

## ABSTRACT

PARSONS, CAMERON TYLER. Functional Genomics of Heavy Metal Resistance in the Foodborne Pathogen *Listeria monocytogenes*. (Under the direction of Dr. Sophia Kathariou).

*Listeria monocytogenes* is a Gram-positive facultative intracellular pathogen that is found ubiquitously in nature. To be able to survive in diverse environments, *L. monocytogenes* has acquired adaptations such as the ability to grow in the cold, to form biofilms, and to tolerate a variety of toxic compounds. *L. monocytogenes* also possesses adaptations that enable it to survive and proliferate inside an animal host, such as the ability to evade the host immune system, spread cell-to-cell and invade normally sterile sites such as the placenta and central nervous system. One of the adaptations that plays a role in both environmental and *in vivo* survival of *L. monocytogenes*, is its tolerance to heavy metals. In the environment *L. monocytogenes* can encounter extremely low levels of the essential metals it needs to function, or extremely high levels of both essential and toxic metals; either can prove to be fatal for the cell. Inside an animal host, *L. monocytogenes* can also encounter extremely high or low levels of essential metals, making the ability to tightly regulate intracellular metal levels of key importance to both saprophyte and pathogen.

Here, transposon (*mariner*-based) mutagenesis was employed to investigate the genetic basis for the tolerance of *L. monocytogenes* to both essential, as well as toxic metals. Mutant libraries for two produce-related strains F8027 and 20111-2858 were screened for mutants that had an increased susceptibility to arsenic, cadmium, and copper. Four mutants were isolated with decreased tolerance to cadmium, and one mutant with a decreased tolerance to copper. One of the cadmium-sensitive mutants was found to have a transposon insertion in *cadA4*, a novel member of the *CadA* family of cadmium-effluxing P-type

ATPases, harbored on a larger mobile genetic element (MGE). Genetic complementation of this mutant with chromosomally integrated *cadA4* restored cadmium tolerance. Furthermore, heterologous expression in two unrelated cadmium-susceptible *L. monocytogenes* strains conferred cadmium tolerance comparable to that of F8027, confirming the role of *cadA4* in cadmium resistance. Assessments in the *Galleria mellonella* model suggested that *cadA4* was negatively correlated with virulence, serving to suppress virulence.

Another transposon mutant of strain F8027 with decreased tolerance to cadmium was found to have a transposon insertion in an intergenic region between a previously uncharacterized cell wall-associated protein and a gene of unknown function, on the same MGE as *cadA4*. This mutant was found to have reduced tolerance to zinc as well.

Construction of deletion mutants suggested that the cell wall-associated protein was responsible for the decreased tolerance to zinc, and genetic complementation suggested that the inactivation of one of the downstream genes was responsible for the decreased tolerance of this mutant to cadmium. Assessments in the *Galleria mellonella* model suggested that, in addition to its role in zinc tolerance, the cell wall-associated protein was required for full virulence potential. The cell wall-associated protein was also found to mediate wild type levels of biofilm formation.

The one mutant found to have a decreased tolerance to copper, was a derivative of strain 2011L-2858, and harbored a transposon insertion in the intergenic region just before the start codon of *pbp4*, encoding a penicillin binding protein. While previous work had associated this gene with susceptibility to  $\beta$ -lactam antibiotics and bacteriocins, this was the first time that a link between this gene and copper tolerance was noted. This gene was also found to be required for full virulence potential in the *Galleria mellonella* model, and to

impact biofilm formation at 25 and 37°C. In summary, three novel determinants associated with metal homeostasis have been identified, and a previously unknown association has been established between a well-characterized gene for a penicillin-binding protein and copper homeostasis.

Functional Genomics of Heavy Metal Resistance in the Foodborne Pathogen *Listeria*  
*monocytogenes*.

by  
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## **DEDICATION**

To my parents Gary and Judy, who for as long as I can remember told me to go out and be whatever I wanted to be, and have never wavered in their love and support of that aim.

## BIOGRAPHY

Cameron Parsons was born in Raleigh, NC. He graduated from Broughton High School, and subsequently graduated from North Carolina State University with a degree in History in 2003. After working for the North Carolina Department of Transportation for a number of years he returned to North Carolina State University to pursue an undergraduate degree in Food Science. It was at that time he was introduced to microbiology and had the good fortune of being able to conduct undergraduate research in the lab of Dr. Sophia Kathariou. After the completion of his Food Science degree in 2012, he had the even better fortune to be able to pursue his PhD in Food Science, also in the lab of Dr. Sophia Kathariou. His doctorate work focused on the genetic basis of metal tolerance in *Listeria monocytogenes*.

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**Chapter 1 : Central role of metal homeostasis in the diverse adaptations of the foodborne pathogen *Listeria monocytogenes*: a minireview.**

## SUMMARY:

*L. monocytogenes* is ubiquitous in the environment and the causative agent of the disease listeriosis. Metal homeostasis is one of the key processes utilized by *L. monocytogenes* in its roles as either saprophyte or pathogen. In the environment as well as within an animal host *L. monocytogenes* needs not only to acquire essential metals when they are limited, but also mitigate the effects of toxic levels of metals. While the mechanisms associated with acquisition and detoxification of essential metals such as copper, iron, and zinc have been recently reviewed, a review of the mechanisms associated with heavy metals such as arsenic and cadmium is lacking. Resistance to both cadmium and arsenic is frequently encountered in *L. monocytogenes*, including isolates from human listeriosis. In addition, a growing body of work indicates the association of these determinants with other cellular functions such as virulence, suggesting the importance of further study in this area.

## INTRODUCTION:

*Listeria monocytogenes* is a Gram-positive facultative intracellular pathogen, and the causative agent of the disease listeriosis. In healthy individuals listeriosis manifests as febrile gastroenteritis; however, in the at-risk individuals including those who are elderly, pregnant or immunocompromised listeriosis can result in much more severe symptoms such as septicemia, meningitis, encephalitis, stillbirths and abortions (Kathariou 2002). Listeriosis is responsible for approximately 1,455 hospitalizations and 255 deaths in the United States annually (Scallan *et al.* 2011). *L. monocytogenes* is found ubiquitously in the environment, is capable of growing in the cold, and can persistently colonize food production facilities

(Kathariou 2002). This, along with the severe outcomes and life-threatening potential of listeriosis, makes *L. monocytogenes* a major cause for food safety and public health concern.

*L. monocytogenes* is well adapted to survive both in the environment as well as within the body of humans and other animals (Gray, Freitag and Boor 2006; Freitag, Port and Miner 2009). One of the adaptations that is key to these dual survival modalities is metal homeostasis. Certain metals such as copper, zinc, and iron are essential for key cellular functions but become toxic at higher concentrations; on the other hand, metals such as arsenic and cadmium appear to serve no cellular function and are considered toxic at any concentration (Jesse, Roberts and Cavet 2014). In the environment metals are typically found at low levels, but their concentrations can increase due to various anthropogenic interventions, including industrial pollution or agricultural practices (Alloway and Alloway 2013; Nunes *et al.* 2016). In an animal host metal concentrations are dependent on various factors such as diet and tissue type. The immune system can also utilize metals, either by restricting metal availability, or via recruitment of metals against pathogens in the course of infection (Zalewski *et al.* 2006; Carrigan *et al.* 2007; White *et al.* 2009). For these reasons, the ability to import or export metals as needed is essential for *L. monocytogenes* to survive in the diverse environments in which it finds itself.

**Essential yet potentially toxic metals.** Metals such as copper, iron, and zinc are cofactors for essential enzymes and are key for cellular function. Insufficient amounts of these metals can result in cellular death. However, at excessive concentrations these metals become toxic to the cell, disrupting membrane potential, interfering with enzyme function and creating reactive oxygen species, (Argüello, Raimunda and Padilla-Benavides 2013; Jesse, Roberts

and Cavet 2014; McLaughlin, Hill and Gahan 2011). *L. monocytogenes* has several determinants to acquire these metals in low abundance conditions, and to expel, sequester, or convert and detoxify these metals when they are in excess. Both of these conditions can be experienced inside of an animal host. Substantial work has been done to elucidate the function of these systems for essential metals, with several reviews (McLaughlin, Hill and Gahan 2011; Jesse, Roberts and Cavet 2014; Lechowicz and Krawczyk-Balska 2015). In relation to iron, recent findings have clarified the role of FrvA (Pi *et al.* 2016), which was initially implicated in haem toxicity and pathogenicity (McLaughlin *et al.* 2012). It was found that FrvA is a high affinity Fe(II) exporting P-type ATPase, which has specificity for elemental iron. In regard to copper, recent findings have elucidated the likely mechanism underlying copper toxicity in *L. monocytogenes* (Yousuf, Ahire and Dicks 2016). Findings suggest that copper disrupts the cell membrane through lipid peroxidation and protein oxidation similar to findings in other organisms (Santo, Quaranta and Grass 2012; Singh *et al.* 2015). Also of note from this study was the fact that *L. monocytogenes* had the most pronounced resistance to copper of all of the Gram-positive organisms tested (*L. monocytogenes*, *Streptococcus* spp., *Enterococcus* spp., and *Bacillus cereus*), which was suggested for further investigation by the authors (Yousuf, Ahire and Dicks 2016).

**Cadmium and arsenic: metals toxic at any concentration.** In contrast to the multiple reviews of *L. monocytogenes* adaptations related to essential metals, no comprehensive reviews are available on this pathogen's adaptations to toxic metals such as arsenic and cadmium. Resistance to arsenic and cadmium were two of the earliest documented phenotypes of *L. monocytogenes* (Buchanan *et al.* 1991; Lebrun *et al.* 1992). Such resistance

was encountered frequently enough to even be utilized as subtyping tool before the advent of higher-resolution techniques (McLauchlin *et al.* 1997). Determinants mediating resistance to these heavy metals are widely distributed within *L. monocytogenes*, being harbored both on the chromosome and on plasmids (Glaser *et al.* 2001; Elhanafi, Dutta and Kathariou 2010; Nelson *et al.* 2004; Kuenne *et al.* 2010, 2013; Lee *et al.* 2013).

**Arsenic resistance.** Whole genome sequencing has revealed genes putatively associated with arsenic detoxification, mostly on the chromosome but occasionally also on plasmids in *Listeria* spp. (Glaser *et al.* 2001; Kuenne *et al.* 2010, 2013); however, direct experimental evidence for the involvement of these genes in arsenic detoxification has been lacking. Plasmid-borne arsenic resistance has only been documented in one plasmid, pLI100, harbored by *L. innocua* CLIP 11262 (Glaser *et al.* 2001; Kuenne *et al.* 2010), but has not yet been detected in *L. monocytogenes*. This is consistent with earlier studies, which found that arsenic resistance was chromosomally mediated (McLauchlin *et al.* 1997). A putative arsenic resistance cassette was identified on a genomic island that also harbored the novel cadmium resistance determinant *cadA4* (Briers *et al.* 2011; Kuenne *et al.* 2013; Lee *et al.* 2013; Parsons *et al.* in review) (Fig. 1-1). Another arsenic resistance cassette has also been identified on a transposon (Tn554-like) in *L. monocytogenes* (Kuenne *et al.* 2013). The sequence analysis suggests this cassette was acquired through horizontal gene transfer with *Enterococcus faecalis*, indicating the importance of these determinants that they are even acquired from other species.

Arsenic resistance overall was found to be much less prevalent than cadmium resistance in previous studies of *L. monocytogenes* from food and food processing

environments, and was primarily encountered among isolates of serotype 4b (Mclauchlin *et al.* 1997; Mullapudi, Siletzky and Kathariou 2008; Ratani *et al.* 2012; Xu *et al.* 2014; Ferreira *et al.* 2011). A survey of 136 clinical 4b isolates from sporadic human listeriosis in the United States showed that approx. 17% were arsenic-resistant, while approx. 33% were resistant to cadmium (Lee *et al.* 2013). This is consistent with the greater prevalence of cadmium resistance as opposed to arsenic resistance, particularly in light of the fact that 100% of that isolates that were arsenic resistant were also resistant to cadmium. Also worthy of note is that only approx. 50% of the isolates that were resistant to cadmium were also resistant to arsenic, demonstrating a greater instance of cadmium resistance in the absence of arsenic resistance, particularly in clinical strains, suggesting that there may be a more significant *in vivo* involvement of cadmium resistance genes.

**Plasmid-associated cadmium resistance determinants.** In a survey of *L. monocytogenes* plasmids from strains of diverse origins (food, environmental, clinical), 95% of the plasmid-harboring strains were found to be cadmium-resistant (Lebrun *et al.* 1992). This was consistent with a later survey of plasmids in the genus *Listeria* that spanned multiple species, serogroups, and origins, which found that the only common plasmid-borne element besides the origin of replication was the presence of a cadmium resistance cassette (Kuenne *et al.* 2010). These plasmids were found to encode a cadmium efflux P-type ATPase (*cadA*) and its putative repressor *cadC*. Many of the *cadA*-harboring plasmids also harbored putative copper resistance determinants (Kuenne *et al.* 2010). *CadA* in conjunction with a cassette of genes for arsenic detoxification has only been encountered once, in pLI100 of *L. innocua* CLIP 11262, discussed above (Glaser *et al.* 2001; Kuenne *et al.* 2013).

*CadA* in *L. monocytogenes* was found to be genetically similar to the *cadA* earlier characterized in *Staphylococcus aureus* (Lebrun, Audurier and Cossart 1994). While the *cadA* in *S. aureus* conferred resistance to cadmium and zinc, the plasmid-harbored *cadA* in *L. monocytogenes* was found to be specific to cadmium (Lebrun, Audurier and Cossart 1994). *CadA1* was found on Tn5422 (Fig. 1-1) (Lebrun, Audurier and Cossart 1994), a mobile genetic element that has been identified on numerous plasmids (Lebrun, Audurier and Cossart 1994; Canchaya *et al.* 2010). One study identified *cadA1* on all of the plasmids tested in that study (Lebrun, Audurier and Cossart 1994). That same study also found that while Tn5422 was actively mobile, that it was never detected chromosomally, but that it was shown to integrate extensively with plasmids, leading the authors to speculate that this element may be responsible for much of the size variation encountered in plasmids in *L. monocytogenes*. A second putative *cad* (*cadA2*) was first identified on the large plasmid pLI100 of *L. innocua* CLIP 11262 (Glaser *et al.* 2001), and on the approx. 80 kb plasmid pLM80 of *L. monocytogenes* H7858 (Nelson *et al.* 2004) (Fig. 1-1). The latter plasmid was later experimentally confirmed to confer not only cadmium resistance but also resistance to the quaternary ammonium compound benzalkonium chloride and to toxic dyes (Elhanafi, Dutta and Kathariou 2010; Dutta *et al.* 2014) (Fig. 1-1).

Plasmids harboring *cadA1* and *cadA2* have been observed in both pathogenic *L. monocytogenes* as well as non-pathogenic other *Listeria* spp. (Kuenne *et al.* 2010; Katharios-Lanwermyer *et al.* 2012). *CadA1* was encountered more frequently than *cadA2* in several studies (Mullapudi, Siletzsky, and Kathariou 2010; Ratani *et al.* 2012; Xu *et al.* 2014), and is more commonly found in strains of serotype 1/2a, while *cadA2* was encountered more frequently in serotype 1/2b strains (Mullapudi, Siletzsky, and Kathariou 2010). While some

strains do harbor both *cadA1* and *cadA2* simultaneously (Mullapudi, Siletzsky, and Kathariou 2010; Xu *et al.* 2014), it was not encountered in every study, with some studies reporting little to no occurrence (Ratani *et al.* 2012; Lee *et al.* 2013), suggesting specific environments may be more conducive to this occurring. Overall *cadA1* and *cadA2* were far more prevalent in serotype 1/2a and 1/2b strains from food and food processing plants, as opposed to serotype 4b (Ratani *et al.* 2012). This could potentially be explained by the overall greater prevalence of plasmids in serogroup 1 than in serogroup 4 (Kuenne *et al.* 2010). In clinical 4b isolates *cadA1* was not encountered in either EC1a or EC1b, while *cadA2* was not found in any of the EC1 isolates from sporadic human cases suggesting a genetic preference in relation to the cadmium resistance determinant harbored. Another key point is that these isolates were all serotype 4b, which harbored *cadA1* and *cadA2* less frequently in other studies (Mullapudi, Siletzsky, and Kathariou 2010; Ratani *et al.* 2012), suggesting that this occurrence and distribution may well be different in serogroup 1 clinical strains.

There have been several surveys of cadmium resistance prevalence within strains isolated from food and food processing facilities (Mullapudi, Siletzky and Kathariou 2008; Ratani *et al.* 2012; Xu *et al.* 2014; Ferreira *et al.* 2011). Prevalence of cadmium resistance among these isolates ranged from 50 to 66%, suggesting that cadmium resistance is highly prevalent in these environments. One study also noted that isolates repeatedly isolated from milk and dairy foods in Northern Ireland were more likely to be cadmium resistant than those that were isolated sporadically (Harvey and Gilmour 2001). These findings are from surveillance of foods and food processing facilities studies in diverse locations in Asia, Europe, and North America, suggesting widespread prevalence of resistance.

**Chromosomal cadmium resistance determinants.** The proliferation of whole genome sequencing resulted in the discovery of several cadmium resistance determinants harbored chromosomally in *L. monocytogenes*. The first to be identified was *cadA3*, harbored on a mobile conjugative element in strain EGDe (Glaser *et al.* 2001). While direct experimental evidence for its role in cadmium resistance is still lacking, the presence of this gene has been associated with a cadmium tolerance of >140 µg/ml (Lee *et al.* 2013). Thus far this determinant has been encountered infrequently, having been identified only in EGDe and a few additional strains (Lee *et al.* 2013; Ratani *et al.* 2012; Kuenne *et al.* 2013). Another chromosomal *CadA* family member (*cadA4*) was identified on a larger genomic island in the chromosome of strain Scott A (Briers *et al.* 2011; Kuenne *et al.* 2013) (Fig. 1-1). While *cadA1-3* are all associated with cadmium tolerance of >140 µg/ml, *cadA4* only confers resistance of approximately 50 µg/ml (Lee *et al.* 2013; Parsons *et al.* in review). This difference in resistance level is likely due to the divergent nature of the sequence of *cadA4* (*cadA1-3* approx. 70% aa identity, *cadA4* approx 36% aa identity) (Lee *et al.* 2013; Parsons *et al.* in review). Expression of *cadA1,2,4* is induced in the presence of even trace amounts of cadmium (Lebrun *et al.* 1994; Elhanafi, Dutta and Kathariou 2010; Parsons *et al.* in review). Due to the more recent characterization of *cadA4*, prevalence of this determinant has not been surveyed as extensively as *cadA1* and *cadA2*; however, it was identified in approx. 10% of serotype 4b isolates in a survey of sporadic cases of human listeriosis in the United States (Lee *et al.* 2013). *CadA4* harboring strains accounted for 28% of the cadmium resistant isolates in that study, all of these isolates were also resistant to arsenic. The presence of *cadA4* was confirmed in these isolates via PCR, which also confirmed the presence of several arsenic detoxification genes (*arsA1* and *arsA2*) that were known to be

present on LGI2 (Fig. 1-1), suggesting that MGE is the source of both arsenic and cadmium resistance in these strains, and also suggesting a potential *in vivo* involvement of the element, given its prevalence in clinical strains (Lee *et al.* 2013). Since many of the prior studies screened for cadmium resistance at a level of 70 µg/ml (McLauchlin *et al.* 1997; Mullapudi, Siletzky, and Kathariou 2008; Ratani *et al.* 2012), the presence of *cadA4* would have been undetected. This also suggests that prevalence of cadmium resistance at lower MICs (e.g. those conferred by *cadA4*) has likely been underreported.

**Impacts on other adaptations, including virulence.** In addition to the prevalence of these determinants in isolates from food and food processing environments, there have been several more direct links between heavy metal resistance and the ability of *L. monocytogenes* to cause disease. As previously mentioned, a survey of clinical isolates from sporadic human listeriosis in the United States found that 33% of the 136 serotype 4b isolates were cadmium-resistant while 17% were resistant to arsenic, while 100% of arsenic resistant strains were also cadmium resistant. The relatively high prevalence of these resistance attributes among isolates from human disease may suggest the involvement of heavy metal resistance genes with either greater *in vivo* fitness, or greater ability to cause disease. Given that isolates from food, and food processing environments are frequently found to be cadmium resistant (Mullapudi, Slietzky, and Kathariou 2008; Ratani *et al.* 2012; Xu *et al.* 2014), and most cases of listeriosis result from contaminated food, the selection for cadmium resistant strains in the food processing environment could be the reason for the prevalence of these strains clinically, as these are more likely to be the strains that people are exposed to in food.

The first experimental evidence for possible involvement of cadmium resistance-related determinants in virulence was obtained from an *in vivo* transcriptional analysis of *L. monocytogenes* from the livers of mice infected with strain EGDe, which harbors *cadA3* (Camejo *et al.* 2009). The putative repressor *cadC* was markedly upregulated in the liver of the infected animals during infection, and deletion of this gene resulted in a decrease in virulence in a murine intravenous infection model (Camejo *et al.* 2009). Interestingly, a transposon insertion mutant of *cadA4* (Parsons *et al.* in review), actually showed an increase in virulence in the *Galleria mellonella* model, suggesting an inverse relationship between *cadA* and virulence. This study found that a functional *cadA4* decreased virulence potential (Parsons *et al.* in review), which is consistent with the previous study which demonstrated that the putative repressor *cadC* was required for full virulence (Camejo *et al.* 2009). Taken together the results of both studies suggest an association between metal resistance determinants and the ability of *L. monocytogenes* to cause disease.

The cadmium and arsenic resistance genes of *L. monocytogenes* discussed here are accompanied by transcriptional regulators of the ArsR family. These regulators have been found to regulate single genes in some circumstances, while mediating a global transcriptional response in others (Osman and Cavet 2010). Most often they regulate expression of genes directly involved in metal detoxification, they can also impact expression of genes with a variety of other functions, such as oxidative stress resistance, acid adaptation, respiration, and ribosome biogenesis (Osman and Cavet 2010). CadC is a member of the ArsR family of metal associated transcriptional regulators (Osman and Cavet 2010), and its involvement in virulence in *L. monocytogenes* has already been mentioned. Other members of the ArsR family have also been found to be involved in the regulation of virulence-

associated genes in other species. Specifically, the PhoPR two-component regulatory system, which is responsible for the regulation of virulence and persistence genes in *Mycobacterium* spp. (Gao, Yang and He 2011), was found to be under the control of an ArsR transcriptional factor. Such findings suggest a role of the ArsR family of transcriptional regulators in virulence, not only of *L. monocytogenes*, but other species as well.

Metal resistance genes have also been found to co-localize with antibiotic and other antimicrobial resistance genes on mobile genetic elements such as plasmids, genomic islands, and transposons (Farias *et al.* 2015; Kuenne *et al.* 2010; Elhanafi, Dutta, and Kathariou 2010) (Fig. 1-1). Whole genome sequencing has revealed evidence suggesting that these elements are potentially transferable to *L. monocytogenes* from other species (Briers *et al.* 2011), while there is direct experimental evidence for the transfer of such elements from *L. welshimeri* or *L. innocua* to *L. monocytogenes* (Katharios-Lanwermyer *et al.* 2012). This creates the possibility that metal contamination, and/or metal resistance genes could facilitate the acquisition and transfer of antibiotic resistance genes to *L. monocytogenes*, or from *L. monocytogenes* to other agents of public health concern. It has also been shown that extremely low levels of metals can induce transcription of metal resistance genes (Lebrun *et al.* 1994; Parsons *et al.* in review), and that extremely low levels of metal exert sufficient selective pressure to result in the retention of these elements (Gullberg *et al.* 2014). These data suggest that in minute amounts either in the environment or in an animal host, heavy metals can potentially exert selective pressure which in turn could drive the acquisition or transfer of mobile genetic elements, which can drastically impact the environmental and *in vivo* survival of *L. monocytogenes*.

## CONCLUSIONS:

Metals play a key role in the survival of *L. monocytogenes* both in the environment and in animal hosts. Essential metals must be acquired, and toxic effects of excess metals must be mitigated. While the *L. monocytogenes* cellular functions associated with essential metals have been extensively studied and reviewed, those involved with exclusively toxic metals such as cadmium and arsenic are poorly understood. The significance of these determinants is shown by their wide distribution within *L. monocytogenes*, as well as their association with food, food processing plants, clinical strains, and clonal groups involved in outbreaks. There is increasing evidence to suggest the involvement of metal resistance genes with a variety of functions beyond just metal detoxification in other organisms (Singh *et al.* 2015; Binopal *et al.* 2016). Several studies mentioned here (Camejo *et al.* 2009; Parsons *et al.* in review) would also suggest that there are alternate functions for these genes in *L. monocytogenes* as well. Given their prevalence, potential involvement in selection and population dynamics, as well as, their growing implication in important alternate cellular functions such as virulence, toxic metal resistance genes are an ideal candidate for further study.

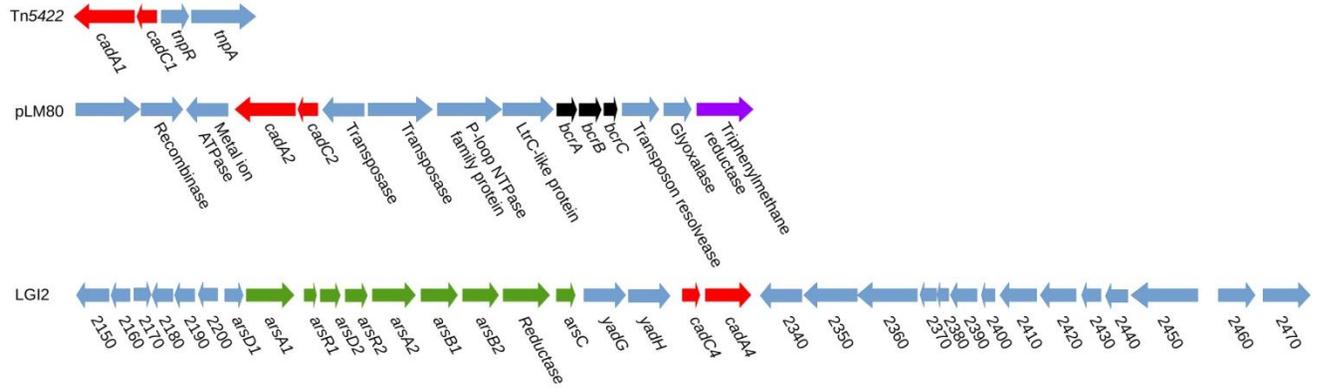
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**Figure 1-1.** Resistance gene distribution across mobile genetic elements for *cadA1*, *cadA2* and *cadA4*. *Cad* family members are indicated in red. Benzalkonium chloride resistance determinants are in black. Toxic dye determinants are in purple. Putative arsenic detoxification determinants are in green.

**Chapter 2 : Characterization of a novel cadmium resistance determinant in *Listeria monocytogenes*.**

## **SUMMARY:**

*Listeria monocytogenes* is a foodborne pathogen that can cause severe disease (listeriosis) in susceptible individuals. It is ubiquitous in the environment and often exhibits resistance to heavy metals. One of the determinants that enables *Listeria* to tolerate exposure to cadmium is the *cadAC* efflux system, with *CadA* being a P-type ATPase. Three different *cadA* genes (designated *cadA1-cadA3*) have previously been characterized in *L. monocytogenes*. A novel putative cadmium resistance gene (*cadA4*) was recently identified through whole genome sequencing, but experimental confirmation for its involvement in cadmium resistance is lacking. In this study, we characterized *cadA4* in *L. monocytogenes* strain F8027, a cadmium-resistant strain of serotype 4b. Screening of a *mariner*-based transposon library of this strain identified a mutant with reduced tolerance to cadmium and harboring a single transposon insertion in *cadA4*. Tolerance to cadmium was restored by genetic complementation with the cadmium resistance cassette (*cadA4C*), and enhanced cadmium tolerance was also conferred to two unrelated cadmium-sensitive strains via heterologous complementation with *cadA4C*. Cadmium exposure induced *cadA4* expression, even at non-inhibitory levels. Virulence assessments in the *Galleria mellonella* model suggested that a functional *cadA4* suppressed virulence, potentially promoting commensal colonization of the insect larvae. Biofilm assays suggested that *cadA4* inactivation resulted in reduced biofilm formation. These data not only confirm *cadA4* as a novel cadmium resistance determinant in *L. monocytogenes*, but also provide evidence for roles in virulence and biofilm formation.

**Importance.** *Listeria monocytogenes* is an intracellular foodborne pathogen causing the disease listeriosis, which is responsible for numerous hospitalizations and deaths every year.

Among the adaptations that allow *Listeria* to survive in the environment is the ability to persist in biofilms, grow in the cold and tolerate toxic compounds such as heavy metals. Here we characterize a novel determinant that was recently identified on a larger mobile genetic island through whole genome sequencing. This gene (*cadA4*) was found to be responsible for cadmium detoxification and to be a divergent member of the Cad family of cadmium efflux pumps. Virulence assessments in a *Galleria mellonella* model suggested that *cadA4* also plays a role in virulence, acting to suppress virulence. Additionally, *cadA4* was implicated in *Listeria*'s ability to form biofilms. Beyond the role of *cadA4* in cadmium detoxification, its involvement in other cellular functions, potentially explains its retention and wide distribution in *L. monocytogenes*.

Keywords: *Listeria monocytogenes*, cadmium resistance, *cadA*, biofilm, virulence

## **INTRODUCTION**

*Listeria monocytogenes* is a Gram-positive rod-shaped bacterium widely distributed in nature (Vivant *et al.* 2013). As a facultative intracellular foodborne pathogen causing the disease listeriosis, *L. monocytogenes* is responsible for numerous hospitalizations and deaths in the United States every year (Scallan *et al.* 2011). To facilitate its survival in a diverse array of conditions, *L. monocytogenes* possesses an equally diverse array of determinants, including the ability to persist in biofilms, grow in the cold and tolerate a variety of toxic compounds such as heavy metals, disinfectants and dyes (Dutta *et al.* 2011, Lebrun *et al.* 1994, Gandhi *et al.* 2007, Romanova *et al.* 2002). In particular, heavy metals in the environment can exert long-term selective pressure on bacteria (Alam *et al.* 2010), as heavy metals do not degrade over time. It is worthy of note that several major foodborne outbreaks of listeriosis have involved cadmium-resistant *L. monocytogenes* strains (Lee *et al.* 2014). Interestingly,

cadmium resistance in *L. monocytogenes* has been found to occur more commonly among strains repeatedly isolated from foods than those encountered only sporadically (Harvey and Gilmour 2001).

One of the determinants shown to mediate cadmium resistance in *L. monocytogenes* is the *CadAC* efflux cassette, comprised of the heavy metal-translocating P-type ATPase *CadA* and its putative repressor, *CadC* (Lebrun *et al.* 1994a, Lebrun *et al.* 1994b). Three members of the *CadAC* family have previously been identified in *L. monocytogenes*. Of these, *CadA1* and *CadA2* have been found to be harbored by plasmids and to be widely distributed among different strains and serotypes of *L. monocytogenes* (Lebrun *et al.* 1994a; Lebrun *et al.* 1994b; Lee *et al.* 2013; Mullanpudi, Siletsky, and Kathariou 2010; Kuenne *et al.* 2013), while *CadA3* is encoded chromosomally and has only been identified in strain EGD-e and a few others (Lee *et al.* 2013; Kuenne *et al.* 2013; Glaser *et al.* 2001). The *cadC* (*Lmo1102*) of strain EGD-e was markedly upregulated *in vivo* in a murine infection model, and experimentally confirmed to be required for virulence in that model (Camejo *et al.* 2009). Another P-type ATPase associated with iron homeostasis has been shown to be required for virulence in *L. monocytogenes* (McLaughlin *et al.* 2013). However, overall limited information is available on the potential impacts of heavy metal tolerance in virulence or persistence of *L. monocytogenes*.

Genome sequencing of *L. monocytogenes* strain Scott A, a clinical isolate from a 1979 listeriosis outbreak in Boston in which contaminated vegetables were epidemiologically implicated (Ho *et al.* 1986), identified a putative new member of the *CadA* family (tentatively termed *CadA4*) within a large (~35kb) genomic island in the chromosome of this strain (Briers *et al.* 2011). *CadA4* was also detected in another strain from this outbreak

(Chen *et al.* 2011) and in additional strains (Kuenne *et al.* 2013), including 29% of serotype 4b isolates from sporadic human listeriosis cases in the United States, 2005-2008 (Lee *et al.* 2014). The majority of these *cadA4*-harboring isolates were members of the same clonal complex as Scott A (CC2, previously also referred to as epidemic clone Ia [ECIa] or ECIV) (Lee *et al.* 2014). An unusual attribute of *cadA4* is that it was associated with growth at 35 µg/ml cadmium chloride but not at 70 µg/ml, whereas strains harboring *cadA1-3* grew at cadmium concentrations up to and exceeding 140 µg/ml (Lee *et al.* 2014; Mullapudi, Siletsky and Kathariou 2010).

In spite of the intriguing attributes of *cadA4* in *L. monocytogenes*, direct experimental evidence for its involvement in cadmium resistance has been lacking. In addition, no information has been reported on potential impacts of this determinant in virulence or other adaptations, to account for its presence and retention by *L. monocytogenes* CC2 and other strains, even in the absence of exposure to cadmium. In the current study, we characterize the role of *cadA4* in cadmium resistance of the serotype 4b, *cadA4*-harboring strain *L. monocytogenes* F8027, originally isolated from celery, and described other functional characteristics associated with this cadmium resistance gene cassette.

## **MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** Bacterial strains used in this study are listed in Table 2-1. Unless otherwise specified, *L. monocytogenes* was grown in brain heart infusion (BHI) (Becton, Dickinson & Co., Sparks, MD, USA) at 37°C. Erythromycin (5 µg/ml), kanamycin (10 µg/ml), chloramphenicol (8 µg/ml), or ciprofloxacin (0.05 µg/ml) (Sigma-Aldrich, St. Louis, MO, USA) were added as indicated. *Escherichia coli* strains were grown in Luria-Bertani (LB) (Becton, Dickinson & Co.) broth or agar medium supplemented with

chloramphenicol (25 µg/ml). Growth monitoring of selected strains in the presence and absence of cadmium (0.1, 1.6, 5 and 10 µg/ml CdCl<sub>2</sub> anhydrous [Fisher Scientific, Fair Lawn, NJ, USA]) employed a Biotek Elx808 absorbance microplate reader (Biotek, Winooski, VT, USA). Recorded changes in optical density (630 nm) were used to plot a growth curve for each strain.

**Heavy metal resistance and MIC determinations.** Tolerance to cadmium chloride, sodium arsenate dibasic heptahydrate (Sigma), and sodium arsenite (Sigma) was assessed on Iso-Sensitest agar (ISA) (Oxoid, Hampshire, England) as previously described (Mullapudi, Siletzsky and Kathariou 2010). To determine cadmium MICs, cell suspensions were spotted in duplicate at increasing concentrations (2.5, 5, 10, 15, 25, 35, 70, 140 µg/ml cadmium chloride, 500-4000 µg/ml sodium arsenate, and 50-1000 µg/ml sodium arsenite). The MIC was designated as the lowest concentration that prevented confluent growth following incubation at 37°C for 48 hours. Copper (II) sulfate pentahydrate (Acros Organics, NJ, USA) and zinc sulfate hydrate (Alfa Aesar, Heysham, England) MICs were determined in BHI (5-12.5 mM copper sulfate, and 1-30 mM zinc sulfate). MICs were determined in at least two independent trials. MICs for a panel of antibiotics (ampicillin, penicillin, oxacillin, pilimycin, penicillin mixed with novobiocin, tetracycline, erythromycin, cephalothin, ceftiofur and sulphadimethoxine) were kindly determined by Dr. Megan Jacob, North Carolina State University School of Veterinary Medicine Clinical Microbiology Laboratory, via A<sub>600</sub> measurements after 48 and 72 hr in Mueller Hinton Broth. The efflux inhibitor reserpine (Sigma-Aldrich) was used at 40 µg/ml. MICs in the presence of reserpine were

determined in duplicate and in two independent trials, with MICs in the absence of reserpine as controls.

**Sequencing of *cadAC* cassette.** Primer pairs were designed based on the genome sequence of strain Scott A (Briers *et al.* 2011) to amplify five overlapping regions of *cadAC* from F8027. The PCR products were gel-purified (QIAquick Gel Extraction Kit, Qiagen, Valencia, CA, USA) and sequenced at the North Carolina State University's Genomic Sciences Laboratory. Nucleotide and deduced polypeptide sequences were aligned using Clustal W2 (<https://www.ebi.ac.uk/Tools/msa/clustalw2/>) (Larkin *et al.* 2007) and the alignments used to compare the four known *L. monocytogenes* *CadA* sequences (*CadA1-CadA4*). Analysis by Softberry BPROM software (<http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb>) was used to identify putative promoter sequences and mFold (<http://mfold.rna.albany.edu/?q=mfold>) (Zuker 2003) was used to identify inverted repeat sequences in the intergenic region upstream of *cadAC*, indicative of putative repressor binding sites.

**RNA extraction and transcriptional assessments.** *L. monocytogenes* F8027 was grown in BHI at 25 and 37°C until mid to late logarithmic phase (OD<sub>600</sub> of 0.7-1.0, as measured by SmartSpec 3000; Bio-Rad, Hercules, CA, USA). The culture was then divided into two portions, one of which was exposed to sublethal levels of cadmium (0.1, 1.6, or 5 µg/ml) and incubated for an additional 30 min at 25 or 37°C while the other was similarly incubated without addition of cadmium. RNA was extracted using the SV total RNA isolation system

(Promega, Madison, WI, USA) and purified with Turbo DNA-free DNase (Ambion, Austin, TX, USA) to remove any contaminating DNA. For RT-PCR, RNA was reverse-transcribed to cDNA with the ImProm-II reverse transcription system (Promega) according to the manufacturer's protocol, using either primer P4 (*cadA4*) or P21 (*spoVG*) (Table 2-2); *spoVG* was used as reference gene for each trial, as previously described (Kim *et al.* 2012). PCR was then performed with cDNA as template using primers P3/P4 to assess *cadA4* transcription and primers P16/P19 and P19/P18 to assess co-transcription of *cadA4* with *LMOSA\_2320* and *cadC*, respectively. Negative controls (RNA and primers without reverse transcriptase) were included each time. For quantitative real-time PCR (qPCR), the BIO-RAD iTaq Universal SYBR Green One-Step Kit (Bio-Rad Laboratories Hercules, CA, USA) was used, per the manufacturer's instructions with a LightCycler 96 system (Roche Diagnostics, Indianapolis, IN, USA). Primers P22-P29 (Table 2-2) were designed using Primer3Plus (<http://primer3plus.com/>) to measure expression of *cadA4*, *cadC* and *LMOSA\_2320*, with *spoVG* used as control. All experiments were repeated in at least three independent trials. Fold change calculations were carried out using the  $\Delta\Delta C_T$  method, normalizing for the expression of *spoVG* as described previously (Kim *et al.* 2012; Baae, Crowley and Wang 2011).

### **Identification of cadmium-susceptible mutants and determination of transposon copy**

**number and insertion in *cadA4*.** A *mariner*-based transposon mutant library of *L.*

*monocytogenes* strain F8027 was constructed using pMC38 (Cao, Bitar and Marquis 2007).

The mutants were grown in individual wells of 96-well plates and screened in groups of 48 using a replicating device as described (Kim *et al.* 2012; Azizoglu and Kathariou 2010) to

identify those that failed to grow on ISA supplemented with cadmium (35 µg/ml). The number of transposon copies in the cadmium susceptible-mutants was determined using Southern blots as previously described (Cao, Bitar and Marquis 2007; Azizoglu and Kathariou 2010). To determine whether the transposon was inserted in *cadA4*, PCR was performed using the *cadA4*-targeting primers P3 and P4 (Table 2-2, Fig. 2-1) and the size of the resulting PCR products was determined by electrophoresis. To identify the transposon insertion site, two rounds of PCR were employed as described previously (Cao, Bitar and Marquis 2007), followed by sequencing (Genewiz Inc., South Plainfield, NJ, USA). The sequencing data were aligned using Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) (Sievers *et al.* 2011) and sequences were analyzed with BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Altschul *et al.* 1990) to differentiate transposon from flanking chromosomal sequences.

**Genetic complementation.** Primers P1 and P2 (Table 2-2) were designed with restriction sites for XmaI and SacI, respectively, and used to amplify a DNA fragment that included 333 nt of the intergenic region upstream of *cadC* (*LMOSA\_2321*), harboring the putative promoter, as well as the coding sequences for *cadC* and *cadA4*. The PCR product was digested with XmaI and SacI (New England Biolabs, Ipswich, MA, USA), gel-purified and ligated using T4 DNA ligase (Promega) to similarly digested shuttle vector pPL2 (Lauer *et al.* 2002), yielding the recombinant plasmid pPL2\_*cadA4*. This plasmid was electroporated into *E. coli* S17-1 (Simon, Prierer and Pühler 1983) resulting in *E. coli* S17-1\_pPL*cadA4*, and transferred into the *cadA4* transposon mutant I1A2 via conjugation as described (Azizoglu and Kathariou 2010), yielding strain I1A2::*cadA4*. The plasmid was additionally

transferred via conjugation to cadmium-susceptible *L. monocytogenes* strains F2365 and H7550-Cd<sup>s</sup>, yielding F2365::*cadA4* and H7550-Cd<sup>s</sup>::*cadA4*, respectively (Table 2-1). I1A2, F2365 and H7550-Cd<sup>s</sup> were also transformed with the empty vector pPL2, yielding I1A2::pPL2, F2365::pPL2 and H7550-Cd<sup>s</sup>::pPL2, respectively (Table 2-1). Putative transconjugants were selected on media containing chloramphenicol (8 µg/ml) and ciprofloxacin (0.05 µg/ml) since pPL2 confers chloramphenicol resistance and *L. monocytogenes* (but not *E. coli* S17-1) is naturally resistant to ciprofloxacin at this concentration (Stock and Wiedmann 1999). The chromosomal insertion site of pPL2 or pPL\_*cadA4* was confirmed via PCR with P11 and either P14 or P15 (Table 2-2).

**Site directed mutagenesis.** To convert the alanine residue (GCA codon) in the metal-binding site of the deduced *CadA4* of F8027 to threonine (ACA, conserved in *cadA1-A3*), primers P32 and P33 (Table 2-2) were designed and the corresponding *cadAC* region was then PCR-amplified in two segments (P1 and P33, P32 and P2). Both P32 and P33 incorporated the desired base change. Resulting PCR products were cleaned up, mixed 1:1 and used as template for PCR with primers P1 and P2, generating a complete *CadAC* cassette identical to the above complementation, except for the presence of the desired substitution. The PCR product was sequenced to confirm the mutation and cloned into pPL2 yielding pPL\_*cadA4*SDM, which was then integrated into the *cadA4* transposon mutant I1A2 (Table 2-1) as described above.

**Adherence and growth on produce.** Strains were grown at 37°C overnight in BHI, washed twice in an equivalent volume of sterile water and diluted in sterile water as needed. Whole

fresh celery (bunch) was purchased at retail and maintained at 4°C until used, typically on the same day. Celery fragments (2x1x0.5 cm) were prepared by aseptically cutting individual celery stalks with a flame-sterilized knife, placed in sterile petri dishes and stored at 4°C until use. To assess adherence, cell suspensions were spot-inoculated (25 µl) in a series of droplets on the fragments and allowed to dry for 1 hr at room temperature in a laminar flow cabinet. Each inoculated fragment was then placed in a sterile microcentrifuge tube containing 1 ml sterile water and subjected to a gentle rinse by manually inverting the tube four times to remove loosely adherent cells. The fragment was then aseptically removed, placed in a new tube with 1 ml sterile water and vortexed for one minute to remove tightly adherent cells. *Listeriae* in the water were enumerated by plating appropriate dilutions on selective media (Modified Oxford Agar [MOX], Becton, Dickinson & Co.) and incubating at 37°C for 48 hr. For assessment of *Listerial* growth on celery, fragments were dip-inoculated in a cell suspension of approx. 10<sup>5</sup> CFU/ml, air-dried in the laminar flow cabinet for 1 hr and incubated at 25°C for up to 48 hr. At 5, 24 and 48 hr post-inoculation, fragments were vortexed in sterile water and suspensions plated on MOX for enumeration. Adherence and growth assays were done in duplicate and in at least three independent trials.

**Virulence assessments in *Galleria mellonella*.** Live fourth-instar larvae of the greater wax moth *Galleria mellonella* were purchased (Vanderhorst Wholesale, Inc., Saint Mary's, OH, USA), kept in their original shipping containers at room temperature in the dark and used within 10 days. *L. monocytogenes* cell suspensions were prepared as described above for inoculation of produce and resuspended in PBS. Larvae were inoculated with 10 µl of cell suspension (10<sup>6</sup> CFU in PBS), that had been spotted on the sterile lid of unused petri dish

inside a biosafety hood, then picked up with a sterile 1 ml syringes and 30G needles (Becton, Dickinson & Co.) and injected into the last left proleg of each larvae. Ten larvae were inoculated per strain, and 10 control larvae were injected with the same volume of sterile PBS. Larvae were incubated at 37°C for up to seven days. Larval death was monitored daily based on lack of movement in response to touch and dead larvae were promptly removed. At the completion of the study, remaining living larvae were euthanized by placing them at -20°C for 20 minutes. Virulence assessments were done in at least three independent trials. For competitive fitness trials, larvae were inoculated with 10<sup>5</sup> CFU/larva of 1:1 mixtures of I1A2 and F8027 in PBS. After ten days surviving larvae were crushed in 3 ml sterile water with a sterile glass rod and vortexed for 1 min. The resulting suspension was diluted and plated on MOX. Colonies (n=96) were then tested on media with and without erythromycin and strain ratios were determined.

**Assessment of biofilm formation.** Biofilm formation was assessed in 96-well PVC plates (Greiner Bio-One, #655-185, VWR, Suwanee, GA) as described (Pan, Breidt, and Kathariou 2009). Overnight cultures were diluted 1:50 in tryptic soy broth (TSB; Becton, Dickinson and Co.) in individual wells, and the 96-well plates were incubated at either 25 or 37°C for 48 hr. Plates were washed three times with sterile water, stained with 0.8% crystal violet (Acros Organics), de-stained with 95% ethanol and monitored for absorbance at 580nm in a Tecan Safire<sup>®</sup> 96 well microplate reader (Tecan, Crailsheim, Germany). Biofilm assessments were done in at least three independent trials.

**Statistical analyses.** Fold change calculations for qPCR experiments employed the  $\Delta\Delta C_T$  method, with statistical evaluation of Ct values using a paired Student's *t*-test. For statistical analysis of growth and adherence on produce, one-way analysis of variance (ANOVA) with a Tukey's test was used at  $p < 0.05$ , with SPSS version 22 (IBM Corporation Software Group, Somers, NY, USA). For virulence data, a non-parametric proportional hazards survival model in Program JMP (SAS Institute, Cary, NC) was used to test for significant differences in survival of *Galleria melonella* when inoculated with different strains of *L. monocytogenes*. All strains were analyzed in a pairwise fashion and determined significance at the  $p < 0.05$  level. Biofilm data were analyzed using a paired Student's *t*-test ( $p < 0.05$ ).

**Nucleotide Sequence Accession Number.** Sequence of *L. monocytogenes* F8027 *cadA4C* was deposited in NCBI under accession number KT946835.

## **RESULTS AND DISCUSSION**

**Insertional inactivation of *cadA4* is accompanied with pronounced decrease in cadmium tolerance.** *L. monocytogenes* strain F8027 (hereafter F8027) was originally derived from fresh celery and has been used as model in studies for produce colonization by *L. monocytogenes* (Lang, Harris, and Beuchat 2004). It grew at 35 but not at 50  $\mu\text{g/ml}$  cadmium chloride, hence its MIC (50  $\mu\text{g/ml}$ ) is below the 70  $\mu\text{g/ml}$  threshold for resistance used in previous studies (Lee *et al.* 2013; Mullapudi, Siletsky and Kathariou 2010; McLauchlin *et al.* 1997). PCR with previously described primers (Lee *et al.* 2014) indicated that F8027 was negative for previously identified cadmium resistance determinants *cadA1*, *cadA2* and *cadA3* (data not shown). However, PCR with primers P30 and P31 (Lee *et al.* 2014), specific to *cadA4* identified through the genome sequencing of Scott A (Briers *et al.*

2011), yielded the expected product (data not shown). Cadmium MICs of F8027 in the presence of the efflux inhibitor reserpine were reduced to 20 µg/ml, suggesting that the observed cadmium tolerance of this strain was at least partly mediated by efflux mechanisms, as also noted for another cadmium resistance determinant in *L. monocytogenes*, *cadA2* (Xu *et al.* 2014).

Screening of a *mariner*-based library (approx. 2,000 mutants) on ISA with cadmium (35 µg/ml), identified four mutants with markedly reduced or no visible growth. PCR with *cadA4*-targeting primers P3 and P4 (Table 2-2) revealed that one mutant, I1A2, generated a larger PCR product (~2.5 kb) compared with F8027 (~1 kb) (data not shown), suggesting that it harbored a transposon insertion in *cadA4*. I1A2 also exhibited the most noticeable impairment of growth in the presence of cadmium (MIC, 10 µg/ml) (Table 2-1) while the other three mutants had higher (15-25 µg/ml) MICs for cadmium and harbored insertions in unrelated loci (data not shown). Sequencing of the *cadA4*-derived PCR amplicon from mutant I1A2 localized the transposon at nt 1059 of the *cadA4* coding region (2103 nt). Southern blot analysis showed that I1A2 harbored a single copy of the transposon (data not shown).

No difference in growth was observed between F8027 and I1A2 at 37°C in BHI (Fig. 2-2A). However, growth of I1A2 was markedly inhibited in the presence of 5 µg/ml cadmium (Fig. 2-2B), with complete inhibition at 10 µg/ml (Fig. 2-2C). Growth at the lower, more environmentally relevant levels of 1.6 and 0.1 µg/ml showed no differences in growth between F8027 and I1A2 at 37 or 28°C (data not shown). MICs of I1A2 to arsenic, zinc or copper were not affected (data not shown), suggesting that *cadA4* was specifically associated with cadmium tolerance. Previous studies with the plasmid-associated *cadA* determinant

*cadA1* also revealed that it mediated resistance to cadmium but not zinc, in spite of its homology with *cadA* from *Staphylococcus aureus*, which conferred resistance to both cadmium and zinc (Lebrun *et al.* 1994a). Additionally, neither *cadA2* nor *cadA3* were associated with resistance to zinc (C. Parsons and S. Kathariou, unpublished).

I1A2 could not be distinguished from its parental counterpart in other routine phenotypic assays. It exhibited no discernible difference from F8027 in motility, growth at 4°C, hemolytic activity on blood agar plates, cell shape or colony morphology. With the exception of resistance to erythromycin (conferred by the transposon), I1A2 could not be distinguished from F8027 in susceptibility to a panel of antibiotics, tested as described in Materials and Methods (M. Jacob, personal communication).

***CadA4* is a *CadA* family member divergent from *CadA1*, *CadA2* and *CadA3*, and associated with lower cadmium MICs.** Analysis of the results from sequencing of the F8027 *cadAC* revealed 100% identity to its counterpart in Scott A. Furthermore, PCR with primers derived from sequences flanking *cadA4* in Scott A indicated that in F8027 the *cadAC* cassette was flanked by the same genes as in Scott A (Fig. 2-1 and data not shown). Additional analysis, to be described in a separate publication, suggested that in F8027 *cadA4* was harbored by a genomic island with several arsenic detoxification genes and highly conserved with its Scott A counterpart but inserted in a different chromosomal location (data not shown).

BLAST analysis showed highly conserved (99-100% at the nt sequence level) *cadA4* homologs in several other *L. monocytogenes* genomes. There were also similarly conserved (90-99% identity) homologs in *Enterococcus cecorum* strains CE1 and CE2 and *E. faecalis*

strain KB1 (87% identity). Less conserved (71-72% identity at the nt sequence level and 63-61% identity of the deduced polypeptides) *cadA4* homologs were identified in *Clostridium botulinum* strain BTK015925, several strains of *Bacillus cereus* (FORC\_005, B4264, J17, A1, and S2-8) and the insect pathogen *B. thuringiensis*, strain Bt185 (data not shown). The genomic island harboring *cadA4* in Scott A was highly similar to a mobile genetic element (MGE) in *Enterococcus faecalis* JH1 (Briers et al. 2011) (data not shown). However, the *E. faecalis* MGE lacked *cadA4*, suggesting that *cadA4* in *L. monocytogenes* was incorporated into an arsenic resistance genomic island similar to that detected in *E. faecalis* strain JH1. The GC content of the Scott A and F8027 *cadAC* (33.6%) is noticeably lower than the average (38%) for *L. monocytogenes* genomes (Glaser et al. 2001; Nelson et al. 2004), suggesting horizontal gene transfer-mediated acquisition of this cadmium resistance cassette.

As mentioned earlier, *cadA4*-harboring strains of *L. monocytogenes* tolerate lower amounts of cadmium (MIC 50 µg/ml) than those harboring *cadA1*, *cadA2* or *cadA3* (MIC ≥ 140 µg/ml), unless they concurrently harbor one of the latter determinants (Lee et al. 2014). The underlying reasons remain unknown, but it is tempting to speculate that they are attributable to the sequence diversity of *cadA* (Fig. 2-3). While *cadA1*, *cadA2* and *cadA3* share ca. 70% identity at the aa sequence level, *cadA4* is noticeably divergent, exhibiting only ca. 36% identity with these other *cadA* determinants (Lee et al. 2013). Nonetheless, the deduced *CadA* encoded by *cadA4* harbored all three key motifs characteristic of the *CadA* protein family (Bal et al. 2003): CXXC, responsible for metal binding; DKTGT, associated with ATP binding; and CXC, specifically CPC, serving as the metal binding domain of the membrane-bound ion transport channel (Fig. 2-3). These key motifs were also present in the *B. cereus* and *B. thuringiensis* homologs (data not shown). It is noteworthy that, in contrast

to the DKTGT and CPC motifs that were conserved in *CadA1-A4*, the metal binding motif CXXC was different in *CadA4* (CANC) than in *CadA1-A3* (CTNC, in all three sequences).

We hypothesized that the observed amino acid substitution in such a key motif may account for the reduced tolerance to cadmium associated with *CadA4*, in comparison to *CadA1-3*. However, site directed mutagenesis-based alteration of the CANC sequence to the consensus CTNC found in *CadA1-3* actually resulted in a modest reduction of the MIC (40 µg/ml, vs. 50 µg/ml for F8027). It is conceivable that the unique CANC motif in *CadA4* works together with other elements of the polypeptide sequence to mediate the observed levels of tolerance to cadmium and that other *CadA4C* features may also contribute to the difference in tolerance to cadmium conferred by *CadA4C*, in comparison to *CadA1C*, *CadA2C* and *CadA3C*. This is also supported by findings from heterologous expression of *cadA4C* in unrelated *L. monocytogenes* strains, to be discussed below.

**Genetic complementation and heterologous expression confirm involvement of *cadA4* in cadmium tolerance.** A putative promoter was identified in the intergenic region upstream of *LMOSA\_2321* (*cadC*) in Scott A, along with a pair of inverted repeats ( $\Delta G$  -33.6), one of which overlapped the putative -10 box of the promoter (Fig. 2-1). These inverted repeats were similar (identity in 11/13 nucleotides) with those previously identified in the -35 putative promoter region of *cadA1C* and hypothesized to be involved in transcriptional regulation of *cadA1C* (Lebrun *et al.* 1994a). Mobilization of *cadA4C* together with the upstream intergenic region harboring the putative promoter into the cadmium-sensitive mutant I1A2 resulted in complete restoration of cadmium tolerance (cadmium MIC, 50 µg/ml) (Fig. 2-4).

Heterologous expression of *cadA4C* in two unrelated cadmium-susceptible *L. monocytogenes* strains, F2365 and H75550-Cd<sup>S</sup>, also conferred similar levels of cadmium tolerance (MIC 50 µg/ml, in contrast to 10 µg/ml for the respective parental strains) (Fig. 2-4). Strains F2365 and H75550-Cd<sup>S</sup> chosen for heterologous expression of *cadA4C* belong to clonal complexes CC1 (also known as ECI) and CC6 (also known as ECII), respectively, markedly distinct from either F8027 (CC315) or Scott A (CC2). H75550-Cd<sup>S</sup> is a plasmid (pPL80)-cured derivative of strain H7550, implicated in the 1998-1999 hot dog outbreak (Elhanafi, Dutta and Kathariou 2010); pLM80 harbors *cadA2C* (Nelson *et al.* 2004), and H7550 grew at cadmium concentrations >140 µg/ml, similar to other *cadA2*-harboring strains (Lee *et al.* 2013). The fact that upon acquisition of *cadA4C* cadmium MICs for both F2365 and H7550-Cd<sup>S</sup> increased only to the level typical for *cadA4*-harboring strains (50 µg/ml) suggests that their lower cadmium tolerance threshold reflects properties of the *cadA4C* cassette itself, and not differences in the genomic background of the strains.

**Expression of *cadA4* is induced by cadmium, and *cadA4C* is a bicistronic unit transcribed independently from the upstream arsenic resistance gene cluster.** RT-PCR of cDNA produced with primer P4 indicated that basal levels of *cadA4* were low but detectible, both at 25 and 37°C, but markedly increased upon exposure to cadmium (Fig. 2-5). When this cDNA was used as template with primers P18 and P19 (Fig. 2-1) the expected PCR product was obtained (data not shown), suggesting that *cadA4* and *cadC* were co-transcribed. In contrast, PCR with primers P16 and P19 (Fig. 2-1) failed to yield any product, suggesting that *cadA4C* was a bicistronic unit and not co-transcribed with the upstream *LMOSA\_2320* (data not shown). These data were supported by qPCR assays, which

showed that *cadA4* was induced in the presence of 5 µg/ml cadmium at both 25 and 37°C with a 65 and 26 fold increase, respectively (Fig. 2-6A). At more environmentally-relevant cadmium levels, e.g. 1.6 µg/ml, there was 53 fold increase in *cadA4* expression at 25°C, with a lower (15 fold) increase at 37°C (Fig. 2-6A). Increases were also noted at even lower cadmium levels (0.1 µg/ml) even though they were lower than at 1.6 µg/ml, i.e. 14 and 9 fold at 25 and 37°C, respectively (Fig. 2-6A). *cadC* was also induced in the presence of cadmium (0.1, 1.6 and 5 µg/ml) at both 25 and 37°C (Fig. 2-6B).

*LMOSA\_2320*, immediately upstream of the *cadA4C* cassette, was minimally induced (3 fold increase) with exposure to cadmium (5 µg/ml) at 25°C, with no evidence for induction at 37°C (data not shown). Several other ORFs upstream of *cadA4* (*arsB2*, *arsB1*, *arsA2* etc.) appear to be involved in arsenic detoxification and are transcribed in the same direction as *cadA4C* (Fig. 2-1). However, exposure to arsenic did not induce expression of *cadA4* (data not shown). Taken together, the transcriptional data suggest that *cadA4* was transcribed independently from *LMOSA\_2320* and other upstream sequences, and induced by exposure to cadmium, even at trace levels.

Two elements in the cadmium-dependent induction of *cadA4C* are worthy of note. First, induction of expression at all tested cadmium levels, and of both *cadA4* and *cadC*, was higher at 25 than at 37°C, suggesting especially high induction at environmental temperatures. Nonetheless, the finding that both genes were still induced by cadmium at 37°C also suggests potential involvement in virulence of warm-blooded animals, which remains to be further investigated. Second, induction was noted at cadmium levels low enough to not result in detectable growth inhibition, similarly to the earlier reports of trace amounts of cadmium inducing expression of *cadA1C* (Lebrun et al. 1994a). While all levels

of cadmium tested here would represent high levels of contamination in most environments, cadmium levels can be unusually high in certain habitats via not only anthropogenic contamination but also bioaccumulation in plant and animal tissues (United Nations 2010). This suggests a potentially significant role of determinants such as *cadA4C* in environmental survival and adaptation of *L. monocytogenes*, particularly in habitats impacted by natural or anthropogenic cadmium contamination.

**Inactivation of *cadA4* enhances virulence in *Galleria mellonella*.** While *G. mellonella* larvae inoculated with ca.  $10^6$  CFU of I1A2 exhibited higher mortality than those inoculated with the parental strain F8027, this trend was found to not be significant ( $p=0.4095$ ). This trend for enhanced larval mortality following inoculation with I1A2 was noted on each of the first five days of incubation of the inoculated larvae at  $37^\circ\text{C}$  (Fig. 2-7A). Genetically complemented I1A2 and I1A2 harboring the empty vector pPL2 were also tested using the *Galleria* model. However, the presence of pPL2 alone appeared to enhance virulence of I1A2, and while the *cadA4C*-complemented I1A2 was less virulent than I1A2 harboring pPL2 alone, the difference between the two did not reach statistical significance (Fig. 2-7B). In addition to the apparent impact of the vector, another potential complication was that the complemented I1A2 harbored the entire *cadA4C* cassette and thus possessed two functional *cadC* copies but only one functional *cadA4*.

To avoid the latter issue and further test the trend observed in I1A2, we employed infections by *cadA4C* constructs heterologously expressed in strains F2365 and H7550-Cd<sup>S</sup> (Fig. 2-8a and 8b). Larvae inoculated with either construct exhibited higher survival than those inoculated with the corresponding parental strains (F2365  $p=0.0402$ ; H7550-Cd<sup>S</sup>

p=0.0334) or the parental strains harboring the empty vector alone, though these differences were not statistically significant (F2365 p=0.4471; H7550-Cd<sup>S</sup> p=0.4607) (Fig. 2-8).

Furthermore, virulence of the parental strains did not differ significantly from virulence of the strains harboring the empty vector (F2365 p=0.25; H7550-Cd<sup>S</sup> p=0.351) (Fig. 2-8).

Such findings suggest that *cadA4* may function as a virulence modulator, with *cadA4*-harboring *L. monocytogenes* likely to be tolerated as a commensal microbe instead of a frank pathogen. Similar studies of metal resistance P-type ATPases in *L. monocytogenes* not only found that inactivation of these determinants reduced virulence, but these effects were present in both the *Galleria mellonella* and murine models, suggesting that these impacts were not an artifact of the model system (McLaughlin *et al.* 2013). Interestingly, reduced virulence in *Galleria* was earlier reported for strain Scott A, which harbors the same *cadA4*-containing island as F8027. Compared to other strains tested in the *Galleria* model in that study, Scott A exhibited the least virulence (Schrama *et al.* 2013). Intriguing virulence impacts were also earlier identified for another *L. monocytogenes* gene implicated in cadmium tolerance: in strain EGD-e which harbors a chromosomal *cadA3C* cassette, *cadC* was markedly induced (10, 5, and 5 fold over 24, 48, and 72 hours respectively) in the livers of infected mice and was also required for normal virulence levels in a murine intravenous infection model (Camejo *et al.* 2009). Since CadC is a putative repressor, such data may suggest that enhanced levels of *CadA* adversely impact virulence, similarly to our findings of enhanced virulence upon insertional inactivation of *cadA4*. As *cadC* is upstream of *cadA4* (Fig. 2-1), its expression is not expected to be impacted in I1A2; this, together with evidence from infections with the complemented I1A2, suggests that the enhanced virulence of I1A2 can be attributed to *cadA4* inactivation.

Further evidence for a role of *cadA4* in virulence modulation was obtained from a fitness assessment following co-infection of larvae with 1:1 mixtures of F8027 and I1A2. The parental strain accounted for most (98%) of the *L. monocytogenes* population in larvae surviving on day 10 post-infection, reflecting a potential fitness advantage of *cadA4* for larval survival.

**Inactivation of *cadA4* reduces biofilm formation at 25°C.** Biofilm formation is critical to the ability of *L. monocytogenes* to persist on abiotic surfaces, and thus of direct relevance to food safety. Assessments of biofilm formation by F8027 and the *cadA4* mutant I1A2 on 96-well PVC plates indicated that the two strains produced similar amounts of biofilm at 37°C (data not shown). However, at 25°C biofilm formation by I1A2 was reduced in comparison to its parental counterpart ( $p < 0.001$ ) (Fig. 2-9). The complemented I1A2 was not significantly different from I1A2 or I1A2 harboring pPL2 alone (Fig. 2-9), possibly reflecting the presence of two functional *cadC* copies, similarly to virulence findings described above. When heterologously expressed constructs of strains F2365 and H7550-Cd<sup>S</sup> were tested, the F2365 construct produced more biofilm than the parental strain ( $p < 0.001$ ) or parental strain with the empty vector ( $p = 0.01$ ) (Fig. 2-9). However, H7550-Cd<sup>S</sup> constructs were not impacted in biofilm formation ( $p = 0.14$ ) (Fig. 2-9). These data suggest a strain-dependent role of *cadA4* in biofilm formation at 25°C, which may impact the ecology of *L. monocytogenes* not only in food processing plants but also in natural environments.

**Inactivation of *cadA4* does not impact survival and growth of *L. monocytogenes* F8027 on produce.** F8027 was originally derived from raw celery and has been used in several

studies related to *L. monocytogenes* adherence and growth on produce (Lang, Harris and Beuchat 2004a; Lang, Harris and Beuchat 2004b; Beuchat, Adler and Lang 2004). However, special attributes potentially associated with the produce colonization potential of this strain remain unidentified. We were therefore interested in determining the potential involvement of strain-specific attributes such as cadmium tolerance in the ability of F8027 to colonize fresh produce. Furthermore, we wished to determine whether the biofilm formation impairment of I1A2 observed at 25°C might also result in impaired growth on produce. However, adherence and growth assessments on celery fragments at 25°C failed to identify significant differences between I1A2 and F8027. The two strains exhibited similar adherence (approx. 10%) and growth on the surface of celery fragments, with ca. 1.4 and 2 log increases after 24 and 48 hr, respectively (data not shown). Colonization of cantaloupe rind also failed to reveal differences between the strains (data not shown). Taken together, the findings suggest that, under the tested conditions, *cadA4* was not required for produce colonization.

Multiple factors may be responsible for the observed lack of correspondence between the biofilm assay data at 25°C and produce colonization, including peculiarities of the biotic surface of living plant tissue and the presence of other microbiota. For instance, *L. monocytogenes* and *Salmonella enterica* can bind to cellulose-derived polymers (Bae et al. 2013) and *lcp*, encoding a *Listeria* cellulose binding protein, has been implicated in adherence of *L. monocytogenes* F2365 to various types of fresh produce (Bae et al. 2013).

## CONCLUDING REMARKS AND PERSPECTIVES.

In addition to the previously identified plasmid-borne *cadA1C* and *cadA2C*, and the chromosomal *cadA3*, we have confirmed the cadmium detoxification involvement of a fourth *cadAC* cassette, *cadA4C*, harbored chromosomally in Scott A and in the produce-derived strain F8027. Additional work is needed to further characterize the evolution and other potential fitness impacts of *cadA4C*. Assessments of virulence in *Galleria mellonella* suggest that *cadA4* may serve to repress virulence. While possible impacts in other animal models remain to be determined, it is tempting to speculate that this virulence modulation may accompany commensal persistence of *cadA4*-harboring strains in insect hosts. This may contribute to amplification of *L. monocytogenes* in invertebrate reservoirs, which are currently poorly characterized but may play important roles in the natural ecology of *L. monocytogenes*. *L. monocytogenes* is present as a presumed saprophyte in soil (Gray and Killinger 1966), which also harbors a rich assortment of invertebrates including the larvae of insects that may serve as hosts and reservoirs. This, together with the observed impact of *cadA4* in biofilm formation at environmental (25°C) temperatures, reinforces the concept that this cadmium resistance determinant has wider functionality than just conferring tolerance to cadmium. The potential of these adaptations to impact colonization of food processing environments and virulence warrants further studies to elucidate the roles of *cadA4C* in *L. monocytogenes*. Since *cadA4C* is harbored by numerous strains of *L. monocytogenes* in addition to Scott A and the F8027 strain investigated here, and several other bacterial pathogens previously mentioned, a better understanding of this resistance cassette could have far-reaching impacts.

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**Table 2-1.** Bacterial strains used in this study

Strain	Source or Reference
<i>Listeria monocytogenes</i>	
F8027	Serotype 4b strain from celery (Lang, Harris, and Beuchat 2004))
I1A2	<i>Mariner</i> -based transposon mutant of F8027 with transposon insertion in <i>cadA4</i>
I1A2_pPL2	I1A2 with empty shuttle vector pPL2 integrated in the chromosome
I1A2:: <i>cadA4</i>	Genetically complemented I1A2, with pPL2_ <i>cadA4</i> integrated in the chromosome
H7550-Cd <sup>S</sup>	Cadmium-susceptible derivative of <i>L. monocytogenes</i> H7550 (Elhanafi, Dutta and Kathariou 2010)
H7550-Cd <sup>S</sup> _pPL2	H7550-Cd <sup>S</sup> with empty shuttle vector pPL2 integrated in the chromosome
H7550-Cd <sup>S</sup> :: <i>cadA4</i>	H7550-Cd <sup>S</sup> with pPL2_ <i>cadA4</i> integrated in the chromosome
F2365	<i>Listeria monocytogenes</i> serotype 4b strain (cadmium-susceptible) (Nelson <i>et al.</i> 2001)
F2365_pPL2	F2365 with empty shuttle vector pPL2 integrated in the chromosome
F2365:: <i>cadA4</i>	F2365 with pPL2_ <i>cadA4</i> integrated in the chromosome

**Table 2-1** continued

I1A2::*cadA4SDM*

I1A2 with pPL2\_*cadA4SDM* integrated in  
the chromosome

*Escherichia coli*

DH5 $\alpha$

Invitrogen

DH5 $\alpha$ \_pPL*cadA4*

*E. coli* DH5 $\alpha$  harboring pPL2\_*cadA4*

DH5 $\alpha$ \_pPL*cadA4SDM*

*E. coli* DH5 $\alpha$  harboring pPL2\_*cadA4SDM*

S17-1\_pPL*cadA4*

*E. coli* S17-1 (30) harboring pPL2\_*cadA4*

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**Table 2-2.** Primers used in this study

Primer	Sequence (5' to 3')
P1 <sup>1</sup>	CTA <u>ACCCGGGA</u> AAGGAATATCAAAGGTTGAGAG
P2 <sup>1</sup>	GTAAG <u>AGCTC</u> TTATTTATATCTTATGACACGTG
P3	ATGAAGAAAGAGTATATTTAGAG
P4	ACCAATGCACATGGACAAGA
P5	ATGTTGATTGTGGCAGGTGT
P6	GAACTTCCACTAGTGGACCA
P7	ATGAGCTATGCACTAGATTTAAA
P8	TTCTCCTCCTTTTGATGTTTG
P9	CAACCAGATCAGTTACCATTAAC
P10	TGCTTCTCCAGAGATTTCTTCTG
P11	GTCAAACATACGCTCTTATC
P12	ATGGATTATGAATTAACAGCAAA
P13	CCTCACAAGAACATGCAGCA
P14	GACAATTGGAAGAGAAAAGAG
P15	CACGTGTCATAAGATATAAATAA
P16	CTGTTTGTGGTATCTTGTTG
P17	TACACAATCTTACCCTCACG
P18	GGCTGTGTTGCTGAATATGA
P19	TCATCAAATACTTGGCCCTTA
P20	ATTACAGATGTGAGATTACGACG

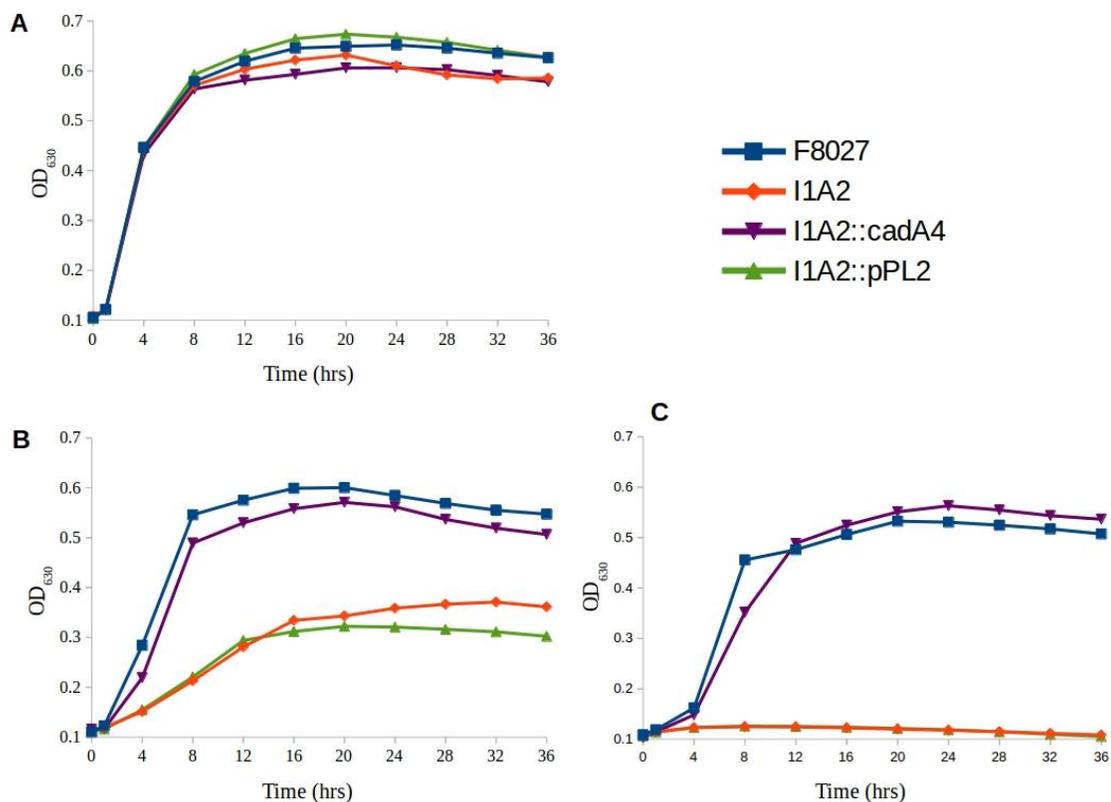
**Table 2-2.** Continued

P21	ACGTTTACTTGGCATAGCTAC
P22	TGGTGTGACGGTGAATTCC
P23	TGCTTCTTCTTCACCAACGC
P24	GTGAATCGGTCCCACGAAAAC
P25	TTTGCCACCGTTGATTCACC
P26	GTGATTTGGCTGTGTTGCTG
P27	CACAATCTTACCCTCACGTCTG
P28	ACCACCTTTTTGGCAAGGAG
P29	AATGACGAGTGGCACATCAG
P30	GCATACGTACGAACCAGAAG
P31	CAGTGTTTCTGCTTTTGCTCC
P32	ACTTGTACAACTGCGCAGG
P33	CCTGCGCAGTTTGTACAAGT

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<sup>1</sup> Underlined CCCGGG and GAGCTC sequences are restriction sites for XmaI and SacI, respectively.

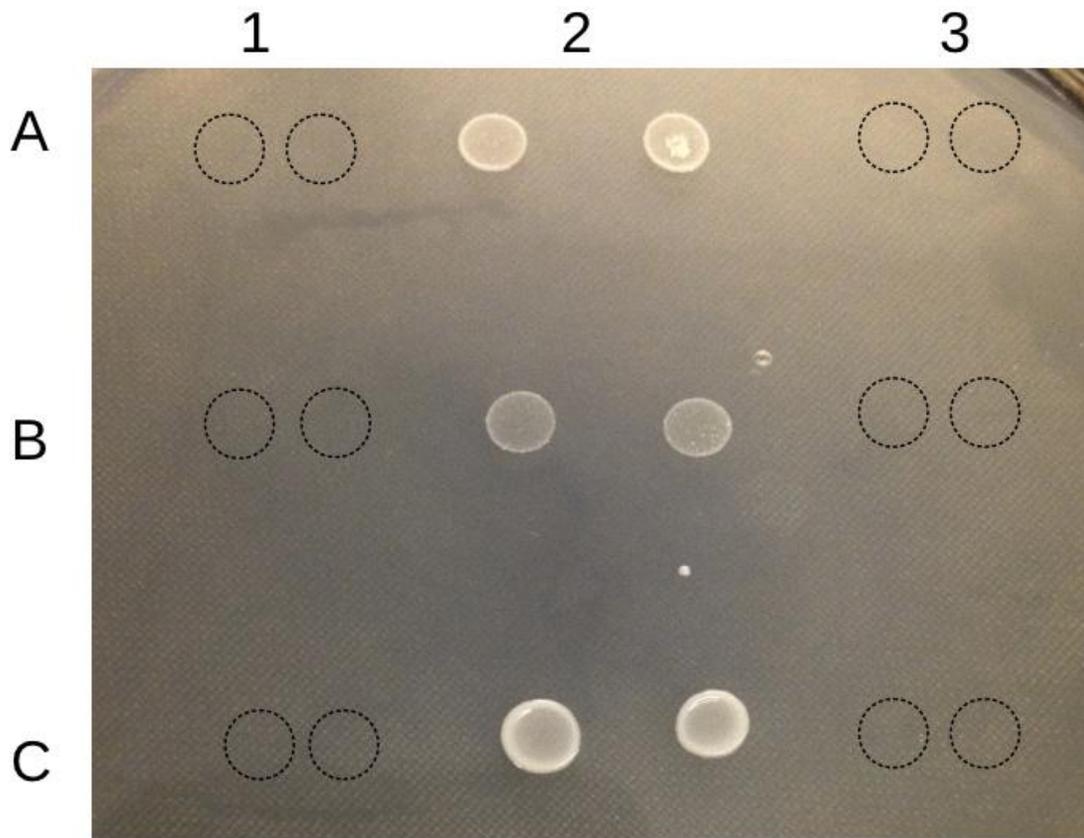




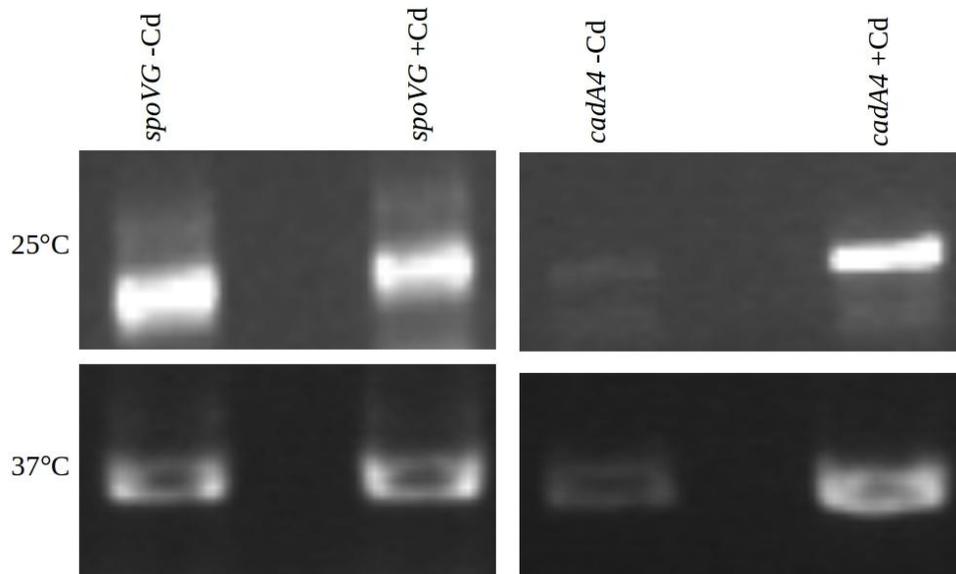
**Figure 2-2.** Impact of *cadA4* inactivation on growth of *L. monocytogenes* F8027 in the presence of cadmium. Growth curves for F8027, I1A2, I1A2 harboring the empty vector (I1A2::pPL2) and genetically complemented mutant (I1A2:: pPL2\_ *cadA4*), were determined in (A) BHI, (B) BHI with 5 μg/ml CdCl<sub>2</sub> and (C) BHI with 10 μg/ml CdCl<sub>2</sub>. Bacteria were grown in 96-well plates at 37°C for 36 hours, and OD<sub>630</sub> was monitored using a Biotek Elx808. Data represent averages of readings from six separate wells, from one representative experiment.



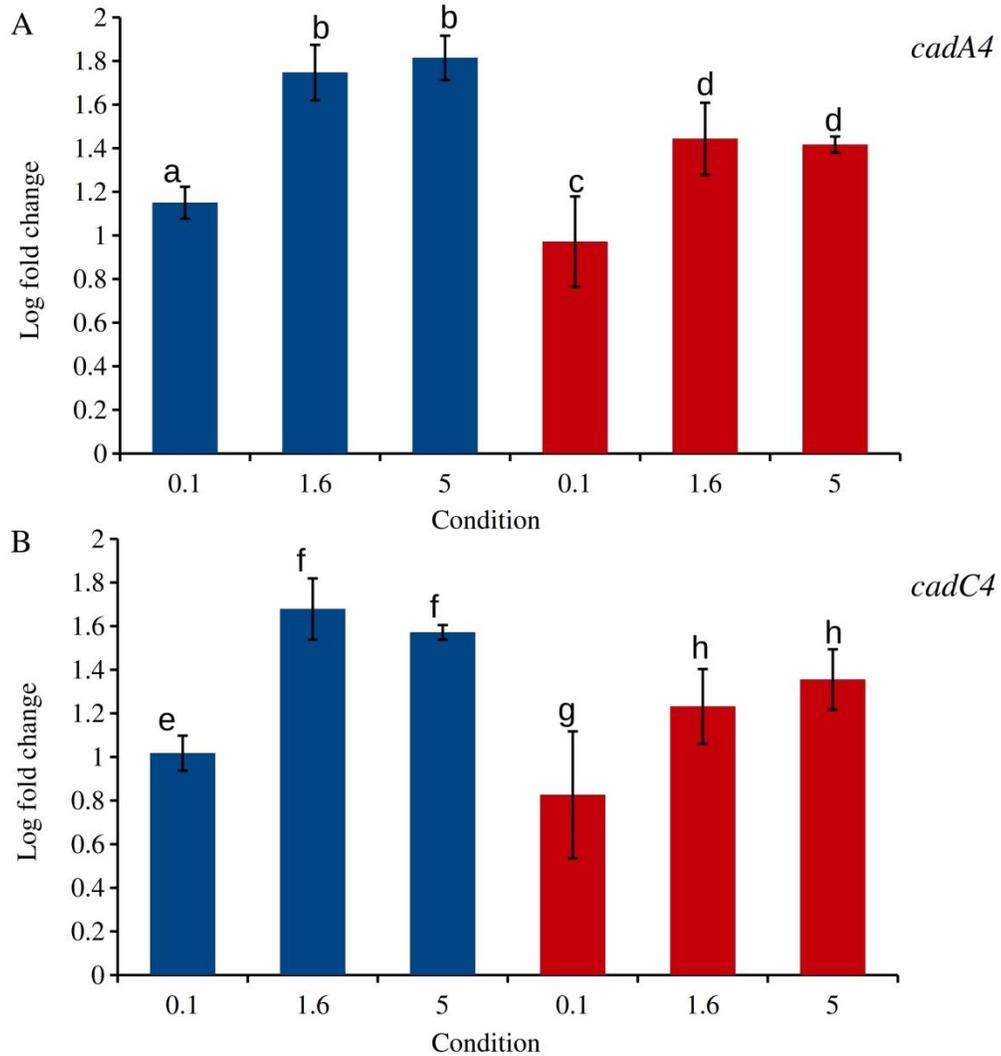
**Figure 2-3.** Amino acid alignment of four identified members of the *CadA* family in *Listeria*. Key functional motifs are outlined in red. CXXC, metal binding domain; CXC, ion channel; DKTGT, ATPase.



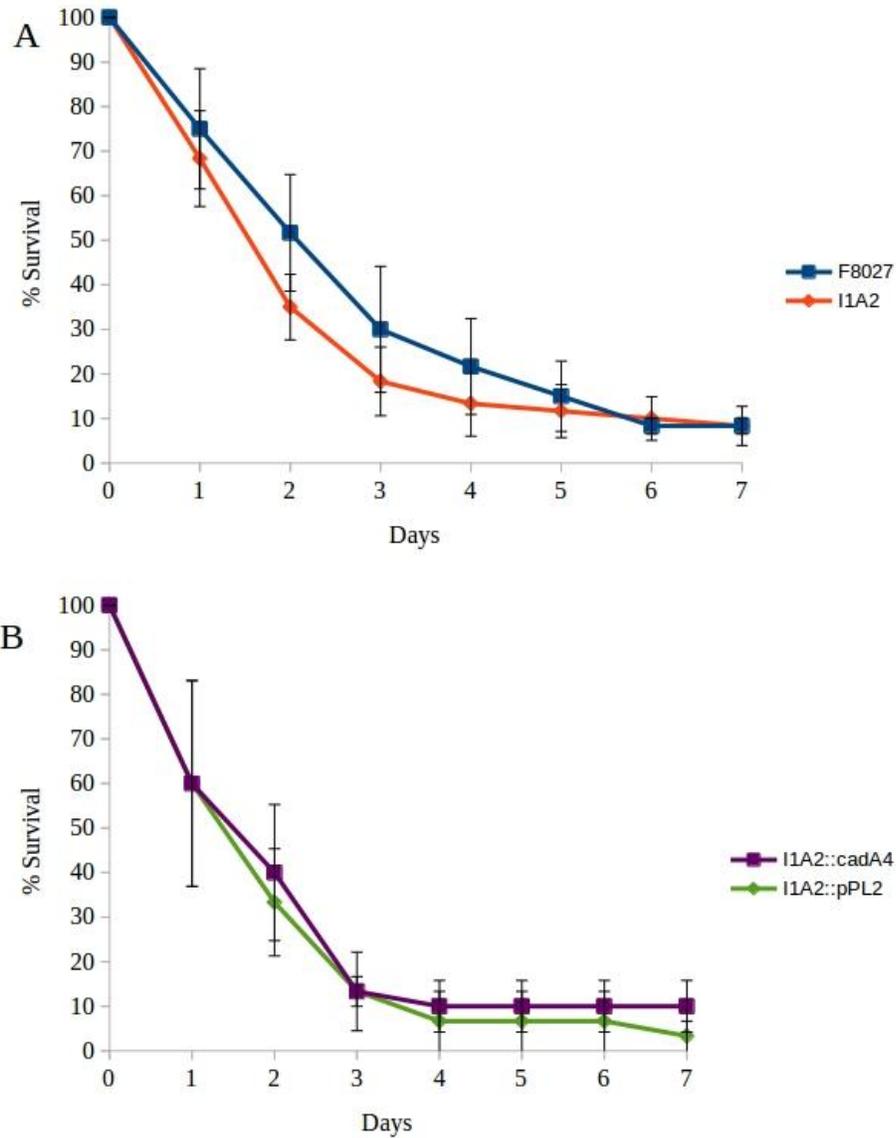
**Figure 2-4.** Growth of genetically complemented strains on ISA supplemented with 35  $\mu\text{g/ml}$   $\text{CdCl}_2$ . A, mutant I1A2, complemented I1A2 (I1A2::pPL2\_cadA4) and I1A2 harboring empty vector (I1A2::pPL2), respectively; B, F2365, complemented F2365 (F2365::pPL2\_cadA4) and F2365 harboring empty vector (F2365::pPL2), respectively; C, H7550-Cd<sup>S</sup>, complemented H7550-Cd<sup>S</sup> (H7550-Cd<sup>S</sup>::pPL2\_cadA4) and H7550-Cd<sup>S</sup> harboring empty vector (H7550-Cd<sup>S</sup>::pPL2), respectively. Cell suspensions (4 $\mu\text{l}$ ) were spotted in duplicate on the surface of the plate which was dried and incubated at 37°C for 48hr. Dotted circles indicate location of spots with no observed growth.



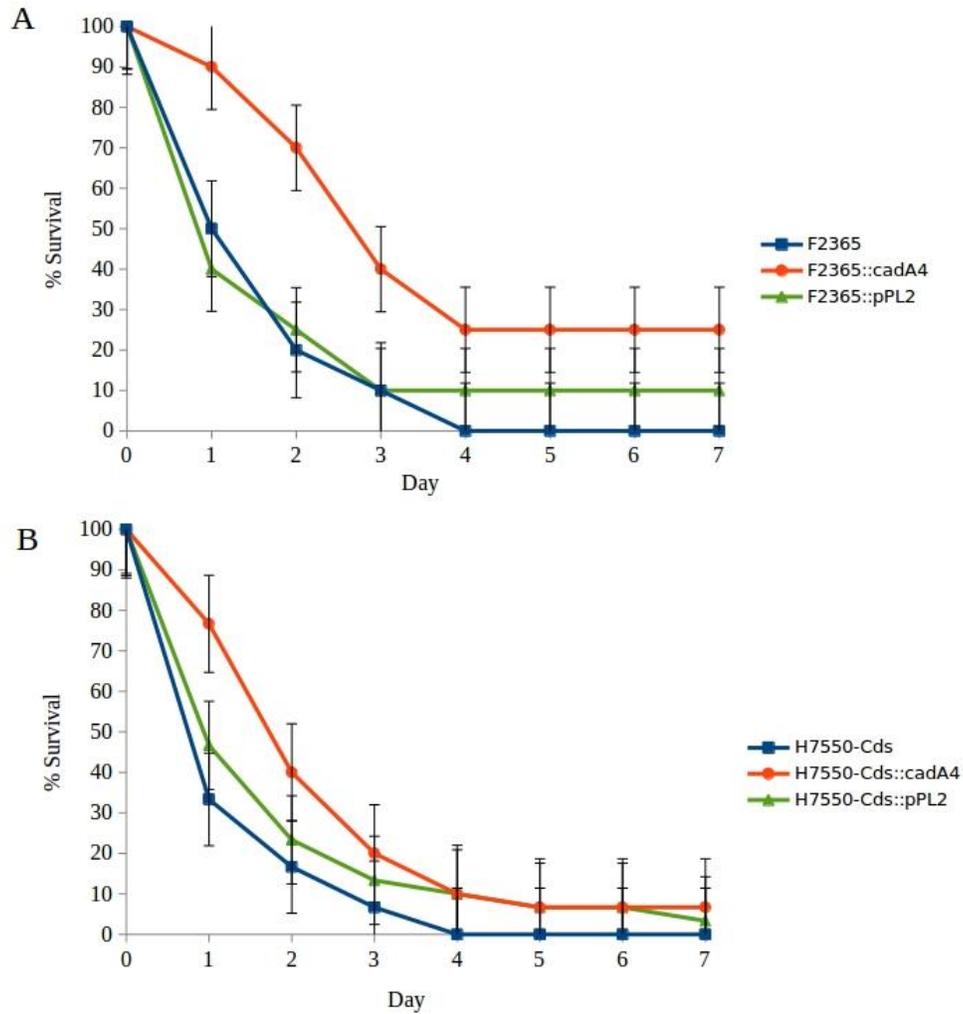
**Figure 2-5.** Reverse transcription assesment of *cadA4* induction by cadmium at 25° and 37°C. Cultures were exposed to 5 µg/ml cadmium (lanes indicated +Cd) or left untreated (lanes indicated -Cd) for 30 min at 25° (top) or 37° C (bottom). House keeping gene *spoVG* is used as control, and experiments were done as described in Materials and Methods. Data are from one representative experiment.



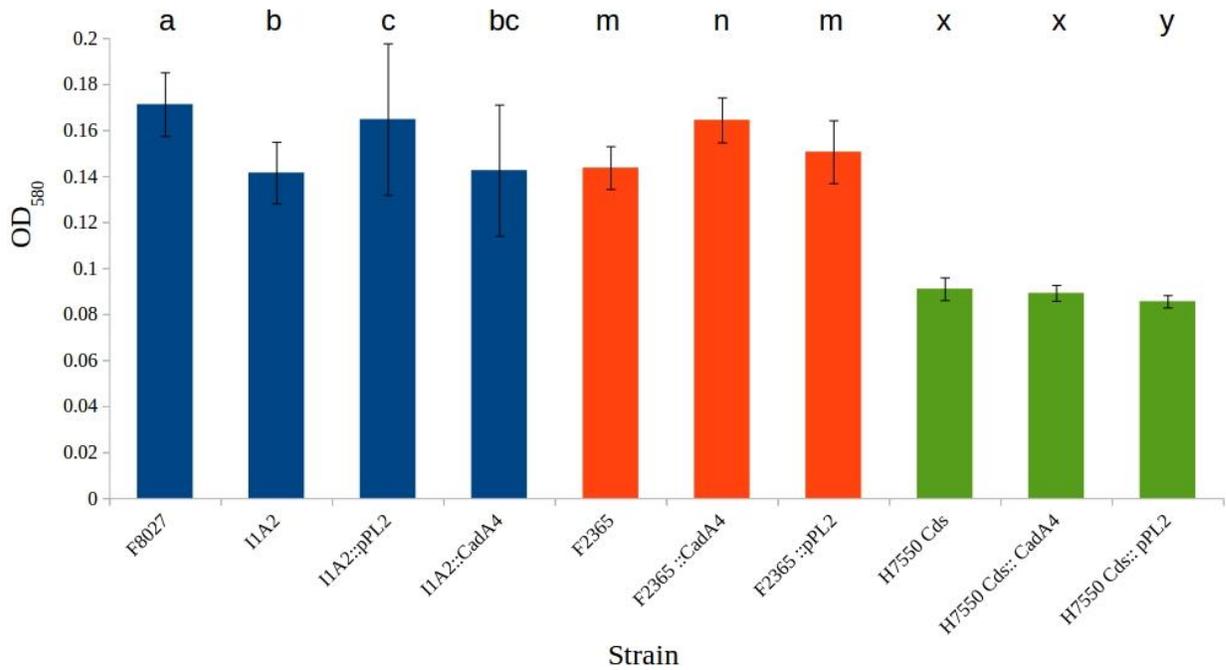
**Figure 2-6.** Relative gene expression of *cadA4* and *cadC4* at 25° and 37°C at varying concentrations of cadmium. Columns in blue indicate 25°C treatments, while those in red indicate treatments at 37°C. Numbers below columns are representative of the cadmium concentration (µg/ml). Data were from at least three independent trials of real-time PCR carried out as described in Material and Methods. For each gene comparisons were made across temperature and concentration, and significant differences ( $p < 0.05$ ) are indicated by differing letters.



**Figure 2-7.** Impact of *cadA4* in the *Galleria mellonella* model. Ten larvae per treatment were inoculated with A, F8027 and I1A2; B, I1A2::pPL2\_*cadA4* and I1A2::pPL2, incubated at 37°C and monitored daily for seven days as described in Materials and Methods. Data are averages from at least three independent trials. Strains were as described in legend of Fig. 2-5 and Table 2-1.



**Figure 2-8.** Impacts of heterologously expressed *cadA4C* constructs of F2365 (A) and H7550-Cd<sup>S</sup> (B) in the *Galleria mellonella* model. Data are from three independent trials. Strains were as described in legend of Fig. 2-5 and Table 2-1.



**Figure 2-9.** Impact of *cadA4* on biofilm formation. Biofilms were established in wells of 96-well plates (15 wells/strain) and measured following staining with crystal violet, as described in Material and Methods. Differing letters within each group indicate statistically significant differences ( $p < 0.05$ ). Strains were as described in legend of Fig. 2-5 and Table 2-1.

**CHAPTER 3 : Novel locus mediating metal homeostasis in *Listeria monocytogenes*.**

## **SUMMARY:**

*Listeria monocytogenes* is a ubiquitous facultative intracellular pathogen responsible for the disease listeriosis. One key element to *Listeria's* survival in both the environment and an animal host is its ability to tolerate frequently high levels of metals. To better understand the functional role that genes associated with heavy metal homeostasis play in *Listeria*, a *mariner*-based mutant library of the cadmium and arsenic-resistant serotype 4b strain F8027 was screened for loss of cadmium resistance. In one of these mutants, E2G4, the transposon insertion was localized to the intergenic space between *LMOSA\_2450* (wall-anchored protein with the LPXTG motif) and *LMOSA\_2460-2470* (hypothetical proteins). While E2G4 was initially identified by its reduced tolerance to cadmium, this mutant also exhibited reduced tolerance to zinc. Genetic complementation of E2G4 with the *LMOSA\_2460-2470* cassette restored tolerance to cadmium, and to a lesser extent zinc. A deletion mutant of *LMOSA\_2450* ( $\Delta$ lpxtg) also exhibited reduced tolerance to zinc, but not cadmium. Quantitative real time PCR indicated that there was no induction of these genes in the presence of cadmium and zinc. In addition to their reduced tolerance to cadmium and/or zinc, E2G4 and  $\Delta$ lpxtg produced significantly more biofilm than the wildtype. Virulence assessments in a *Galleria mellonella* model revealed no significant impact on virulence for E2G4, while reduced virulence was noted for  $\Delta$ lpxtg. These findings suggest that while the functions of *LMOSA\_2450*, *LMOSA\_2460*, and *LMOSA\_2470* have not been fully elucidated, that this locus is required for zinc and cadmium tolerance in F8027, with impacts on biofilm formation, and virulence.

## INTRODUCTION:

*Listeria monocytogenes* is a facultative intracellular pathogen that is ubiquitous in the environment and capable of survival and growth in an array of conditions (Gray and Killinger 1966; Romanova, Favrin and Griffiths 2002; Vivant, Garmyn and Piveteau 2013). One phenotype that plays a key role in almost all of these conditions is heavy metal homeostasis. Heavy metal contamination is becoming an increasing problem both due to naturally occurring and anthropogenic sources (Alloway and Alloway 2013). Heavy metals can accumulate in water and soils and bioaccumulate in plants and other organisms (Morin *et al.* 2008). Such accumulation can fundamentally alter the natural microbiota, selecting for microorganisms that are capable of tolerating or mitigating the toxic effects of these metals (Nunes *et al.* 2016). *Listeria* can be resistant to copper, zinc and to extremely high levels of cadmium and arsenic, creating the possibility that high environmental levels of these metals could potentially select for this pathogen. While a variety of determinants have been identified in *Listeria monocytogenes* that function in heavy metal transport and detoxification (Lee *et al.* 2013; Parsons *et al.* in review; Pi *et al.* 2016; Francis and Thomas 1997), much remains to be known in regard to how intracellular metal levels are maintained, especially in varying environments.

Adding complexity to this relationship is the fact that many essential enzymes require metals such as iron, copper, and zinc as cofactors for proper cellular function (Jesse, Roberts and Cavet 2014), requiring that metals must be actively imported for cells to function. However, excess levels of these metals are cytotoxic, either by competitively binding with essential enzymes, or through their ability to perform fenton chemistry generating reactive oxygen species (Xu and Imlay 2012; Varghese *et al.* 2007). Metals can also be utilized by

the host immune system to combat invading microorganisms (Jesse, Roberts and Cavet 2014), so pathogens that are inured to high levels of these metals could potentially be better able to tolerate host immune responses. Whether present in the environment, or within the body of a host, *L. monocytogenes* must acquire essential metals for adequate cellular function, and mediate their toxic effects when they are in high concentrations.

Links have been established between heavy metal homeostasis and other adaptations, suggesting that heavy metal detoxification genes do not function solely in metal resistance. Certain findings suggest a relationship between heavy metal resistance genes and virulence in *L. monocytogenes* (Francis and Thomas 1997; Pi *et al.* 2016; Parsons *et al.* in review). There is also a growing body of work to suggest a relationship between heavy metals and biofilm formation. Exposure to heavy metals has been shown to induce biofilm formation in some organisms (Perrin *et al.* 2009; Wu, Santos and Fink-Gremmels 2015), while biofilm formation was inhibited in others (Koechler *et al.* 2015), and metal homeostasis genes have been implicated in the ability to form biofilms (Binepal *et al.* 2016). This study examines three previously uncharacterized genes in a heavy metal resistance genomic island of *L. monocytogenes* for their role in resistance to cadmium and zinc, as well as the impacts on other adaptations, such as virulence and biofilm formation.

## **MATERIALS AND METHODS:**

**Bacterial strains and growth conditions.** Bacterial strains used in this study are listed in Table 3-1. Growth conditions were as described in the previous chapter.

**Mutant library construction.** Random mutagenesis vector pMC38 (Cao, Bitar and Marquis 2007) was introduced into *L. monocytogenes* strain F8027 via electroporation as described previously (Cao, Bitar and Marquis 2007; Azizoglu and Kathariou 2010; Parsons *et al.* in review).

**Metal resistance and MIC determination.** The mutant library was screened for growth on Iso-Sensitest agar (ISA) (Oxoid, Hampshire, England) supplemented with cadmium chloride (Fisher Scientific) (35 µg/ml). Mutants exhibiting decrease tolerance to cadmium were tested for minimum inhibitory concentrations (MICs) to a variety of metals and antibiotics, including the efflux inhibitor reserpine as described previously (Parsons *et al.* in review).

**Determination of transposon location and copy number.** Southern blotting was performed to determine the number of chromosomal transposon insertions in each cadmium sensitive mutant, as previously described (Cao, Bitar and Marquis 2007; Azizoglu and Kathariou 2010). To localize the transposon insertion site in E2G4, two rounds of PCR were performed using non-specific primers and primers specific to the transposon, as described previously (Cao, Bitar and Marquis 2007). The resulting PCR product was then sequenced (Genewiz Inc., South Plainfield, NJ, USA). Based on these data primers E2G4F and E2G4R (Table 3-2) were designed flanking the putative insertion site, PCR with these primers was conducted on E2G4 and sequenced (Genewiz Inc.).

**RNA extraction and transcriptional assessments.** *L. monocytogenes* F8027 and E2G4 were grown in TSBYE at 25 and 37°C until mid to late logarithmic phase (OD<sub>600</sub> of 0.7-1.0,

as measured by SmartSpec 3000; Bio-Rad, Hercules, CA, USA). The culture was then divided into two portions, one of which was exposed to either sublethal levels of cadmium (0.1, 1.6 µg/ml) or zinc (7 mM) and incubated for an additional 30 min at 25 or 37°C while the other was similarly incubated without addition of metals. RNA extraction, RT-PCR, and qPCR were carried out as described previously (Parsons *et al.* in review). For RT-PCR, RNA was reverse-transcribed to cDNA with the ImProm-II reverse transcription system (Promega) according to the manufacturer's protocol, using either primer 2430F, LMOSA\_2450F or spoVGR (Table 3-2); *spoVG* was used as reference gene for each trial, as previously described (Kim *et al.* 2012). For quantitative real-time PCR (qPCR), the BIO-RAD iTaq Universal SYBR Green One-Step Kit (Bio-Rad Laboratories Hercules, CA, USA) was used, per the manufacturer's instructions with a LightCycler 96 system (Roche Diagnostics, Indianapolis, IN, USA). Primers qLPXF-R and q2460F-R (Table 3-2) were designed using Primer3Plus (<http://primer3plus.com/>) to measure expression of *LMOSA\_2450* and *LMOSA\_2460*, with *spoVG* used as control.

**Genetic complementations and deletions.** For genetic complementation, primers 2460compF and 2470compR (Table 3-2) were designed with KpnI and NotI restriction sites respectively, and used to amplify the *LMOSA\_2460 - LMOSA\_2470* cassette, as well as 148nt of the upstream region (including predicted promoter region) and 284nt of the downstream region. Primers 2460compF and 2460compR (Table 3-2) were designed with KpnI and NotI restriction sites respectively, and used to amplify *LMOSA\_2460* and 148nt of the upstream intergenic region which contained a predicted promoter. Primers 2470compF and 2470compR (Table 3-2) were designed with restriction sites for KpnI and NotI

respectively, and used to amplify *LMOSA\_2470*, as well as 143nt of the coding region of *LMOSA\_2460* immediately upstream of *LMOSA\_2470*, which contained a predicted promoter sequence. Primers LPXcompF and LPXcompR (Table 3-2) were designed with Sall and NotI restriction sites respectively and used to amplify *LMOSA\_2450* as well as the entire predicted intergenic regions both upstream and downstream of *LMOSA\_2450*. PCR products were digested with indicated enzymes (New England Biolabs, Ipswich, MA, USA), and then ligated into similarly digested shuttle vector pPL2 (Lauer *et al.* 2002) using T4 DNA ligase (Promega, Madison, WI, USA). Plasmids were electroporated into *E. coli* SM10 (Simon, Priefer and Pühler 1983) and transferred into indicated strains of *L. monocytogenes* as previously described (Elhanafi, Dutta and Kathariou 2010).

For deletion of *LMOSA\_2450*, primers LPXTGdel1F and LPXTGdel1R (Table 3-2) were designed with restriction sites for HindIII and NheI respectively, to amplify a 655nt region which includes 88nt of the upstream region and the initial portion of the coding region of *LMOSA\_2450*; and primers LPXTGdel2F and LPXTGdel2R (Table 3-2) were designed with restriction sites BamHI and EcoRI respectively, to amplify a 649nt region just downstream from the coding region of *LMOSA\_2450*. PCR products were digested with indicated enzymes (NEB) and then sequentially ligated into vector pGFem (Li and Kathariou 2003) as described previously. Constructed deletion vector was electroporated into *E. coli* S17-1 (Simon, Priefer and Pühler 1983), and introduced into *L. monocytogenes* strain F8027 through conjugation. Vector integration was then carried out as described previously (Li and Kathariou 2003) and deletion of 2150nt of the coding sequence of *LMOSA\_2450* was confirmed via PCR and sequencing (Genewiz Inc.).

In-frame deletions were made of *LMOSA\_2460*, *LMOSA\_2470*, as well as the tandem region of *LMOSA\_2460/2470* using splicing by overlap extension (SOE) PCR as described previously (Cotter *et al.* 2005). Two pairs of primers (2460soeA-2460soeB, 2460soeC-2460soeD; 2470soeA-2470soeB, 2470soeC-2470soeD; 2460soeA-B, UNKsoeC-2470soeD respectively) (Table 3-2) were used to amplify flanking regions of the sequence to be deleted. The resulting PCR products were mixed 1:1 and used as template for PCR with primers soeA-D, effectively merging the two regions. The spliced PCR product was then digested with XbaI and HindIII (NEB), ligated into shuttle vector pCON1 (Behari and Youngman 1998), and introduced into *L. monocytogenes* strain F80287 via electroporation. Transformants were selected on BHI agar with Cm (10 µg/ml), with integration and dual-crossover carried out as described previously (Cotter *et al.* 2005). Putative deletions were confirmed via PCR and sequencing (Genewiz Inc.).

**Virulence assessments in *Galleria mellonella*.** Inoculations, data collection and analysis were carried out as described previously (Parsons *et al.* in review).

**Assessment of biofilm formation.** Biofilm formation was assessed in 96-well PVC plates (Greiner Bio-One, #655-185, VWR, Suwanee, GA) as described previously (Parsons *et al.* in review).

**Peroxide killing assay.** Overnight cultures of F8027, E2G4, and  $\Delta$ lpxtg grown at 37°C were diluted into LB soft agar and poured as an overlay on BHI plates. Sterile paper discs (6mm, BD, Sparks MD) were then deposited on the surface of the agar in triplicate for each strain

and 20 µl of 30% H<sub>2</sub>O<sub>2</sub> was deposited on each disc. Plates were then incubated overnight at 37°C, and the zone of inhibition around each disc was measured and recorded for at least two independent trials.

**Growth and adherence on the surface of cantaloupe.** Cantaloupe growth and adherence was assessed as described in the previous chapter.

**Statistical analyses.** Fold change calculations for qPCR experiments employed the  $\Delta\Delta C_T$  method, with statistical evaluation of Ct values using a paired Student's *t*-test. For statistical analysis of growth and adherence on cantaloupe, bacterial counts were compared using a paired Student's *t*-test  $p < 0.05$ . For virulence data, a non-parametric proportional hazards survival model in Program JMP (SAS Institute, Cary, NC) was used to test for significant differences in survival of *Galleria melonella* when inoculated with different strains of *L. monocytogenes*. All strains were analyzed in a pairwise fashion and determined significance at the  $p < 0.05$  level. Biofilm data were analyzed using a paired Student's *t*-test ( $p < 0.05$ ).

## **RESULTS AND DISCUSSION:**

**Transposon insertion in a novel intergenic region within LGI2 results in substantially decreased tolerance to cadmium and zinc.** Mutant E2G4 (Table 3-1) was initially isolated with three other cadmium sensitive mutants during mutant library screening on ISA with cadmium (35 µg/ml)(Table A-1). Through PCR and sequencing, the transposon in E2G4 was localized to the intergenic region between *LMOSA\_2450* and *LMOSA\_2460* (Fig. 3-1). The other three mutants C1A6, I1A2, and G2E6 had transposon insertions in the coding

region of differing loci, putative membrane protein (*lmo1695*), *cadA4*, and *iap* respectively (Table A-1). After initial isolation from a screening on cadmium, MIC determinations were made on arsenic, cadmium, copper, and zinc. E2G4 was found to have substantially reduced tolerance to cadmium (F8027 MIC 50 µg/ml, E2G4 MIC 25 µg/ml), and zinc (F8027 MIC 22 mM, E2G4 MIC 12 mM) (Table 3-1). Additionally, each of the other mutants with decreased cadmium tolerance only exhibited sensitivity to cadmium but not zinc or other metals tested. Southern blot revealed that all four mutants harbored a single copy chromosomal transposon insertion (data not shown).

Cadmium and zinc MICs of F8027 in the presence of the efflux inhibitor reserpine were reduced to 25 µg/ml and 12 µg/ml respectively, suggesting that the observed tolerances of this strain were at least partly mediated by efflux mechanisms. Cadmium and zinc tolerances of E2G4 in the presence of reserpine were reduced to 25 and 10 µg/ml, respectively. The differential in zinc tolerance between mutant and wild type were minimal in the presence of reserpine, suggesting that efflux was not contributing to the mutant's increased susceptibility to zinc or cadmium. Growth in the presence of arsenate, arsenite, and copper was unaffected under the conditions tested. No difference in growth was observed between E2G4 and F8027 at 37°C (data not shown). E2G4 and F8027 were also indistinguishable in hemolytic activity on blood agar, motility, colony morphology, susceptibility to phage, or growth in the cold (4°C), (data not shown).

***LMOSA\_2450*, *LMOSA\_2460*, and *LMOSA\_2470* are highly conserved, and associated with *Listeria* Genomic Island 2 (LGI2).** The transposon insertion in E2G4 was mapped to 135 nts downstream from the start codon of *LMOSA\_2450*, and 44 nts upstream from the

start codon of *LMOSA\_2460* (Fig. 3-1). Both genes are harbored on a chromosomal island, *Listeria* Genomic Island 2 (LGI2) which also harbors *cadAC* (*cadA4*) and a cluster of genes putatively mediating resistance to arsenic (Briers *et al.* 2011; Lee *et al.*, 2013). LGI2 is a mobile genetic element (MGE) first discovered through whole genome sequencing of strain Scott A (Briers *et al.* 2011; Kuenne *et al.* 2013). *LMOSA\_2450* encodes a putative cell wall-associated protein with an LPXTG cell wall anchor domain (LPDTG). It is annotated as a collagen-binding protein due to three Cna protein B type domains, which have been found to bind collagen in *Staphylococcus aureus* (Xu *et al.* 2004). Sequence analysis revealed highly conserved homologs within *L. monocytogenes* (99-100%) strains harboring LGI2. Homologs were also identified in numerous strains of *Enterococcus faecalis* (93-99%), which also harbor a LGI2-like MGE, in *Listeria welshimerii* strain SLCC 5334 (74%) and *Enterococcus silesiacus* strain LMG 23085 (68%). A protein BLAST yielded highly similar matches (>90%) with *Listeria* and *Enterococcus faecalis*, and homologs with lower similarity in *Carnobacterium gallinarium* (54%) and *Carnobacterium divergens* (49%). Nucleotide BLAST analysis of *LMOSA\_2460* revealed highly conserved (99-100%) homologs in several other strains of *L. monocytogenes*. Interestingly, there were no significant homologies outside of *L. monocytogenes* strains harboring LGI2. The deduced polypeptide is annotated as a hypothetical protein with only one conserved domain, which was described as having unknown function. A protein BLAST revealed close homologies with other *Listeria* strains, but also *Carnobacterium maltaromaticum* (89%), and *Enterococcus faecalis* strain JH1 (100%); lower homologies were found with *Dehalobacter*, *Clostridium difficile*, and *Streptococcus pneumoniae* (38, 37, and 35% respectively).

**Neither *LMOSA\_2450* nor *LMOSA\_2460* are induced by cadmium or zinc.** Attempts to ascertain cotranscription of *LMOSA\_2450* with upstream genes, or assess induction in the presence of cadmium or zinc via RT-PCR were unsuccessful. Despite the use of a variety of primer combinations, no evidence of transcription was found (data not shown). Real-time PCR revealed that *LMOSA\_2450* was expressed at very low levels, and not induced by either cadmium or zinc (data not shown).

*LMOSA\_2460* was expressed at higher levels than *LMOSA\_2450*, but was similarly not induced by either cadmium or zinc. This may suggest that these genes are not part of a metal specific detoxification response but instead mediate other cellular functions that may be critical in the presence of excess zinc or cadmium, such as DNA repair, or antioxidant functions. When RNA from E2G4 was compared to F8027 it was found that expression levels of *LMOSA\_2460* were significantly decreased in E2G4, with little to no impact on the levels of *LMOSA\_2450*. In the current study, RNA was extracted from cultures in exponential phase. Further studies will be needed to determine whether *LMOSA\_2450* may be expressed at different phases, e.g. during stationary phase.

**Genetic complementations confirm role *LMOSA\_2470* in cadmium tolerance.** Given the intergenic nature of the transposon insertion in E2G4, genetic complementations and deletions of ORFs on either side of the insertion were pursued. Sequence analysis suggested that *LMOSA\_2460* and *LMOSA\_2470* (Fig. 3-1) are transcriptionally linked, as the coding sequence of *LMOSA\_2460* is predicted to overlap with that of *LMOSA\_2470*. Therefore, complementation was initially pursued with the entire two-gene *LMOSA\_2460* / *LMOSA\_2470* cassette. The complemented construct, E2G4 :: 2460/2470, showed full

restoration of cadmium resistance and partial restoration of resistance to zinc (Table 3-1) (Fig. 3-2). Complementation with *LMOSA\_2470* alone fully restored cadmium resistance, while zinc tolerance was not restored. On the other hand, neither cadmium nor zinc tolerance was restored by complementation with *LMOSA\_2460* (Table 3-1).

**Deletion of *LMOSA\_2450* suggests role in zinc homeostasis.** Deletion of *LMOSA\_2450* ( $\Delta$ lpxtg) resulted in decreased tolerance for zinc (MIC 12mM), while cadmium tolerance was not impacted in the  $\Delta$ lpxtg construct (Table 3-1). Resistance to arsenic or copper was also not impacted in  $\Delta$ lpxtg. However, when  $\Delta$ lpxtg was complemented with *LMOSA\_2450* there was no restoration of the phenotype. PCR and sequencing of the *LMOSA\_2450* in the complemented construct revealed 100% homology with wild type F8027, suggesting that errors in replication or spontaneous mutations of *LMOSA\_2450* were not responsible for the lack of complementation. Interestingly, after prolonged incubation in the presence of zinc small colonies began to form. One of these colonies ( $\Delta$ lpxtg +Zn15) was isolated and found to have wild type levels of zinc tolerance (Table 3-1). PCR confirmed that this isolate still harbored the *LMOSA\_2450* deletion (data not shown), suggesting that suppressor mutations had restored zinc tolerance in  $\Delta$ lpxtg.

To attempt to rule out unintended downstream impacts of the deletion of *LMOSA\_2450*, a complementation construct ( $\Delta$ lpxtg::pPL2040) containing *LMOSA\_2420*-*LMOSA\_2430*-*LMOSA\_2440* was constructed. However, complementation of  $\Delta$ lpxtg with these upstream genes also failed to restore zinc tolerance (Table 3-1). This would suggest that the observed base change in  $\Delta$ lpxtg::lpxtg might mitigate a loss of function, or that with the integration and curing of the deletion vector there could possibly have been as yet

unidentified mutations in other regions of the chromosome. Alternatively, one of the genes even farther upstream of *LMOSA\_2450* may be responsible for the observed zinc sensitivity. Results of the peroxide killing assay were comparably sized zones of inhibition for both E2G4 and  $\Delta$ lpxtg in comparison to the wild type (data not shown), suggesting that the nature of the mechanism underlying the increased sensitivity to both cadmium and zinc does not involve increased susceptibility to reactive oxygen species.

**Impacts on virulence in an insect model.** While larvae inoculated with E2G4 exhibited decreased mortality in comparison to the wild type (Fig. 3-3), it was found to not be significant ( $P=.534$ ).  $\Delta$ lpxtg exhibited even greater reduction in mortality in the *Galleria mellonella* model in comparison to F8027, these differences were found to be significant ( $P=.0194$ ). These findings are consistent with previous studies, which implicated surface associated LPXTG proteins in the ability of *L. monocytogenes* to cause disease (Reis *et al.* 2010), though *LMOSA\_2450* is previously uncharacterized in *L. monocytogenes*. Similar to results seen in metal testing, complementation constructs in E2G4 and  $\Delta$ lpxtg failed to restore the phenotype in the *Galleria mellonella* model (data not shown).

**Impacts on biofilm formation.** E2G4 was found to produce significantly more biofilm than F8027 ( $P=.032$ ) (Fig. 3-4). Similar to results for metal resistance, the complemented construct E2G4::*2460/2470* did not show restoration of the wild type biofilm levels (Fig. 3-4) and was not significantly different from E2G4 ( $P=.8831$ ).  $\Delta$ lpxtg exhibited an even larger increase in biofilm formation (Fig. 3-4), which was also significantly increased over F8027 ( $P=.0002$ ). Also similar to the results of the zinc resistance testing, complementation with

*LMOSA\_2450* did not restore wild type levels of biofilm formation to  $\Delta$ lpxtg ( $P=.7359$ ) (Fig. 3-4). While the putative suppressor mutant  $\Delta$ lpxtg +Zn15 exhibited wild type levels of zinc tolerance, this mutant still exhibited significantly increased levels of biofilm formation in comparison to wild type ( $P=.0019$ ) at 25°C, and levels that were not significantly different from  $\Delta$ lpxtg ( $P=.4629$ ) (Fig. 4-4). Given the presentation of this phenotype at 25°C but not 37°C, it suggests that temperature-associated genes, such as flagella that are more highly expressed at 25 than 37°C, are likely also implicated in the mechanism. These findings were consistent with previous work, that found that inactivation of cell wall associated internalins, which also contain an LPXTG motif resulted in increased biofilm formation at 15 and 32°C (Piercey, Hingston, and Truelstrup 2016; Franciosa *et al.* 2009).

**Impacts on growth and adherence on produce.** In adherence assessments on the surface of cantaloupe at 25°C, both E2G4 and  $\Delta$ lpxtg performed comparable to the wild type (43.2, 53.6, and 47.7% adherence respectively) (data not shown). E2G4 and  $\Delta$ lpxtg were also indistinguishable from F8027 in growth on the surface of cantaloupe at 25°C (0.6, 0.9, and 0.8 log increase after 5 h respectively) (1.7, 1.8, 1.6 log increase after 24 h respectively) (1.6, 1.6, and 1.7 log increase after 48 h respectively).

## **CONCLUSIONS:**

Transposon insertion in the intergenic region between *LMOSA\_2450* and *LMOSA\_2460* was shown to reduce tolerance to both cadmium and zinc. Complementation with *LMOSA\_2450*, *LMOSA\_2460*, *LMOSA\_2470*, or *LMOSA\_2460-LMOSA\_2470* were all unsuccessful at restoring the wild type levels of zinc tolerance. The fact that deletion of

*LMOSA\_2450* also resulted in the loss of zinc tolerance, but had no impact on cadmium tolerance indicates that *LMOSA\_2450* is associated with tolerance to zinc, and could potentially suggest that both upstream and downstream regions were impacted by the transposon insertion in E2G4. This is further supported by the fact that when *LMOSA\_2460* and *LMOSA\_2470* were deleted individually or as a cassette in F8027 that there was no observed impact on tolerance to either cadmium or zinc. However, when either *LMOSA\_2470* or the *LMOSA\_2460- LMOSA\_2470* cassette were cloned into E2G4 they restored cadmium resistance (and the *LMOSA\_2460- LMOSA\_2470* cassette partially restored resistance to zinc). The zinc-sensitive phenotype of E2G4 and the lack of impact of deletions of *LMOSA\_2460* and *LMOSA\_2470*, together with the association of *LMOSA\_2470* with cadmium tolerance suggest that the transposon insertion in E2G4 may impact additional genes, besides the ones characterized here.

This hypothesis is supported by the results of both the infections in the *Galleria* model, as well as the biofilm data. In the case of the *Galleria* infections, there was reduced mortality in E2G4 and  $\Delta$ lpxtg, but with a greater reduction observed in  $\Delta$ lpxtg. Biofilm production was greater in  $\Delta$ lpxtg than in E2G4, but E2G4 still produced more biofilm than wild type, potentially explained by the inactivation of the same gene in both constructs (*LMOSA\_2450*), but with the additional inactivation of *LMOSA\_2460* and *LMOSA\_2470* in E2G4. The fact that there was a more significant impact on virulence in  $\Delta$ lpxtg than in E2G4, suggests that *LMOSA\_2460* and *LMOSA\_2470* may actually work to suppress virulence and that the dual inactivation on both sides of the transposon resulted in an antagonistic response.

*LMOSA\_2450*, *LMOSA\_2460*, and *LMOSA\_2470* are all harbored on LGI2, a MGE that has been found to be widely distributed and highly conserved in *L. monocytogenes* and *E. faecalis* (Kuenne *et al.* 2013; Lee *et al.* 2013). Subsequent works have identified associations between LGI2 and heavy metal detoxification (Lee *et al.* 2013; Parsons *et al.* in review). While previous work identified *cadA4* as primarily responsible for cadmium resistance in the LGI2-harboring strain F8027 (Parsons *et al.*, in review), transposon insertion between *LMOSA\_2450* and *LMOSA\_2460* also significantly decreased cadmium tolerance. In the previous work, heterologous expression of *cadA4*, cloned from F8027, conferred comparable levels of cadmium tolerance to cadmium sensitive strains F2365, and H7550-Cd<sup>s</sup>. This demonstrated that full cadmium tolerance was conferred by *cadA4*. Here we saw that even with a functional *cadA4*, cadmium tolerance was reduced by the likely inactivation of both *LMOSA\_2450* and *LMOSA\_2470* suggesting that even though *cadA4* is sufficient for heterologous expression of cadmium tolerance, in F8027 the observed levels of cadmium tolerance require *cadA4* as well as additional LGI2-harbored genes. While qPCR results showed no impact on *cadA4* expression in untreated E2G4, assessments would need to be done in the presence of cadmium to know if these genes truly impact *cadA4* expression. It is possible that heterologous expression of cadmium tolerance in heterologous constructs may be the result of yet unidentified genes mediating similar functions to *LMOSA\_2450* and *LMOSA\_2470* that are present in F2365 and H7550-Cd<sup>s</sup> but absent in F8027. A comparative genomic assessment of their respective genomes could yield insight into the potential functions of *LMOSA\_2450* and *LMOSA\_2470*.

The data, in conjunction with the presence of a putative arsenic resistance cassette on LGI2, a proven cadmium resistance gene, and the results from the study of the E2G4 mutant

indicate this island is strongly associated with metal metabolism. Interestingly, MIC determinations for serotype 4b strains both containing and lacking LGI2 were made for both zinc and cadmium (data not shown), and while cadmium resistance was strongly correlated with the presence of LGI2, zinc tolerance did not appear to vary, again suggesting genes of similar function elsewhere in the genome of strains lacking LGI2.

This is the first work to establish a link between an LPXTG protein and zinc homeostasis. While the mechanism for this relationship has yet to be established, this increased susceptibility to zinc could form the basis for the reduced virulence of  $\Delta$ lpxtg. *LMOSA\_2450* is a cell wall-associated protein, and cell wall integrity or reduced cell membrane potential may underlie zinc sensitivity of the *LMOSA\_2450* deletion mutant. While it does not appear that that *LMOSA\_2460* and *LMOSA\_2470* are directly involved in metal homeostasis, *LMOSA\_2470* does appear to have an effect on resistance to cadmium. The involvement of these genes in metal tolerance, biofilm formation, and virulence gives new insights into the role of LGI2 and potentially explains its distribution and retention in *L. monocytogenes*.

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**Table 3-1.** Strains used in this study

Strains used in this study	Significance	MIC Cd ( $\mu\text{g/ml}$ )	MIC Zn (mM)
F8027	Serotype 4b strain isolated from celery (Lang, Harris and Beuchat 2004)	50	22
E2G4	<i>Mariner</i> -based transposon mutant of F8027 with transposon insertion in intergenic region between <i>LMOSA_2450</i> and <i>LMOSA_2460</i>	25	10
E2G4 :: 2460/2470	E2G4 complemented with the <i>LMOSA_2460</i> - <i>LMOSA_2470</i> cassette	50	15
E2G4::Lpxtg	E2G4 complemented with <i>LMOSA_2450</i>	25	10
E2G4::2460	E2G4 complemented with <i>LMOSA_2460</i>	25	10
E2G4::2470	E2G4 complemented with <i>LMOSA_2470</i>	50	10
E2G4::pPL2	E2G4 with chromosomal insertion of the empty shuttle vector pPL2 (Lauer <i>et al.</i> 2002)	25	10
$\Delta$ lpxtg	F8027 with deletion of <i>LMOSA_2450</i>	50	10
$\Delta$ lpxtg::lpxtg	$\Delta$ lpxtg complemented with <i>LMOSA_2450</i>	50	10
$\Delta$ lpxtg::2040	$\Delta$ lpxtg complemented with <i>LMOSA_2420</i> - <i>LMOSA_30</i> - <i>LMOSA_40</i>	50	10
$\Delta$ lpxtg::pPL2	$\Delta$ lpxtg with chromosomal insertion of the empty shuttle vector pPL2	50	10
$\Delta$ lpxtg +Zn15	Spontaneous zinc-tolerant mutant of $\Delta$ lpxtg isolated on BHI with zinc (15 mM)	50	22
$\Delta$ 2460	F8027 with deletion of <i>LMOSA_2460</i>	50	22
$\Delta$ 2470	F8027 with deletion of <i>LMOSA_2470</i>	50	22
$\Delta$ 2460/2470	F8027 with deletion of <i>LMOSA_2460</i> - <i>2470</i>	50	22

**Table 3-2.** Primers used in this study

Primer	Sequence (5' to 3')
E2G4F	TAATCAGCAATTCTTGTGTAGTT
E2G4R	GAAGCCTTTTCTACTGCTCTA
2460compF <sup>1</sup>	CTAAG <u>GTACC</u> TTTAGAGTTATGAGAGGGAC
2460compR <sup>1</sup>	GTAAG <u>GCGCCGC</u> ATCATCTTCTGTAGTAATGATG
2470compF <sup>1</sup>	CTAAG <u>GTACC</u> CTTGGAGAAATATGATGTTGAAG
2470compR <sup>1</sup>	GTAAG <u>GCGCCGC</u> CTTAATCGTTGGTATAACAG
LPXcompF <sup>2</sup>	CTAAG <u>GTCGAC</u> GCTTGTGACACGATTGGACA
LPXcompR <sup>2</sup>	GTAAG <u>GCGCCGC</u> AGTTATACCTCCTCAAGTAATA
242040compA <sup>3</sup>	GACT <u>GCGCCGC</u> GCGGGTCACCTGCCTTGTGTT
242040compB	ATGTCCAATCGTGTCAACAAG
242040compC	CTTGTGACACGATTGGACATTGTTTCTTTTCCTCCTTGAATT
242040compE <sup>3</sup>	AGTC <u>GGTACC</u> AGTTATACCTCCTCAAGTAATAA
LPXTGdel1F <sup>4</sup>	GCATA <u>AAGCTT</u> GGAGTTGAACCAAAACCCTT
LPXTGdel1R <sup>4</sup>	CGTAGCTAGCAGCTAGATTGCCATACTTACT
LPXTGdel2F <sup>5</sup>	GCAT <u>GGATCC</u> GAGGCAGTTCAATGTCTCT
LPXTGdel2R <sup>5</sup>	CGTAGAATT <u>CAAGTGCAGT</u> GACTTCTCCTG
2470soeA <sup>6</sup>	AGTCT <u>CTAGAG</u> AAAAAACTGATAGAGCAGTAG
2470soeB	ACTTCGCTTTATTTTCAAATGTT
2470soeC	AACATTTGAAAATAAAGCGAAGTTAGTGATTAATTAGTCTTGCTGA
2470soeD <sup>6</sup>	GACTA <u>AAGCTT</u> AAGGTTAGCTTTTCTCAGAGC
2460soeA <sup>6</sup>	AGTCT <u>CTAGAT</u> CGTCTGTCCAGTGAAGACC
2460soeB	AGTTATACCTCCTCAAGTAATAA
2460soeC	TTACTTGAGGAGGTATAACTATGAATACTCTGACATTTAATAA
2460soeD <sup>6</sup>	GACTA <u>AAGCTT</u> TTTAAACAGCTTATCCTGCCTA
UNKsoeC	TTATTACTTGAGGAGGTATAACTTAGTGATTAATTAGTCTTGCTGA

<sup>1</sup> Underlined GGTACC and GCGGCCGC sequences are restriction sites for KpnI and NotI, respectively.

<sup>2</sup> Underlined GTCGAC AND GCGGCCGC sequences are restriction sites for SalI AND NotI, respectively.

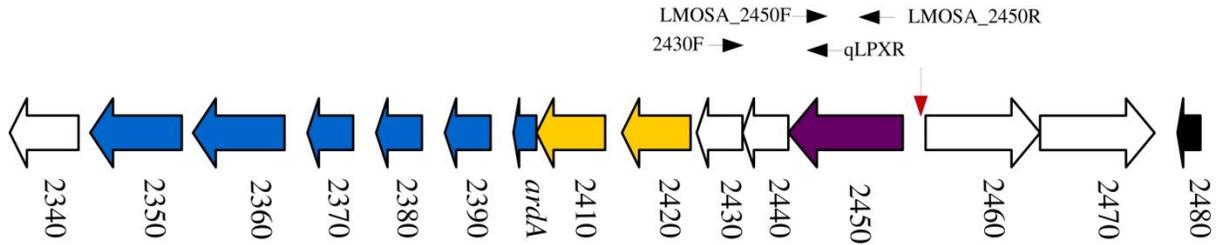
<sup>3</sup> Underlined GCGGCCGC and GGTACC sequences are restriction sites for NotI and KpnI, respectively.

**Table 3-2** continued

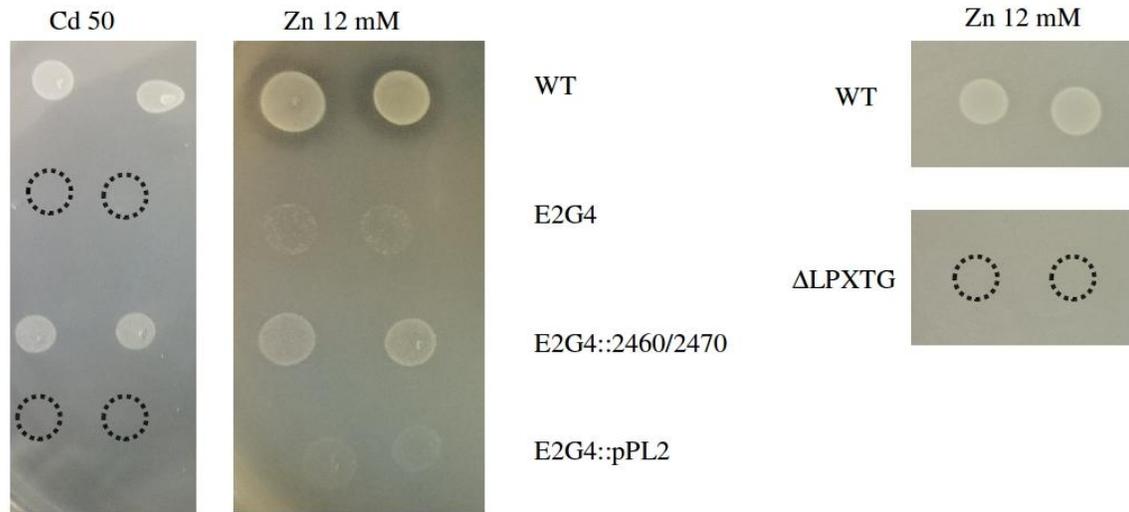
<sup>4</sup> Underlined AAGCTT and GCTAGC sequences are restriction sites for HindIII and NheI, respectively.

<sup>5</sup> Underlined GGATCC and GAATTC sequences are restriction sites for BamHI and EcoRI, respectively.

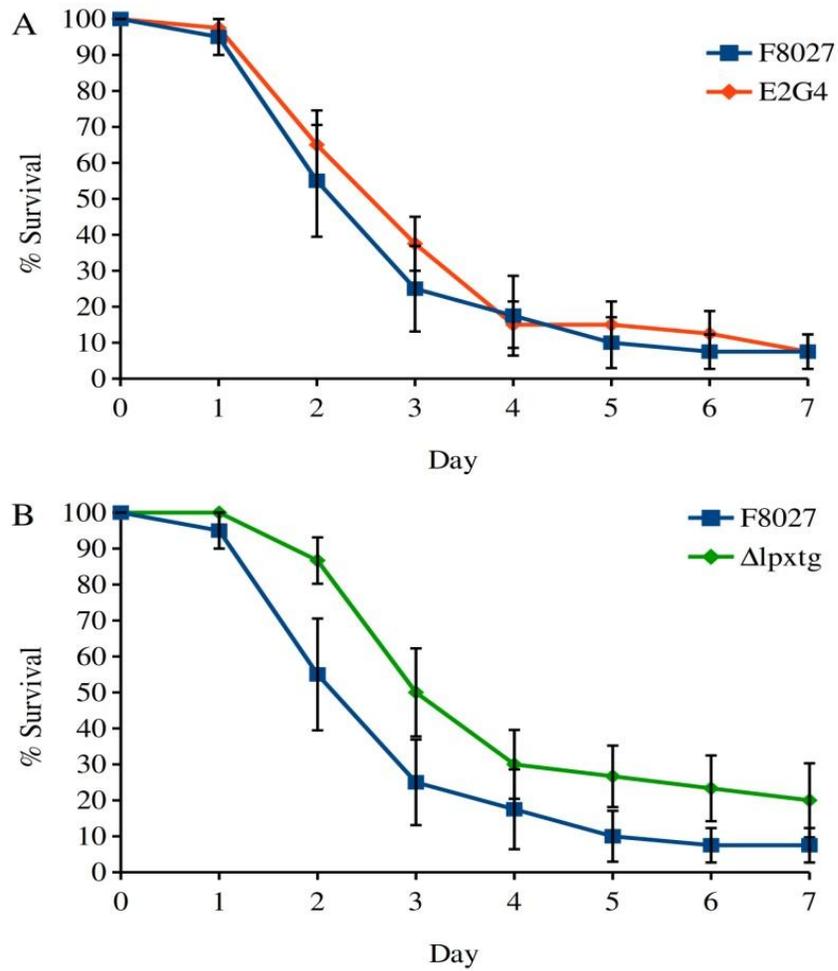
<sup>6</sup> Underlined TCTAGA and AAGCTT sequences are restriction sites for XbaI and HindIII, respectively.



**Figure 3-1.** Genomic organization of the region harboring the transposon insertion site in E2G4. Insertion site is noted with a red arrow. Genes in white are annotated as conserved domains of unknown function. The gene in purple is a putative cell wall-associated protein with the LPXTG motif. The genes in orange are putatively associated with replication, and the genes in blue are putatively associated with conjugative or antirestriction functions. Primer locations indicated by small arrows. Arrow orientation of genes indicates direction of transcription.



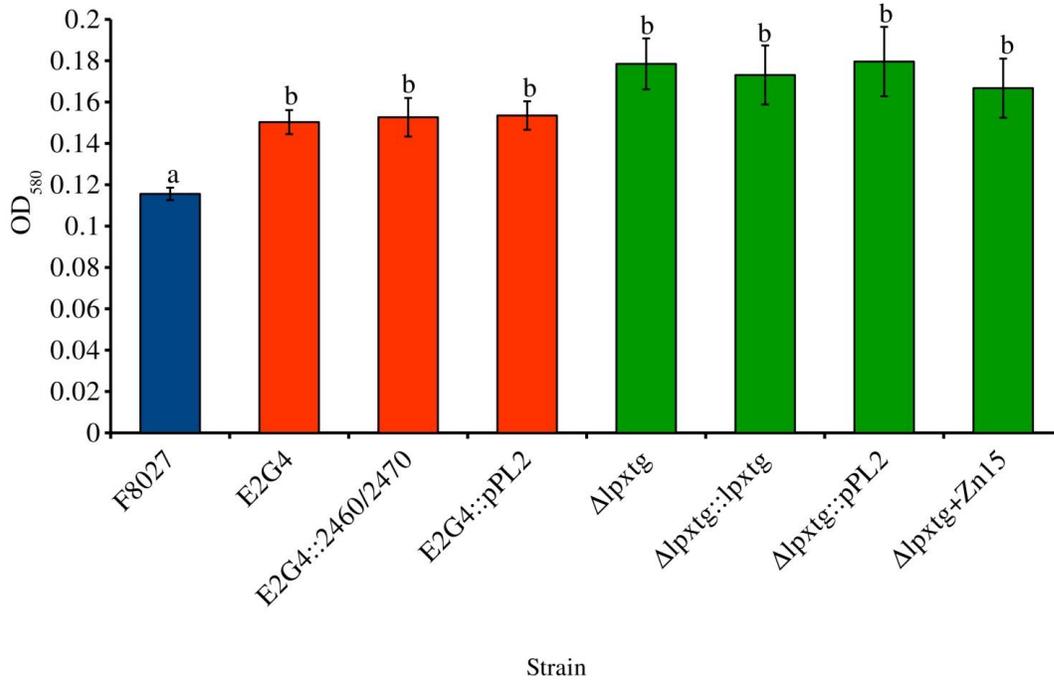
**Figure 3-2.** Growth of complementation and deletion constructs in the presence of cadmium (50 µg/ml) or zinc (12 mM). F8027 the wild type (WT), mutant E2G4, E2G4 complemented with both *LMOSA\_2460* and *LMOSA\_2470* (E2G4::2460/2470), E2G4 with a chromosomal insertion of the empty shuttle vector pPL2 (E2G4::pPL2), or F8027 with a deletion of the coding region of *LMOSA\_2450* ( $\Delta$ lpxtg). Cell suspensions (4µl) were spotted in duplicate on the surface of the plate which was dried and incubated at 37° C for 48h. Dotted circles indicate location of spots with no observed growth.



**Figure 3-3.** Impacts of transposon insertion in intergenic region between *LMOSA\_2450* and *LMOSA\_2460* (A) and deletion of *LMOSA\_2450* (B) in the *Galleria mellonella* model.

Larvae were inoculated with F8027, E2G4, or  $\Delta$ lpxtg, and monitored daily for seven days as described in Materials and Methods. Data are averages from at least three independent trials.

Strains were as described in Table 3-1 and legend of Fig. 3-2.



**Figure 3-4.** Impact of transposon insertion in intergenic region between *LMOSA\_2450* and *LMOSA\_2460*, and deletion of *LMOSA\_2450* on biofilm formation at 25°C. Biofilms were established in wells of 96-well plates (15 wells/strain) and measured following staining with crystal violet, as described in Material and Methods. Differing letters within each group indicate statistically significant differences ( $p < 0.05$ ). Strains were as described in Figure 3-2 and Table 3-1.

**Chapter 4 : Penicillin binding protein encoded by *pbp4* mediates copper homeostasis in *Listeria monocytogenes*.**

## **SUMMARY:**

*Listeria monocytogenes* has long been a cause for major concern with regard to food safety and public health. Safe and cost effective control strategies are needed to prevent colonization of food processing facilities and food contamination. Copper is often employed to control pathogens in agriculture as well as healthcare facilities, but mechanisms mediating tolerance of *L. monocytogenes* to copper are poorly understood. A *mariner*-based mutant library of *L. monocytogenes* strain 2011L-2858 was screened for growth on sublethal levels of copper, yielding mutant G2B4 with decreased copper tolerance. The transposon was localized in *pbp4* (*lmo2229* homolog), encoding a penicillin-binding protein. In addition to reduced copper tolerance, G2B4 exhibited increased susceptibility to  $\beta$ -lactam antibiotics, reduced biofilm formation, and reduced virulence in the *Galleria mellonella* model. All G2B4 phenotypes were fully restored via genetic complementation with *pbp4*. Findings suggest that *pbp4* inhibition may enable the use of lower levels of copper and possibly other compounds, or enhance effectiveness of levels currently in use. Given the wide distribution of penicillin binding proteins and their highly conserved nature, this could have profound impacts in regard to control of *L. monocytogenes* and other microorganisms.

## **INTRODUCTION:**

The foodborne pathogen *Listeria monocytogenes* is the causative agent of the disease listeriosis. This disease causes febrile gastroenteritis in healthy individuals, with much more severe symptoms in at-risk populations. For this reason *L. monocytogenes* is a leading contributor to death from domestically acquired foodborne illness in the United States (Scallan *et al.* 2011). *L. monocytogenes* can be found ubiquitously in nature and possesses

numerous adaptations for survival in a wide range of conditions, either as saprophyte or as facultative intracellular pathogen (Gray and Killinger 1966; Gandhi and Chikindas 2007; Vivant, Garmyn and Piveteau 2013). For these reasons many control strategies have been employed to exclude *L. monocytogenes* from the food supply and protect at-risk populations.

Copper has been investigated for use in reducing levels of *L. monocytogenes* and other pathogenic or spoilage microorganisms on poultry carcasses (Russell 2008), and for incorporation into metal surfaces of hospitals and food production facilities to mitigate pathogen colonization and transmission (Wilks, Michels and Keevil 2006; Mikolay *et al.* 2010). Copper has long been used for pathogen control in agriculture, being one of the few chemicals approved for use in organic agriculture (Jesse, Roberts and Cavet 2014; Nunes *et al.* 2016). *In vivo* copper is also employed in immune responses against certain pathogens, including *L. monocytogenes* (Samanovic *et al.* 2012; Jesse, Roberts and Cavet 2014). While excessive extracellular copper can be lethal to *L. monocytogenes* and other microbes, copper is an essential element at trace levels for many key bacterial enzymes (Corbett *et al.* 2011; Latorre *et al.* 2015). Thus, maintaining appropriate intracellular copper levels is critical for cell survival.

Even though intracellular levels of copper must be tightly regulated to ensure bacterial survival, the mechanisms by which this is accomplished in *L. monocytogenes* are poorly understood (Waldron and Robinson 2009; Jesse, Roberts and Cavet 2014). One of the determinants mediating copper homeostasis in *L. monocytogenes* is the *csoR-copA-Z* cassette. It consists of the copper sensing transcriptional regulator CsoR, the P-type ATPase CopA, and the metallochaperone CopZ. This cassette is widely distributed in *L. monocytogenes* and functions to maintain appropriate levels of intracellular copper by sensing cytoplasmic levels

of copper and activating or suppressing the efflux pump (CopA) accordingly (Corbett *et al.* 2011; Chang *et al.* 2014). CtpA, another copper transporting P-type ATPase mediating copper efflux has also been identified in *L. monocytogenes*, it was found to impact not only copper tolerance but virulence as well (Francis and Thomas 1997). A survey of *L. monocytogenes* plasmids revealed genes putatively associated with copper detoxification and transport, including a copper translocating P-type ATPase and a multi-copper oxidase (Kuenne *et al.* 2010). While functional confirmation for these genes is lacking, at least one was present in eight of the 14 plasmids studied, though premature stop codons were detected in certain cases. These putative copper resistance genes were located on plasmids that also contained the cadmium resistance gene *cadAI* (Kuenne *et al.* 2010).

In another Gram-positive pathogen, *Streptococcus mutans*, genes associated with copper homeostasis have impacts not only in copper tolerance but additional adaptations, such as genetic competence, biofilm formation and acid tolerance (Singh *et al.* 2015). Considering the critical importance of copper homeostasis in bacteria, there is currently a lack of relevant information related to mechanisms mediating such homeostasis in *L. monocytogenes*. For that reason, in the current study we sought to better characterize the mechanisms involved in copper homeostasis in *L. monocytogenes* 2011L-2858, implicated in a major outbreak of listeriosis in 2011 via contaminated cantaloupe.

## **MATERIALS AND METHODS:**

**Bacterial strains and growth conditions.** The *L. monocytogenes* strains used in this study were all derivatives of 2011L-2858, a serotype 1/2b isolate from the 2011 cantaloupe outbreak (McCollum *et al.* 2013; Rakic-Martinez *et al.* 2016). Strains were grown in either

brain heart infusion broth (BHI) (Becton, Dickinson & Co., Sparks, MD, USA) or on BHI agar (1.2% Bacto agar [Becton Dickinson]). *Escherichia coli* strains used were grown at 30°C in Luria Bertani (LB) broth (Becton Dickinson) or on LB broth supplemented with 1.2% Bacto agar. Unless otherwise indicated the antibiotics used for *L. monocytogenes* were erythromycin (5 µg/ml), ciprofloxacin (0.05 µg/ml) and chloramphenicol (8 µg/ml), while chloramphenicol (25 µg/ml) was used for *E. coli*. The antibiotics were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Mariner-based transposon mutagenesis and screening.** A transposon mutant library of approximately 2000 mutants was generated for strain 2011L-2858 as described previously (Cao, Bitar and Marquis 2007; Azizoglu and Kathariou 2010). Individual mutants were grown in wells of a 96-well plate, then each mutant was stamped on to a BHI plate containing 10 mM copper sulfate using a metal transfer device, and incubated for 48 h at 37°C to assess copper tolerance.

**Localization of transposon insertion and copy number.** Southern blots were performed as previously described (Cao, Bitar and Marquis 2007; Azizoglu and Kathariou 2010) to assess transposon copy number in the chromosome. Two rounds of PCR with primers specific to the transposon were conducted as previously described (Cao, Bitar and Marquis 2007; Azizoglu and Kathariou 2010) and the amplicon was sequenced (Genewiz, Inc. South Plainfield, NJ, USA) to determine the location of the transposon in the chromosome. To confirm transposon location, PCR primers were designed flanking the implicated region, tF (5'-

AATCGTGGCTGACTCTAAGC-3') and tR (5'-ATGGTAACAGTAATATCTACCC-3') (Fig. 4-1). The resulting PCR product was sequenced (Genewiz, Inc.).

**Resistance determination for metals and antibiotics.** Minimum inhibitory concentrations (MIC) were determined as described previously (Mullapudi, Siletzky and Kathariou 2010). Tolerance to cadmium chloride, sodium arsenate, and sodium arsenite was assessed on Iso-Sensitest agar (ISA) (Oxoid, Hampshire, England), at concentrations of (2.5-10 µg/ml cadmium chloride, 500-4000 µg/ml sodium arsenate, and 50-1000 µg/ml sodium arsenite). All other agents were tested in BHI (2,5, 6,7.5, 8, 10, 11, 12.5,15 mM copper sulfate, and 1-30 mM zinc sulfate, 0.06, 0.12, 0.18, 0.25, 0.5, 0.75 µg/ml penicillin G, 1,4,8,16 µg/ml vancomycin, 6-100 µg/ml tetracycline, 0.5-4 µg/ml trimethoprim, 1-20 µg/ml benzalkonium chloride, and 0.3-2 M NaCl). MIC was established as the lowest concentration that prevented confluent growth following incubation at 37°C for 48 h. MIC for copper and penicillin were also assessed at 4°C except plates were monitored for growth over the course of three weeks. Cell suspensions were spotted in duplicate on each plate, and each assessment was done in at least two independent trials. MICs for a panel of antibiotics were kindly determined by M. Jacob at North Carolina State University's School of Veterinary Medicine Clinical Microbiology Laboratory via A<sub>600</sub> measurements after 48 and 72 h in Mueller Hinton Broth in the presence of varying concentrations of ampicillin, penicillin, oxacillin, pirlimycin, penicillin in combination with novobiocin, tetracycline, cephalothin, ceftiofur and sulphadimethoxine. The efflux inhibitor reserpine (Sigma-Aldrich) was used at 40 µg/ml in at least two independent trials, with MICs also determined in the absence of reserpine as controls.

**RNA extraction and transcriptional assessments.** *L. monocytogenes* 2011L-2858 was grown in TSBYE at 37°C until mid to late logarithmic phase (OD<sub>600</sub> of 0.7-1.0, as measured by SmartSpec 3000; Bio-Rad, Hercules, CA, USA). The culture was then divided into two portions, one of which was exposed to sublethal level of copper (3 mM) and incubated for an additional 30 min at 37°C while the other was similarly incubated without addition of copper. RNA was extracted using the SV total RNA isolation system (Promega, Madison, WI, USA) and purified with Turbo DNA-free DNase (Ambion, Austin, TX, USA) to remove any contaminating DNA. For quantitative real-time PCR (qPCR), the BIO-RAD iTaq Universal SYBR Green One-Step Kit (Bio-Rad Laboratories Hercules, CA, and USA) was used per the manufacturer's instructions with a LightCycler 96 system (Roche Diagnostics, Indianapolis, IN, USA). Primers qPBPF (5'-AGTGGCTTCATTGTGGAACC-3') and qPBPR (5'-CATTAGAGCGCTTGTGGTG-3') were designed using Primer3Plus (<http://primer3plus.com/>) to measure expression of *pbp4*, with *spoVG* used as control. All experiments were repeated in at least three independent trials. Fold change calculations were carried out using the  $\Delta\Delta C_T$  method, normalizing for the expression of *spoVG* as described previously (Dutta *et al.* 2014).

**Genetic complementation.** Primer cF (5'-GATCCCCGGGATTCCAATTCCTTTCCGGCG-3') with XmaI restriction site (underlined) and primer cR (5'-GATCGGTACCTGTACTCACCTCATTCTTTTGT-3') with KpnI restriction site (underlined) were used to generate a fragment consisting of 2142 nts of the coding sequence of *pbp4* as well as 219 and 109 nts upstream and downstream, respectively. The PCR product was then digested with XmaI and KpnI (New England

Biolabs, Ipswich, MA, USA), ligated using T4 DNA ligase (Promega) into similarly digested shuttle vector pPL2 (Lauer *et al.* 2002), and electroporated into *E. coli* S17-1 (Simon, Prierer and Pühler 1983). This allowed for the mobilization of the vector into G2B4 via conjugation yielding strain G2B4::pbp. The empty vector was similarly mobilized into G2B4 yielding G2B4::pPL2.

**Peroxide killing assay.** Cultures of 2011L-2858 and G2B4 were grown overnight at 37°C. Overnight cultures were then mixed with liquid LB soft agar, poured as an overlay on BHI plates and allowed to solidify. Sterile paper discs (6mm, BD, Sparks MD) were then deposited on the surface of the overlay in triplicate for each strain and 20 µl of 30% H<sub>2</sub>O<sub>2</sub> was deposited on each disc. Plates were then incubated overnight at 37°C, and zone of inhibition around each disc was measured and recorded for at least two independent trials.

**Assessment of biofilm formation.** Biofilm formation for each strain was assessed in 96-well PVC plates as described previously (Pan, Breidt and Kathariou 2009). Each strain was grown for 48 h in TSB at either 25 or 37°C. Cultures were then removed, stained with 0.8% crystal violet, destained with 95% ethanol, and absorbance of destained ethanol measured at 580nm in a Tecan Safire® 96 well microplate reader (Tecan, Crailsheim, Germany). Biofilm assessments were repeated for at least three independent trials.

**Growth and adherence on the surface of cantaloupe.** Strains were grown overnight at 37°C in BHI, and cells were washed twice in an equivalent volume of sterile water. Produce was purchased at retail and maintained at 4°C until used. Cell suspensions were spot-

inoculated (100  $\mu$ l) on the surface of cantaloupe fragments (approx. 2 x 2 x 1 cm) that had been aseptically cut and allowed to dry in a biological safety cabinet for one hour. For adherence, fragments were first placed into 10 mL of sterile water and washed gently to remove loosely adherent bacteria, the fragment was then moved into a fresh tube of 10 mL sterile water and vortexed for one minute to remove any adherent or remaining bacteria. Rinsates were appropriately diluted and plated on Modified Oxford Agar (MOX, Becton Dickinson), plates were incubated at 37°C for 48 h and counted. To monitor growth, at specific time points the inoculated produce fragments were vortexed in 10 mL sterile water and the cell suspension was diluted appropriately and plated on MOX. Plates were then incubated at 37°C for 48 h and counted. Assessments were conducted for at least three independent trials, with three fragments for each condition per time point.

**Virulence assessment in *Galleria mellonella* model.** Relevant strains were grown in BHI, washed twice, and resuspended in sterile PBS, then inoculated into larvae of the greater wax moth *Galleria mellonella* as described previously (Joyce and Gahan 2010; Mukherjee *et al.* 2010; Parsons *et al.* in review). Larvae were monitored for survival over seven days. All assessments were conducted in at least three independent trials.

**Statistical analyses.** Fold change calculations for qPCR experiments employed the  $\Delta\Delta C_T$  method, with statistical evaluation of Ct values using a paired Student's *t*-test. For statistical analysis of growth and adherence on produce, one-way analysis of variance (ANOVA) with a Tukey's test was used at  $p < 0.05$ , with SPSS version 22 (IBM Corporation Software Group, Somers, NY, USA). For virulence data, a non-parametric proportional hazards survival

model in Program JMP (SAS Institute, Cary, NC) was used to test for significant differences in survival of *Galleria melonella* when inoculated with different strains of *L. monocytogenes*. All strains were analyzed in a pairwise fashion and determined significance at the  $p < 0.05$  level. Biofilm data were analyzed using a paired Student's *t*-test ( $p < 0.05$ ).

## **RESULTS AND DISCUSSION:**

**Copper-sensitive phenotype of *mariner*-based transposon mutant in penicillin-binding protein *pbp4* (*lmo2229*).** Screening of a *mariner*-based transposon mutant library (approx. 2,000 individual mutants) on BHI with 10 mM copper sulfate yielded one mutant (G2B4) that failed to grow. Subsequent MIC testing of G2B4 confirmed that it had reduced copper tolerance with a MIC of 6 mM, in contrast to the parental strain 2011L-2858 for which the copper MIC was 12 mM (Table 4-2). Southern blots confirmed that there was a single insertion in the chromosome of G2B4 (data not shown). PCR and sequencing localized the insertion site of the transposon to four nucleotides prior to the start codon of *pbp4* (*lmo2229*), in the intergenic space between an annotated penicillin-binding protein (*pbp4*) and an annotated arsenate reductase (Fig. 4-1). While *pbp4* is located at the beginning of a series of genes transcribed in the same direction (Fig. 4-1), the sequence data suggest the presence of a Rho-independent terminator at the end of the coding region of *pbp4* (Fig. 4-1), and previous studies (Gravesen *et al.* 2004) have established that the transcript size associated with *pbp4* correlates closely with the size of *pbp4* coding region (~2.1 kb), suggesting that, despite its location and orientation *pbp4* is a monocistronic unit.

Nine penicillin-binding proteins (PBPs) have been identified in the *L. monocytogenes* genome (Krawczyk-Balska *et al.* 2012). These proteins are divided into three classes (A, B

and C). All three classes contain a C-terminal module that has transpeptidase activity catalyzing cross-linking between glycan chains. Class A PBPs also harbor an N-terminal glycosyltransferase residue that is responsible for glycan chain elongation, while Class B PBPs have N-terminal residues that interact with other proteins. Class C PBPs are monofunctional, harboring only transpeptidase activity. Pbp4 is a Class B penicillin-binding protein which is capable of both transpeptidase and glycosyltransferase activity (Zawadzka-Skomial *et al.* 2006). NCBI BLASTp analysis of the deduced amino acid sequence of *pbp4* harbored by 2011L-2858 revealed domains for the transpeptidase and the glycosyltransferase activity indicated in previous studies. Protein sequence alignment between 2011L-2858 and the extensively characterized *pbp4* of EGDe (*lmo2229*) showed 99.86% homology, with a single nucleotide polymorphism (SNP) mediating the one amino acid divergence between their two sequences. An alignment of their respective nucleotide sequences indicated 96.78% homology. *Lmo2229* has been experimentally proven to exhibit the previously mentioned transpeptidase and glycosyltransferase activity, but to also bind penicillin (Zawadzka-Skomial *et al.* 2006; Korsak *et al.* 2010). Interestingly, there were no metal binding domains detected within this protein, there were not even any cysteine residues within this sequence, which are key elements of known metal binding domains (Banci *et al.* 2006). The lack of metal binding domains may suggest that *pbp4* does not interact directly with metal cations.

***Pbp4* is not induced in the presence of copper.** Real-time PCR indicated that *pbp4* was not induced in the presence of 3 mM copper at 37°C (data not shown). While *lmo2229* was found to be highly upregulated in the presence of the cell wall-active antibiotic cefuroxime

(Nielsen *et al.* 2012), copper does not seem to elicit the same response. There were similar findings in a global transcriptional study of *Enterococcus faecalis* with exposure to heavy metals (zinc, manganese, and copper), none of the penicillin-binding proteins were found to be differentially expressed in response to these metals (Abrantes *et al.* 2011). However, the cells were only grown until mid-exponential phase (Abrantes *et al.* 2011). Prior studies speculated that growth phase may play a role in the expression of these penicillin-binding proteins (Begley *et al.* 2006). Thus, if the copper exposure was for a longer duration, or in a different growth phase, it is possible that induction might be observed.

Previous studies with strain have found that this gene is directly regulated by alternative sigma factor Sig B and the two-component system LiaSR (Begley *et al.* 2006; Collins *et al.* 2012). If copper is not an activator of these systems then that would be one likely explanation for the lack of differential expression in response to copper. In one study of genes differentially expressed in murine macrophages in comparison to growth in BHI, *pbp4* was not found to be differentially expressed (Schultze *et al.* 2015). Interestingly, only one of the nine identified penicillin-binding proteins (*lmo1438*) was found to be differentially expressed, though the difference was only found to be 1.32 fold. A previous study in living mice also found that *pbp4* was not differentially expressed, though three other penicillin-binding proteins (*lmo0540*, *2039*, and *1438*) were (Camejo *et al.* 2009). The study by Schultze *et al.* 2015 examined murine macrophages four hours post infection, while Camejo *et al.* 2009 extracted RNA 24, 48, and 72 h post infection from mouse livers. The difference in time after infection, as well as extracting RNA from mouse livers as opposed to macrophages could explain the difference in results. An earlier study using murine macrophages did find upregulation of *pbp4*, with 3 fold directly after infection and 2.16 fold

4 h after infection, consistent with previous findings that upregulation of penicillin-binding proteins in cell culture was below detection or minimal (Chatterjee *et al.* 2006).

*Pbp4* inactivation in 2011L-2858 produced similar phenotypes to previous studies. There were no observed differences between 2011L-2858 and G2B4 in colony morphology, hemolysis, phage susceptibility, or cold growth (data not shown). There were also no observed differences in growth in TSBYE or BHI (data not shown). G2B4 exhibited an elongated cell morphology under phase contrast microscopy, and the tendency to form chains of cells (data not shown). MIC testing of cadmium and zinc, as well as the disinfectant benzalkonium chloride, showed no difference in MICs between G2B4 and wild type (data not shown). Resistance to salt-induced osmotic stress also showed no observed difference from wild type (data not shown). At 4°C copper was highly inhibitory, with no growth observed by the wild type parental strains at copper concentrations as low as 6mM, which was congruous with previous findings (Latorre *et al.* 2015). This suggests that several of the factors that assist in metal detoxification may be downregulated at the reduced temperature, increasing susceptibility. There were similar findings for penicillin at 4°C, with MIC of wild type being reduced to 0.12 µg/ml and the MIC of G2B4 being reduced to 0.06 µg/ml. MIC testing in the presence of the efflux inhibitor reserpine showed a reduction of wild type tolerance to copper from 12mM to 10mM, and a similar reduction in the tolerance of G2B4 tolerance to copper from 6mM to 4mM, suggesting that efflux does play a role in the copper homeostasis of this strain. This may be expected as there are several annotated chromosomal copper transport determinants in *L. monocytogenes*. A global transcriptional study of *E. faecalis* upon exposure to toxic metals showed that transport proteins were the most significantly upregulated class of genes (Abrantes *et al.* 2011).

Conflicting data have been obtained in previous studies on the impacts of the inactivation of *lmo2229* on cell morphology and growth (Gravesen *et al.* 2004; Guinane *et al.* 2006; Zawadzka-Skomial *et al.* 2006; Rismondo *et al.* 2015). Similar to our findings, Guinane *et al.* 2006 and Gravesen *et al.* 2004 noted elongated cell morphology and the tendency of *lmo2229*-deficient mutants to form chains of three or more cells, while Rismondo *et al.* 2015 and Zawadzka-Skomial *et al.* 2006 noted no cell morphology changes. While we did not observe any impact on growth under standard laboratory conditions, Zawadzka-Skomial *et al.* 2006 noted reduced growth for *pbp4* mutants across a range of temperatures, while Rismondo *et al.* 2015 only noted a slight growth defect at 42°C. One possible explanation for these conflicting findings is that Zawadzka-Skomial *et al.* 2006 were monitoring for growth in TSB, while Rismondo *et al.* 2015 was using BHI, which is a more rich medium. Several studies also noted an increased susceptibility to the bacteriocin nisin (Graveson *et al.* 2003; Guinane *et al.* 2006), while Zawadzka-Skomial *et al.* 2006 observed no impact on tolerance to nisin. Nisin susceptibility of G2B4 was not assessed in the current study.

**Inactivation of *pbp4* decreases tolerance to cell wall-active antibiotics.** Given the transposon insertion in the intergenic space between a gene associated with penicillin binding, and a gene putatively associated with arsenic detoxification, testing for tolerance to both arsenic and penicillin was pursued to assess potential impacts on transcription on either side of the transposon insertion. MICs for both arsenate and arsenite showed no difference between G2B4 and the wild type (data not shown). MIC to penicillin G was noticeably (4 fold) reduced in G2B4 (0.12 µg/ml, in comparison to 0.5 µg/ml for 2011L-2858) (Table 4-2

and Fig. 4-2) suggesting that the transposon insertion functionally impacted the upstream gene, but not the one downstream. When tested for tolerance to antibiotics, impairment was found to the cell wall-active antibiotic vancomycin (G2B4 MIC 1 µg/ml, compared to 2011L-2858 MIC of 2 µg/ml) (Table 4-2); however, no perceived differences were found in MICs of other antibiotics (tetracycline, trimethoprim, pirlimycin, sulphadimethoxilne). Inactivation of *pbp4* in strains EGDe and EGD resulted in an increased susceptibility to  $\beta$ -lactam antibiotics including cephalosporins (Guinane *et al.* 2006; Zawadzka-Skomial *et al.* 2006), while Zawadzka-Skomial *et al.* (2006) found that the inactivation in EGD did not alter susceptibility to  $\beta$ -lactams. Our findings showed increased sensitivities to certain  $\beta$ -lactams (penicillin, ampicillin and oxacillin) (Table 4-2), but no impacts on susceptibility to cephalosporins, in contrast to previous findings (Guinane *et al.* 2006; Zawadzka-Skomial *et al.* 2006). Differences in observed phenotypes among different studies might be the result of growth phase-dependent differences in gene expression (Begley *et al.* 2006), type of mutation, or strain-specific impacts.

**Genetic complementation restores resistance phenotypes.** Genetic complementation of *pbp4* via a chromosomally integrated pPL2-derived construct restored not only copper tolerance (Fig. 4-2) but also tolerance to penicillin (Fig. 4-2, Table 4-2). These results were in agreement with the *in silico* analysis which suggested that *pbp4* is a monocistronic gene and indicated that the mutant phenotypes were due to inactivation of *pbp4*, and not to possible polar effects from the transposon insertion.

**Peroxide tolerance is not impacted by inactivation of *pbp4*.** One mode of copper toxicity is through the use of Fenton chemistry to generate oxygen radicals within the cell. There were no significant differences in the H<sub>2</sub>O<sub>2</sub> zones of inhibition between G2B4 and the parental strain (data not shown), suggesting that this mutant does not exhibit increased susceptibility to peroxide. In Gram-positive organisms, peptidoglycan functions in conjunction with teichoic acids to bind mono and divalent metal ions, facilitating their trafficking to the membrane under normal conditions, and potentially serving to sequester excess metal ions under toxic conditions (Neuhaus and Baddiley 2003; Abrantes *et al.* 2011). Thus, it is possible that alterations brought about by *pbp4* inactivation interfered with copper ion levels in the cell, leading to the observed increase in copper susceptibility in G2B4.

***Pbp4* inactivation reduces biofilm formation but does not impact adherence or survival on produce.** Inactivation of *pbp4* resulted in reduced biofilm formation at both 25 and 37°C (Fig. 4-3). In both instances G2B4 produced significantly less biofilm (P=0.03 and <.005, respectively), even though was considerably less biofilm formation at 25 than 37°C (Fig.4-3), making the differences less pronounced at the lower temperatures. Full restoration in the complemented mutant was seen at 37°C, with no significant difference between wild type and complemented strain (P=0.46). The vector alone was excluded as a contributing factor as there was no significant difference between G2B4 and G2B4 with the empty vector alone (P<0.005). Similarly, at 37°C there was a significant difference in biofilm formation between the complemented G2B4 and G2B4 with the empty vector (P<.005). At 25°C, while G2B4 appeared to produce less biofilm than the complemented G2B4, the differences did not reach statistical significance (P=0.06), potentially due to the overall reduced biofilm formation at

25°C. Our findings on the involvement of *pbp4* in biofilm formation agree with results from previous transposon mutant screens for biofilm-impaired mutants of strain EGDe, which also identified *pbp4* among genes required for biofilm formation at 32°C (Chang *et al.* 2012).

Given the involvement of *pbp4* in biofilm formation and the fact that the parental strain was isolated from cantaloupe (McCollum *et al.* 2013), assessments for impacts of *pbp4* on growth and adherence on cantaloupe were pursued. Despite the observed deficiencies in biofilm formation, there was no significant difference in adherence or growth on the surface of cantaloupe between 20111-2858 and G2B4. Following 48 h at 25°C, 20111-2858 and G2B4 exhibited approx. 1.6 and 1.8 log increase, respectively, while after 24 h at 37°C both strains exhibited approx. 1.8 log increase (data not shown). This suggests that, under the conditions employed, *pbp4*-mediated biofilm formation was not required for produce colonization.

**Inactivation of *pbp4* reduces virulence in *Galleria mellonella*.** Rismondo *et al.* 2015 found a six fold reduction in the ability of *pbp4* deletion mutants to invade HeLa cells, while intracellular growth was comparable to wild type in cell culture assays. Guinane *et al.* 2006, however, found that insertional inactivation of *pbp4* did not impair the ability to invade or grow within cultured cells, but that there was significantly less recovery of the deletion mutant after intraperitoneal injection in a murine model. In our study, G2B4 was found to cause significantly less mortality in *Galleria mellonella* than the parental strain (P=0.0375) (Fig. 4-4). Virulence was fully restored upon genetic complementation of G2B4 (Fig. 4-4), with the complemented mutant causing significantly more mortality than G2B4 (P=0.0109) and exhibiting no significant difference from the parental strain (P=0.74). The presence of

the vector alone in G2B4 was not different from G2B4 (P=0.3161), while also exhibiting significantly more mortality than the complemented strain (P=0.0334) (Fig. 4-4).

A previous study on the copper efflux ATPase *ctpA* (Francis and Thomas 1997) also supported *ctpA* involvement in virulence. The *ctpA* deletion mutant was indistinguishable from the wild type in cell culture models but was much more rapidly cleared from the liver of infected mice than the parental strain. While *ctpA* and *pbp4* are different determinants and tested in different animal infection models, their mutants exhibit similar behavior, suggesting that their copper-sensitive phenotype may contribute to their observed impaired virulence. It has been speculated that metal concentrations remained relatively constant, and at optimum concentrations for cell growth in cell cultures, while in live tissues they may fluctuate significantly in response to bacterial infection (Francis and Thomas 1997). This would explain the reported differences in virulence of *pbp4* mutants between cell culture models and *in vivo* (Guinane *et al.* 2006). Given both the increased sensitivity to copper and the decreased virulence it is conceivable that the increased copper sensitivity is responsible for the decrease in virulence observed in murine infections and in the *Galleria mellonella* model.

## **CONCLUSIONS:**

This is the first work establishing a link between a penicillin binding protein and copper homeostasis in *L. monocytogenes*. By inactivation of even one of the 9 known PBPs in *L. monocytogenes*, copper tolerance was decreased by approximately half. To date the focus on the roles of PBPs has focused on tolerance to penicillin and other cell wall-active antimicrobials (Rismondo *et al.* 2016), and the extent to which other PBPs may also play roles in copper homeostasis remains to be investigated. Inactivation of additional PBPs

could result in even more pronounced impacts on copper tolerance than noted here, or in increased susceptibility to other mono and divalent cations. Such studies would further elucidate the mechanism behind this increased sensitivity of the *pbp4* mutant to copper. While the underlying mechanism has yet to be characterized, complementation with a functional *pbp4* did restore copper tolerance, confirming the copper tolerance role of this gene. Given that there is no corresponding increase in susceptibility to salt stress, other metals, disinfectant and antibiotics other than  $\beta$ -lactams, this phenotype does not appear to generally interfere with cell wall integrity and permeability as a result of inhibited peptidoglycan synthesis. The increased susceptibility to cell wall-active antibiotics, and the observed differences in cell morphology indicate fundamental effects on cell wall synthesis. Similar to previous findings, virulence in an *in vivo* model is reduced with the inactivation of *pbp4*, possibly as a result of the cell being unable to mitigate the toxic effects of immune response-associated copper.

Copper has been shown to kill *L. monocytogenes* and other organisms by inducing damage to the cell membrane, disrupting membrane potential and via oxidative damage of lipids and proteins (Santo, Quaranta and Grass 2012; Singh *et al.* 2015; Yousuf *et al.* 2016). Given the lack of increased sensitivity of G2B4 to peroxide, the increased susceptibility of G2B4 to copper does not appear to be due to a transposon-mediated reduced capacity to mitigate oxidative damage. However, the increased sensitivity to cell wall-active antibiotics and bacteriocins observed in G2B4 and other *pbp4* mutants (Guinane *et al.* 2006; Rismondo *et al.* 2015) would seem to suggest that the mutation causes cell wall-specific alterations. The altered binding of metal ions to teichoic acid / peptidoglycan complexes (Neuhaus and Baddiley 2003; Abrantes *et al.* 2011), would be consistent with the known mechanism of cell

membrane disruption in copper toxicity of *L. monocytogenes* (Yousuf *et al.* 2016). A possible explanation is that mutation-induced differences in peptidoglycan composition, consistent with increased susceptibility to cell wall-active antibiotics, were sufficient to alter metal binding capacity of the peptidoglycan-teichoic acid complex, but not drastic enough to alter cell growth or resistance to other cell wall associated stresses such as exposure to quaternary ammonium disinfectant or salt. The increased interest in the use of copper as an antibacterial agent, and the continuing problem of antibiotic resistance in bacterial pathogens render further study and elucidation of the mechanisms encountered here of importance to the control and mitigation of *L. monocytogenes*.

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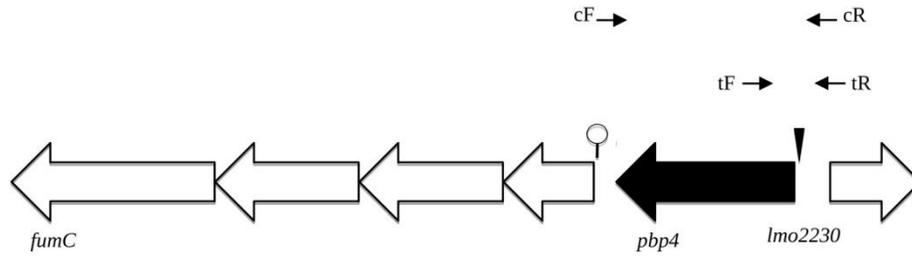
**Zawadzka-Skomial J, Markiewicz Z, Nguyen-Distèche M, Devreese B, Frère J-M, Terrak M.** 2006. Characterization of the bifunctional glycosyltransferase/acyltransferase penicillin-binding protein 4 of *Listeria monocytogenes*. *J Bacteriol* 188:1875–81.

**Table 4-1.** Strains used in this study

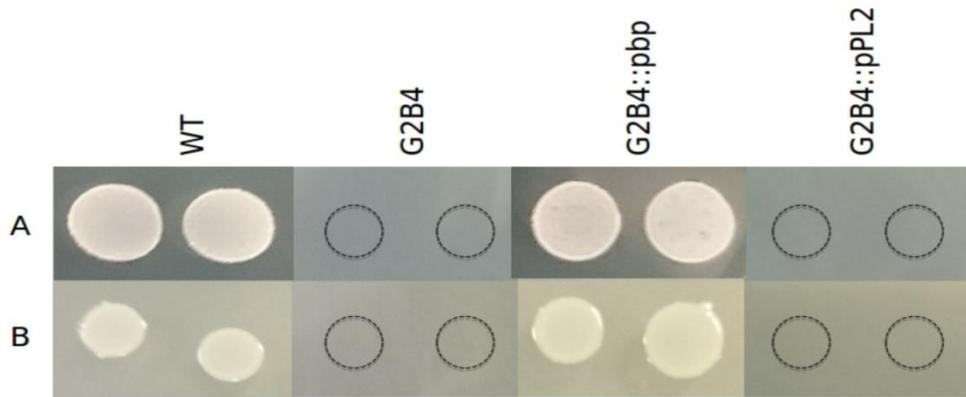
Strains used in this study	Phenotype	Source / Reference
2011L-2858	Serotype 1/2b, from the 2011 Jensen Farms cantaloupe outbreak	McCollum <i>et al.</i> 2013
G2B4	Copper and penicillin sensitive transposon mutant of 2011L-2858	This study
G2B4::pbp	Complemented derivative of G2B4 with cloned functional <i>lmo2229</i>	This study
G2B4::ppl2	G2B4 derivative with the empty shuttle vector pPL2 inserted chromosomally	This study
<i>E. coli</i> S17-1	Conjugation donor	Simon <i>et al.</i> 1983

**Table 4-2.** Increased sensitivity to copper and cell wall active antibiotics due to inactivation of lmo2229

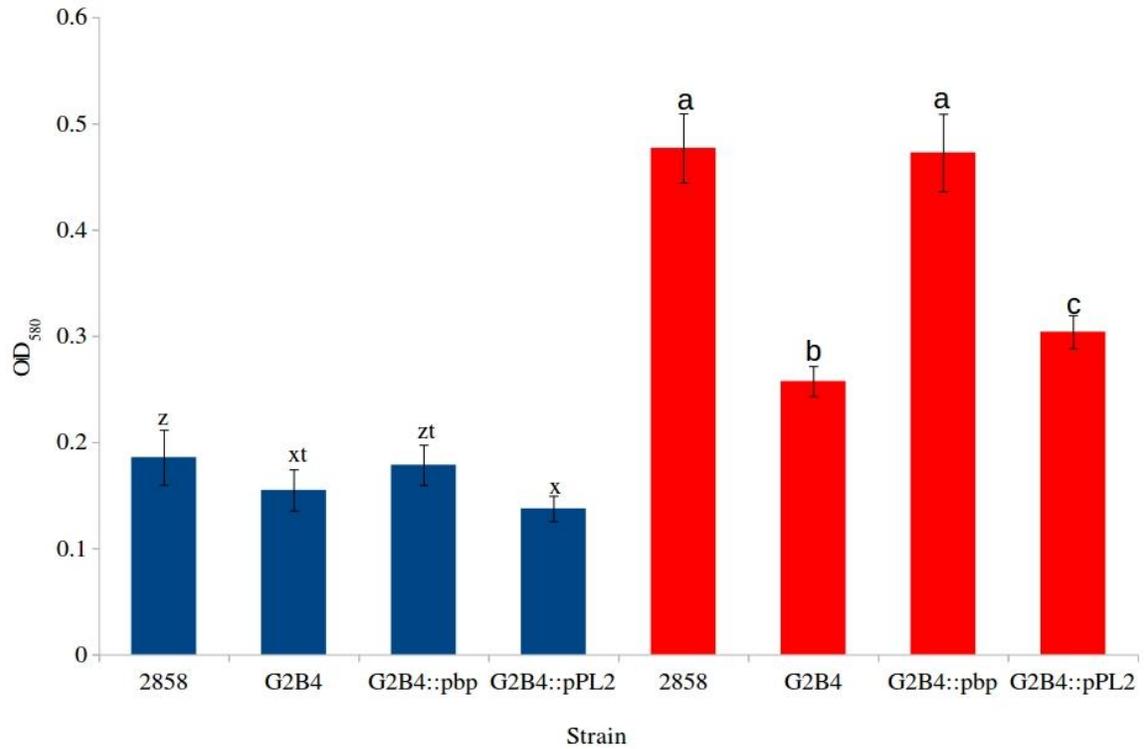
Compound (medium)	Function	MIC WT	MIC G2B4
Copper	Heavy metal required for healthy cell function.	12 mM	6 mM
Vancomycin	Antibiotic that impedes cell wall synthesis	2 ug/ml	0.75 ug/ml
Penicillin (plate)	$\beta$ -lactam antibiotic that inhibits cell wall synthesis	0.5 ug/ml	0.12 ug/ml
Penicillin (broth)	See above	0.25 ug/ml	0.12 ug/ml
Ampicillin	Member of penicillin group of $\beta$ -lactam antibiotics	0.25 ug/ml	0.12 ug/ml
Oxacillin	Member of penicillin group of $\beta$ -lactam antibiotics	4 ug/ml	2 ug/ml



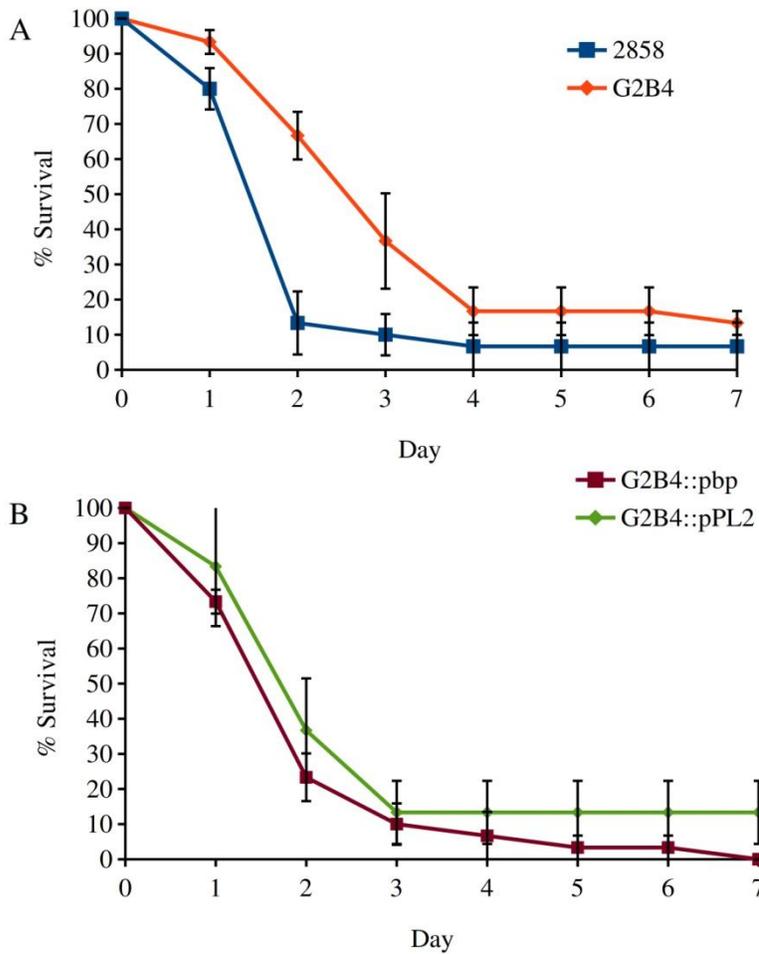
**Figure 4-1.** Genomic region harboring transposon insertion in mutant G2B4. Transposon insertion site is indicated with a downward arrow. Putative Rho-independent terminator is marked with a lollipop symbol. Primer locations are indicated by horizontal arrows. Direction of ORF arrows indicates direction of transcription. Black arrow is penicillin binding protein 4 (*pbp4*). *Lmo2230* is annotated arsenate reductase.



**Figure 4-2.** Growth of genetically complemented strains on BHI supplemented with 10 mM Cu (A) or 0.12 µg/ml penicillin (B). Wild type 2011L-2858 (WT), *pbp4* inactivated mutant G2B4, complemented G2B4 (G2B4::pbp), and G2B4 harboring empty shuttle vector pPL2 (G2B4::pPL2), grown for 48 h at 37°C. Cell suspensions (4µl) were spotted in duplicate, dotted circles indicate location of spots with no observed growth.



**Figure 4-3.** Biofilm formation at 25(blue bars) and 37(red bars)°C. Data are from at least three independent trials. Statistically significant differences are denoted by differing letters above each bar. Strain designations can be found in Table 4-1.



**Figure 4-4.** Impact of *pbp4* in the *Galleria mellonella* model. Larvae inoculated with 2011L-2858, mutant G2B4 (A), G2B4::pbp, or G2B4::pPL2 (B), as described in Materials and Methods, incubated at 37°C and monitored daily for seven days. Data are averages from at least three independent trials. Strains were as described in Fig. 4-2 and Table 4-1.

## Appendices

## Appendix A

**Library screening reveals arsenic sensitive mutants.** In addition to the cadmium sensitive mutants, screening of the mutant library on arsenic (500 µg/ml) identified two mutants (A2F3 and D1C6) with increased susceptibility to arsenic. Sodium arsenite MIC for A2F3 was found to be decreased to 100 µg/ml, while the MIC for D1C6 was reduced to 500 µg/ml, in comparison to arsenite of wild type F8027, which was found to be >1500 µg/ml (Table A-1).

In mutant A2F3 the transposon localized to the dihydrolipoamide dehydrogenase gene, a homolog of *lmo1371* in EGDe. This mutant was not observed to have any increased susceptibility to other metals (cadmium and zinc), or any impacts on hemolysis or motility. However, this mutant did exhibit formation of visibly smaller colonies, and a general growth deficiency, growing poorly at all tested temperatures (4, 25, and 37°C), in comparison to wild type (Fig. A-1). When this strain was grown anaerobically growth remained impaired, suggesting that the transposon insertion was not impeding the ability to mediate potentially toxic effects of oxygen. It was observed that propagation of this mutant at 42°C allowed for comparable levels of growth to wild type on an agar plate.

In mutant D1C6, the transposon localized to the coding region of the foldase *prsA2* (*lmo2219*). This mutant was observed to have an increased susceptibility to arsenic, but no increased susceptibility to any of the other metals tested (cadmium and zinc). Growth of this mutant was not impacted 4 or 25°C, but did exhibit reduced growth in comparison to wild type at 37°C (Fig. A-1). There were no observed impacts on hemolysis or motility.

**Table A-1. *Mariner*-transposon mutants of strain F8027**

Mutant	Phenotype	Transposon insertion site	Tn copy #	MIC ug/ ml
C1A6	Cd-susceptible	Putative membrane protein ( <i>lmo1695</i> )(nt 7090 EGDe 9/12)	1	25 Cd
I1A2	Cd-susceptible	Cad A4 (LMOSA_2330)	1	10 Cd
G2E6	Cd-susceptible	<i>Iap</i> P60 ( <i>lmo0582</i> ) (nt 259757 in EGDe 3/12)	1	35 Cd
E2G4	Cd-susceptible	Intergenic space between LMOSA_2450 and LMOSA_2460	1	35 Cd
A2F3	As-susceptible	similar to branched-chain alpha-keto acid dehydrogenase E3 subunit ( <i>lmo1371</i> )	1	100 As
D1C6	As-susceptible	similar to post-translocation molecular chaperone ( <i>lmo2219</i> )	1	500 As
E1F3	Cold sensitive	ND	ND	NA
C1H6	Cold sensitive	ND	ND	NA
F1F2	Cold sensitive	NA	ND	NA
T2A8	Cold sensitive	ND	ND	NA
R1F2	Cold sensitive	ND	1	NA
P1C2	Cold sensitive	ND	ND	NA
P1C6	Cold sensitive	ND	ND	NA
I2C8	Cold sensitive	ND	ND	NA
N2D3	Cold sensitive	ND	ND	NA
M1A5	Cold sensitive	ND	ND	NA
N1A2	Cold sensitive	ND	ND	NA
J1A5	Cold sensitive	ND	ND	NA
B2H2	Cold sensitive	ND	ND	NA
A1F2	Reduced growth on blood agar	<i>PurA</i> ( <i>lmo0055</i> )	1	NA
C1B2	Hemolytically Impaired	ND	ND	NA
C2F12	Hemolytically Impaired	ND	ND	NA
A1E1	Hemolytically Impaired	ND	1	NA

\*tested for arsenic tolerance on sodium arsenite

\*\*tested for cadmium tolerance on cadmium chloride

\*\*\*NA indicates not applicable

\*\*\*\*ND indicates not done

**Table A-2. *Mariner*-transposon mutants of strain 2011L-2858**

Mutant	Phenotype	Tn Insertion site	Tn Copy #
L1E4	Non-motile, cold sensitive/ growth on metals inhibited	RNA DEADbox Helicase ( <i>lmo0617</i> ) (nt 16943 EGDe 5/12)	1
M1A5	Non-motile	Conserved hypothetical protein, located in operon of flagellar/chemotaxis genes ( <i>lmo0694</i> ) (nt 88747 EGDe 4/12)	ND
F1F3	Non-motile	Flagellar biosynthesis FlhA ( <i>lmo0680</i> )(nt 75898 EGDe 4/12)	ND
R1B3	Non-motile	Flagellin protein FlaA ( <i>lmo0689</i> ) (nt 85175 EGDe 4/12)	ND
Q1B2	Non-motile	Conserved hypothetical protein, located in operon of flagellar/chemotaxis genes ( <i>lmo0701</i> )(nt 95219 EGDe 4/12)	ND
F1A3	Non-motile	Flagellar hook protein <i>flgK</i> ( <i>lmo0705</i> ) (nt 98821 EGDe 4/12)	ND
C1D2	Non-motile	Conserved hypothetical protein, located in operon of flagellar/chemotaxis genes ( <i>lmo0687</i> ) (nt 81631 EGDe 4/12)	ND
J2E1	Non-motile / clearing in motility spot	Similar to branched-chain fatty-acid kinase ( <i>lmo1370</i> )(nt 233775 EGDe 6/12)	1
J2E3	Non-hemolytic	<i>prfA</i> (nt 65 coding region)	1
B2G6	Non-hemolytic	<i>hly</i> (nt 225 coding region )	1
L1B6	Non-hemolytic	<i>hly</i> (confirmed by size of pcr)	1
G2B4	Copper susceptible	<i>pbp4</i> ( <i>lmo2229</i> ) (4 nt prior to start codon of <i>lmo2229</i> )	1
D1D5	Cd-susceptible	<i>tktB</i> ( <i>lmo1365</i> )( nt 227497 EGDe 6/12)	1
E2D5	Cd-susceptible	similar to glycosidase ( <i>lmo2444</i> ) (nt 117388 EGDe 11/12)	ND

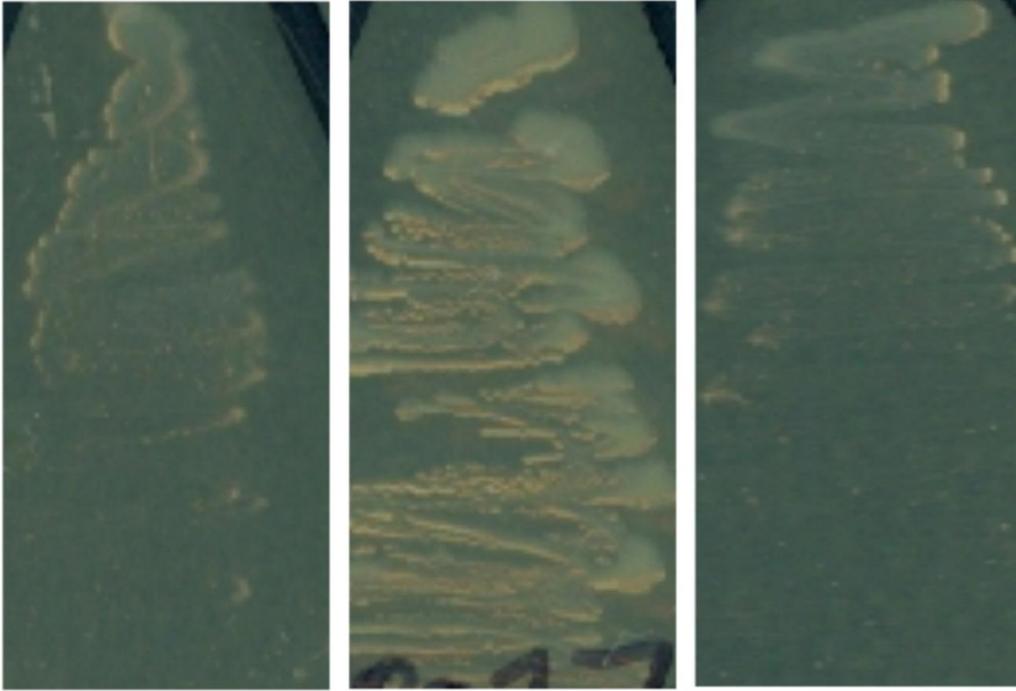
\*ND indicates not done

\*\*unless otherwise specified tn locations listed as sites in the EGDe chromosome with specific locus being specified out of 12

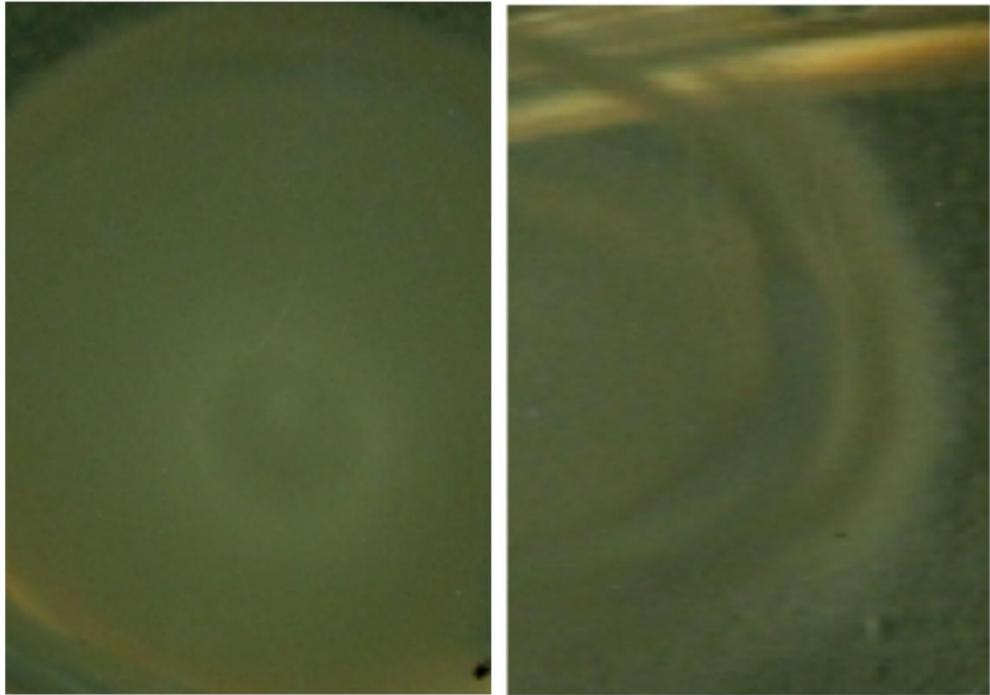
**A**

**B**

**C**



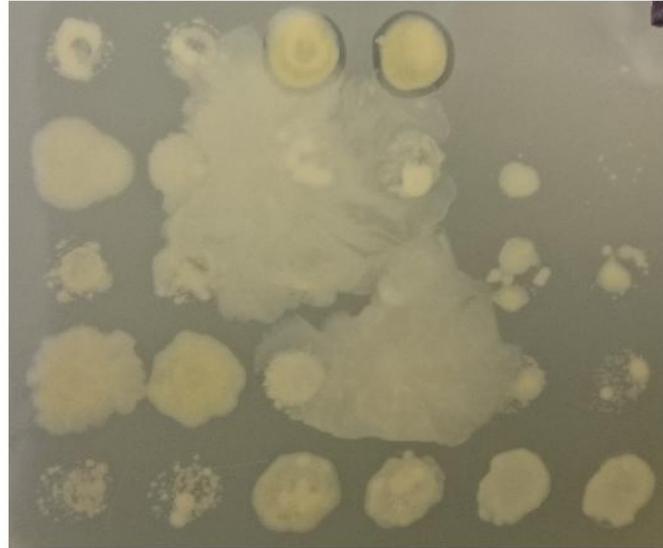
**Figure A-1.** Inhibited growth of mutants A2F3 (A) and D1C6 (C) in comparison to wild type F8027 (B) with growth at 37°C.



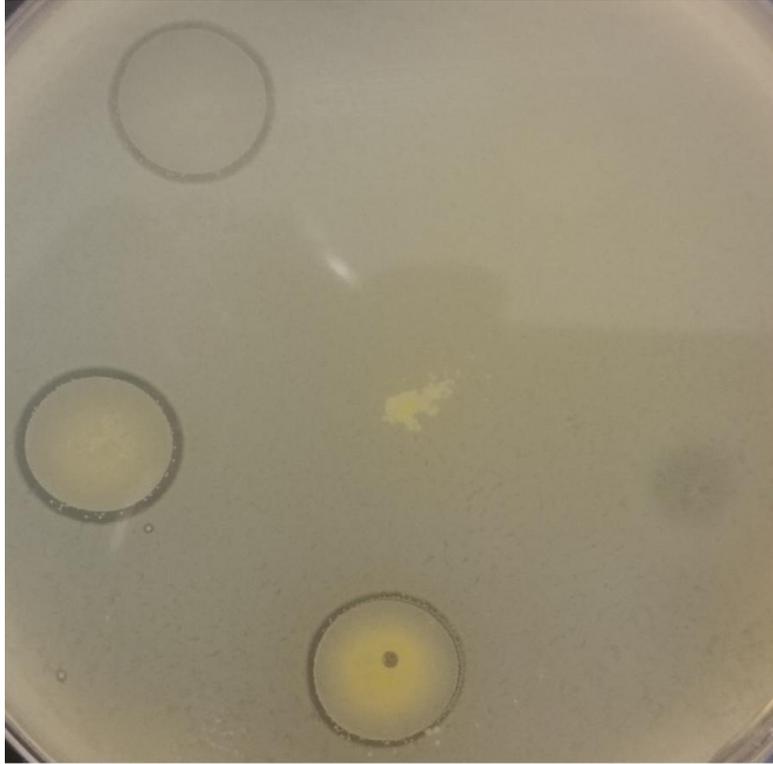
**Figure A-2.** Clearing in motility spot of J2E1 (right) in comparison to 2011L-2858 (left).

## Appendix B

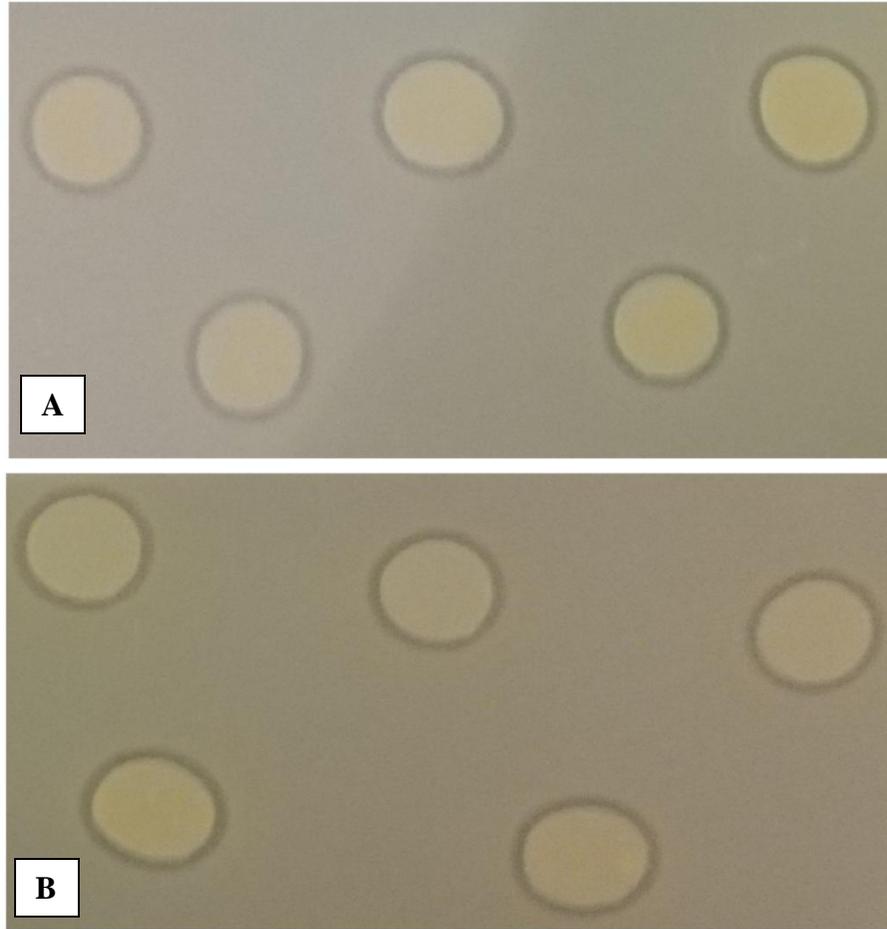
**Isolation of listericidal bacteria.** Cantaloupes were harvested from the field (collaboration with Dr. E. Guttierrez and Dr. C. Gunter) and brought directly to the lab. A flame-sterilized knife was used to remove six equally sized fragments from different regions of the cantaloupe rind, which were then submerged in 10 ml of sterile water in a 50-ml tube. The suspended fragments were then vortexed for one minute to remove adherent bacteria. Approx. 200 ul of cell suspensions were loaded into the wells of sterile PVC 96 well plates which were then stamped onto the surface of plates that had been overlaid with *L. monocytogenes*, incubated overnight at 37°C and monitored for zones of inhibition in the lawn of *L. monocytogenes*. Rinsate from one sample was found to inhibit growth of *L. monocytogenes* (Fig. B-1). Rinsate from this sample was streaked on TSAYE and multiple individual organisms were isolated. Individual organisms were then tested against lawns of *L. monocytogenes* to confirm inhibitory properties (Fig. B-2). From five individual isolates tested only one was found to be responsible for the observed inhibition. A fragment of the 16S rRNA gene of this isolate was then PCR amplified (primers 8f: 5' AGAGTTTGATCCTGGCTCAG 3' and u1492r: 5' GGT-TACCTTGTTACGACTT 3'), purified, and sent for sequencing. Sequencing results revealed 99% sequence homology with *Exiguobacterium acetylicum*. A panel of mutants was tested for growth in the presence of *E. acetyliicum* with no discernable difference in the zone of clearing between mutant and (Fig B-3).



**Figure B-1.** Inhibition of F8027 by rinsate from field-harvested cantaloupes



**Figure B-2.** Individual isolates from cantaloupe rinsate inhibit growth of strain 2011L-2858.



**Figure B-3.** *Exiguobacterium* spp. (putative *E. acetylicum*) inhibits *L. monocytogenes* 2011L-2858 (A) and *pbp4* mutant G2B4 (B) during growth at 37°C.