to search for a positive transformant. A successful clone for each of the constructs was found and named pKSV7:: $\Delta gadD1$, pKSV7:: $\Delta gadD2$, pKSV7:: $\Delta gadD3$ and pKSV7:: $\Delta lmo0913$.

Each of the plasmid clones was purified (Section 2.11.4) and sent for sequencing with a commercial company (MWG Eurofins) to ensure fidelity in each construct. Sequencing confirmed the presence of an in-frame deletion construct as expected from the design of the primers. Three nucleotide bases were found to be changed as a result of the PCR in *gadD1*. One of these was a silent mutation resulting in the same amino acid. Two resulted in changes of A59V and Q92R in the amino acid sequence for the downstream antiporter GadT1. As this is a non functional protein in EGD-e the changes were deemed acceptable. Two changes were observed in the *gadD2* cassette however these did not occur in a known open reading frame. A single change occurred in an intragenic region upstream of *lmo0913* although not in any known promoter sequence. A mutation in the construct for *gadD3* resulted in a phenylalanine to serine substitution in the

upstream gene *lmo2433*. To prevent this the *gadD3* cassette was reconstructed through a new round of SOEing PCR and cloning. The eventual successful clone did not have any changes from the published sequence.

3.2.3 Transformation of *L. monocytogenes* EGD-e with plasmid constructs and chromosomal integration

Once all cloned deletion cassettes were confirmed, the next stage was to introduce them into the EGD-e host strain. Electrocompetent *L. monocytogenes* EGD-e cells were produced as described in Section 2.13.2. Approximately 2 μl (100 ng μl^{-1}) of either pKSV7:: Δ *gadD1*, pKSV7:: Δ *gadD2*, pKSV7:: Δ *gadD3* or pKSV7:: Δ *lmo0913* was mixed with the EGD-e cells and transformed (Section 2.14). Following incubation on BHI agar plates supplemented with 10 $\mu\text{g ml}^{-1}$ of Chl for 24 - 48 h, around 150 - 200 chloramphenicol resistant colonies were screened for each transformation using M13 FOR and REV primers. Positive transformants were successfully identified for each plasmid clone (Fig. 3.4 c).

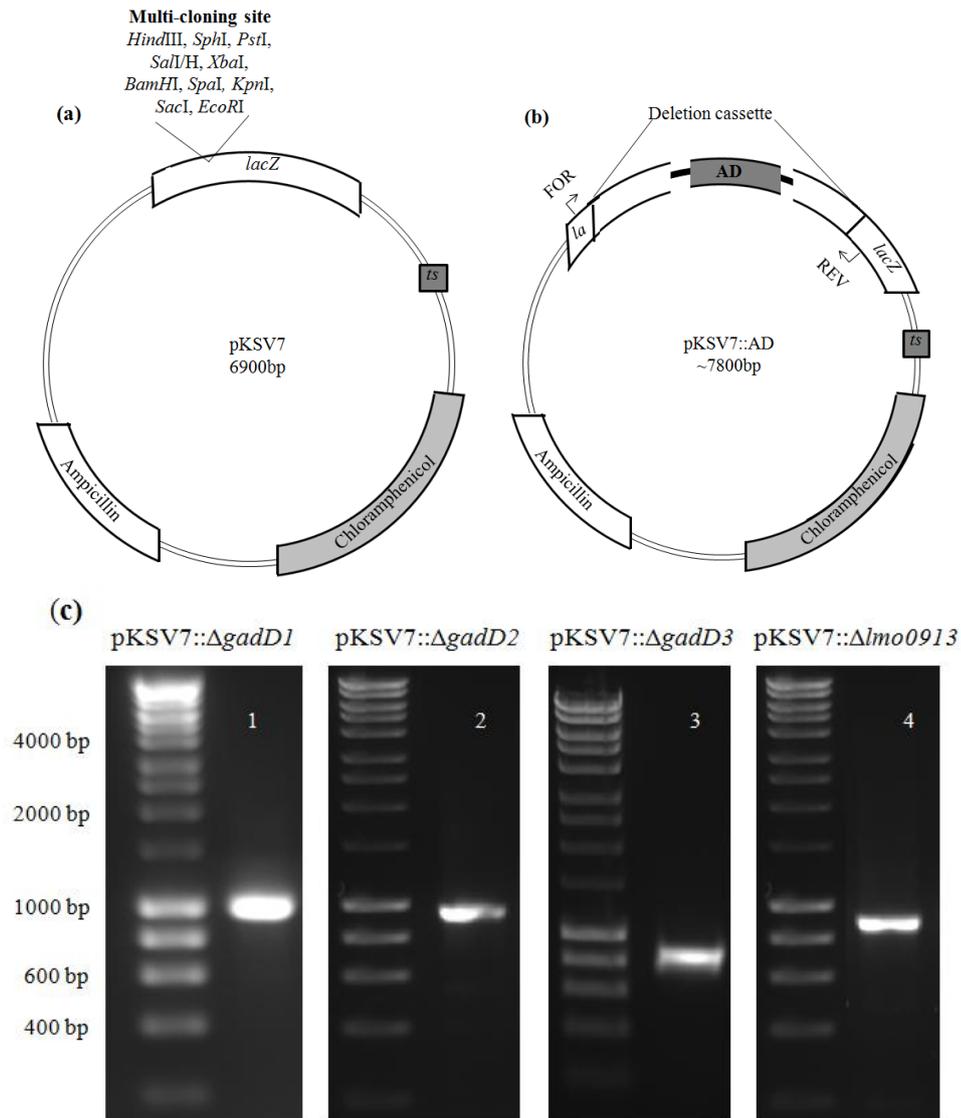


Figure 3.4. Transformation of EGD-e with pKSV7 deletion cassettes. (a) The schematic depicts the pKSV7 plasmid indicating the location of the multi-cloning site in the *lacZ* gene, the chloramphenicol and ampicillin resistance genes and the temperature sensitive origin of replication (b) pKSV7 plasmid containing a cloned AD fragment disrupting the *lacZ* gene. (c) PCR with M13 FOR/REV primers indicating correct cloned AD product size for (1) pKSV7:: Δ *gadD1* [1024 bp] (2) pKSV7:: Δ *gadD2* [974 bp] (3) pKSV7:: Δ *gadD3* [872 bp] (4) pKSV7:: Δ *lmo0913* [905 bp].

The shuttle vector pKSV7 contains a temperature sensitive origin of replication (Fig. 3.4 **a**). In order to replicate at high temperatures it must integrate into the host chromosome. All positive EGD-e transformants were grown at 45°C on BHI agar plates supplemented with chloramphenicol (Section 2.15) until isolated colonies grew without fail. This growth was indicative that the plasmid had integrated into the region of the EGD-e chromosome homologous to that of the cloned deletion cassette. Integration of the plasmid can occur either upstream or downstream of the target gene (Fig. 2.2). Primers were designed to anneal to nucleotide regions outside of the AD region targeted for deletion (Fig. 2.2). To confirm integration a PCR was carried out using a combination of the outside deletion primers with M13 FOR or REV primers. From each PCR it appeared that pKSV7::*ΔgadD1*, pKSV7::*ΔgadD3* and pKSV7::*Δlmo0913* integrated downstream of the wild type copy of its respective gene (Fig. 3.8 **a,c,d**). Integration of pKSV7::*ΔgadD2* occurred upstream of *gadD2* (Fig. 3.8 **b**).

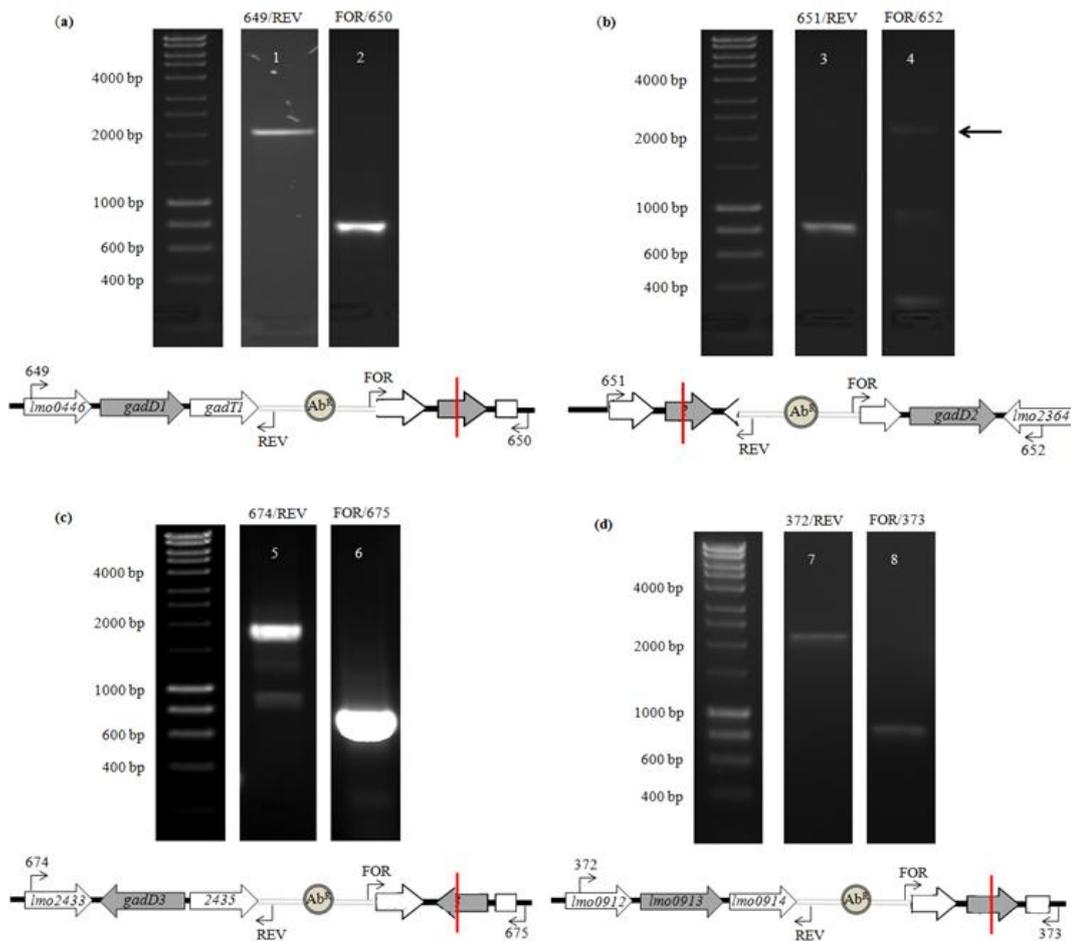


Figure 3.5. Integration of deletion cassettes into *L. monocytogenes* chromosome. Lanes 1 & 2: Downstream integration of pKSV7:: Δ *gadD1* with primers 649/REV producing a 2191 bp product and primers FOR/650 producing a 784 bp product (a). Lanes 3 & 4: Upstream integration of pKSV7:: Δ *gadD2* with primers 651/REV producing a 979 bp product and primers FOR/652 producing a 2324 bp product (b). Lanes 5 & 6: Downstream integration of pKSV7:: Δ *gadD3* with primers 674/REV producing a 1907 bp product and primers FOR/675 producing a 927 bp product (c). Lanes 7 & 8: Downstream integration of pKSV7:: Δ *lmo0913* with primers 372/REV producing a 2341 bp product and primers FOR/373 producing a 873 bp product (d).

3.2.4 Second homologous recombination and mutant confirmation

All four successful integrants were subsequently passaged at 30°C in the absence of antibiotic selection in order to select for excision of the integrated plasmid. The excision of the plasmid can result in two outcomes, the first involves the plasmid removing the deletion cassette and as a result the strain reverts to a wild type genotype. The second scenario results in plasmid excision along with the wild type gene. In this case the allele that remains is the deletion cassette and a deletion mutant has been obtained. Loss of the plasmid from the cell was identified for each isolate by sensitivity to chloramphenicol. For EGD-e pKSV7:: Δ *gadD3* this occurred after 3 passages (see Section 2.15; Chapter 2) however sensitive colonies were not identified for the other deletion strains until after 6-8 passages. Over 350 colonies were screened by PCR for each deletion from 3-9 passages. The percentage of wild type reversions to mutants increased as the number of passages increased.

Eventually, following the PCR screening of sensitive colonies with outside deletion primers, mutants possessing a truncated allele for each *gadD1*, *gadD2*, *gadD3* and *lmo0913* gene were identified (Fig. 3.9). The generation of each of these mutants indicated that they do not play an essential role in EGD-e.

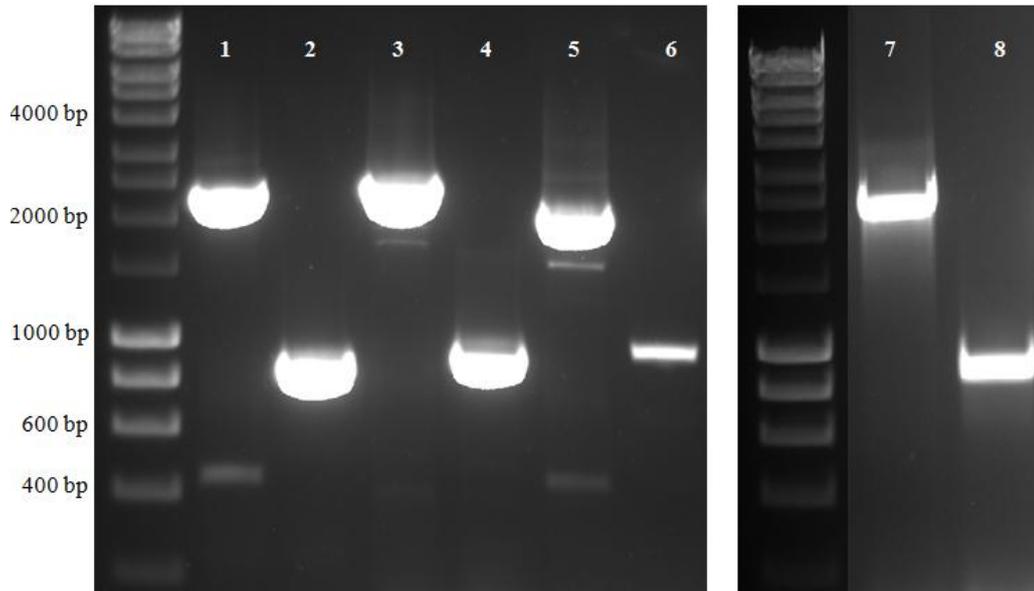


Figure 3.6. *L. monocytogenes* EGD-e deletion mutants. PCR analysis showing the successful construction of each gene deletion. The wild-type strain is compared against the corresponding deletion mutant. EGD-e wt *gadD1* (2171 bp; Lane1), EGD-e Δ *gadD1*(845 bp; Lane 2), EGD-e wt *gadD2* (2285 bp ;Lane 3), EGD-e Δ *gadD2* (920 bp; Lane 4), EGD-e wt *gadD3* (1962bp; Lane 5), EGD-e Δ *gadD3* (933bp; Lane 6), EGD-e wt *lmo0913* (2310 bp; Lane 7), EGD-e Δ *lmo0913* (903 bp; Lane 8).

3.3 Phenotypic characterisation of EGD-e deletion mutants

3.3.1 Viability of the EGD-e deletion mutants

Growth curve experiments were undertaken to determine the growth properties of the deletion mutants compared to the parent strain. The growth of each strain was measured as outlined in section 2.4 with the strains cultured in BHI medium. All of the strains were seen to enter stationary phase after 5 h of growth. There were no significant differences seen in the exponential growth for the strains (Fig. 3.7) with an average specific growth rate (SGR) of 1.25 h^{-1} observed.

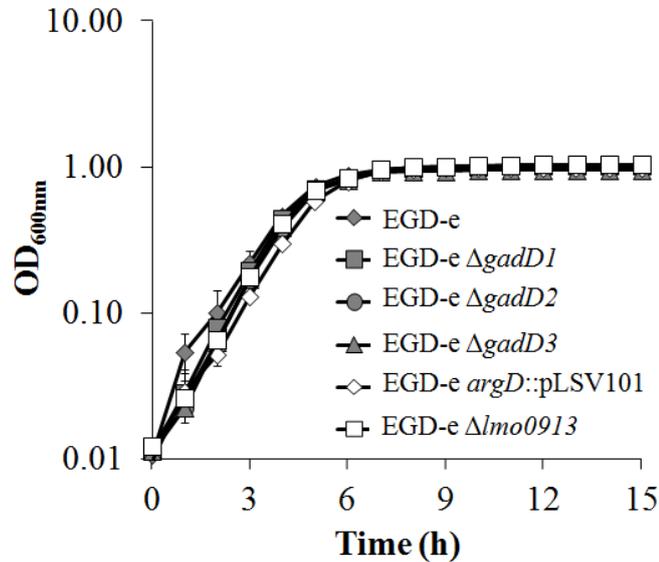


Figure 3.7. Growth curve analysis for each EGD-e strain. The growth of each strain in BHI medium was recorded at OD_{600nm} for 15 h. Measurements were taken every 1 h. Error bars represent the standard deviation from the mean of three independent biological replicates including two technical replicates of each.

3.3.2 Survival and growth of EGD-e GAD mutants at low pH

As the GAD system is involved in acid resistance, each strain lacking a single glutamate decarboxylase gene was tested for survival when exposed to pH 2.5 for 1 h. The acid survival and other phenotypes of EGD-e Δ *lmo0913* and EGD-e *argD*::pLSV101 are presented later in Chapter 5. As expected the EGD-e wild-type strain was seen to reduce by over 5 log-cycles after 1 h of exposure to acid stress (Fig. 3.8). Interestingly deletion of either *gadD1* or *gadD2* increased the survival of the strain, with an increase in acid resistance of over 3 log-cycles compared to the parent strain. EGD-e Δ *gadD3* was observed to have a similar survival pattern as EGD-e with a reduction close to 5 log-cycles by the end of the experiment (Fig. 3.8).

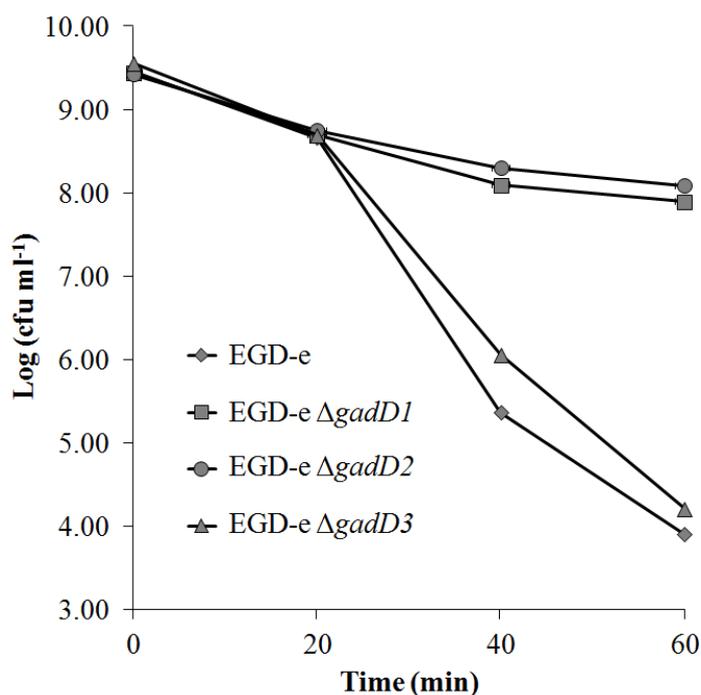


Figure 3.8. Acid survival of *L. monocytogenes* EGD-e gad mutants. Stationary phase EGD-e gad mutants mutants were challenged at pH 2.5. Cell counts were taken every 20 min. Values are the means of data from three individual cultures, with the cell counts for each culture being the means of counts from three platings.

Following from the acid survival experiments, the role that the GAD system may play in growth at low pH was examined. Work by Ryan *et. al* (2009) revealed that *gadDIT1* is in fact part of a 5-gene islet. Deletion of this islet was shown to impair the growth of the mutant strain under a combination of osmotic and acidic stress (Ryan *et al.*, 2010). The three EGD-e GAD deletion strains and the parent strain were grown across a pH range from 7.0 to 4.0 in BHI medium. As expected, a reduction in SGR was observed for all strains as the pH was reduced. At pH 7.0, 4.5 and 4.0 there were no significant differences seen in the SGR between any of the four strains. At pH 5.0 however there was small but significant reduction (*p* value <0.02; Students *t*-test) in the SGR for EGD-e Δ gadD3 (Fig. 3.9). No altered acid growth phenotype was detected for EGD-e Δ gadD1.

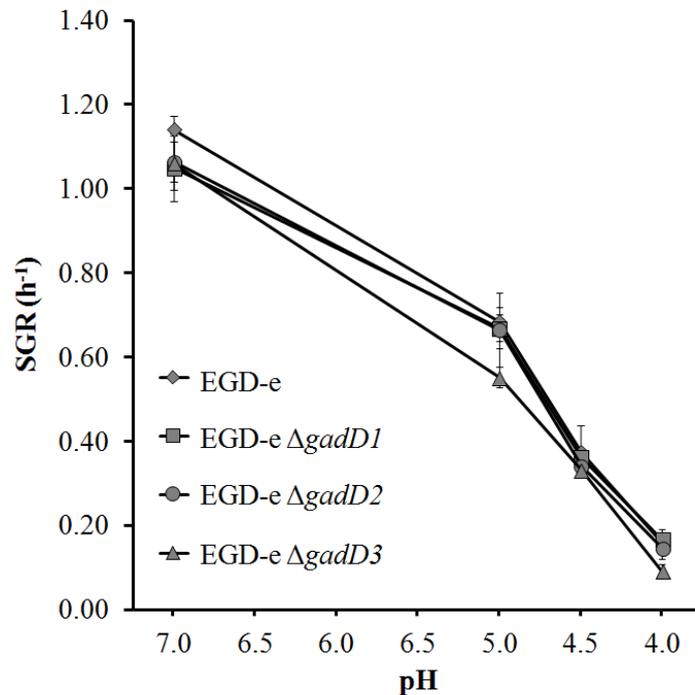


Figure 3.9. Comparison of EGD-e GAD mutant growth rates as a function of pH. The standard growth rates (SGR) of each strain grown in BHI with the pH adjusted to either 7.0, 5.0, 4.5 or 4.0. Error bars indicate the standard deviation from the mean value of three biological replicates for each strain at each pH.

3.3.3 The production of GABA in response to acid treatment by EGD-e GAD mutant strains

A product of the GAD system is GABA (Section 1.6). Using an enzymatic assay developed to detect GABA from bacteria (Section 2.7.3; O’Byrne *et al.*, 2011), all EGD-e GAD mutant strains were tested for their ability to produce GABA in response to an acid shock. The cultures analysed were grown to stationary phase (~16 h) in BHI medium and subsequently treated with HCl to pH 4.0. As expected, the EGD-e parent strain only produced intracellular GABA (~1.5 mM; GABA_i) and no extracellular GABA (GABA_e; Fig. 3.10) after the treatment.

Deletion of *gadD1* or *gadD2* however resulted in the production of concentrations of GABA_i almost 4-times greater than that of the parent strain after acid treatment. This equated to between 3.80 and 4.10 mM of GABA_i, respectively. The deletion of *gadD3* halved the amount of GABA_i that the bacteria could produce (Fig. 3.10). In all deletion strains there was still no GABA_e produced in response to acid stress. Taken together, a deletion in any of the three decarboxylase genes significantly disrupted the normal production levels of GABA_i in EGD-e (*p*-value <0.02).

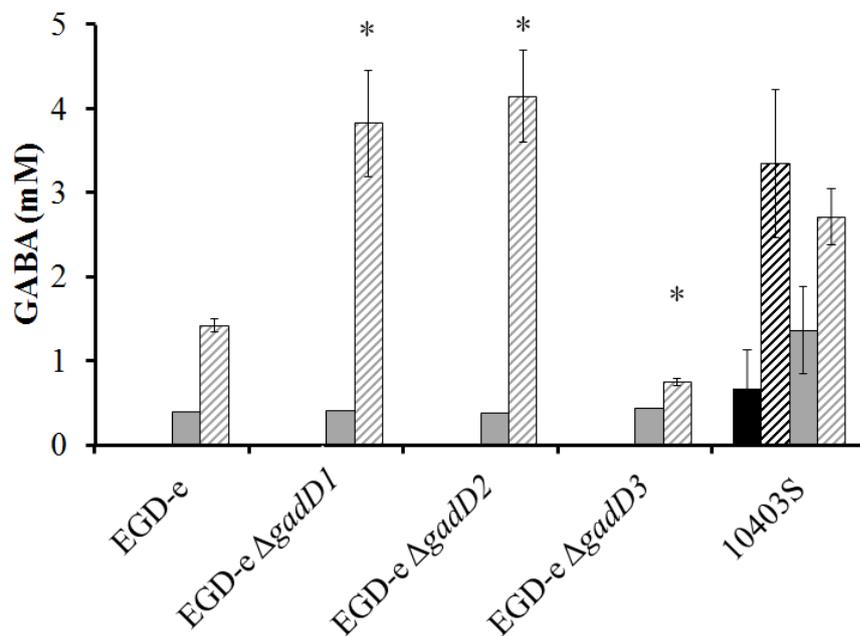


Figure 3.10. GABA production by EGD-e strains in response to low pH. Both the extracellular (GABA_e; Black) and intracellular (GABA_i; Grey) concentration of GABA was measured for each strain before acid exposure (Filled) or after acid exposure of pH 4.0 (Striped) in BHI. 10403S, a control strain that produces both GABA_e and GABA_i was treated to pH 3.5 in BHI to highlight GABA_e production. Error bars represent the standard deviation from the mean of three biological replicates for each strain. An asterix (*) represents a significant difference in GABA_i production between mutant and parent strain as determined by a Students *t*-test (*p*-value <0.02).

These data suggest that a correlation exists between increased survival at low pH and an increase in GABA_i (Fig. 3.8, 3.10). If such an accumulation is beneficial for the cells at low pH then the question arises as to why such a strain is not selected for over its parent strain EGD-e in nature. To address this point, growth experiments were conducted to determine whether the apparent increased load of GABA_i would have a negative impact on growth for either EGD-e Δ *gadD1* or EGD-e Δ *gadD2*. Stationary phase cultures of all four strains were treated to pH 4.0 for 1 h as occurs during the GABA experiments. Following the 1 h exposure, each culture was inoculated into 20 ml fresh BHI broth at an OD_{600nm} of 0.05 and the subsequent growth of each strain measured by recording increases in OD_{600nm}. None of the strains tested had difficulty in reaching stationary phase after 8 h (Fig. 3.11 a). There was no significant differences seen in the SGR between EGD-e and any of the three deletion strains after exposure to pH 4.0 (Fig. 3.11 b). There was however a reduced SGR for all strains that were treated to pH 4.0 for 1 h compared to the control set that was not exposed to a reduction in pH.

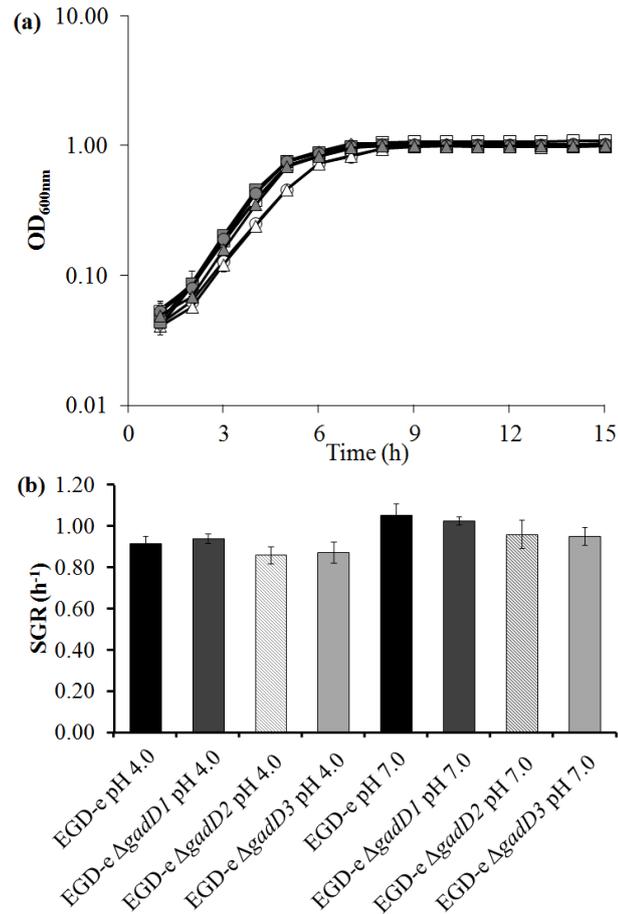


Figure 3.11. Growth and recovery of GAD mutants after acid exposure. (a) Growth curve of EGD-e GAD mutants in BHI pH 7.0 after prior exposure to pH 4.0 (empty symbols) or pH 7.0 (filled symbols) in BHI for 1 h. EGD-e (diamonds), EGD-e Δ gadD1 (squares), EGD-e Δ gadD2 (circles) and EGD-e Δ gadD3 (triangles) were all inoculated to a starting OD_{600nm} of 0.05. (b) The specific growth rate per hour for each strain over the first 5 hours of growth. Error bars for both graphs represent the standard deviation from the mean of three biological replicates for each strain.

3.3.4 Relative transcription for the GAD genes in response to low pH

The transcription of each of the three GAD genes was measured in each of the four strains over the course of a pH 4.0 acid challenge. As a reporter of σ^B activity, *lmo2230* (similar to arsenate reductase) transcript levels were also

measured. This gene was selected as it has been shown to be under the positive control of σ^B across numerous stresses, including low pH (Utratna *et al.*, 2011). Among the GAD genes it appeared that *gadD3* was the dominantly expressed gene at stationary phase for EGD-e, EGD-e Δ *gadD1* and EGD-e Δ *gadD2* (> 1 log; Fig. 3.12). *gadD1* only appeared to respond to acid treatment in EGD-e Δ *gadD2* and expression was not seen to significantly change expression in either the wild-type or EGD-e Δ *gadD3*. The expression of *gadD2* was greatest in EGD-e Δ *gadD3* before acid treatment and after 15 min however transcript levels appeared to reduce after 30 min of exposure to acid. *gadD3* had a marked increase in expression after 15 min in EGD-e Δ *gadD2*, however it remained unchanged in EGD-e Δ *gadD1* and displayed a reduction in transcription over 30 min in EGD-e. Interestingly expression of the σ^B reporter *lmo2230* had increased expression in both EGD-e Δ *gadD1* and EGD-e Δ *gadD2*. The expression of this gene was greater than that of any of the GAD genes although it did not appear to be affected by acid treatment, suggesting that SigB activity was already maximal under these conditions (Utratna *et al.*, 2011). Overall, the expression of all the GAD genes varied between each strain, and dependent on the strain the GAD genes showed a different response to the low pH. From this data no direct correlation was observed between survival of a particular strain and the expression of a *gad* gene.

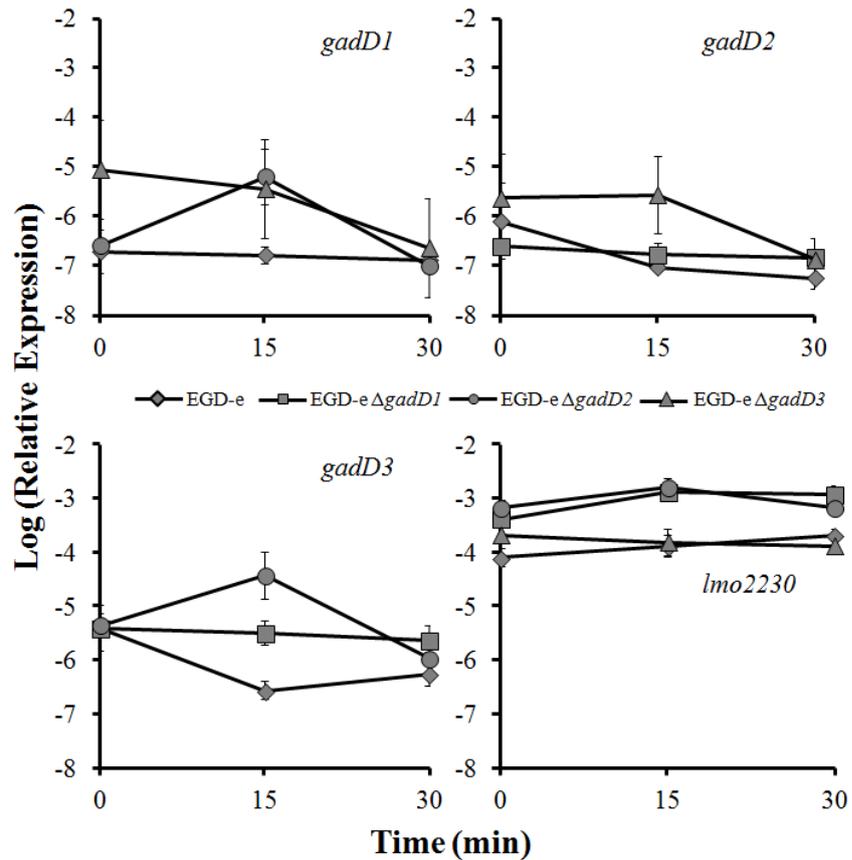


Figure 3.12. Relative gene expression levels of GAD genes in EGD-e. The transcript levels of each gene are expressed relative to the measured levels of 16S rRNA in each strain. Measurements were taken before exposure of stationary phase cultures in BHI to pH 4.0 and at 15 and 30 min after exposure. Error bars represent the standard error in the mean value for three biological repeats for each strain and two technical replicates of each.

3.3.5 Survival of EGD-e GAD system mutants in THP-1 macrophages

During infection *L. monocytogenes* cells can be taken up by macrophages. Engulfed in the phagolysosome, the bacterium is exposed to oxidative stress as well as a drop in pH (de Chastellier & Berche, 1994). To ascertain if the GAD system in *L. monocytogenes* EGD-e plays a role in survival in this environment, the three deletion mutants and the parent strain were analysed for their ability to

persist inside human-derived THP-1 macrophages (Section 2.9). Both EGD-e $\Delta gadD1$ and EGD-e $\Delta gadD2$ displayed a reduced ability to replicate inside the macrophages. After 5 hours, both of these strains were recovered at lower numbers (~ 0.5 log reduction; p -value 0.0034), compared to EGD-e (Fig. 3.13). After seven hours however both of these strains reached a similar recovered cell count as the parent strain, EGD-e. There was no significant difference seen for the strain carrying a deletion in $gadD3$. Overall, it appeared that the GAD system was not playing a major role in growth inside these macrophages as all 4 strains grew, although longer lags for EGD-e $\Delta gadD1$ and EGD-e $\Delta gadD2$ were observed.

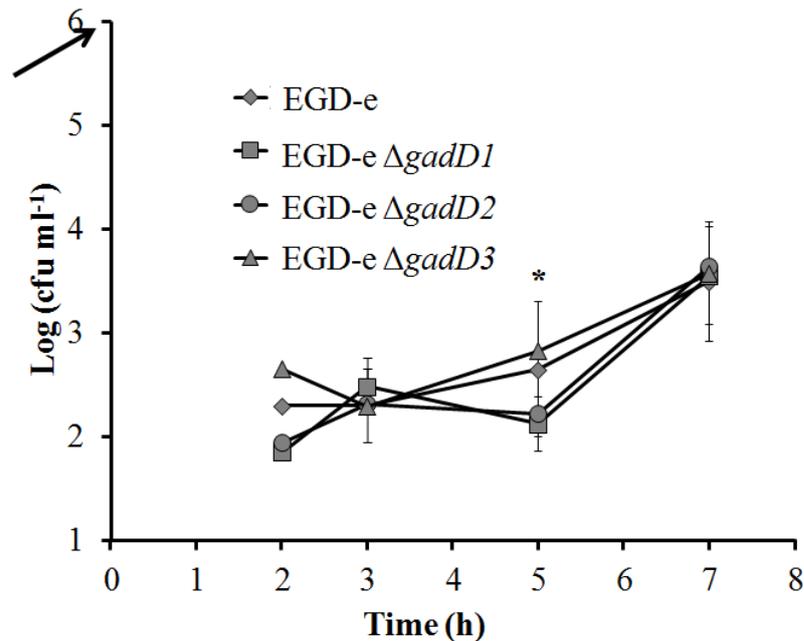


Figure 3.13. Growth of EGD-e GAD mutants in THP-1 macrophages. Growth of EGD-e GAD mutants inside THP-1 macrophages was measured over 7 h. Counts were recorded 2 h post co-incubation of THP-1 with bacteria at an MOI of 10 (10^6 bacteria; black arrow). Error bars represent the standard deviation from the mean of at least 4 biological replicates for each strain and time-point. Significant differences (*; $p < 0.05$) were determined using a Kruskal-Wallis test.

3.4. Generation of single and double GAD mutant strains in EGDm

One of the main aims for this work was to analyse the role that the GAD system plays in gastric transit and virulence using Balb/C mice as a model. To infect mice with *L. monocytogenes*, the strain must first be murinised by altering the InlA in order to improve its interaction with murine E-cadherin (Wollert *et al.*, 2007). To this end a murinised EGDm strain was obtained from Dr. Cormac Gahan at University College Cork (Monk *et al.*, 2010). All single mutants obtained previously in EGD-e were subsequently introduced into EGDm using the deletion cassettes constructed above (Section 3.2.2). Following integration of these plasmids into EGDm and after selecting second homologous recombination events, EGDm $\Delta gadD1$, EGDm $\Delta gadD2$, EGDm $\Delta gadD3$, and EGDm $\Delta lmo0913$ were successfully generated (data not shown).

Competent cells of EGDm $\Delta gadD1$ and EGDm $\Delta gadD3$ were prepared and transformed with pKSV7:: $\Delta gadD2$ and/or pKSV7:: $\Delta gadD3$ (Fig 3.14). Both pKSV7:: $\Delta gadD2$ and pKSV7:: $\Delta gadD3$ were successfully introduced into EGDm $\Delta gadD1$ while pKSV7:: $\Delta gadD1$ was successfully introduced into EGDm $\Delta gadD3$.

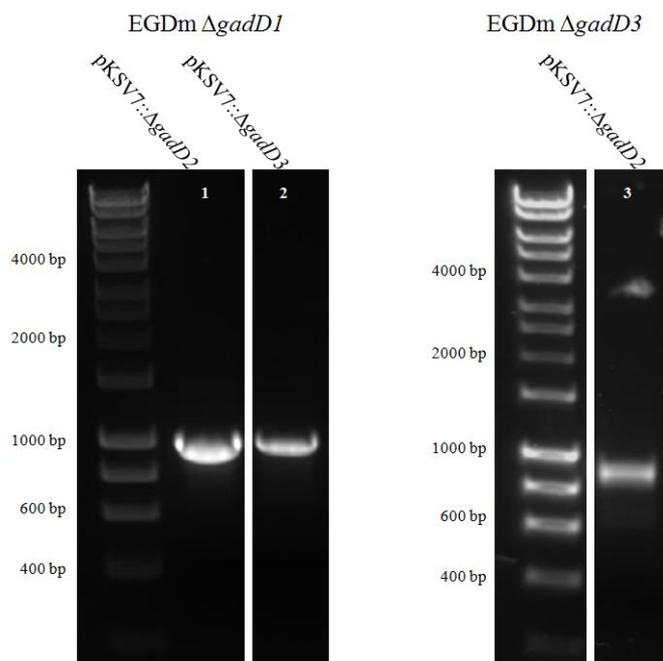


Figure 3.14. Transformation of single mutants with second deletion plasmids. M13 FOR/REV primers were used to confirm the presence of the deletion cassette. Lane 1: EGDm $\Delta gadD1$, pKSV7:: $\Delta gadD2$. Lane 2: EGDm $\Delta gadD1$, pKSV7:: $\Delta gadD3$. Lane 3: EGDm $\Delta gadD3$, pKSV7:: $\Delta gadD2$.

After confirmation of transformations, the three strains were grown at 45°C in order to select for the integration of the plasmid. Despite repeated attempts it was not possible to obtain the integration of pKSV7:: $\Delta gadD2$ into EGDm $\Delta gadD1$. Successful integrants however were detected for the two remaining strains (Fig. 3.15). An upstream integration was detected for pKSV7:: $\Delta gadD3$ into EGDm $\Delta gadD1$ while pKSV7:: $\Delta gadD2$ integrated downstream of the native *gadD2* gene in EGDm $\Delta gadD3$.

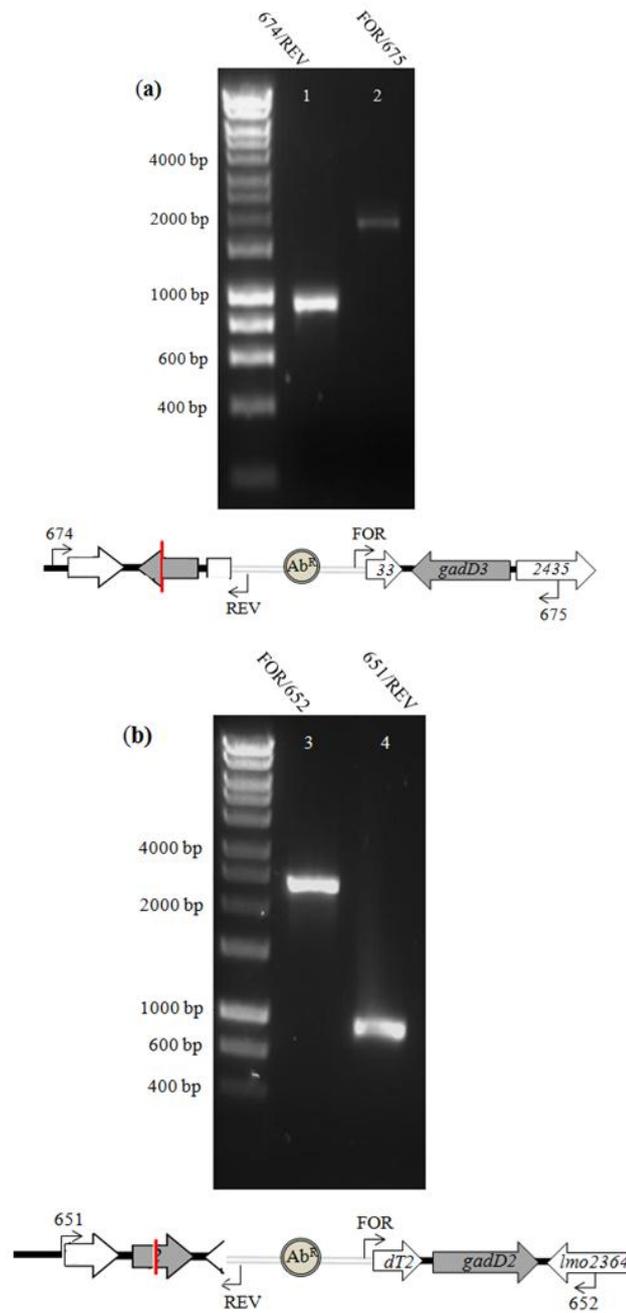


Figure 3.15. Integration of plasmids for double mutants. (a) Lanes 1 & 2: Upstream integration of pKSV7:: Δ *gadD3* into EGDm Δ *gadD1* with primers 674/REV producing a 938 bp product and primers FOR/675 producing a 1896 bp product. **(b)** Lanes 3 & 4: Downstream integration of pKSV7:: Δ *gadD2* into EGDm Δ *gadD3* with primers FOR/652 producing a 2318 bp product and primers 651/REV producing a 941 bp product.

Both EGDm $\Delta gadD1::pKSV7::\Delta gadD3$ and EGDm $\Delta gadD3::pKSV7::\Delta gadD2$ were passaged to select a second homologous recombination event as before (Section 3.2.4). Following four to seven passages, chloramphenicol sensitive colonies were identified. Following PCR screening with primers outside the deletion region, double mutants were identified for both EGDm $\Delta gadD1D3$ and EGDm $\Delta gadD2D3$ (Fig. 3.16). As no integrant was obtained for pKSV7:: $\Delta gadD2$ into EGDm $\Delta gadD1$ and due to time constraints no double mutant for EGDm $\Delta gadD1D2$ could be constructed.

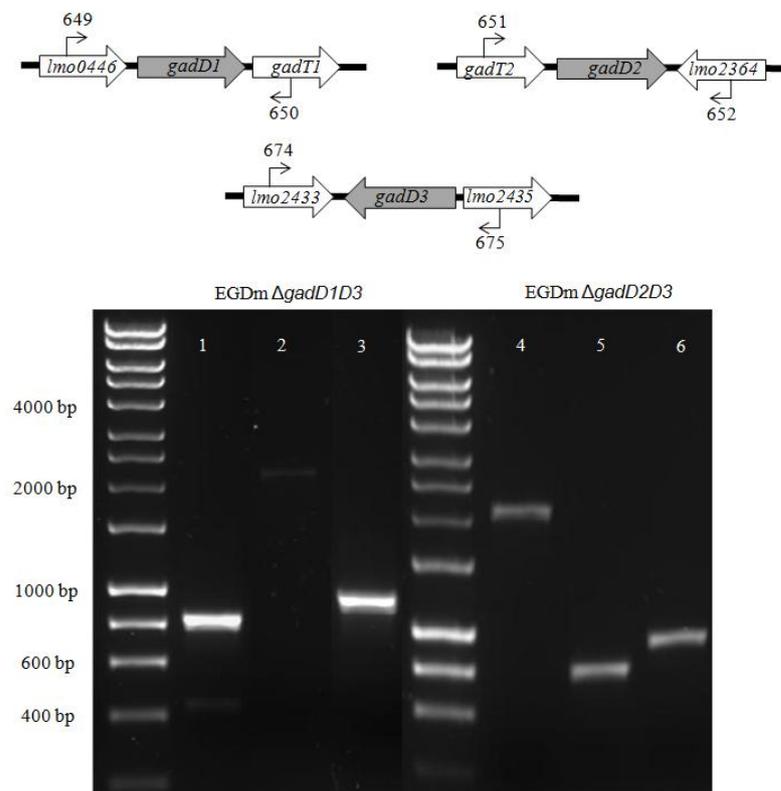


Figure 3.16. Confirmation of double EGDm GAD mutants. Lanes 1,2&3: EGDm $\Delta gadD1D3$ with truncated *gadD1* (Lane 1; 845 bp) from primers 649/650, native *gadD2* (Lane 2; 2285 bp) from primers 651/652 and truncated *gadD3* (Lane 3; 933 bp) with primers 674/675. Lanes 4,5&6: EGDm $\Delta gadD2D3$ with native *gadD1* (Lane 4; 2171 bp) from primers 649/650, truncated *gadD2* (Lane 5; 920 bp) from primers 651/652 and truncated *gadD3* (Lane 3; 933 bp) with primers 674/675.

3.4.1 Growth and viability of all EGDm strains

To identify that all the newly constructed mutants in the EGDm background were able to grow at an equal rate, the growth of each strain in BHI medium was measured. There was no difference observed for the growth of any strain with a mean SGR of 1.27 and entry into the stationary phase of growth occurred at 7 h (Fig. 3.17).

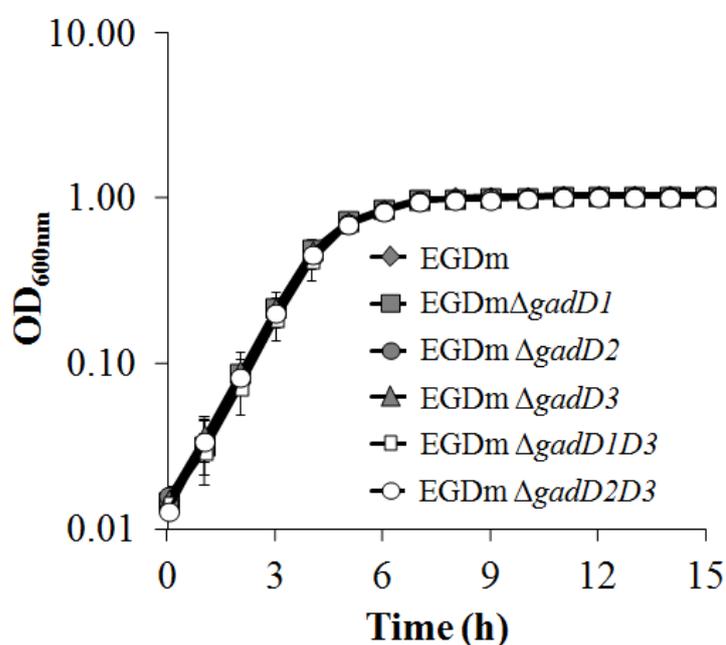


Figure 3.17. Growth curves for all EGDm strains. The OD_{600nm} was recorded during growth for each strain in BHI. Error bars represent the standard deviation from the mean of three independent biological replicates and two technical replicates.

3.4.2 Comparison of GABA production from EGD-e and EGDm

The EGD-e strain discussed above (Section 3.3) was a part of the lab collection and was originally received from Prof Simon Foster, University of Sheffield. This well-studied strain is one of the first isolates of *L. monocytogenes* and in 2001 was the first strain to be fully sequenced (Glaser *et al.*, 2001). As mentioned above,

EGDm is a murinised version of EGD-e. While the *inlA* gene has been altered, the strain as a whole should be identical to our own strain. To confirm that the same GABA production phenotype existed, both EGD-e and EGDm were compared for production of GABA_i after exposure to low pH. Somewhat surprisingly, EGDm produced much greater amounts of GABA_i than EGD-e (Fig. 3.18). Neither strain was able to produce GABA_e. These data suggest that EGD-e and EGDm are not isogenic, since it is highly unlikely that the modified *inlA* gene contributes to altered GABA production.

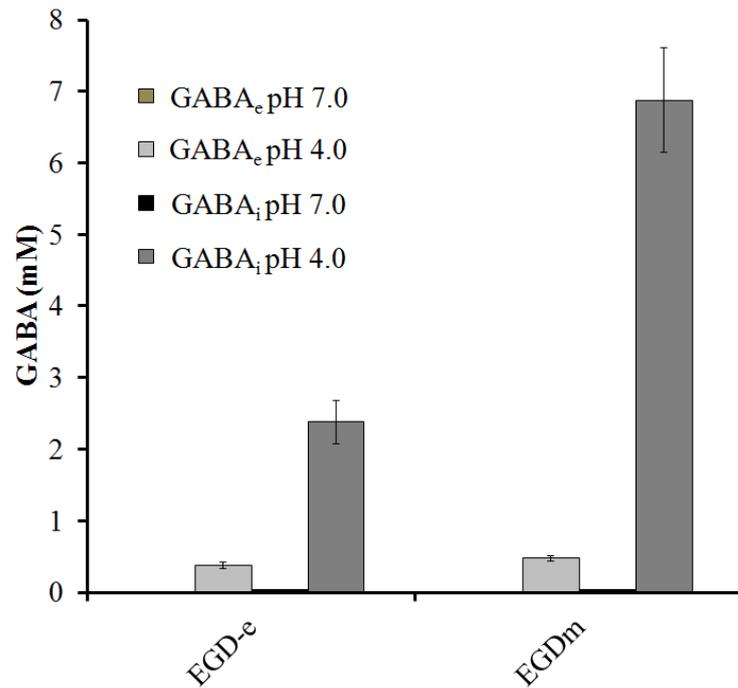


Figure 3.18. GABA production by EGD-e and EGDm. Both GABA_i and GABA_e was measured in cultures treated or not treated with HCl to pH 4.0. Measurements for GABA_i represent the concentration of GABA found in 19.5 ml of culture. Measurements for GABA_e represent the concentration of GABA found in 0.5 ml of culture. Error bars represent the standard deviation from the mean values of three biological repeats for each strain.

3.5. Construction of GAD mutants in 10403S

To fully understand the role that the GAD system plays in *L. monocytogenes* a comparison of the system between two strains was undertaken. The strain 10403S, unlike EGD-e has the ability to produce GABA_e in response to acid (Karatzas *et al.*, 2012). Therefore this strain was selected as a representative of a strain that utilises both GAD_i and GAD_e. In order to directly compare this strain with EGD-e, single deletions in the three glutamate decarboxylase genes was necessary. As before, the method of homologous allelic exchange was used. Both the pKSV7:: Δ *gadD1* and pKSV7:: Δ *gadD3* deletion cassettes that were constructed for EGD-e (Section 3.2.3) were deemed appropriate for use due to similar nucleotide sequences between these strains in the designed deletion region. Alignment of both the EGD-e and 10403S *gadD2* sequences however revealed that use of the pKSV7:: Δ *gadD2* cassette from EGD-e would introduce several amino acid changes in the region of the deletion. To prevent this, the entire deletion cassette was reconstructed using 10403S as a template in the SOEing PCR step. Furthermore, prior to amplification, the D primer (618) was redesigned due to a single nucleotide difference in the EGD-e primer sequence.

Following successful cloning of the new AD fragment into pKSV7, all three deletion cassettes, pKSV7:: Δ *gadD1*, pKSV7:: Δ *gadD2* and pKSV7:: Δ *gadD3* were successfully transformed into 10403S (Fig. 3.19 a). These transformants were subsequently grown at 43°C and integrants were identified after 3 rounds of growth passage. In order to identify the integration of the 10403S specific pKSV7:: Δ *gadD2* into the chromosome, new outside deletion primers had to be designed (695/696), once more due to a sequence difference between those previously designed for EGD-e. An integration event was identified downstream of *gadD1* for pKSV7:: Δ *gadD1* (Fig. 3.19 b; 1&2) while integration of pKSV7:: Δ *gadD3* also occurred downstream of *gadD3* (Fig. 3.19 b; 5&6). From screening the integration of pKSV7:: Δ *gadD2* it appeared that the AD fragment had arranged itself in a reverse orientation during cloning into the plasmid. Nonetheless, integration was confirmed to have occurred upstream of the native *gadD2* gene on the chromosome (Fig. 3.19 b; 3&4).

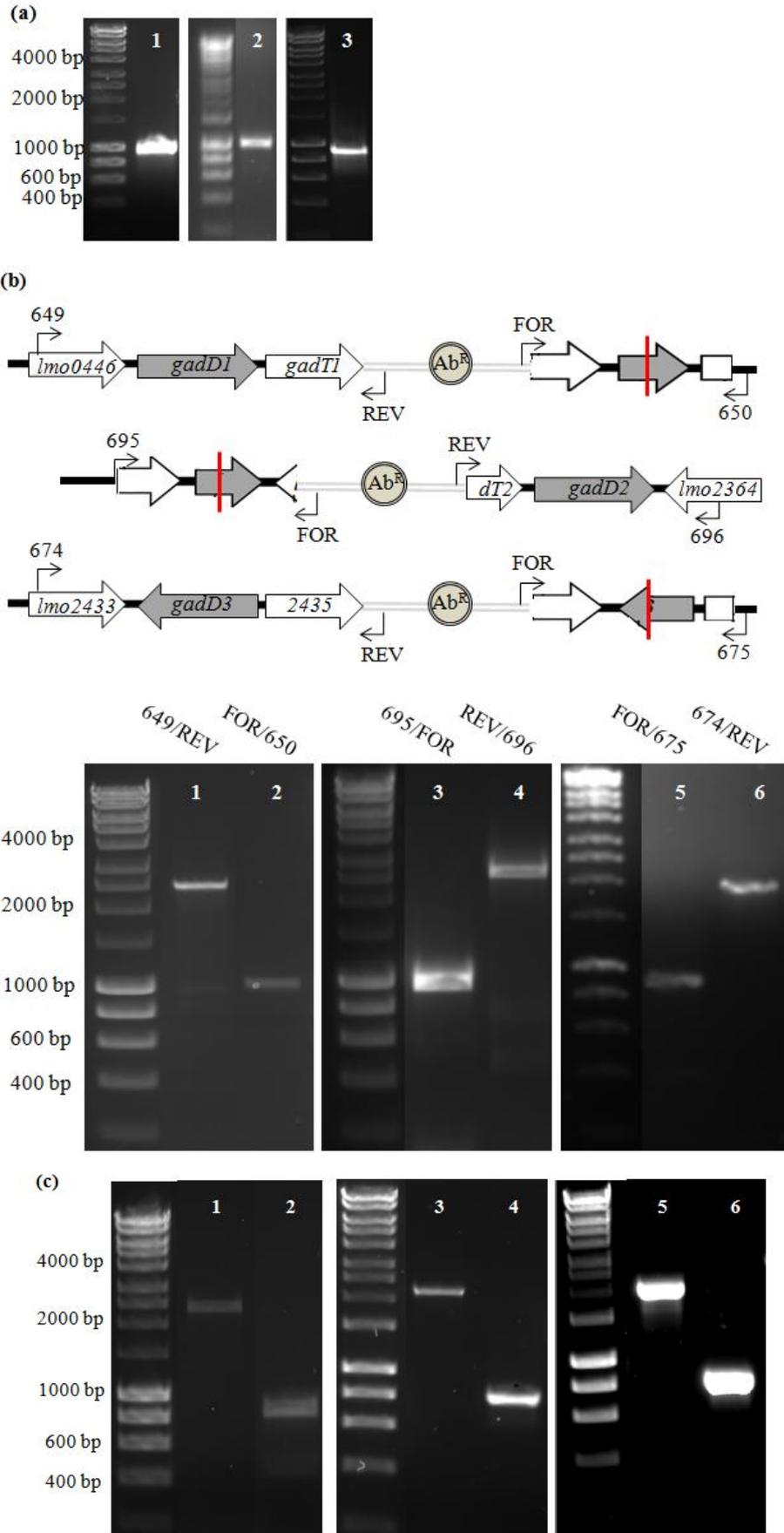


Figure 3.19. Construction of GAD mutants in 10403S. (a) Positive transformation of 10403S with pKSV7:: Δ *gadD1* (1), pKSV7:: Δ *gadD2* (2) and pKSV7:: Δ *gadD3* (3) identified using M13 FOR/REV primers binding each side of the multi-cloning site for pKSV7. (b) Confirmation of integration of all cassettes into the 10403S chromosome using a combination of plasmid specific and chromosome specific, outside deletion region primers. Lanes 1&2 indicate downstream integration of *gadD1* deletion cassette as indicated on above schematic. Lanes 3&4 indicate upstream integration of *gadD2* deletion cassette as indicated on above schematic. Lanes 5&6 indicate downstream integration of *gadD3* deletion cassette as indicated on above schematic. (c) Confirmation of the generation of deletion mutants. Lanes 1,3 and 5 show the wild-type copy of each *gadD1*, *gadD2*, and *gadD3* genes in 10403S while Lanes 2,4 and 6 show the truncated gene copy present in 10403S Δ *gadD1*, 10403S Δ *gadD2* and 10403S Δ *gadD3* respectively using primers 649/640, 695/696 and 674/675 respectively.

After the growth of each integrant at 30°C without antibiotic selection, chloramphenicol sensitive colonies were isolated. Between 50 and 150 sensitive colonies were screened for each strain. Eventually the three strains, 10403S Δ *gadD1*, 10403S Δ *gadD2* and 10403S Δ *gadD3* were identified (Fig. 3.19 c). To confirm that all the GAD mutants in 10403S were fully viable and comparative to the wild-type in terms of growth, the growth of each strain was analysed. As seen in Fig. 3.20, all strains grew at similar rates to each other, possessing a mean SGR of 1.25 h⁻¹. The strains were seen to enter stationary phase growth after 5 h into the experiment (Fig. 3.20). A complete physiological comparison of these strains is described in Chapter 4.

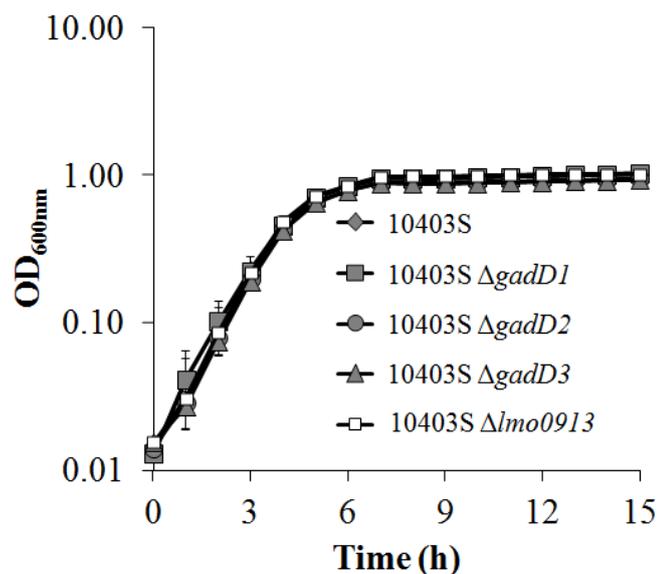


Figure 3.20. Growth curves for 10403S GAD mutants. The recorded OD_{600nm} over the course of growth for each strain in BHI are shown. Error bars represent the standard deviation from the mean of three independent biological replicates and two technical replicates.

3.6. Discussion

In total three separate strain backgrounds of *L. monocytogenes*, EGD-e, EGDm and 10403S have been used to generate single deletions in the three glutamate decarboxylase genes of each. Successful double knockout mutants were constructed in EGDm for *gadD1/gadD3* and *gadD2/gadD3*. Unfortunately a double deletion with *gadD1* and *gadD2* was not made due to the fact that a successful integrant could not be obtained for pKSV7::*ΔgadD2* into the EGDm *ΔgadD1* chromosome. An attempt at introducing the pKSV7::*ΔgadD1* cassette into EGDm *ΔgadD2* might prove successful however this approach was not attempted due to time constraints. To aid in the elucidation of a pathway for GABA catabolism in *L. monocytogenes* a deletion of the putative succinic semialdehyde dehydrogenase gene, *lmo0913*, was generated in both EGD-e and EGDm. The characterisation of EGD-e *Δlmo0913* is presented in Chapter 5.

Although not described above a knockout mutant was also generated in *gsaB* in EGD-e (Appendix 1). This putative transaminase encoding gene was thought to be a secondary GABA-AT and analysis of the EGD-e Δ *gsaB* strain is described in Chapter 5. A full analysis of the GAD systems of EGDm and 10403S is covered in the following chapter (Chapter 4). This allows for a direct comparison between a strain using both GAD_i and GAD_e with one using only GAD_i.

The role of *gadD2* has previously been implicated in the survival of *L. monocytogenes* strain LO28 under severely acidic conditions (Cotter *et al.*, 2001). It was expected that deletion of this gene from EGD-e would result in a strain highly sensitive to exposure to a pH of 2.5. Unexpectedly both this strain, EGD-e Δ *gadD2* and EGD-e Δ *gadD1* had a far greater survival rate at low pH compared to the wild-type EGD-e strain (Fig. 3.8). The reasons for this are still unclear, however it would appear that this increase in acid resistance is coupled with an apparent increased capacity to generate GABA_i when exposed to low pH (Fig. 3.10). It may be that accumulation of large amounts of GABA_i benefits the cell indicating an important role for intracellular glutamate decarboxylase action (GAD_i). LO28 does however possess GAD_e activity (Karatzas *et al.*, 2010) suggesting that the difference seen here with *gadD2* is due to strain differences in overall GAD activity.

Alternatively this increase in acid tolerance may be due to an overcompensation by the two remaining GAD enzymes within the strain. Unfortunately very little is known about the regulation of the GAD system in *L. monocytogenes*. From analysis of transcript levels for each of the GAD genes (Fig. 3.12), it would appear that at a transcriptional level there is a direct correlation between increased gene transcription and increased acid survival. While *gadD3* shows increased expression in the EGD-e Δ *gadD2* strain, it is not increased in EGD-e Δ *gadD1* (Fig. 3.12). Therefore, increased compensation by *gadD3* cannot be a direct cause for the increased GABA/acid survival. The fact that σ^B activity appears to be increased in both EGD-e Δ *gadD1* and EGD-e Δ *gadD2* (Fig. 3.12) suggests that

another σ^B regulated pathway may be playing a role in the response to acid or that σ^B is in some way having a role to play in regulation of the activity of the GAD enzymes themselves.

Despite finding that both EGD-e $\Delta gadD1$ and EGD-e $\Delta gadD2$ accumulate increased concentrations of GABA, there was no negative effect of this on cell growth. Both of these strains were able to grow equally when compared to EGD-e when first exposed to pH 4.0 and subsequently pH 7.0 (Fig. 3.11). Even at pH 4.0, growth of all four strains remained similar (Fig. 3.10). The only gene that appeared to play a role in growth at low pH was *gadD3*. The strain carrying a deletion in this gene has a reduced growth rate at the mild pH of 5.0 (Fig. 3.10). This suggests that *gadD3* is important for the ability of this strain to grow in slightly acidic environments. A role for this gene had not previously been identified with work in LO28 as the gene had not been deleted (Cotter *et al.*, 2005).

The role that the GAD system plays in survival inside human derived macrophages appears to be limited. After a 7 h co-incubation period, both the wild-type EGD-e and the three GAD mutants grew in number by over 2-log cycles (Fig. 3.13). It would however appear that EGD-e $\Delta gadD1$ and EGD-e $\Delta gadD2$ possess a longer lag phase in their intracellular growth. In order to replicate inside the host macrophage, *L. monocytogenes* cells must first escape the phagolysosome. It is tempting to predict that the extended lag period for EGD-e $\Delta gadD1$ and EGD-e $\Delta gadD2$ may be due to a delay in escaping from the phagolysosome. An attempt to visualise compartmentalised bacteria over the course of the experiment would perhaps answer this question. Preliminary efforts to perform this were tried without success however a full study was beyond the scope of the project. Despite the initial lag seen, it would appear that there is no overall net deficiency that would hinder these two strains in continuing their virulence cycle.

An important discovery from this work was the clear difference between EGD-e and EGDm in terms of acid induced GABA production. While several publications have highlighted the different phenotypes between different strains of *L. monocytogenes* even within the same serotype, it is generally assumed that lab reference strains from the same source behave the same and can be compared like for like. The EGD-e from our laboratory and the EGDm from UCC should therefore in theory be identical, albeit that EGDm possesses a murinised copy of *inlA*. Despite this, the EGDm strain was seen to generate almost 3.5 times (p value <0.02) the amount of GABA_i than EGD-e after acid treatment (Fig. 3.18). It is unlikely that this is due to the altered *inlA* although this has not been ruled out. It is clear that care must be taken when drawing experimental conclusions from lab strains as it appears that years of passaging of the strain may have led to unidentified changes. The first published genome of *L. monocytogenes* in 2001 was of this same EGD-e strain (Glaser *et al.*, 2001). As such genetic manipulation of this strain is based upon the published sequence. However if this varies greatly between different EGD-e types then it may lead to difficulty with future manipulation.

Overall it appears that the deletion of a single GAD gene from EGD-e does not have a negative impact on survival at low pH. In this strain however there appears to be a strong correlation between increasing accumulation of GABA_i with an increase in acid survival. As EGD-e solely utilises GAD_i it would appear that the GABA derived from this pathway is capable of protecting the cell. This GABA however is not essential as the EGD-e Δ *gadD3* strain can survive equal to the EGD-e strain and yet produces roughly half the concentration of GABA_i. Despite being considered an acidic stress hurdle for *L. monocytogenes* to overcome en route to infection, it appears that the GAD system does not play a significant role in survival inside host macrophages. The role that the GAD system may play in gastric passage and virulence *in vivo* is dealt with in the following chapter.

CHAPTER 4

Divergent evolution of the activity and regulation of the glutamate decarboxylase systems in *Listeria monocytogenes* EGD-e and 10403S: Roles in virulence and acid tolerance.

Note

Much of the material presented in this chapter forms part of a paper prepared for publication, however the methods section has been removed to prevent duplication with Chapter 2.

4.1 Abstract

The glutamate decarboxylase (GAD) system has been shown to be important for the survival of *Listeria monocytogenes* in low pH environments. The bacterium can use this faculty to maintain pH homeostasis under acidic conditions. The accepted model for the GAD system proposes that the antiport of glutamate into the bacterial cell in exchange for γ -aminobutyric acid (GABA) is coupled to an intracellular decarboxylation reaction of glutamate into GABA, that consumes protons and so facilitates pH homeostasis. Most strains of *L. monocytogenes* possess three decarboxylase genes (*gadD1*, *D2* & *D3*) and two antiporter genes (*gadT1* & *gadT2*). Here, we confirm that the *gadD3* encodes a glutamate decarboxylase dedicated to the intracellular GAD system (GAD_i). We also compare the functionality of the GAD system between two common reference strains, EGD-e and 10403S. Through functional genomics we show that EGD-e that is unable to use the antiport only utilises the GAD_i and heavily relies on the action of the GadD3 decarboxylase for this, confirming that it is an integral part of GAD_i. In contrast 10403S relies upon GadD2 to maintain both an intracellular and extracellular GAD system (GAD_i/GAD_e). Through experiments with a murinised strain of EGD-e (EGDm) in macrophages and mice we found that the GAD system might play only a minor role in gastric passage and survival during phagocytosis although overall it does contribute significantly to colonization of the host. Most importantly, EGDm utilises only GAD_i and it is possible that in another background the GAD system might play an important role in virulence. In conclusion the evidence presented indicates a separate line of evolution in the GAD system between two commonly used reference strains.

4.2 Introduction

Survival in sometimes harsh environmental conditions is vital for any pathogens en route to infection of the host. The foodborne pathogen *Listeria monocytogenes* is well noted for an ability to withstand high salt environments (Abram *et al.*, 2008a; Cole *et al.*, 1990; Utratna *et al.*, 2011), survive high pressure (Karatzas *et*

al., 2003; Wemekamp-Kamphuis *et al.*, 2004a), grow at low temperature (Walker *et al.*, 1990) and has a broad pH range (Cole *et al.*, 1990; Heavin *et al.*, 2009; Young KM, 1993). This makes it a major concern for the food industry where preservation methods are often inadequate. In order for *L. monocytogenes* to survive low pH environments, the bacterium has evolved several mechanisms that allow it to maintain pH homeostasis. These include the arginine deiminase system (Ryan *et al.*, 2009a), an F₀F₁ ATPase (Cotter *et al.*, 2000), the adaptive acid tolerance response (ATR; Davis *et al.*, 1996) and the glutamate decarboxylase (GAD) system (Cotter *et al.*, 2001). The GAD system has been shown in *L. monocytogenes* to be important for survival both in synthetic gastric fluid (Cotter *et al.*, 2001) and organic acids (Heavin *et al.*, 2009) commonly found in foods.

The accepted model for the GAD system (Fig. 4.1) involves the combined action of a membrane bound antiporter (GadT) and a cytosolic glutamate decarboxylase (GadD). During exposure to low pH, the bacterium can exchange an extracellular molecule of glutamate for an intracellular molecule of GABA via the GadT antiporter. This imported glutamate then undergoes a decarboxylation to form GABA via the GadD enzyme. At pH > 4.5, glutamate is imported in a deprotonated state (Tsai *et al.*, 2013), which allows the removal of intracellular H⁺ when glutamate is converted to GABA. This consumption of intracellular protons helps maintain a tolerable intracellular pH. GABA generated via this reaction is expected to exit the cell via the antiporter in exchange for further glutamate, allowing a cycling process to continue (Fig. 4.1). In previous work we have shown that GAD activity can take place independently of the antiporter, a finding that prompted a revision of the previous model by introducing the concepts of extracellular GAD system that relies on the Glu/GABA antiport and the intracellular GAD system that relies on intracellular pools of glutamate (Karatzas *et al.*, 2012).

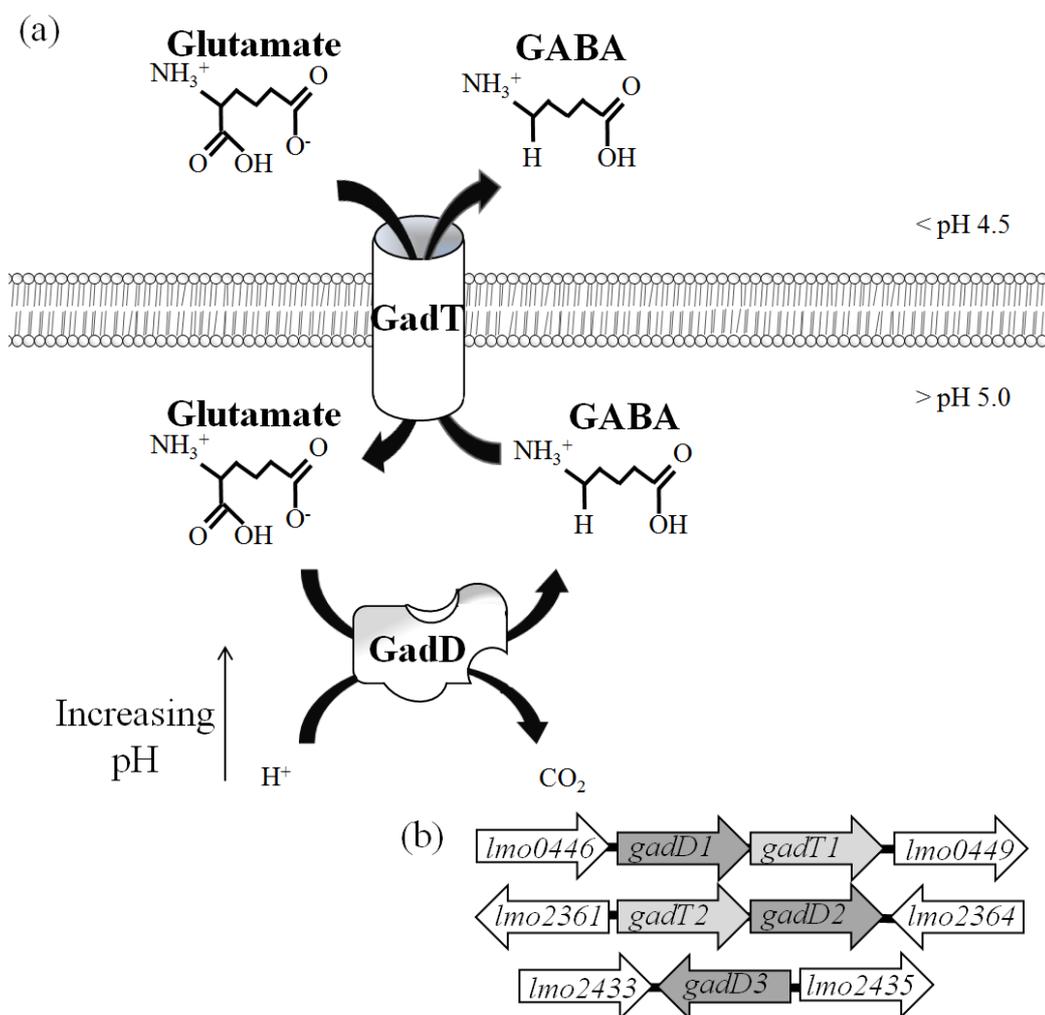


Figure 4.1. (a) The standard model for the action of the GAD system. A membrane bound antiporter carries glutamate into the cell in exchange for GABA. A cytosolic decarboxylase enzyme converts glutamate to GABA, with a consumption of H^+ . **(b)** The genomic structure of the genes encoding the GAD system in *L. monocytogenes* EGD-e.

For *L. monocytogenes*, the glutamate/GABA antiporter can be encoded by one of two genes (*gadT1*, *T2*) while it possesses up to three decarboxylase encoding genes (*gadD1*, *D2*, and *D3*; Glaser *et al.*, 2001). Previously, *gadD3* has been considered as a putative glutamate decarboxylase encoding gene and some lines of evidence suggest that this glutamate decarboxylase may form an integral part of the GAD_i (Karatzas *et al.*, 2012). These genes are encoded in three transcriptional units *gadD1T1*, *gadT2D2* and *gadD3* (Cotter *et al.*, 2005; Fig. 4.1 b). While all strains possess both *gadT2D2* and *gadD3*, strains from serotype 4 do not possess

the *gadD1T1* operon (Cotter *et al.*, 2005). Previously, genetic studies on the GAD system have shown that *gadD2* is critical for survival of *L. monocytogenes* at low pH, while *gadD1* is important at a milder pH (Cotter *et al.*, 2001, 2005). This work was carried out in the strain LO28 (serotype 1/2c), however recent work has shown that the GAD system can behave differently depending on both strain and media type. Analysis of strain EGD-e has shown that the antiporters are non functional, which results in a GAD system (which we call GAD_i) that produces intracellular GABA from the cytoplasmic pool of glutamate (Karatzas *et al.*, 2012). Furthermore, in a minimally defined media, the strain 10403S also relies solely on the activity of GAD_i, despite having a functional antiport mechanism (Karatzas *et al.*, 2010).

Despite what has been learned so far it is apparent that the accepted model of the GAD system in *L. monocytogenes* is not complete and certain knowledge gaps remain including elucidating the role that the GAD system plays *in vivo*. While studies have shown that the GAD system is important for survival in porcine gastric juice no attempt has been made to define the role of this system during pathogenesis in a live animal model. In order to study *L. monocytogenes* infection in a mouse model, the surface ligand InlA must first be engineered to generate a version that is capable of productive interactions with the murine E-cadherin receptor. The native InlA for *L. monocytogenes* cannot bind to E-cadherin expressed on mouse epithelial cell due to the substitution of a proline with a glutamic acid on position 16 of the amino acid sequence for murine E-cadherin (Wollert *et al.*, 2007). This hinders the use of a mouse model in studying *L. monocytogenes* infection. To overcome this, the InlA protein of EGD-e was murinised, by targeted mutagenesis, resulting in the changing of two key amino acids (Ser192Asn and Tyr369Ser; Monk *et al.*, 2008).

Here we compare the GAD system of two important reference strains of *L. monocytogenes*, EGDm (a murinised form of EGD-e) and 10403S. We describe an extensive genetic and physiological analysis of the GAD system in these strains and elucidate the role of the system in pathogenesis within a mouse model.

4.3 Results

4.3.1 Deletion of *gadD1* or *gadD2* increases acid survival in EGDm

Strains lacking either a single or pair of GAD genes were tested for survival at low pH *in vitro*. After 20 min the numbers of surviving wild-type EGDm cells began to rapidly reduce as did those of the strain with a deletion in *gadD3*. Sixty minutes post treatment both EGDm and EGDm Δ *gadD3* had reduced by over 4.5 log-cycles (Fig. 4.2 a). In contrast the numbers of both EGDm Δ *gadD1* and EGDm Δ *gadD2* strains reduced by only ~2 log-cycles after 60 min. Measurements of the transcript levels of each GAD gene prior to acid exposure showed that *gadD3* was expressed by almost 2 orders of magnitude greater than either *gadD1* or *gadD2* (Fig. 4.2 c). Deletion of any of the GAD genes did not appear to significantly affect the base levels of any of the remaining two GAD genes. Thus the increased acid resistance observed in the EGDm Δ *gadD1* and EGDm Δ *gadD2* strains cannot be explained by an increase in the transcription of the remaining GAD genes.

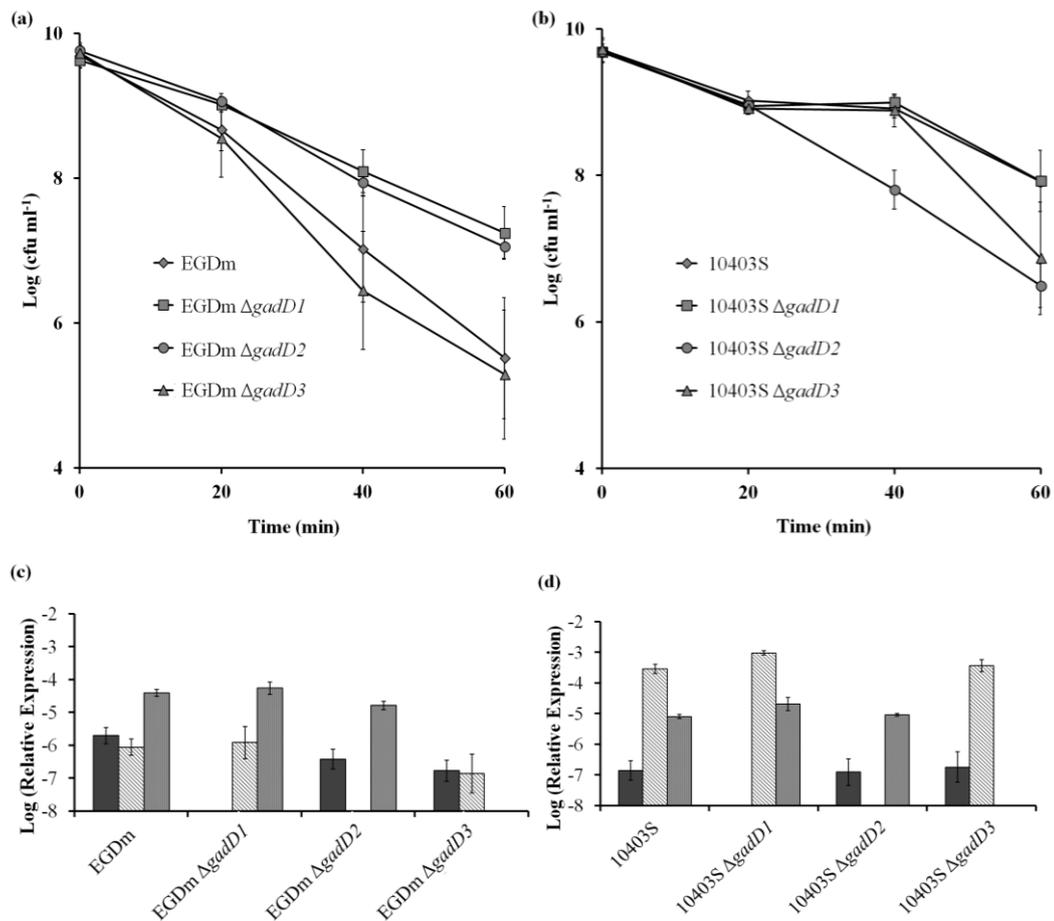


Figure 4.2. Acid survival of *L. monocytogenes* gad mutants. Stationary phase EGDm (a) and 10403S (b) Δ gad mutants were challenged at pH 2.5. Cell counts were taken every 20 min. Values are the means of data from three individual cultures, with the cell counts for each culture being the means of counts from three platings. Relative transcript levels of EGDm (c) or 10403S (d) *gadD1* (dark grey fill), *gadD2* (hatched) and *gadD3* (grey) genes to 16S gene prior to acid exposure in each mutant strain. Error bars represent the standard error from the mean value of three individual biological repeats.

4.3.2 Deletion of *gadD2* and *gadD3* reduces acid tolerance in 10403S

Following from the results observed for EGDm, deletion mutants were constructed for the GAD system genes in a second well-studied reference strain, 10403S. Once more the effect of acid treatment was tested on stationary phase cultures for 1 h in BHI. After 60 min wild-type 10403S showed a reduction in cell numbers by over 1.5 log-cycles (Fig. 4.2 b). This level of survival was much

greater than that seen in EGDm (Fig. 4.2 **a**). Deletion of *gadD1* did not affect survival at this pH compared to the wild-type. For 10403S Δ *gadD2* a steady decrease in cell numbers was observed over 60 min with a final reduction of about 3 log-cycles, 100-fold greater than the parent strain. 10403S Δ *gadD3* did not show a significant reduction in cell numbers compared to 10403S until after 40 min of acid treatment and reached a final reduction of about 3 log-cycles after 60 min (Fig. 4.2 **b**). A profile of the GAD gene transcript levels prior to acid exposure in 10403S showed that *gadD2* is dominantly expressed (Fig. 4.2 **d**). As in EGDm, deletion of one GAD gene did not significantly affect the expression of any remaining gene. Together these results suggest that the two strains have evolved different transcriptional controls over the GAD genes and further indicate that GAD gene transcript levels *per se* don't determine the intrinsic level of acid tolerance.

4.3.3 EGDm relies on *gadD3* for GABA production while 10403S requires *gadD2*

GABA is the main product of the GAD system and therefore measurement of its production could give insights into how the systems work in the strains. To this end both strains were challenged to mild acidic pH for one hour and both the extracellular and intracellular GABA (GABA_e/GABA_i) production was measured. Previous work has indicated that EGD-e does not produce GABA_e while 10403S produces both GABA_e and GABA_i (Karatzas *et al.*, 2012). As expected, GABA_e was not produced here by any EGDm strain in response to low pH (Fig. 4.3 **a**). After 1 h at pH 4.0 however EGDm produced over 5 mM GABA_i. An important finding was that EGDm produced higher concentrations of GABA_i compared to our previously studied EGD-e strain (Fig. 3.18; Section 3.4.2). Since the parent EGD-e strains came from separate laboratories it may be that some mutation had arisen in one of the lines that impacts on the GAD system. Deletion of either *gadD1* or *gadD2* however did not affect GABA_i production compared to the parent strain (Fig. 4.3 **b**). GABA production in EGDm Δ *gadD3* however was almost abolished, with production just above detectable levels for the assay (0.45 mM; Fig. 4.3 **a**). Unlike EGDm, 10403S produced both GABA_i (2.72 mM) and

GABA_e (3.35 mM) after acid treatment. Deletion of *gadD1* or *gadD3* in did not significantly affect the acid-induced GABA production. 10403S Δ *gadD2* did have a significant decrease in both GABA_i (0.64 mM; Fig. 4.3 b) and GABA_e (0.75 mM; Fig. 4.3 a). Thus the GAD gene with the highest transcript levels in each strain also contributed the most to GABA production in that strain; *gadD3* for EGDm and *gadD2* for 10403S.

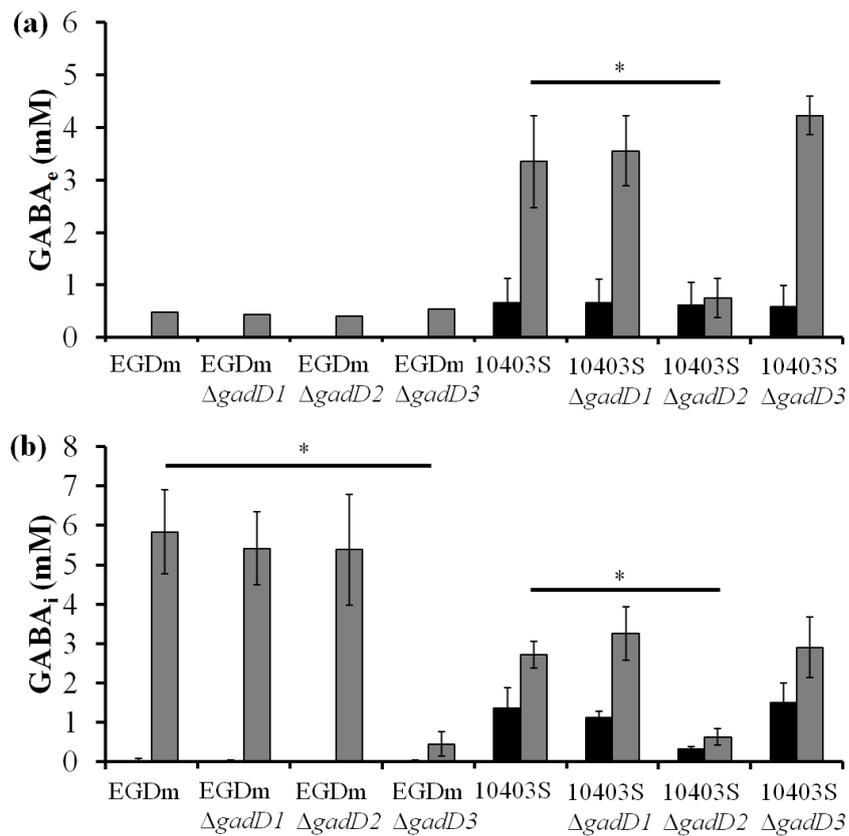


Figure 4.3. GABA production from *L. monocytogenes* gad mutants. (a) Production of GABA_e by EGDm and 10403S gad mutants with (grey) or without (black) 1 h exposure to acid at pH 4.0 (EGDm) or pH 3.5 (10403S). (b) Production of GABA_i by EGDm and 10403S *gadD* mutants with (grey) or without (black) 1 h exposure to acid at pH 4.0 (EGDm) or pH 3.5 (10403S). Error bars represent the standard deviation from the mean of three individual biological repeats for each sample. An asterisk represents significant difference of less than 0.05 between a given mutant and respective wild-type as determined by a student's *t*-test.

4.3.4 Expression of the GAD system is pH-independent

To determine the transcriptional response of EGDm and 10403S to both low pH exposure and GAD system mutation, the expression levels of all three decarboxylase genes was compared to the expression of 16S rRNA in each strain. Overall expression of the GAD genes did not change in EGDm in response to low pH (Fig. 4.4 a). The expression of *gadD3* remained higher than both *gadD1* and *gadD2* in the parent EGDm strain and EGDm Δ *gadD1* and EGDm Δ *gadD2* throughout the course of the acid challenge. In 10403S, the relative expression of *gadD2* was significantly greater than the other genes at stationary phase (Fig. 4.2 d). Deletion of any of the GAD genes did not affect the expression of the remaining two in stationary phase. The expression of the GAD genes did not appear to change in response to acid challenge for any of the four 10403S strains (Fig. 4.4 b). σ^B activity as measured via *lmo2230* appeared to remain stable in all strains throughout the acid challenge (Fig. 4.4 a & b; bottom right). The use of *lmo2230* as a reporter of σ^B activity has been described previously (Utratna *et al.*, 2011) Overall it appeared that regardless of pH *gadD3* was the predominately expressed GAD system gene in EGDm while for 10403S it was *gadD2*.

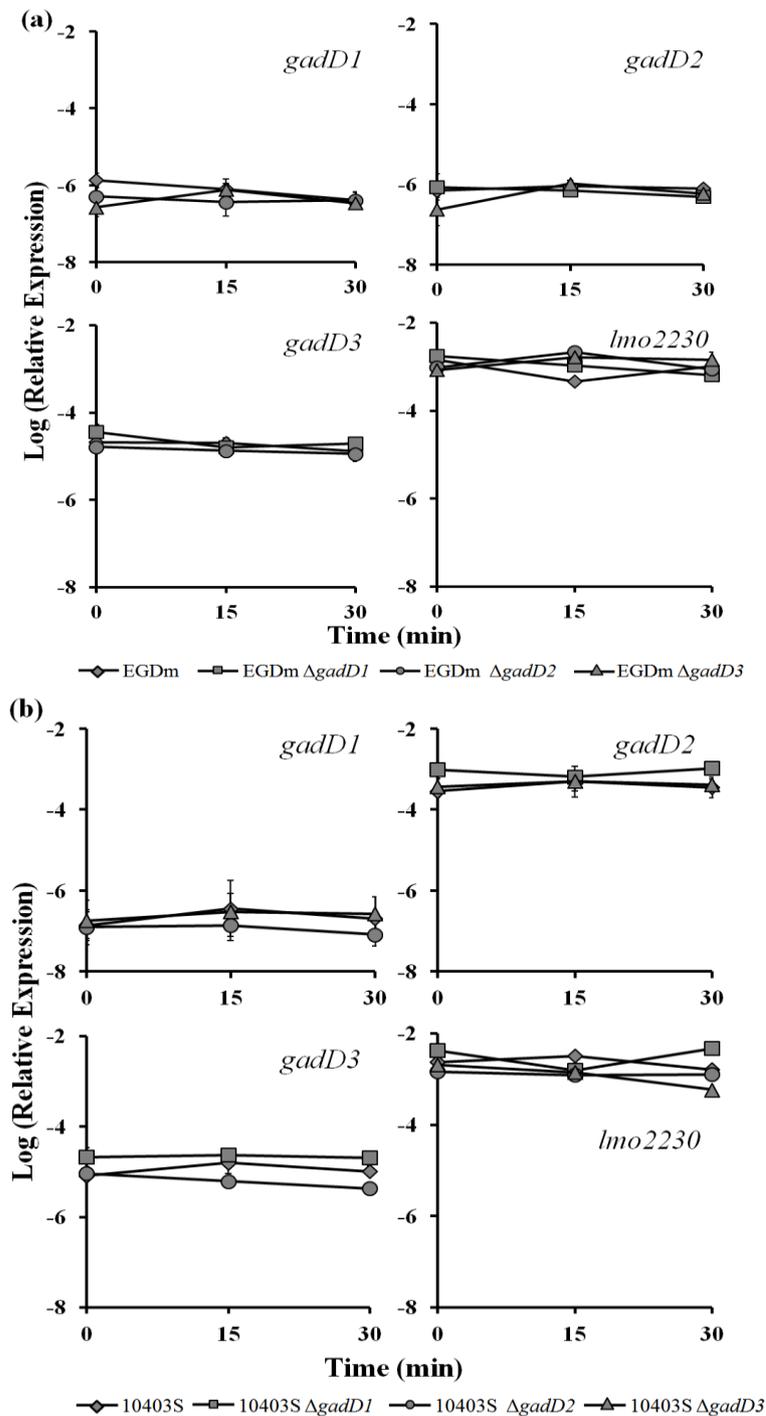


Figure 4.4. Relative expression of gad genes in response to acid treatment. Expression of *gadD1*, *gadD2*, *gadD3* and *lmo2230* relative to expression of the 16S rRNA gene prior to, 15 min and 30 min after exposure in BHI broth to pH 4.0 (EGDm (a)) or pH 3.5 (10403S (b)). Error bars represent the standard error in the mean of 3 independent biological repeats. Differences found to be significant between the genes at any time-point for each strain are shown with an asterisk [*]. Significance was determined where $p < 0.05$ as determined by a Student's *t*-test.

4.3.5 Growth of *L. monocytogenes* GAD system mutants in human THP-1 macrophages

In order to cause infection *L. monocytogenes* must be able to survive inside phagocytic macrophages. The environment that the bacteria encounters inside these cells is reported to be acidic (de Chastellier & Berche, 1994) and thus we investigated the role that the GAD system may play in survival. None of the mutants of EGDm showed a significant difference in uptake rate by THP-1 cells compared to the wild type after 2 h (Fig. 4.5 a). Furthermore, there was no significant difference seen in the ability of any strain to grow inside the THP-1 cells over a 7 h time-course. EGDm Δ *gadD1* and EGDm Δ *gadD2* did however show a significant lag until 5 h but eventually reached similar numbers to both EGDm and the *gadD3* mutant (Fig. 4.5 a). Similarly, 10403S and its isogenic GAD system mutants grew inside THP-1 macrophages with only an apparent lag seen for 10403S Δ *gadD2* after 3 h. Otherwise no significant differences were seen between the wild-type and mutants for the duration of the experiment.

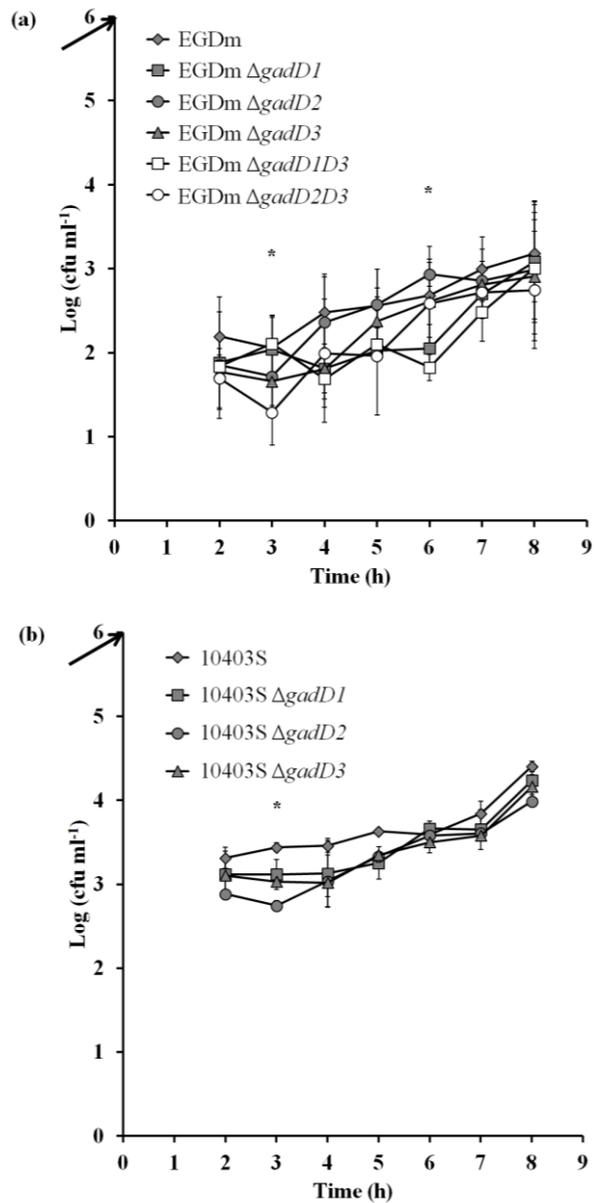


Figure 4.5. Growth of GAD system mutants in THP-1 macrophages. Growth of EGDm (a) and 10403S (b) GAD system mutants inside THP-1 macrophages over 7 h. Counts are recorded 2 h post co-incubation of THP-1 with bacteria at an MOI of 10 (10^6 bacteria; black arrow). Error bars represent the standard deviation from the mean of at least 4 biological replicates for each strain and time-point. Significant differences (*; $p < 0.05$) were determined using one-way ANOVA.

4.3.6 EGDm GAD mutants survive gastric passage in mice

In order to analyse the role that the GAD system plays *in vivo*, survival through a live animal gastric passage was carried out. Three days post oral inoculation of female Balb/C mice with each of the EGDm *gad* mutants, the animals were sacrificed and dissemination of the strains was analysed. Counts ranged from 6.50×10^3 to 3.80×10^7 cfu ml⁻¹ and were similar in the liver, spleen and intestinal content for all single deletion strains and the respective wild-type. The counts for EGDm Δ *gadD1* and EGDm Δ *gadD3* however were significantly lower in the mesenteric lymph node (MLN) compared to the wild-type (1-log; Fig. 4.6). Double deletion of either *gadD1* with *gadD3* or *gadD2* with *gadD3* resulted in reduced counts from both the liver and spleens of mice 3 days post infection. There was no significant reduction seen in the MLN counts for the double mutants. These data suggest that while the GAD system may play a minor role within the host during the development of an infection it is not essential for virulence even when the bacteria are administered orally.

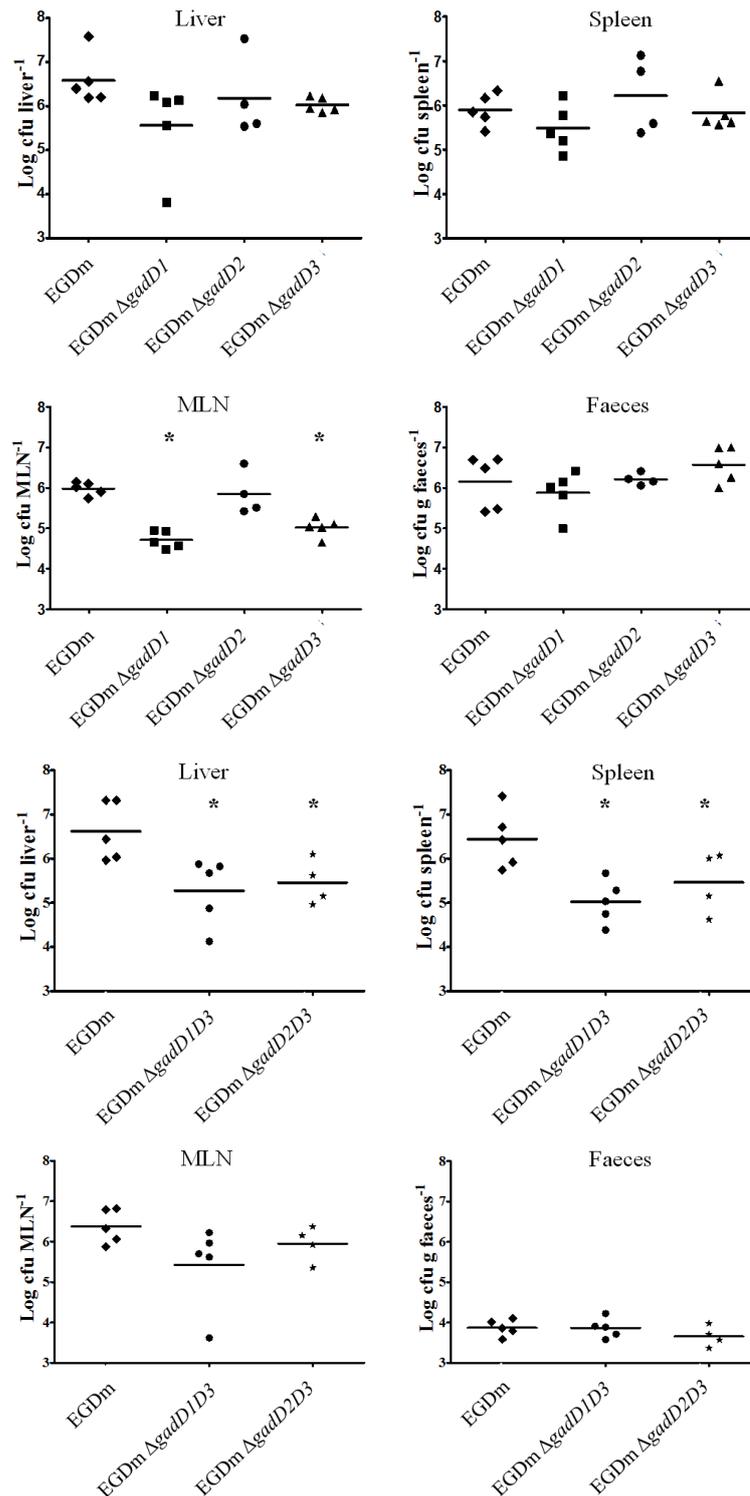


Figure 4.6. Infection of Balb/C mice with EGDm GAD system mutants. Plate counts of surviving EGDm GAD system mutants 3 days post infection from female Balb/C mice (n=5). Isolated from the liver, spleen, mesenteric lymph node (MLN) and faeces. Significant differences (*) between wild-type and mutants were determined using one-way ANOVA.

4.3.7 Deletion of *gadD3* together with either *gadD1* or *gadD2* reduces acid tolerance seen in single mutants and abolishes GABA production

To account for the increase in acid survival seen in both EGDm Δ *gadD1* and EGDm Δ *gadD2* (Fig. 4.2 a) double knockout mutant were tested for survival to exposure in pH 2.5 in BHI. EGDm Δ *gadD1* Δ *gadD3* counts reduced at a faster rate than EGDm and were about 1-log cycle lower than the wt after 60 min (Fig. 4.7). The presence of *gadD2* on its own did not appear to protect the strain. Deletion of both *gadD2* and *gadD3* however followed a pattern of survival similar to the wild-type but reduced compared to the single knockout of *gadD1*. Overall it appeared that removing the *gadD3* gene from EGDm Δ *gadD1* or EGDm Δ *gadD2* negatively impacted on the increased acid survival seen with these strains, further highlighting the importance of *gadD3* to this strain.

GABA_i was measured in each strain as previously after exposure to pH 4.0. The mutants carrying double deletions both failed to produce GABA_i in response to exposure to the low pH. Possession of either *gadD1* or *gadD2* alone did not bestow an ability to produce GABA. As EGDm is unable to utilise the GAD_e system no GABA_e was produced by any of these strains in response to acid treatment (data not shown).

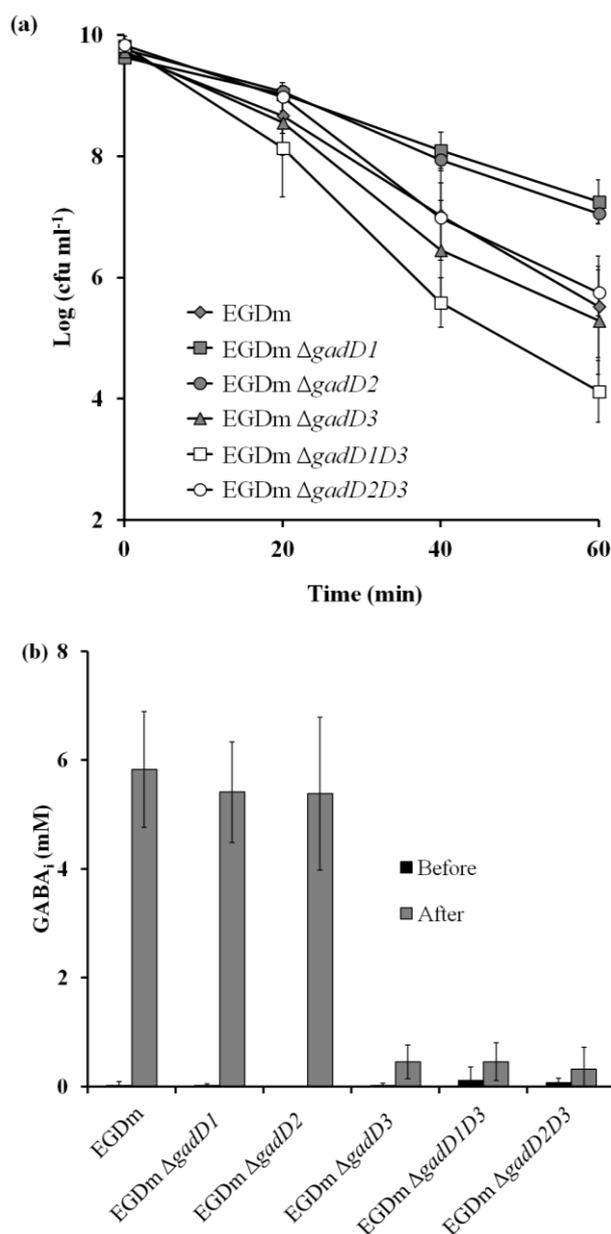


Figure 4.7. Acid survival and GABA_i production of *L. monocytogenes* EGDm double GAD system mutants indicates a key role for *gadD3*. (a) Stationary phase EGDm *gadD* mutants were acidified to pH 2.5 with 3 M HCl in BHI broth. Cell counts were taken every 20 min. Values are the means of data from three individual cultures, with the cell counts for each culture being the means of counts from three platings. Error bars represent the standard deviation from the mean value for each time-point. (b) Stationary phase EGDm *gad* mutants were acidified (grey) or not acidified (black) with 3 M HCl to pH 4.0 and GABA_i accumulation was quantified. Error bars represent the standard deviation from the mean of three independent biological replicates.

4.4 Discussion

Previous studies with *L. monocytogenes* have shown that strains can vary greatly in terms of their ability to cope with low pH (Cotter *et al.*, 2001; Karatzas *et al.*, 2012). Here the GAD system, one of the major mechanisms for acid tolerance was compared between two commonly used reference strains EGDm and 10403S. The survival of 10403S during exposure to low pH is significantly greater than EGDm (Fig. 4.2) and this correlates with an ability of 10403S to utilise both GAD_i and GAD_e in response to low pH. Despite possessing the genes for a putative glutamate/GABA antiporter, EGDm does not export GABA. This may be due to the fact that the amino acid sequences of *gadT2* are different by four amino acids between 10403S and EGDm (V409I, V419I, M438T, I441M, respectively; Appendix 3). However, efforts to clone the *gadT2* gene from 10403S into EGDm were unsuccessful in inducing GABA_e production in EGDm (data not shown). This may be due to an incompatibility between the native glutamate decarboxylase with the introduced antiporter. Under acidic conditions, GAD_e activity occurs after the recruitment of the cytosolic decarboxylase closer to the cytosolic side of the cell membrane (Capitani *et al.*, 2003). If this movement cannot happen then GABA export would be unlikely. It is predicted that expression of *gadT2* together with *gadD2* from 10403S, i.e. the entire *gadT2D2* operon would be required to induce GABA_e production in an EGD-e background.

As EGDm appears to be solely reliant upon intracellular decarboxylation (GAD_i), the three genes encoding glutamate decarboxylases (*gadD1*, *gadD2* and *gadD3*) were deleted. Deletion of *gadD1* or *gadD2* improved the ability of the strain survive low pH (Fig. 4.2 **a**). Surprisingly the increase in acid tolerance was comparable to the levels of resistance seen for 10403S, which is one of the most resistant strains of *L. monocytogenes* (Feehily *et al.*, 2013; Fig. 4.2 **b**). None of the single deletions were seen to reduce the bacterium's ability to survive at low pH, showing that each is dispensable for wild-type acid tolerance levels. However,

deletion of both *gadD1* and *gadD3* together did prevent the increase in survival seen for EGDm Δ *gadD1* and this double mutant strain, EGDm Δ *gadD1D3* was more acid sensitive than EGDm (Fig. 4.7 a). The deletion of *gadD3* along with *gadD2* also appeared to prevent the increase in acid tolerance seen in the Δ *gadD2* mutant. Measurement taken of GABA_i across the strains in response to acid, indicated that only EGDm Δ *gadD3* was impaired in an ability to produce GABA_i. Taken together with the acid survival data, it would appear that this decarboxylase may play a more important role in the GAD system of this strain compared with remaining two. Interestingly, a different effect was observed in 104033S after the deletion of the GAD genes. Here deletion of *gadD2* and to some extent deletion of *gadD3* negatively impacted on survival (Fig. 4.2. b). In 10403S the Δ *gadD2* deletion was also accompanied by a reduction in both GABA_i and GABA_e. It would appear that failure to produce GABA in this strain impacted negatively on its ability to survive at low pH. As *gadD2* forms an operon with *gadT2* it is not surprising that both GABA_i and GABA_e production was affected. The remaining GadT1D1 system apparently could not compensate for the loss of GadT2D2 activity in this strain although this is perhaps not surprising as this pair was shown to play a role in acid growth milder pH (Cotter *et al.*, 2005).

Examination of the transcriptional response for each of the strains in response to acid confirms the differential importance of either *gadD2* or *gadD3* for 10403S and EGDm, respectively. The *gadD2* transcript was the most abundant of the three in 10403S in stationary phase cultures, whereas *gadD3* was dominant in the EGDm background (Fig. 4.4). Overall, neither strain displayed an alteration in GAD gene expression in response to the pH treatment. This may be due to the fact that the cultures have already reached stationary phase and therefore were expressing each gene to a maximal level. Although little is still known about the transcriptional regulation of the GAD system in *L. monocytogenes*, it is known that both the *gadT2D2* operon and *gadD3* are at least partially under the control of σ^B . Using *lmo2230* as a reporter of σ^B activity (Utratna *et al.*, 2011, 2014), there was clearly no change in σ^B activity over the course of the acid treatment or as a result of the deletions in the GAD genes. As shown previously (Utratna *et al.*,

2011, 2014) σ^B is fully active in stationary phase and additional stress doesn't enhance its activity beyond that level. This might indicate that the cells already possess functional GAD system proteins prior to acid shock and that regulation of the GAD system occurs post transcriptionally.

While much of the previous work undertaken on the GAD system in *L. monocytogenes* has focused on *in vitro* models and synthetic gastric fluid, the role of the system *in vivo* has not been addressed previously. In fact very little evidence has been shown in any bacterial pathogen that the GAD system is important for survival in the host. Work with *Brucella microti* has however shown that deletion of the GAD system reduced counts in mice after oral inoculation (Occhialini *et al.*, 2012). In this study we see that in both EGDm and 10403S, the GAD system does not appear to play a major role in survival within human derived macrophages. This may not be surprising seen as the pH generally found inside bone marrow derived macrophages after compartmentalisation of *L. monocytogenes* is about pH 5.5 (de Chastellier & Berche, 1994). Induction of the GAD system normally occurs below pH 4.5 (Karatzas *et al.*, 2010) in *L. monocytogenes* and therefore the milder pH of the macrophages would mean there is no role for the GAD system to play. Interestingly, although GadDIT1 is known to play a role in growth at mild acidic conditions (Cotter *et al.*, 2005) similar to those occurring within macrophages no major effect of these proteins was documented within macrophages.

Due to the extensive use of the EGD-e strain as a model intracellular pathogen (Chatterjee *et al.*, 2006; Joseph *et al.*, 2006; Toledo-Arana *et al.*, 2009), we focused upon the role of the GAD system in the virulence of this strain in a live mouse model. These mice were inoculated via the oral route to simulate gastric passage. No significant role was obtained for the GAD system in gastric passage here. Both the liver and spleen were infected equally by either wild type or single deletion mutants 3 days post infection. However the two double mutants did display a reduced infection for the liver and spleen, indicating that a complete

GAD system may be required for full infection. This correlates with *in vitro* survival assays with these mutants, particularly EGDm Δ *gadD1D3*, which displays a greater sensitivity to low pH than the single mutants. Interestingly, bacterial counts in the faeces for all strains were unchanged which suggests that the deletions did not impair the ability of the bacteria to pass through the stomach of the mice. Taken together with the macrophage data, it would appear that the GAD system does play a role in virulence however due to an apparent inbuilt redundancy, at least two of the genes encoding the GAD system must be deleted in order to observe an effect. EGDm however is one of the most acid sensitive strains tested and importantly it does not possess a functional GAD_e system. The importance of the role for GAD_e therefore cannot be established and may yet prove to be important in virulence. A study identifying genes expressed by *L. monocytogenes* in response to *Lactobacilli* within the mouse gut identified that all three gene systems (*gadD1T1*, *gadD2T2* & *gadD3*) had increased expression (Archambaud *et al.*, 2012). This evidence suggests that the GAD system is active within the host.

Overall, the GAD system in *L. monocytogenes* appears to show a clear divergence in evolutionary functionality between these two well-studied strains. Strains appear to have adapted the use of either a GAD_i system or both a GAD_i/GAD_e system which may reflect an importance based on the strains evolutionary histories. The strain which possessed both GAD_i and GAD_e, 10403S, displayed a more acid resistant phenotype suggesting that a functional antiport is highly beneficial. In contrast, the strain which utilised only GAD_i did not appear to have a sole reliance on any of its three isoforms of decarboxylases for acid tolerance, indicating a more robust system. From comparing these two strains it is clear that the knowledge of the GAD system in *L. monocytogenes* is not complete and in order to fully appreciate the role that the system plays, strain comparison is essential.

CHAPTER 5

***Listeria monocytogenes* has a
functional γ -aminobutyrate (GABA)
shunt: Role in acid tolerance and
succinate biosynthesis.**

Note

Much of the material presented in this chapter has been published (Feehily *et al.*, 2013), however the methods section has been removed to prevent duplication with Chapter 2. Relevant data, obtained since the publication (Sections 5.3.4 and 5.3.5) have also been included and discussed in the final section.

5.1 Abstract

Listeria monocytogenes, the causative agent of human listeriosis, is known for its ability to withstand severe environmental stresses. The glutamate decarboxylase (GAD) system is one of the principal systems utilised by the bacterium to cope with acid stress, a reaction that produces γ -aminobutyrate (GABA) from glutamate. Recently we have shown that GABA can accumulate intracellularly under acidic conditions, even under conditions where no extracellular glutamate-GABA exchange is detectable. The GABA shunt, a pathway that metabolises GABA to succinate, has been described for several other bacteria genera and the present study sought to determine whether *L. monocytogenes* has this metabolic capacity, which if present could provide a possible route for succinate biosynthesis in *L. monocytogenes*. Using crude protein extracts from *L. monocytogenes* EGD-e we show that this strain exhibits activity for the two main enzyme reactions in the GABA shunt, GABA amino-transferase (GABA-AT) and succinic semialdehyde dehydrogenase (SSDH). Two genes were identified as candidates for encoding these enzyme activities, *argD* (GABA-AT) and *lmo0913* (SSDH). Crude protein extracts prepared from a mutant lacking a functional *argD* gene had significantly reduced GABA-AT activity, while an *lmo0913* mutant had lost all detectable SSDH activity. Deletion of *lmo0913* increased the acid tolerance of EGD-e and showed an increased accumulation of intracellular GABA suggesting that this pathway plays a significant role in the survival of this pathogen under acidic conditions. This is the first report of such a pathway in the genus *Listeria*, which highlights an important link between metabolism and acid tolerance and also presents a possible compensatory pathway to partially overcome the incomplete tricarboxylic acid cycle of *Listeria*.

5.2 Introduction

Listeria monocytogenes is a food borne pathogen that causes listeriosis, a disease with a mortality rate of up to 30% (Farber & Peterkin, 1991). The disease has

recently shown an increase in reported cases across Europe (Allerberger & Wagner, 2010). Almost 99% of cases are associated with a contaminated food source (Mead *et al.*, 1999) and as such, *L. monocytogenes* is of major concern to the food industry. This bacterium is quite resilient to environmental stresses including an ability to grow under acidic conditions as low as pH 4.5 and survive at pH values as low as 2.0 for extended periods (Cole *et al.*, 1990; McClure *et al.*, 1989; Young KM, 1993). This characteristic is important for its pathogenicity because it allows the bacterium to survive the acidic environment in various foods and in the stomach of the potential host.

To counteract this threat, strains of *Listeria* can employ a variety of acid resistance mechanisms including the arginine deiminase system (Ryan *et al.*, 2009a), the adaptive acid tolerance response (ATR; Davis *et al.*, 1996) and the glutamate decarboxylase (GAD) system (Cotter *et al.*, 2001). It is clear however that there is a degree of strain-to-strain variation in terms of ability to cope with an acid challenge (Adrião *et al.*, 2008; Cotter *et al.*, 2001; Karatzas *et al.*, 2012). The GAD system operates to maintain the internal pH homeostasis of the bacteria. Upon acid exposure an extracellular molecule of glutamate is taken up by an antiporter (GadT) and decarboxylated intracellularly by a glutamate decarboxylase enzyme (GadD) to form γ -aminobutyric acid (GABA). This reaction consumes a proton thereby increasing the intracellular pH. GABA is then exported via GadT in exchange for a further molecule of glutamate. *L. monocytogenes* can possess up to three genes encoding glutamate decarboxylases (*gadD1*, *gadD2* and *gadD3*) and two encoding antiporters (*gadT1* and *gadT2*) arranged into three unlinked operons (*gadD1T1*, *gadT2D2* and *gadD3*; (Cotter *et al.*, 2005), however strains from serotype 4 lack *gadD1T1*. Recent work from our lab has demonstrated the existence of the intracellular GAD system (GAD_i) which converts intracellular glutamate to GABA and contributes significantly to acid resistance (Karatzas *et al.*, 2010, 2012). The activity of this system results in the accumulation of high levels of GABA_i under acidic conditions even in the absence of GABA export.

In *Escherichia coli*, GABA is used as both a carbon and nitrogen source and is metabolised via the GABA shunt pathway (Fig. 5.1; Dover & Halpern, 1972a, b). This pathway incorporates two further enzymes downstream from glutamate decarboxylase. A GABA/ α -ketoglutarate amino-transferase (GABA-AT) removes the amino group from GABA to form succinic semialdehyde (SSA) and glutamate. The SSA is then oxidised through the activity of succinic semialdehyde dehydrogenase (SSDH) to form succinate (Fait *et al.*, 2008). While the GABA shunt pathway has not been extensively studied in bacteria, it is thought to play a role in glutamate metabolism, anaplerosis and antioxidant defence (de Carvalho *et al.*, 2011). The use of arginine, ornithine and agmatine as nitrogen sources by *E. coli* relies on the GABA shunt pathway as these are first converted to putrescine and subsequently to GABA (Schneider *et al.*, 2002). In plants and mammals, the pathway has been described as an alternative route to produce succinate, bypassing two enzymes of the TCA cycle, namely α -ketoglutarate dehydrogenase and succinyl co-A synthetase (Fig. 5.1; Bouché & Fromm, 2004; Fait *et al.*, 2008). For *L. monocytogenes*, the GABA shunt may represent a potential route to compensate the incomplete TCA cycle identified through sequencing and biochemical analysis (Eisenreich *et al.*, 2006; Glaser *et al.*, 2001). Genome sequencing has shown an absence of genes encoding α -ketoglutarate dehydrogenase, succinyl-CoA synthetase and malate dehydrogenase (Fig. 5.1). The work of Eisenreich *et al.* (2006), supports this with biochemical data showing that oxaloacetate is not derived from α -ketoglutarate by the TCA cycle. Therefore the importance of this pathway cannot be overlooked when trying to establish a complete metabolic profile of this bacterium.

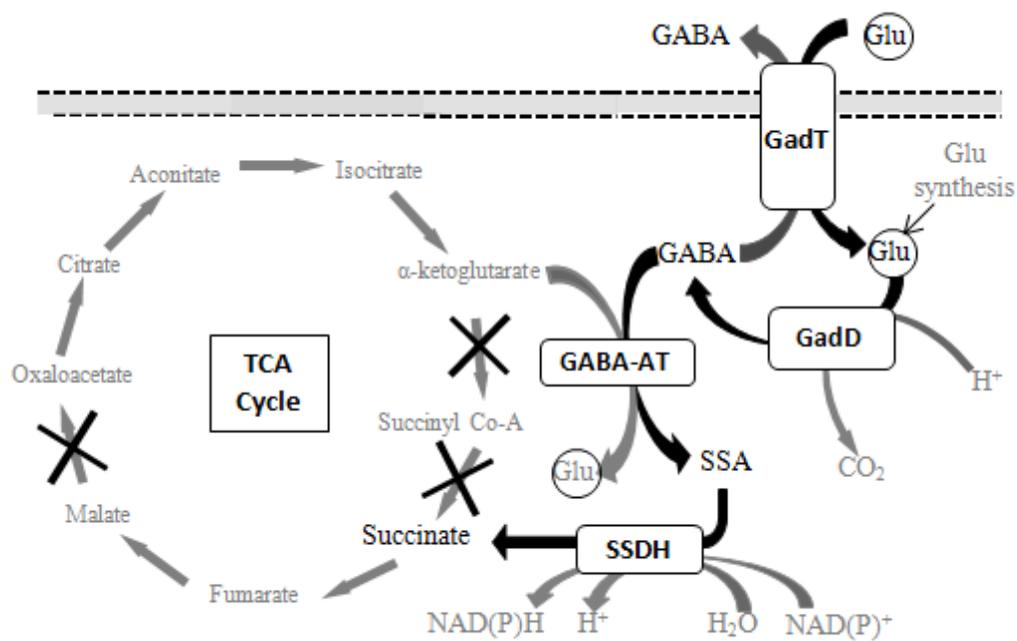


Figure 5.1. Glutamate decarboxylase system and GABA shunt pathway of *L. monocytogenes*. The schematic shows a proposed model for the metabolism of GABA_i in *L. monocytogenes*. Glutamate is decarboxylated to GABA_i by GadD. The GABA can either be exported by GadT in exchange for another Glu or enter the GABA shunt pathway. Here GABA_i donates its amino group to α-ketoglutarate in a reaction catalysed by transaminase enzyme (GABA-AT), resulting in the formation of succinyl semialdehyde (SSA) and Glutamate. The SSA is then oxidised to succinate by a dehydrogenase (SSDH). The incomplete TCA of *L. monocytogenes* is shown with the missing steps marked with 'X'. The GABA shunt pathway can provide an alternative source of succinate for the bacteria.

The aim of this study was to determine if *L. monocytogenes* possesses the two metabolic steps comprising the GABA shunt pathway. Secondly, strains with disruptions or deletions in genes predicted to be involved in this pathway (*argD* and *lmo0913*) were analysed for effects on respective activities in order to confirm their roles. Finally, due to the fact that the GAD system and the GABA shunt are linked, the main metabolites of the pathway were quantified in the cell in response to an acid treatment.

5.3 Results

5.3.1 *L. monocytogenes* possesses activity for both enzymes of the GABA shunt

BLAST analysis showed that ArgD from *L. monocytogenes* EGD-e shared 52% similarity and 34% identity to GabT, a GABA-AT in *E. coli*, while Lmo0913, a predicted SSDH (Cotter *et al.*, 2005) had 67% similarity and 49% identity with its homologue protein, GabD, in *E. coli*. These two proteins appeared frequently throughout the published sequences of *L. monocytogenes*. Twenty four out of 25 strains searched possessed a homologue to Lmo0913 with greater than 98 % identity while 14 out of 25 possessed an ArgD homologue with greater than 93 % identity. Most notable is LO28 which does not appear to have similar proteins.

The first step in the GABA shunt is the conversion of GABA to SSA. Crude protein extracts were tested for the ability to produce SSA from GABA as described in Section 2.10. Co-incubation of these protein preparations (7 mg ml⁻¹) for 1 h with GABA (50 & 100 mM) resulted in the production of SSA (1.79 & 3.94 μM SSA min⁻¹ mg protein⁻¹, respectively; Fig. 5.2). In the wild type EGD-e strain the concentration of SSA produced increased in proportion to the concentration of GABA added. When a mutant with a disruption in a putative GABA-AT gene (*argD*) was used, the production of SSA was reduced (1.57 & 2.34 μM SSA min⁻¹ mg protein⁻¹, with 50 and 100 mM GABA present, respectively; *p* value of < 0.05), indicating an involvement of this gene in the metabolic step. The effect of disrupting *argD* was more apparent at the higher concentration of 100 mM GABA whereas at 50 mM GABA the difference did not appear significant. The production of significant albeit reduced SSA levels in the absence of *argD* suggests that an alternative GABA-AT activity is also present in the cell extracts. GABA-AT activity was also observed in the 10403S strain but at

much lower levels than EGD-e ($0.55 \mu\text{M SSA min}^{-1} \text{ mg protein}^{-1}$ in the presence of 100mM GABA).

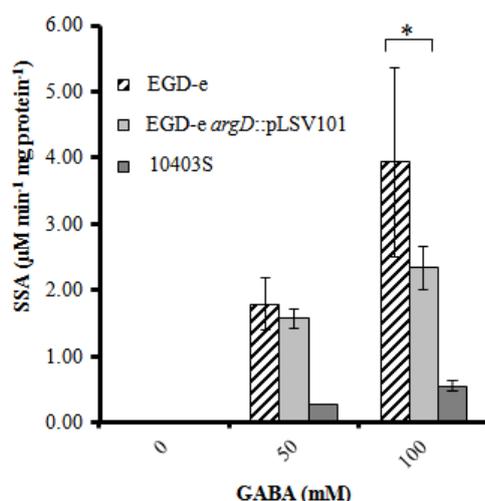


Figure 5.2. GABA-AT activity of *L. monocytogenes*. GABA-AT activities of crude cell extracts harvested after overnight growth (16 h) and incubated with either 0, 50 or 100 mM GABA. Values plotted are the means of three individual culture replicates. Errors bars represent the standard deviations of values from the mean. The asterisk indicates a statistically significant difference ($p < 0.05$) as determined by student's *t*-test.

The oxidation of SSA to succinate is the second step in the GABA shunt. Crude protein extracts were tested for SSDH activity, the enzyme that carries out this step. Production of NADPH (the by-product of the SSDH reaction) was seen in EGD-e protein extract that was incubated with SSA (Fig. 5.3). The production of NADPH did not occur in the absence of substrate and its production increased in proportion to the concentration of substrate (SSA). Deletion of the putative SSDH encoding gene, *lmo0913*, resulted in a complete loss of activity for this reaction. To determine if *Lmo0913* directly acts as an SSDH, the corresponding gene was cloned into pKSV7 generating plasmid pKAK0913 and this was used to transform two strains of *L. monocytogenes* carrying deletions in *lmo0913*; EGD-e Δ *lmo0913*

and 10403S $\Delta lmo0913$. An *E. coli* strain lacking a functional SSDH ($\Delta gabD$) was also transformed with this construct. Crude protein extracts of these strains were analysed for SSDH activity. *E. coli* BW25113 extracts were found to have SSDH activity when incubated with SSA (Fig. 5.4). Disruption of the SSDH for *E. coli*, *gabD*, resulted in a complete loss of this activity. Complementation of this mutant with *lmo0913* from 10403S using pKAK0913 partially restored the SSDH activity (Fig. 5.4).

No SSDH activity could be detected in extracts from either 10403S or the $\Delta lmo0913$ derivative of this strain, however complementation of the knockout mutant with *lmo0913*, resulted in production of NADPH. As expected, complementation of the $\Delta lmo0913$ mutation in EGD-e restored the SSDH activity to this strain. It is interesting to note that Lmo0913 from 10403S has six amino acids difference from that of EGD-e (V2-, F3-, L4-, E251/248D, G312/309A, D352/349E). These data show that Lmo0913 is responsible for the SSDH activity in *L. monocytogenes* and further suggest that baseline SSDH activity varies between strains (Fig. 5.3 & Fig. 5.4).

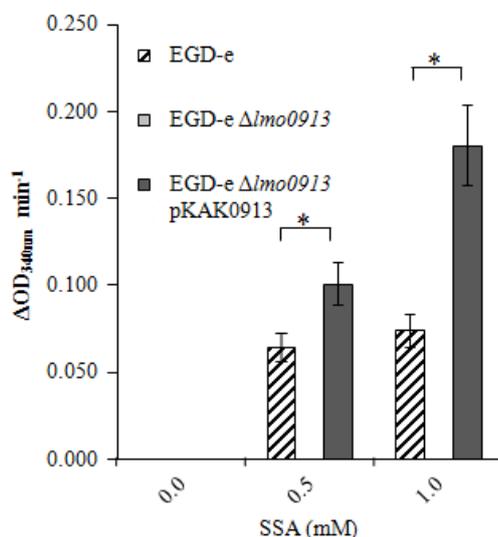


Figure 5.3. SSDH activity of *L. monocytogenes*. Crude cell extracts of EGD-e, EGD-e $\Delta lmo0913$ and EGD-e $\Delta lmo0913$ pKSV7::*lmo0913* were incubated with either 0, 0.5 or 1.0 mM SSA. NADPH production is measured at OD_{340nm}. Values

are the mean of three individual culture replicates. Errors bars shown display the deviation of values seen between each replicate sample.

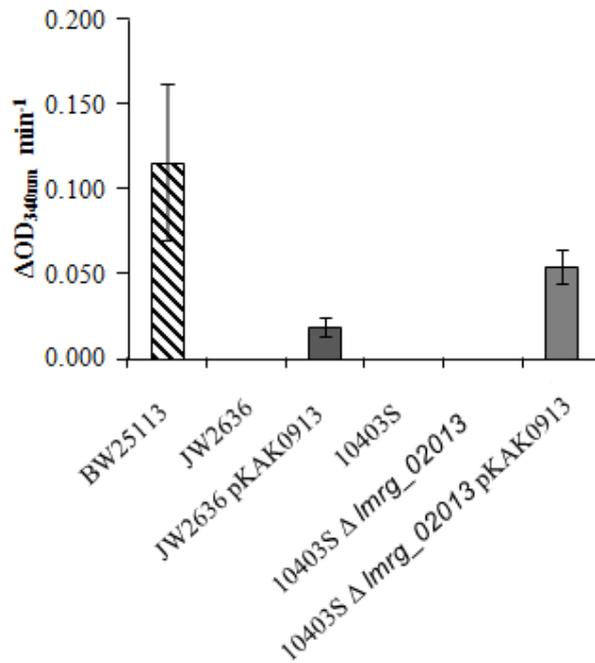


Figure 5.4. SSDH activity in *E. coli* and *L. monocytogenes* 10403S. Crude cell extracts of either *E. coli* K-12, JD23574, JD23574::pKSV7*lmo0913*, 10403S, 10403S Δ *lmg_02013* (*lmo0913*), 10403S Δ *lmg_02013* pKSV7:*lmo0913*, harvested after 16 -18 h growth and incubated with 1.0 mM SSA. NADPH production was measured at OD_{340nm}. *lmg_02013* is the annotation for *lmo0913* as in the NCBI sequence database. Values are the means of two individual culture replicates, each measured twice. Errors bars represent the standard deviation of values from the mean.

5.3.2 Acid exposure affects the intracellular concentrations of GABA shunt intermediates

The levels of glutamate did not appear to significantly change in response to acid in both EGD-e and pLSV101::*argD* (Table 5.1), however deletion of *lmo0913* resulted in a reduction in the levels of glutamate from 0.53 mM to 0.45 mM (p value = 0.03). As expected, GABA_i concentrations are affected in EGD-e in response to acid. Both EGD-e and pLSV101::*argD* showed a 2.6- and 1.2-fold increase in the GABA_i levels reaching 1.1 and 0.6 mM, respectively. GABA_i produced by the *argD* mutant was significantly lower than EGD-e after acid treatment. However a dramatic 9.2-fold increase in GABA_i was observed in Δ *lmo0913*, reaching a concentration of ~3.5 mM following acidification to pH 4.0. This, along with a significant decrease in glutamate (p -value 0.01) suggests a highly active GAD_i system in this strain. The increase seen in SSA before and after acid treatment for EGD-e was not significant and the increase seen in *argD*::pLSV101 was close to the detectable range of the assay but the increase for the Δ *lmo0913* mutant was greater as would be expected for a gene involved in the breakdown of SSA. Succinate increased to a small extent in both EGD-e and *argD*::pLSV101 after acid treatment however the increase in Δ *lmo0913* mutant was not significant (p value of 0.165).

Table 5.1. Intracellular concentrations of intracellular metabolites at stationary phase cells

	Glutamate		GABA	
	pH 7.0	pH 4.0	pH 7.0	pH 4.0
EGD-e	0.55(+/-0.06)	0.53(+/-0.05)	0.43(+/-0.05)	1.14(+/-0.68) ^a
<i>argD</i> ::pLSV101	0.59 (+/-0.02)	0.48 (+/-0.06)	0.51(+/-0.02)	0.60(+/-0.01) ^{ab}
<i>lmo0913</i>	0.53 (+/-0.01)	0.45 (+/-0.02) ^{ab}	0.35(+/-0.06)	3.24(+/-1.83) ^{ab}
	SSA		Succinate	
	pH 7.0	pH 4.0	pH 7.0	pH 4.0
EGD-e	0.41(+/-0.21)	0.52(+/-0.10)	0.24(+/-0.05)	0.44(+/-0.07) ^a
<i>argD</i> ::pLSV101	0.24(+/-0.02)	0.34(+/-0.02) ^a	0.17(+/-0.01)	0.34(+/-0.04) ^a
Δ <i>lmo0913</i>	0.53(+/-0.04)	0.61(+/-0.03) ^a	0.24(+/-0.05)	0.54(+/-0.24)

^aStatistically significant difference between acid and non acid treated cells.

^bStatistically significant difference between wild type and mutant strain for the condition tested. A difference was identified as statistically significant using a paired student *t*-test, $p < 0.05$.

5.3.3 Lmo0913 affects acid resistance of *L. monocytogenes* EGD-e

EGD-e showed a reduction in cell numbers by ~8 log cycles and the *argD* mutant strain demonstrated a similar reduction following an acid challenge at pH 2.5 (Fig. 5.5). However, $\Delta lmo0913$ showed a dramatic increase (~5 log cycles) in the acid resistance compared to the wild type. The increase in acid resistance of the $\Delta lmo0913$ mutant reached levels of resistance just short of those achieved by 10403S, a highly acid resistant strain that utilises both the GAD_i and GAD_e systems with great efficiency (Karatzas *et al.*, 2010). Thus a strong correlation existed between GABA_i accumulation and survival at low pH. Attempts to complement this resistant phenotype failed however as the transformed strain (EGD-e $\Delta lmo0913$ pKSV7::*lmo0913*) did not grow as well as the other strains and thus gave an incomparable acid resistance phenotype (data not shown). This growth defect was not caused by the presence of the *lmo0913* gene on the plasmid since strains containing pKSV7 also grew slower than the wild type.

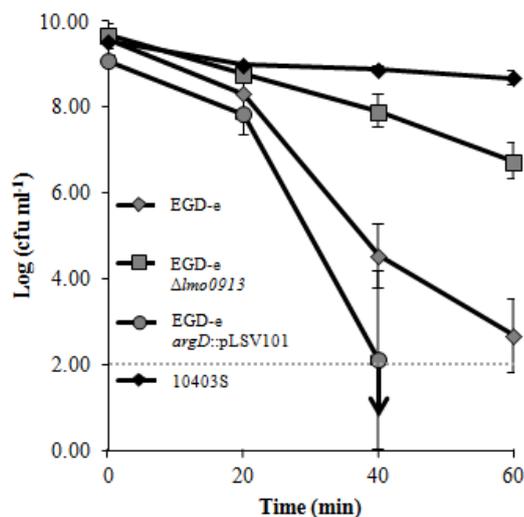


Figure 5.5. Acid survival of *L. monocytogenes* EGD-e and GABA putative shunt system mutants. Stationary phase EGD-e and GABA shunt mutants were challenged at pH 2.5. Cell counts were taken every 20 min. Values are the mean of three individual cultures with the cell counts for each the mean of three platings. Error bars represent the standard deviations from the mean (n=3). 10403S was included for comparison. The down arrow represents that counts were below the limit of detection (100 cfu ml⁻¹ dotted line) after 40 min.

5.3.4 Deletion of *gsaB* does not affect GABA-AT activity (Data not in paper)

The second protein with the highest similarity to *E. coli* GabT in *L. monocytogenes* was GsaB. This protein shared 28% identity and 46 % similarity to the protein sequence of GabT. As disruption of *argD* did not fully remove GABA-AT activity, the gene encoding GsaB, was deleted to identify a possible role in the GABA shunt pathway. Crude protein extracts from both EGD-e and EGD-e Δ *gsaB* displayed GABA-AT activity (Fig. 5.6). This activity increased as the concentration of substrate added to the reaction increased. However there was no significant difference seen between the GABA-AT activity for EGD-e and EGD-e Δ *gsaB*, suggesting that in *L. monocytogenes* GsaB does not catalyse this reaction.

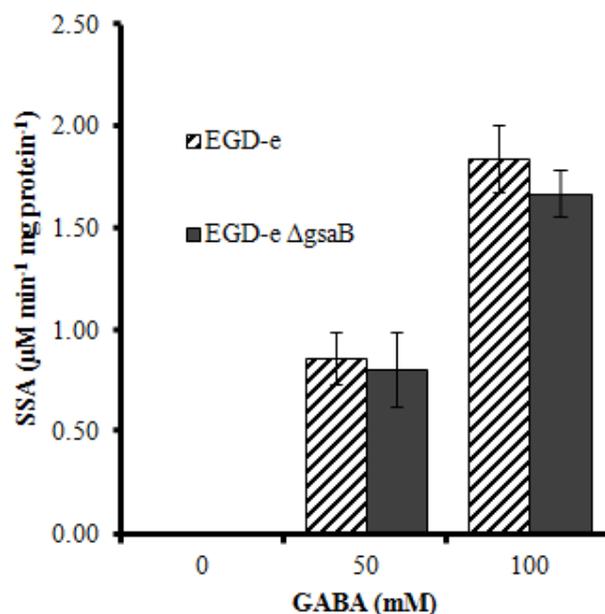


Figure 5.6. GABA-AT activity of *L. monocytogenes*. GABA-AT activities of crude cell extracts harvested after overnight growth (16 h) and incubated with either 0, 50 or 100 mM GABA. Values are mean of three individual culture replicates. Errors bars shown display the standard deviation of values seen between each replicate sample.

5.3.5 Lmo0913 may play a role in biofilm formation in nutrient poor media

(Data not in paper)

A study by (Zhou *et al.*, 2012) on the changes in protein expression between planktonic and biofilm forming *L. monocytogenes* showed that the formation of a biofilm mainly changed the expression of proteins associated with metabolism. Among these there was Lmo0913, which showed an increased expression during biofilm formation in a minimal media (Zhou *et al.*, 2012). To determine if our *lmo0913* mutant was affected in biofilm formation, a simple crystal violet biofilm assay was performed (Section 2.6). After 24 h growth in BHI, there was no significant difference seen between EGD-e and EGD-e Δ *lmo0913*. In defined media (DM), the EGD-e Δ *lmo0913* strain produced a small but significantly greater level of biofilm than the EGD-e wild-type (Fig. 5.7 a). Both strains produced more biofilm in DM than in the richer BHI media. After 48 h of growth there was still no significant difference between EGD-e Δ *lmo0913* and EGD-e and the amount of biofilm was the same as at 24 h. In DM after 48 h there was an increase in biofilm for both strains compared to 24 h and there was a greater increase in biofilm for EGD-e Δ *lmo0913* compared to EGD-e (Fig. 5.7 b).

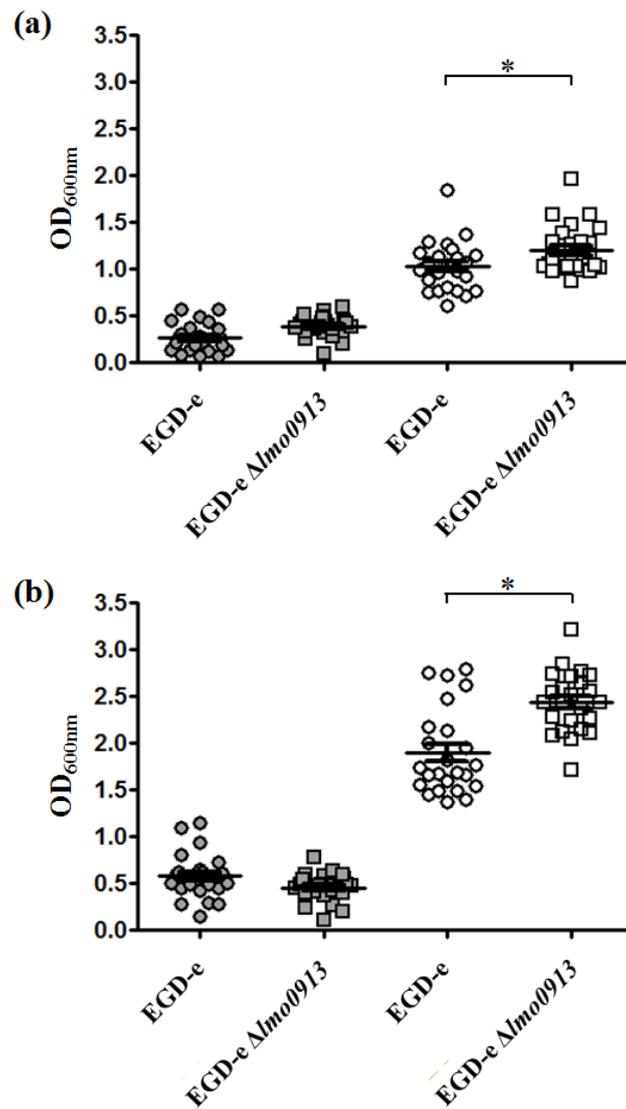


Figure 5.7. Biofilm formation of GABA shunt mutant. The biofilm concentration as measured by OD_{600nm} of each replicate is represented for each strain after 24 h (a) and 48 h (b) growth in BHI (grey-filled) or DM (white-filled). The horizontal line represents the mean value for the pool of replicates while the error bars represent the standard error in the mean value. An asterisk represents significant differences between samples as determined by the Student's *t*-test where significance is *P*-value < 0.05.

5.3.6 EGDm $\Delta lmo0913$ displays an attenuated virulence in both murine liver and mesenteric lymph node (Data not in paper)

The *lmo0913* deletion was introduced into a murinised EGD-e background to generate EGDm $\Delta lmo0913$ and this strain was orally inoculated into Balb/C mice (Section 2.8). Three days post infection both EGDm wild-type and EGDm $\Delta lmo0913$ were recovered from the liver, spleen, mesenteric lymph node (MLN) and faeces (Fig. 5.8) at about 10^6 bacteria per organ. There was no difference in the recovery of either strain from the spleen or in the faeces. A significant reduction in counts was seen for EGDm $\Delta lmo0913$ from both the liver and MLN of about 1-log unit. All mice were displaying signs of acute illness prior to sacrifice including hair standing up and reduced movement. One of the mice infected with EGDm $\Delta lmo0913$ was dead prior to the day 3 sacrifice. Reduced counts in the liver and MLN may suggest a role of *lmo0913* in virulence

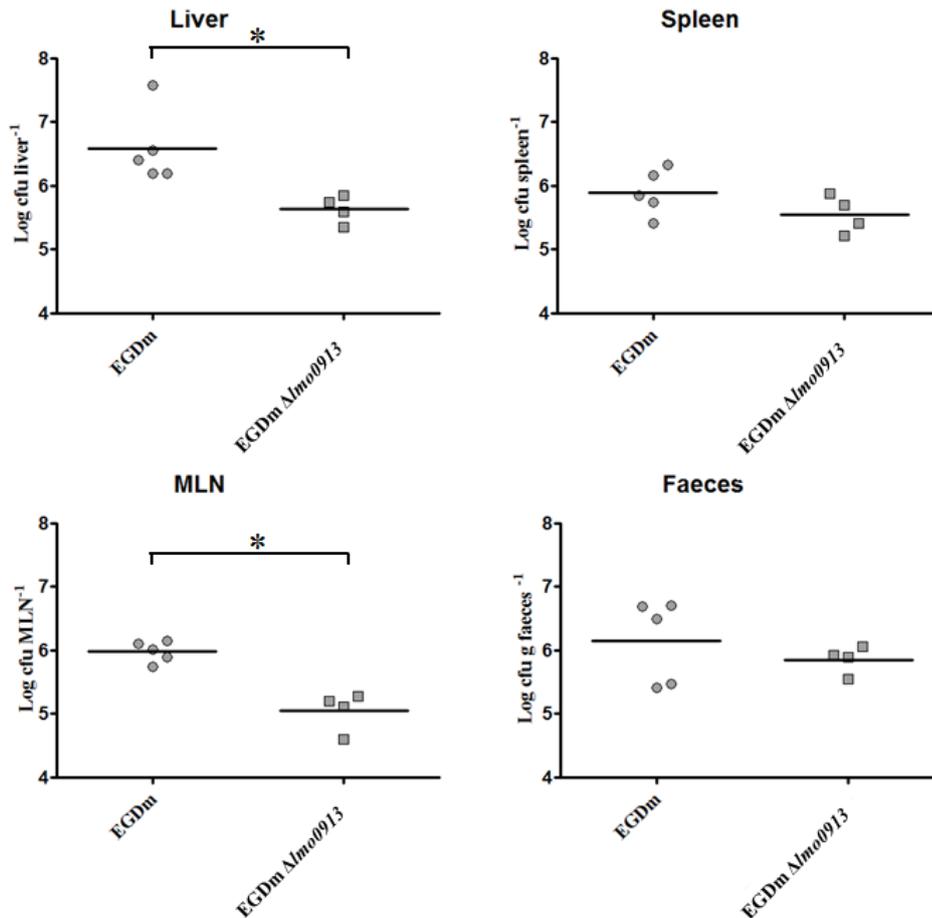


Figure 5.8. Infection of Balb/C mice with EGDm $\Delta lmo0913$. Plate counts of surviving EGDm strains 3 days post infection of female Balb/C mice (n=5). Isolated from the liver, spleen, mesenteric lymph node (MLN) and faeces. Significant differences (*) between wild-type and mutant was determined using Student's *t*-test.

5.4 Discussion

We have previously shown that *L. monocytogenes* possesses a GAD_i system that leads to GABA_i accumulation under acidic conditions (Karatzas *et al.*, 2012). Here we report that this GABA can be metabolised to succinate by ArgD and Lmo0913 via the GABA shunt pathway. As the GABA shunt is coupled to the GAD system, which is the principal mechanism of acid tolerance in *L. monocytogenes*, we investigated the acid tolerance phenotype of both *argD*::pLSV101 and $\Delta lmo0913$ strains. Interestingly these mutants yielded quite

contrasting acid tolerance phenotypes. While disruption of *argD* slightly hindered the cells' ability to cope with acid treatment, deletion of *lmo0913* increased survival by over 5 log cycles (Fig. 5.5). This significant increase highlights a role for the GABA shunt in acid tolerance. The acid resistance phenotype of Δ *lmo0913* was accompanied with 9.2-fold and 1.1-fold increases in GABA_i and SSA concentrations, respectively (Table 1). The increase in these metabolites is likely to be caused by a diminished capacity for GABA metabolism in this mutant (Fig. 5.3). Interestingly, EGD-e does not utilize the GAD_e system but only the GAD_i system. Recently, we have demonstrated that GAD_i contributes to acid resistance independently of the antiport carried out by GAD_e (Karatzas *et al.*, 2012). The fact that deletion of the *lmo0913* gene is able to convert EGD-e, from one of the weakest strains in terms of acid resistance to a moderate one (2 logs lower than 10403S which is the most acid resistant strain we have identified and 5 logs higher than EGD-e; Fig. 5.5) through an increase in GABA_i underpins the importance of this system in acid resistance.

If the disruption of the GABA shunt, by deletion of *lmo0913* is responsible for the GABA_i build up and subsequently of acid resistance a similar result might be expected for the *argD* mutant. This however was not the case, suggesting that in this mutant the transamination activity observed is probably carried out by an enzyme that compensates the loss of *argD*. GsaB has 46% similarity and 28% identity to GabT, a GABA-AT (Bartsch *et al.*, 1990). It has been shown that for several bacteria multiple copies of GABA shunt genes exist (Buell *et al.*, 2003; Kurihara *et al.*, 2010) and this may well be the case with *L. monocytogenes*. BLAST searches for a secondary SSDH also revealed a likely candidate in Lmo0383 which has 54% similarity and 33% identity to the corresponding SSDH (GabD) in *E. coli*. Attempts to define the roles of these genes was carried out since publication of the above paper. Deletion of *gsaB* did not show an apparent role for this gene in the GABA shunt pathway as its deletion did not affect GABA-AT activity in crude protein extracts (Fig. 5.6). Attempts were carried out to delete *lmo0383* however all of these failed as no clone carrying the deletion fragment could be achieved both in our own lab and from *de novo* synthesis using a commercial company.

When comparing the SSDH activity between EGD-e and 10403S it is interesting to note that no SSDH activity was detectable under the conditions measured for 10403S. The difference between the two strains may suggest differing degrees of importance for the GABA shunt between strains (although both belong to lineage II and share the same serotype; 1/2a). Strain-to-strain variation is also exemplified by the fact that previously, a deletion of *lmo0913* in 10403S did not confer acid resistance but instead resulted in a mild sensitivity to acid (Abram *et al.*, 2008a). As mentioned above the main difference between the two strains is that EGD-e utilises only the GAD_i and so unlike 10403S, which utilises both GAD_i and GAD_e, it must rely solely on the GABA shunt to metabolise the GABA generated during acid stress (Karatzas *et al.*, 2012). Thus it appears that the elevated SSDH activity in EGD-e might be related to the higher GABA_i pools that exist in this strain during acid stress.

The existence of the GABA shunt pathway has significant implications for our understanding of the overall metabolism of *L. monocytogenes*. The genome sequence of *L. monocytogenes* lacks a complete set of enzymes for a full TCA cycle (Glaser *et al.*, 2001) and this is consistent with biochemical measurements (Joseph *et al.*, 2008). Two of the enzymes missing from *L. monocytogenes* are α -ketoglutarate dehydrogenase and succinyl Co-A synthetase, which are responsible for the conversion of α -ketoglutarate to succinate (Fig. 5.1). The GABA shunt pathway appears to serve different species of bacteria in a range of ways from sporulation (Aronson *et al.*, 1975) to nitrogen metabolism (Dover & Halpern, 1974). For *L. monocytogenes* it is possible that the pathway can in part substitute for the lack of a functional TCA cycle. It is important to note however that the GABA shunt does not provide a means of completing the TCA cycle since malate dehydrogenase, required to convert malate to oxaloacetate, is also absent in *L. monocytogenes* (Eisenreich *et al.*, 2010; Glaser *et al.*, 2001). According to Trivett and Meyer (1971), *L. monocytogenes* lacks a functional succinate dehydrogenase required to convert succinate to fumarate. This presents yet a further disruption in the classical TCA cycle and would indicate a dead end for the succinate produced by the GABA shunt pathway. However their experiments did show activity for

fumarate reductase. Evidence has been presented that shows fumarate reductase can compensate for a loss of succinate dehydrogenase (Guest, 1981; Hirsch *et al.*, 1963). This could putatively convert succinate to fumarate. From here fumarate can enter the arginine biosynthetic pathway and thus provides a means of recycling the carbon for further biosynthesis. It is also important to note that while the pathway is a means of biosynthesising succinate, the activity of the SSDH enzyme may be important for generating NAD(P)H that could be coupled to electron transport as is the case in plants (Busch *et al.*, 2000). This might provide a means of energy production for the cell.

Several studies have shown that regulation of the TCA cycle is related to biofilm development in several species of bacteria (Kim *et al.*, 2002; Seidl *et al.*, 2008; Vuong *et al.*, 2005; Yamazaki *et al.*, 2006). In *L. monocytogenes* a large proportion of proteins that change expression upon a switch from planktonic lifestyle to biofilm were found to be involved in metabolism including the TCA cycle associated protein CitC (Zhou *et al.*, 2012). Given that Lmo0913 would potentially play a role in feeding into the TCA cycle, it is perhaps not surprising to see that this protein is involved. Although Lmo0913 is shown to increase in expression during biofilm formation in minimal medium, the results above indicate that biofilm formation increased in a strain lacking the gene *lmo0913* (Fig. 5.7). It is possible that the GABA shunt might control the rate of biofilm production by perhaps altering the energy state of the cell.

Despite showing an increased capacity to survive a low pH challenge similar to EGD-e Δ *lmo0913* (data not shown), the EGDm Δ *lmo0913* mutant did not show increased recovery from the faeces after passage through the murine gastric passage. In fact this mutant showed a reduced dissemination through host tissue (Fig 5.8) when compared with the parent strain. Although low pH is a major hurdle that these strains overcome, there will still be several host associated factors that might impair the ability of EGDm Δ *lmo0913* to cause infection. Among these is an oxidative stress, encountered during macrophage uptake. Exposure of *Saccharomyces cerevisiae* to H₂O₂ was shown to increase expression of SSDH (Coleman *et al.*, 2001) and the generation of NADPH during the

conversion of SSA to succinate may protect *Fransicella tularensis* from oxidative stress (Ramond *et al.*, 2014) Comparison of survival of EGD-e and EGD-e $\Delta lmo0913$ within THP-1 macrophages however did not show a significant difference (Appendix 3). More in-depth *in vitro* experiments however might uncover a clear role for the GABA shunt in *L. monocytogenes* oxidative stress protection.

In summary, we have shown that *L. monocytogenes* EGD-e does have the capacity to metabolise GABA_i via the steps of the GABA shunt. Accumulation of GABA_i appears to be important for the survival of the bacteria under acid stress. As seen in the *lmo0913* mutant strain, a high accumulation of GABA_i is coupled with an increase in survival at low pH. Regulation of the metabolism of GABA may therefore be an important aspect in this survival response. A greater understanding of this pathway may in long term give new insights into the mechanisms of acid tolerance in this pathogen that can be used to devise improved food safety regimes. While additional studies are required to elucidate both secondary enzymes in the pathway and the regulatory mechanisms involved we have shown that the GABA shunt pathway is functional in *L. monocytogenes*, providing this pathogen with a means to synthesise the TCA intermediate succinate.

CHAPTER 6

Discussion

The glutamate decarboxylase system, which several species of bacteria possess, is considered to be one of the most important mechanisms to counteract a reduction in pH. In *L. monocytogenes* the GAD system has been identified as important in playing a protective role in synthetic gastric fluid, modified atmosphere foods and mild organic acids.

The standard model for the GAD system involves a membrane bound antiporter complex that imports glutamate and exports the GABA formed by the intracellular decarboxylase enzyme. Initial work to establish the presence of a GAD system in *L. monocytogenes* was performed in the serotype 1/2c strain LO28. This strain displays the characteristic export of GABA as predicted from the standard model of the GAD system. It was from this strain, that roles for each of the two GAD operons (*gadD1T1*, *gadT2D2*;) were established in *L. monocytogenes*. The *gadT1D1* system was seen to be important for survival in mild acid stress, while *gadD2T2* was identified as key to survival in extreme low pHs. At the time, the third decarboxylase system, *gadD3*, had not been successfully deleted and so no role was identified for it (Cotter *et al.*, 2001, 2005).

More recently, and with the help of strain sequencing, work has identified the presence of a GAD system in all strains of *L. monocytogenes*. Despite this, it has become clear that not all strains possess all three decarboxylase genes and two antiporter genes as seen in LO28. Furthermore, it was shown that all strains of *L. monocytogenes* display a unique pattern of GAD activity as measured by the concentration of GABA they produce. Work by Karatzas *et al.*, 2010 , added to the knowledge of the GAD system in *L. monocytogenes* by showing that strains accumulate GABA intracellularly, generating independent concentrations of both GABA_i and GABA_e. This GABA_i appeared to be generated independently of the known antiport mechanism, and in the strain EGD-e, it was shown that glutamate/GABA antiport never occurs. It has become apparent that both an

extracellular GAD system and an intracellular GAD system operate in parallel in *L. monocytogenes*.

6.1 Divergence of GAD system functionality within *L. monocytogenes*

The work in this project aimed to further analyze the full role of the GAD system in *L. monocytogenes* by comparing two representative strains, one possessing GAD_i/GAD_e and the other solely GAD_i. It was hoped that this would help to further define the model of the GAD system in *L. monocytogenes* and determine if one system is more beneficial than the other. Continuing from this, it was necessary to investigate whether the GABA_i produced within the cells could be metabolized as it did not appear to be exported. Successful deletion mutants were constructed in the three glutamate decarboxylase genes for 10403S and two strains of EGD-e. Comparison of acid survival rates and GABA production revealed a clear difference in the relative contributions of each GAD gene between the strains.

The deletion of *gadD1* or *gadD2* in EGD-e unexpectedly increased the ability of this strain to survive at pH 2.5 (Fig. 3.8; Section 3.3.2). This increase in survival of these two mutants was accompanied with an increased ability to produce GABA_i at pH 4.0. For this strain, which cannot establish an antiport, it would appear that both the enzymatic conversion of glutamate to GABA and also the subsequent accumulation of GABA_i help the cell in coping with the low pH stress. Although GABA has been shown to be a poorer buffer than glutamate (Feehily & Karatzas, 2012), the correlation between increased survival and GABA_i accumulation in EGD-e Δ *gadD1* and EGD-e Δ *gadD2* cannot be overlooked. In order to explain the cause of this increased GABA production in both in EGD-e Δ *gadD1* and EGD-e Δ *gadD2*, transcript levels of all three GAD genes were quantified in response to low pH. It was possible that an over-compensation by any of the GAD genes in a mutant may result in the elevated levels of GABA_i and this would be reflected in an alteration of gene transcription. In EGD-e Δ *gadD2*, it

indeed appears that *gadD3* expression increases, which may in part be the cause of this phenotype, however in EGD-e Δ *gadD1*, *gadD3* did not show increased expression. Therefore it is not possible to conclude that expression of a particular GAD gene led to the increased GABA production. (Chapter 3)

In contrast to the findings with EGD-e, a different effect was observed when the decarboxylase genes were deleted from either EGDm or 10403S. Firstly it appears that the murinised strain, while assumed to be isogenic with EGD-e actually shows a much greater generation of GABA_i in response to low pH. The performance of the GAD system may be affected as a result of long term persistent passaging and use of the strain in a lab. This phenomenon of strain variation has already been reported in *E. coli* environmental strains that have undergone significant changes in their phenotypes as a result of subculture in a laboratory setting (Eydallin *et al.*, 2014). In contrast, a recent study comparing the virulence of an EGD-e and EGD strain of *L. monocytogenes* has predicted that the changes seen between these two strains has actually arisen due to historical mislabelling and not as a result of mutation (Bécavin *et al.*, 2014). Therefore care must always be taken when handling and annotating strains in a laboratory culture collection. Interestingly the acid survival phenotypes of each single mutant in EGDm remained similar to EGD-e (Section 4.4.1) despite the aberration in GABA production of the wild-type strain. This may suggest that there is compensation by an alternative acid tolerance mechanism (Section 1.5.5) which is responsible for the survival in acid, independent of the GAD system. Sequencing of these two strains is necessary to determine the exact genetic changes that have occurred.

The finding that strains of *L. monocytogenes* do not produce GABA_e under certain conditions, if at all (Karatzas *et al.*, 2010), suggested that the GAD system might not just vary in terms of overall activity between strains but in fact possess the ability to operate an intracellular GAD system independent of an extracellular one (Karatzas *et al.*, 2012). By comparing a representative strain from each case (Chapter 4), it was clear that the strain mainly utilising GAD_e was dependent on *gadD2* while the strain mainly utilising GAD_i was not dependent on any particular

single *gad* gene for acid survival but did require the presence of *gadD3* in order to generate GABA.

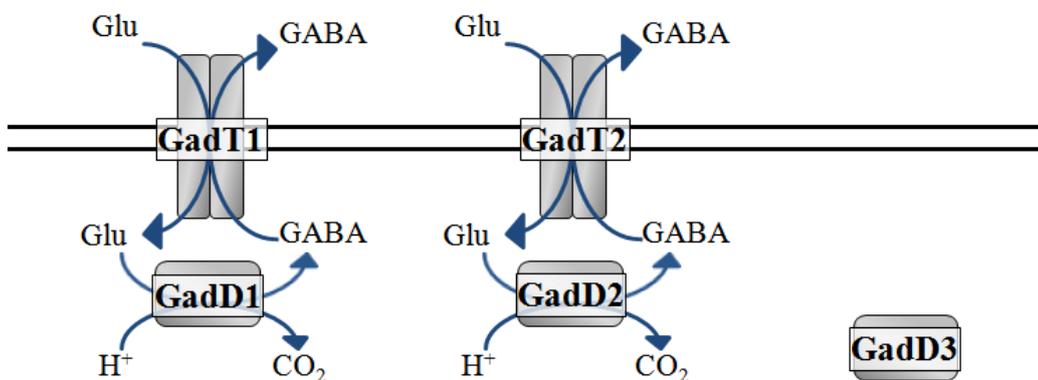
Using the EGDm mutant strains, a comparative study was undertaken with 10403S. The 10403S strain is a serovar 1/2a like EGDm, however it possesses the ability to produce both GABA_e and GABA_i. The ability of 10403S and EGDm to survive at low pH differs and 10403S is generally considered to be one of the most acid resistant strains of *L. monocytogenes* (Karatzas *et al.*, 2012). From both acid survival experiments and measurements of GABA in each of the GAD mutants it became apparent that the two strains utilise a different GAD gene system. Deletion of either *gadD1* or *gadD2* in EGDm produces a strain that was more resistant to low pH than the parent. This phenomenon was previously observed in the EGD-e background, however neither EGDm Δ *gadD1* nor EGDm Δ *gadD2* displayed a subsequent increase in acid induced GABA_i production. As GABA_i production in the parent strain appears to be at a relatively high concentration when comparing strains of *L. monocytogenes*, it may be that a maximum threshold of GABA_i production has been achieved already. If this is the case then EGDm Δ *gadD1* and EGDm Δ *gadD2* would not be able to further increase the GABA_i concentration as occurred in their EGD-e counterparts. Interestingly, deletion of *gadD3* in EGDm dramatically reduced this strain's ability to produce GABA_i in response to low pH. This strain did not however display any increased sensitivity in the acid survival experiments. Unlike in EGD-e, there is no correlation between altered survival at a low pH and a change in GABA_i accumulation in EGDm.

While no single deletion in 10403S increased resistance to low pH, deletion of *gadD2* and to a lesser extent *gadD3* did increase acid sensitivity. In agreement with the reduced survival of 10403S Δ *gadD2* there was a significant reduction in both GABA_i and GABA_e production in this strain. This result clearly indicates that under the conditions tested *gadD2* is important to this strain when dealing with a reduction in pH. As *gadD2* forms an operon with *gadT2*, it is not surprising

to see that export of GABA has also been affected. This result suggests that the remaining *gadDIT1* system is not capable of compensating for the loss of the *gadT2D2* system. Analyses of the nucleotide sequence for *gadDIT1* indicates the presence of a stem-loop like structure, possibly preventing co transcription of *gadD1* with *gadT1* (Wemekamp-Kamphuis *et al.*, 2004a) and the full transcriptional profile of *L. monocytogenes* performed in EGD-e did not identify *gadDIT1* as an operon (Toledo-Arana *et al.*, 2009). It has yet to be proven that GadD1 and GadT1 can work together. The results above would also suggest that the remaining decarboxylases cannot interact with GadT2. However protein analyses would be necessary in order to confirm that GadT2 is still expressed in the mutant. Furthermore as both GABA_i and GABA_e were almost absent in 10403S Δ *gadD2* it suggests that 10403S required the action of both GAD_i and GAD_e.

Overall, the comparative analyses of these strains, confirms the independent action of GAD_i and GAD_e as mechanisms of acid tolerance in *L. monocytogenes*. While some strains may use GAD_e or a combination of GAD_e/GAD_i (Fig. 6.1 a), other strains can utilise GAD_i as a means of counteracting acid stress (Fig. 6.1 b), It is predicted that this mechanism would be useful in conditions of low extracellular glutamate availability. The role that the GAD_i system might play in other micro-organism has yet to be studied. A species of particular interest would be *Mycobacterium tuberculosis*. This bacterium only possesses a single gene to encode for a glutamate decarboxylase (*gadA*; Cotter *et al.*, 2001). It is predicted that in this bacterium the sole action of this decarboxylase may play a role in acid tolerance (Feehily & Karatzas, 2012). Evidence for a GAD_i also has been seen in *E. coli* with GABA accumulation in an antiporter mutant (Castanie-Cornet *et al.*, 1999) and also in *Shigella flexneri* where GAD activity remains despite disruption of GadB (Waterman & Small, 2003).

(a) Extracellular GAD system



(b) Intracellular GAD system

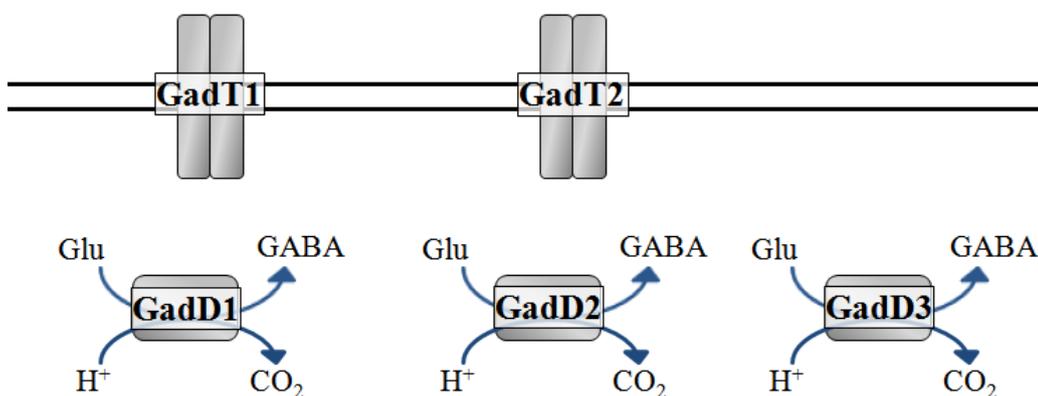


Figure 6.1. The suggested two separate GAD systems in *L. monocytogenes*. (a) The extracellular GAD system (GAD_e) operates in a classical manner, utilising extracellular glutamate to fuel the intracellular decarboxylation reaction. (b) The intracellular GAD system (GAD_i) utilises glutamate from a source other than the antiporters. This generalised schematic does not take into account the different pH activities of each enzyme and does not intend to suggest that all enzymes are active at the same time.

6.2 Is the GAD system regulated differently between strains?

Bioinformatic analyses shows that upstream of both *gadD2T2* and *gadD3* there are predicted promoter sites for σ^B (GTTTTA-N14-GGGTAA), albeit with 3 and 2 mismatches, respectively (Wemekamp-Kamphuis *et al.*, 2004a). This group also showed that in exponential phase cells, expression of *gadT1*, *gadT2*, *gadD2* and *gadD3* all showed increased expression in response to acid treatment of pH 4.5. Interestingly, our work has shown that in stationary phase cells, there is no increase in the expression of any of the three decarboxylase genes in either wild-type EGD-e, EGDm or 10403S in response to the reduction in pH (Fig. 3.12; 4.4). Of the genes showing increased expression in acid treated exponential phase of growth, only *gadD2*, *gadT2* and *gadD3* were expressed in a σ^B dependent manner as predicted from the aforementioned bioinformatics and confirmed by transcriptional analysis (Wemekamp-Kamphuis *et al.*, 2004a). In our own work, through use of the reporter gene *lmo2230*, it would appear that σ^B activity does not increase dramatically in response to acid treatment in stationary phase cells. Taken together, these results demonstrate that the regulation for the GAD system varies greatly between stationary and exponential phase cells. The mechanism by which cells are responding to the acid challenge in stationary phase is currently unclear. How then are stationary phase cells responding to the acid treatment?

During the growth of *L. monocytogenes* cells, it has been shown that σ^B activity increases and reaches a maximum level in stationary phase (Utratna *et al.*, 2014). During this time, the cells are experiencing increasingly stressful conditions including a gradual reduction in pH. Therefore, the cell may already have developed sufficient amounts of GAD system proteins during this time. Measurement of *gad* gene transcription over the course of growth would help clarify this issue. Despite the evidence for σ^B dependency of the *gadD2*, *gadT2* and *gadD3* genes in exponential phase cells treated to low pH, both *gadD1* and *gadT1* were expressed independent of σ^B (Wemekamp-Kamphuis *et al.*, 2004a). This indicates that the regulation of the GAD system is not fully understood. While in stationary phase, the GAD system does not appear to increase transcription in response to acidic pH either σ^B -dependently or independently, regulation of the system could still occur at a post-transcriptional level. Measurement of actual protein concentrations of the GAD proteins before and after acid treatment would help to clarify this, while performing next generation sequencing and generating a full transcriptomic profile of the cell may identify any RNA regulating units that have not been reported thus far. In *Clostridium perfringens* GAD protein production increased to a greater rate during late exponential growth (Cozzani *et al.*, 1975). This supports the idea that cells have already produced the required GAD proteins prior to the phase of growth that we have examined.

6.3 What is the role for succinate in the cell?

As is evident from the experimental results, *L. monocytogenes* possesses the means to convert glutamate to succinate via the GABA shunt pathway (Chapter 5). Furthermore, intracellular succinate concentrations were also recorded (Table 5.1). Accordingly it would be expected that the succinate generated via this pathway could enter the classical TCA cycle as an intermediate as has been suggested to occur in several other species of bacteria and eukaryotic organisms. *L. monocytogenes* however lacks malate dehydrogenase, which prevents completion of the TCA cycle after the entry of succinate (Fig 1.2). If the succinate

cannot be used in the TCA cycle then what use is it to the cell? The reverse reaction of succinyl-CoA synthetase which converts succinate to succinyl-CoA is also absent in *L. monocytogenes* as neither the α - or β - sub-unit genes encoding succinyl-CoA synthetase are present. This means that succinate cannot be utilised in the formation of porphoryins as a means of sensing light (Endarko *et al.*, 2012; Shemin & Kumin, 1952). Furthermore, the pathway identified in *E. coli* that converts succinate to propionate via a propionyl CoA:succinate CoA transferase (YgfH; Haller, Buckel, Rétey, & Gerlt, 2000) is unlikely to be present in *L. monocytogenes*. Alignment of the gene encoding YgfH in *E. coli*, *scpS*, failed to identify any homologue in the published genome sequences of *L. monocytogenes*. As discussed in Section 5.4 succinate could be converted to fumarate via the reverse action of fumarate reductase. Succinate dehydrogenase catalyses the aerobic conversion of succinate to fumarate, while fumarate reductase catalyses the reverse reaction, converting fumarate to succinate under anaerobic conditions. This allows fumarate to act as an electron receptor during anaerobic electron transfer (Iverson, 1999). In the absence of either of these two enzymes, *in vitro* work has shown a compensatory role by the other remaining enzyme complex (Guest, 1981; Maklashina *et al.*, 1998). As no succinate dehydrogenase has been identified in *L. monocytogenes* (Trivett & Meyer, 1971), it is possible that fumarate reductase, which has been identified (Trivett & Meyer, 1971), is carrying out this reaction. This would mean that succinate could be used as an electron donor during respiration. Currently this appears to be the only potential use for succinate in the cell. Measurements of succinate however were only determined intracellularly. By measuring the changes in succinate concentrations in the extracellular medium during growth and also before/after acid treatment, an insight could be gained as to whether succinate is simply exported from the cell as a waste product. Further work measuring the abundance of this dicarboxylic acid is necessary, however it lies outside the scope of the current project.

Another benefit to the generation of succinate via the GABA shunt pathway would be the production of NADPH, which could be used to protect the cell against oxidative stress as seen in *Francisella tularensis* (Ramond *et al.*, 2014).

The NADPH could also be used as a means of electron transport in energy production as seen in plants (Busch *et al.*, 2000). While this does not require succinate *per se*, it does indicate a possible use for the pathway. This however requires further work to clarify these possibilities.

6.4 The GAD system and virulence

Work carried out in *Brucella microti* provided evidence that the GAD system is important for survival during gastric passage of mice for this particular bacteria (Occhialini *et al.*, 2012). In contrast to these findings, deletion of a single glutamate decarboxylase encoding gene from *L. monocytogenes* EGDm did not affect the recovery of bacteria from the liver, spleen or faeces of orally infected mice. Deletion of either *gadD1* or *gadD2* together with *gadD3* did reduce counts in both the liver and spleen, suggesting that there may be a redundancy in the GAD system. It is generally expected that the GAD system would be required for survival through the gastric passage of the host and it was previously shown that the GAD system is required for survival in a synthetic gastric fluid (Cotter *et al.*, 2001). Additionally all GAD genes have shown increased gene expression within the gut of a mouse model (Archambaud *et al.*, 2012). The absence of any major effect on *in vivo* survival by deletion of the GAD genes here might be due in part to the use of the particular animal model. The pH however of Balb/C mice can be as high as pH 4.04 and reaches only as low as pH 2.98 (McConnell *et al.*, 2008). As the pH of the stomach was not known at the time of infection it is not fully clear what conditions the bacteria were exposed to. The higher pH of 4.04 would not be sufficient to kill the bacterium.

Similar to *B. microti*, there was no clear difference seen between any of the GAD mutants within in THP-1 macrophages. This might indicate that the pH inside these macrophages is not low enough to present a challenge to the mutants in the GAD system. Alternatively, work by Conte *et al.*, 2002 suggests that the GAD system would play a role inside macrophages after prior acid adaption. The work

above used bacteria taken directly from stationary phase broth culture, therefore pre adaption of the *gad* mutants might result in a greater difference between them and the wild-type.

Taken together, these data demonstrate that the role of the GAD system in virulence remains to be fully understood. Due to the apparent compensatory redundancy of the system, all three decarboxylase genes may need to be deleted.

6.5 Conclusion

Overall this project has helped to shed further light on the behaviour of the GAD system in *L. monocytogenes*. Importantly, it is now clear that the GAD system behaves differently across the range of strains studied here. This has significant implications for the effort of developing an encompassing model of acid tolerance in *L. monocytogenes*. The previous model of the GAD system, based on an antiport mechanism is not a complete picture and we have shown that GAD_i activity, independent of antiport is important for acid survival.

From the construction of deletion mutants, we have confirmed that *gadD3* is indeed a decarboxylase, and plays an important role in acid tolerance and GABA production. The comparative study between 10403S and EGDm indicates that strains utilising GAD_e/GAD_i favour the use of GadD2 while strain solely utilising GAD_i rely upon GadD3.

This work has also given an insight into the regulation of the GAD system during stationary phase acid exposure. Despite a reduction in extracellular pH, there were no apparent changes in *gad* gene expression. This knowledge highlights the necessity to study this system at both a protein level and also determine whether regulation of the system occurs by regulation of mRNA.

Work with cell extracts of *L. monocytogenes* has given the first evidence of a GABA shunt pathway for this bacterium. The presence of this pathway has implications for the metabolism of GABA produced by the cell. A link between metabolism and acid tolerance was also discovered while analysing this pathway in *L. monocytogenes* as disruption of a gene shown to encode the SSDH of this pathway (*lmo0913*) affected survival in acidic conditions.

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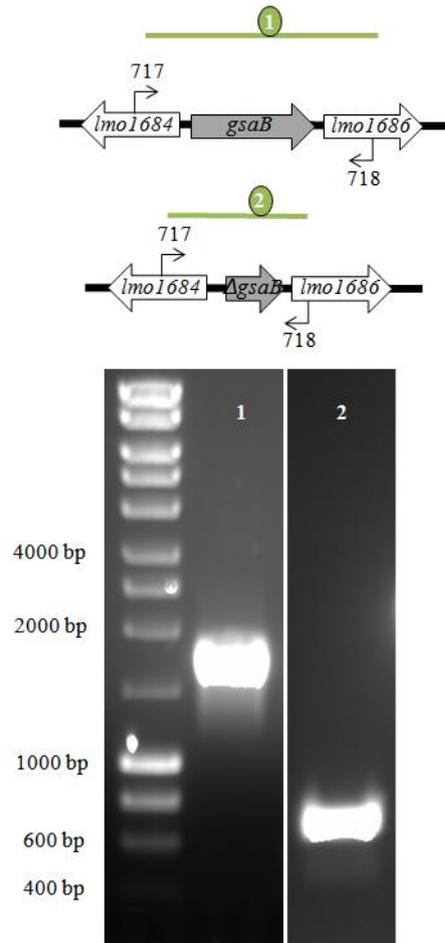
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APPENDICES

Appendix 1



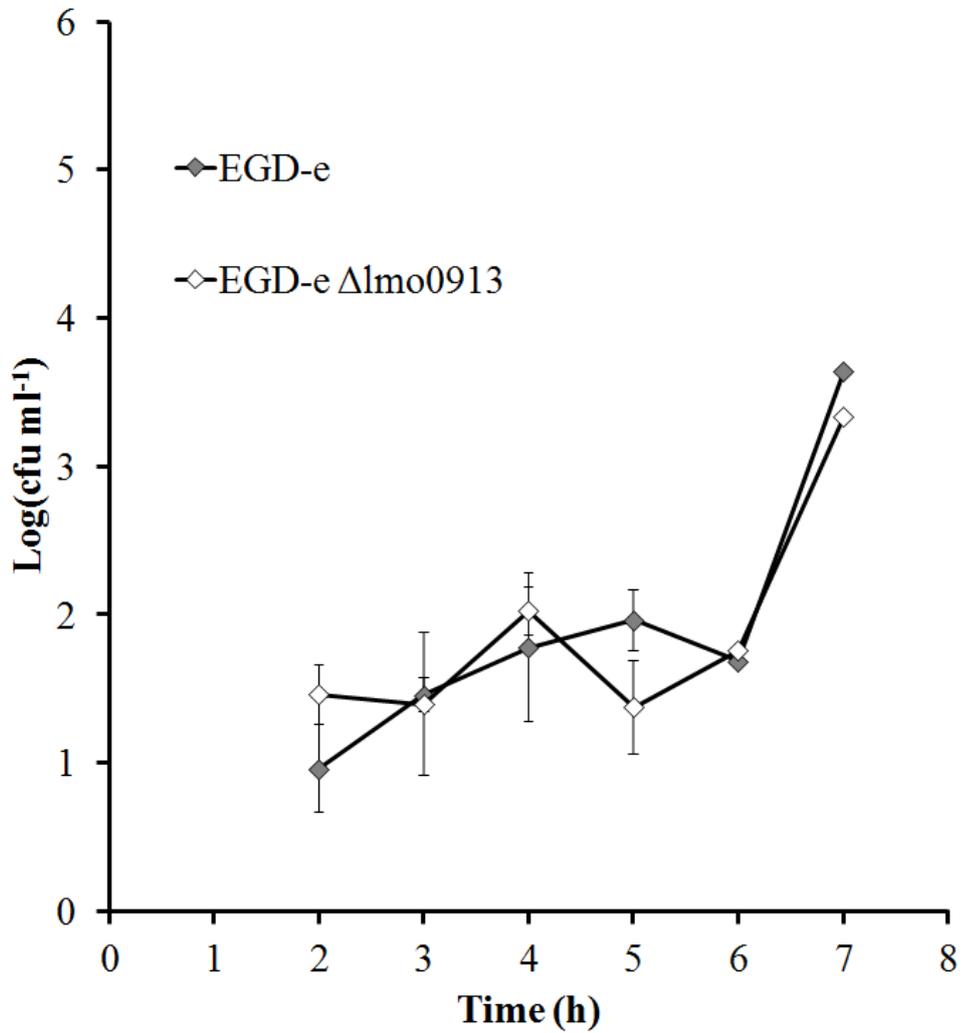
Appendix 1. Confirmation of EGD-e Δ *gsaB* mutant strain. Lane 1 contains a PCR product using primers 717/718 on a colony of EGD-e wild-type giving a band size of 1879 bp. Lane 2 contains PCR product using primers 717/718 on a colony of EGD-e Δ *gsaB* giving a band size of 616 bp.

Appendix 2

		10	20	30	40
EGD-e GadT2		MSKQAKSLTL	FGFFAITASM	VMTVYEYPTF	ATSGFHLVFF
10403S GadT2		MSKQAKSLTL	FGFFAITASM	VMTVYEYPTF	ATSGFHLVFF
		50	60	70	80
EGD-e GadT2		LLLGGLWFL	PVALCAAEMA	TVDGWQEGGI	FSWVGNTLGE
10403S GadT2		LLLGGLWFL	PVALCAAEMA	TVDGWQEGGI	FSWVGNTLGE
		90	100	110	120
EGD-e GadT2		RFGFAAIFQ	WFQITVGFVT	MIYFILGALS	YVFDFPALES
10403S GadT2		RFGFAAIFQ	WFQITVGFVT	MIYFILGALS	YVFDFPALES
		130	140	150	160
EGD-e GadT2		NPFIKFIGVL	VIFWGLTFSQ	LGGTKNTAKI	AKFGFIIIGIL
10403S GadT2		NPFIKFIGVL	VIFWGLTFSQ	LGGTKNTAKI	AKFGFIIIGIL
		170	180	190	200
EGD-e GadT2		IPAILFILG	IAYVAGGNPL	HVTFSKEAFI	PDFSKASTLV
10403S GadT2		IPAILFILG	IAYVAGGNPL	HVTFSKEAFI	PDFSKASTLV
		210	220	230	240
EGD-e GadT2		IFVSFILAYM	GVEASASHVN	EMTNPKRDP	LAMIMLVILA
10403S GadT2		IFVSFILAYM	GVEASASHVN	EMTNPKRDP	LAMIMLVILA
		250	260	270	280
EGD-e GadT2		IALNTIGGLT	ISAVLPLKDL	SLS SGVVQTF	QALILHFGSG
10403S GadT2		IALNTIGGLT	ISAVLPLKDL	SLS SGVVQTF	QALILHFGSG
		290	300	310	320
EGD-e GadT2		LGWAVKLI	MIALGVMEV	SAWVVGPSRG	MYTAAEQGLL
10403S GadT2		LGWAVKLI	MIALGVMEV	SAWVVGPSRG	MYTAAEQGLL
		330	340	350	360
EGD-e GadT2		PEKMKKVNKH	GVPVPLIMVQ	GVVVTIWA	LTFGGGGNNL
10403S GadT2		PEKMKKVNKH	GVPVPLIMVQ	GVVVTIWA	LTFGGGGNNL
		370	380	390	400
EGD-e GadT2		SFLTAISLTV	VIYLVGYLLF	FIGYLVLI	KSSLKRITYQI
10403S GadT2		SFLTAISLTV	VIYLVGYLLF	FIGYLVLI	KSSLKRITYQI
		410	420	430	440
EGD-e GadT2		PGGKIVKVI	ALIGLVTSIF	ALCISFVPPA	SIAAKSDTTY
10403S GadT2		PGGKIVKVV	ALIGLVTSVF	ALCISFVPPA	SIAAKSDMTY
		450	460	470	480
EGD-e GadT2		MVILSISFIV	TVLIPFIIYA	VHDKKDITPK	DITHLDS SDI
10403S GadT2		IVILSISFIV	TVLIPFIIYA	VHDKKDITPK	DITHLDS SDI
		490	500		
EGD-e GadT2		NKFTHPRARG	EHIINPDEKH	IMNQDKL	
10403S GadT2		NKFTHPRARG	EHIINPDEKH	IMNQDKL	

Appendix 3. Alignment of GadT2 sequences. The top line displays the amino acid sequence of EGD-e GadT2 with the pairwise alignment of GadT2 from 10403S below. Amino acid difference are highlighted in blue.

Appendix 3



Appendix 4. Growth of EGD-e *lmo0913* mutant in THP-1 macrophages.

Growth of EGD-e *lmo0913* mutant inside THP-1 macrophages was measured over 7 h. Counts were recorded 2 h post co-incubation of THP-1 with bacteria at an MOI of 10 (10^6 bacteria; black arrow). Error bars represent the standard deviation from the mean of at least 4 biological replicates for each strain and time-point.

PUBLICATIONS