Membrane biophysics of Listeria monocytogenes: analysis of an alternate pathway of branched-chain fatty acid biosynthesis and elasticity of fatty acid utilization

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MEMBRANE BIOPHYSICS OF *LISTERIA MONOCYTOGENES*: ANALYSIS OF AN ALTERNATE PATHWAY OF BRANCHED-CHAIN FATTY ACID BIOSYNTHESIS AND ELASTICITY OF FATTY ACID UTILIZATION

Laura E. Kuczek

45 Pages

The membrane homeostasis of the psychrotolerant foodborne pathogen *Listeria monocytogenes* involves maintaining fluidity in a range of different conditions by incorporation of specific branched-chain fatty acids (BCFAs). BCFAs are synthesized through a well-characterized pathway from branched-chain amino acids into short branched-chain acyl-CoAs followed by elongation by the FAS II system. Branched-chain alpha-keto acid dehydrogenase (Bkd) is a major player in this pathway. MOR401 is a transposon insertion mutant deficient in Bkd and has decreased membrane BFCAs. Low levels of BCFAs in *L. monocytogenes* are linked to diminished growth, less resistance to antimicrobials, and a severe reduction in virulence. Rescued growth and survivability of MOR401 can be achieved by media supplementation with BCFA precursors. Feeding the mutant various branched-chain carboxylic acids induced the production of BCFAs and restored growth. After culturing in the presence of novel BCFA C6 precursors 2-ethylbutyrate and 2-methylpentanoate, MOR401 possessed restored survivability after one hour of incubation with protamine, a cationic antimicrobial peptide.
Production of BCFAs in MOR401 suggests the existence of a bypass pathway of BCFA biosynthesis. Exogenous fatty acid precursors are proposed to be utilized by the activity of the enzymes Ptb and Buk, products of genes located just upstream of the bkd gene cluster, which work to prime exogenous short chain carboxylic acids for BCFA biosynthesis. To further study the role of the proposed pathway, a buk knockout mutant was constructed and grown in BHI medium supplemented with natural and unnatural BCFA precursors to verify its ability to utilize these substrates in Buk’s absence. Lack of novel fatty acid incorporation confirms the bypass pathway enzymes’ importance in the conversion of carboxylic acids into activated acyl CoAs for elongation and addition into the membrane.

BCFAs dominate the membrane of L. monocytogenes, which is unlike other organisms which utilize unsaturated fatty acids to maintain membrane fluidity. How then does L. monocytogenes modulate its membrane fluidity during infection of a host? Serum fatty acids are straight chain fatty acids and straight chain unsaturated fatty acids. To investigate L. monocytogenes’ likely in vivo membrane fatty acid composition and growth, wild type strain and MOR401 were cultured in fetal bovine serum. Following the ex vivo growth studies, fatty acid analysis of the cell membranes was carried out. The results show that even though L. monocytogenes does not possess the machinery needed to synthesize SCUFAs, it is indeed able to utilize and benefit from SCUFA incorporation into the membrane.

KEYWORDS: Branched-chain fatty acids, Listeria monocytogenes, Membrane fluidity, Butyrate kinase
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CHAPTER I

CHARACTERIZATION OF AN ALTERNATE BRANCHED-CHAIN FATTY ACID BIOSYNTHESIS PATHWAY BY KNOCKOUT OF BUTYRATE KINASE IN Listeria monocytogenes

Introduction

Listeria monocytogenes is a Gram-positive, rod-shaped, ubiquitous saprophyte found in environmental habitats containing soil, ground water, and decaying vegetation (Gray et al., 2006). However, L. monocytogenes is also a foodborne pathogen responsible for listeriosis, a potentially fatal infection especially dangerous in pregnant women and immunocompromised individuals. Forms of infection include sepsis, gastroenteritis, meningitis, spontaneous miscarriage, and stillbirth. Mortality rates of listeriosis are relatively high; nearly 20% of admitted hospital cases do not survive infection. This is in contrast to common sources of foodborne illness such as nontyphoidal Salmonella spp. and Clostridium perfringens, with hospitalization rates of roughly 27% and 0.6% respectively, L. monocytogenes-infected patients are hospitalized at a much higher rate of 94% (Scallan et al., 2011).

The switch between L. monocytogenes’ pathogenic and peaceful soil microbe states is mediated by positive regulatory factor A (prfA), which regulates the expression of virulence genes in response to environmental signals (Gray et al., 2006). This microbe most often gains access to the host by the ingestion of food, and following its
consumption it passes through the stomach. Acid tolerance is an important aspect of \textit{L. monocytogenes} in that virulence and acid tolerance are strongly associated (O’Driscoll \textit{et al.}, 1996). If the organism cannot handle the acidity of the stomach or survival within macrophage phagosomes, the chance of proliferation of the host is diminished. Following its journey through the stomach, it travels through the digestive tract to the small intestine where the organism can cross junctions between goblet and epithelial cells. Surface proteins InlA and InlB work to enter host cells via the zipper mechanism by targeting E-cadherin and Met expressed on human cells. Once inside the host cell, \textit{L. monocytogenes} manages to escape the vacuole in which it was engulfed through the activity of listeriolysin. Actin polymerization is hacked by the microbe, allowing for movement in the cytosol as well as infection of other cells. Internalization of macrophages is advantageous for \textit{L. monocytogenes} as this can lead to spreading of the infection to the whole body (Ribet \textit{et al.}, 2015). This also includes access to the brain or any present fetus because of the organism’s ability to cross the blood-brain and placental barrier (Disson \textit{et al.}, 2008). Fortunately, treatment of a \textit{L. monocytogenes} infection does not require intense therapies if caught early. Structural analogs of ampicillin, penicillin G, trimethoprim-sulfamethoxazole, and gentamicin are the drugs of choice, and little to no resistance to these has been detected so far (Temple and Nahata, 2000; Morvan \textit{et al.}, 2010).

Because \textit{L. monocytogenes} is a ubiquitous, saprophytic organism, it does eventually find its way into food processing facilities where biofilms can form and become sources of food contamination. Biofilms have been known to be even more
resistant to stress and antimicrobial methods than individual cells (Frank et al., 1990),
which causes extremely unsusceptible populations of cells to persist. One such strain
isolated at a poultry food plant in the United States was found to be established in the
facility for at least 12 years (Tompkin et al., 2002).

These biofilms are difficult to totally eradicate due to L. monocytogenes’
adaptations to growth in relatively extreme conditions. Outbreaks of L. monocytogenes
continue to be a major problem in the food industry
(http://www.cdc.gov/listeria/outbreaks). The FDA upholds a zero tolerance policy
towards L. monocytogenes in food products; while beneficial in safeguarding the health
of immunodeficient individuals, this rule in turn leads to revenue losses in the hundreds
of millions of dollars in the event of each outbreak. For example, a 2008 outbreak from a
meat processing facility in Canada that caused 57 hospital cases and 24 deaths cost
approximately $242 million Canadian dollars, which included costs to the food facility,
individual case costs, and federal outbreak response costs (Thomas et al., 2015).

L. monocytogenes’ ability to grow in typically unfavorable conditions such as
those of refrigeration temperature, adverse pH, and high salt content makes it a
formidable foodborne pathogen (Shabala et al., 2008) because many of these are utilized
in food preservation. This resilience is due in part to the organism’s ability to modulate
its cell membrane fluidity by the incorporation of specific fatty acids. Homeoviscous
adaptation by addition of particular branched-chain fatty acids (BCFAs), a characteristic
of Gram positive bacteria, is related to virulence and adaptation to outside stresses in L.
monocytogenes (Giotis et al., 2007; Sun et al., 2012).
Membranes lipids, although made up into a relatively simple bilayer structure, play crucial roles in the regulation of membrane-associated enzymes, active and passive transport of solutes across the permeable barrier, energy generation via ion gradients, and cell signaling pathways (Hazel et al., 1995). Without proper maintenance of membrane homeostasis during regularly encountered conditions such as changes in temperature, pH, and osmolarity, a host of vital cell processes are at risk. Maintaining membrane integrity during times of stress involves controlling membrane fluidity by several different mechanisms. For organisms such as *L. monocytogenes*, fatty acid shortening and incorporation of different fatty acids types which confer desired properties into membrane lipids is how fluidity is regulated (Zhu et al., 2005a). The extent of membrane fluidity is related to the type of fatty acids and how they “pack” to differing degrees. Saturated fatty acids pack tightly producing a more rigid membrane, while unsaturated fatty acids and branched-chain fatty acids increase packing distance between membrane fatty acids producing a fluid state. The steric hindrance of the methyl branch on a BCFA causes the membrane to organize into a more fluid arrangement. The same effect can be seen in the kink created by the double bond of an unsaturated fatty acid (Poger et al., 2014). Making use of a variety of fatty acids causes different effects on membrane fluidity, which is the reason they are incorporated to varying degrees depending on external circumstances (Cronan et al., 1975).

The fatty acid profile of *L. monocytogenes* fluctuates with environmental conditions such as temperature and pH, but BCFAs consistently make up greater than 90% of membrane fatty acids whereas straight-chain fatty acids (SCFAs) are part of the
membrane to a much lesser degree (Annous et al., 1997). BCFAs of the iso and anteiso series are common to *L. monocytogenes* and other closely related species (Kaneda, 1963a; Raines et al., 1968) with iso and anteiso BCFAs containing one methyl group at the penultimate and antepenultimate positions, respectively.

At 37°C in *L. monocytogenes* 10403S, anteiso C15:0, anteiso C17:0, and iso C15:0 fatty acids make up the majority of the membrane fatty acids at 40.7, 31.7, and 13.0% respectively. However at 5°C, anteiso C15:0 increases in percentage to 65.7% and iso C17:0 to 17.2% while anteiso C17:0 is reduced to 6.2% of membrane fatty acids (Annous et al., 1997). Anteiso BCFAs possess a lower phase transition temperature than iso BCFAs because the methyl group is located further from the end of the fatty acid chain, which causes looser packing in the membrane (Lindström et al., 2006). The greatest proportion of BCFAs are anteiso C15:0, a fatty acid consisting of a 14-carbon chain with a methyl branch at the 12th position (12-methyltetradecanoic acid). Anteiso C15:0 has been found to confer greater membrane fluidity and thus, plays a significant role in *L. monocytogenes*’ growth at cold temperatures (Edgcomb et al., 2000).

BCFAs are synthesized from the branched-chain amino acids leucine, isoleucine, and valine. Branched-chain amino acid transaminase converts these amino acids into branched-chain α-keto acids which are then oxidatively decarboxylated by branched-chain α-keto acid dehydrogenase (Bkd) into short chain CoA precursors (Zhu et al., 2005b). FabH, which has greater affinity for the anteiso C15:0 and C17:0 precursor 2-methylbutyryl-CoA especially at low temperatures (Singh et al., 2009), initiates the elongation process which is taken over by the FAS II pathway (Oku and Kaneda, 1988).
The resulting BCFAs are anteiso C15:0, anteiso C17:0, iso C14:0, iso C16:0, iso C15:0, and iso C17:0.

The transposon insertions are located within the bkd gene cluster, which consists of four open reading frames encoding the four subunits of Bkd, thus interrupting proper production of Bkd. When BCFA precursors are fed to these Bkd-deficient mutants, in particular short branched-chain carboxylic acids such as 2-methylbutyrate (2-MB), low temperature growth is restored (Annous et al., 1997; Kaneda, 1963b) and membrane fluidity is similar to that of wild type (Jones et al., 2002). These same results have also been replicated in S. aureus and B. subtilis mutants (Singh et al., 2008; Willecke et al., 1971). This suggests the occurrence of a bypass pathway acting to produce the CoA derivatives of the fatty acid precursors and incorporate the generated fatty acids into the cell membrane in the absence of Bkd. Bkd may not be the only enzyme involved in the generation of CoA derivatives for fatty acid biosynthesis.

Recently, it has been found that the medium supplementation of the branched-chain C6 carboxylic acid isomers 2-ethylbutyrate (2-EB) and 2-methylpentanoate (2-MP) with BCFA deficient mutant MOR401 (Sun et al., 2012) yields the production of novel fatty acids in the membrane. Gas-liquid chromatograph traces from Sen et al. (2015) display two major peaks in samples where MOR401 was supplemented with 2-EB and 2-MP. These peaks correspond to the presence of novel fatty acids made through elongation of the added precursors (Figure 1.3 and 1.4) which were further analyzed by electron
ionization mass spectroscopy. Incorporation of novel fatty acids benefitted the mutant; MOR401 shows restored growth patterns and membrane fluidity when fed 1 mM 2-MB, 2-EB, or 2-MP (Sen et al., 2015). BCFA precursors are not confined to the usual substrates, but in fact, L. monocytogenes is able to utilize many more which in turn leads to the creation of unnatural BCFA.

Just upstream of the bkd gene cluster are ptb and buk, whose gene products are phosphotransbutyrylase (Ptb) and butyrate kinase (Buk) (Zhu et al., 2005b). ptb and buk were identified in L. monocytogenes by sequence similarity to genes within the bkd operon of B. subtilis and E. faecalis (Debarbouille et al., 1999; Ward et al., 1999). Although their genes are located in an operon also encoding Bkd, an important part of BCFA generation, Ptb and Buk are known to be involved in the synthesis of butyrate. Most commonly, butyrate production begins with acetyl-CoA and after a series of reactions, butyryl-CoA is converted to butyryl-phosphate by Ptb (Vital et al., 2014). Buk is then responsible for catalyzing the reversible butyryl-phosphate to butyrate reaction (Hartmanis and Gatenbeck, 1984; Hartmanis, 1987; Twarog and Wolfe, 1962). Because of their proximity to the bkd gene cluster and their reversible enzymatic activity, it has been proposed that both Ptb and Buk are able to reverse their physiological directions and act upon exogenous carboxylic acids to yield the corresponding acyl CoA derivatives.

Very recently, a paper on the characterization of Ptb was published from our laboratory in which Ptb is described to possess broad substrate specificity, chain length specificity, and a preference for branched-chain substrates (Sirobhushanam et al., 2016).
Additionally, results of an analysis of Ptb’s activity with unnatural substrates such as 2-EB suggest that Ptb is an important factor in the proposed bypass pathway.

In a manuscript submitted for publication, the second enzyme in the putative alternate pathway of BCFA biosynthesis, Buk, was characterized. Buk also displays broad substrate specificity and a preference for a specific chain length (C3-C5). Notably, Buk demonstrates a strong inclination towards activity with branched chain carboxylic acids at low temperatures and is able to utilize unnatural BCFA precursors just like Ptb.

These findings lead us to consider Ptb and Buk as the main participants in the conversion of exogenous short chain carboxylic acids to short chain acyl CoAs, which can then be elongated into membrane BCFAs by the FAS II system. To further confirm the involvement of this potential bypass pathway in priming available BCFA precursors for membrane incorporation, a buk knockout mutant was created in L. monocytogenes 10403S and analyzed for utilization of BCFA substrates. The lack of incorporation of BCFA precursors observed in this study demonstrates the importance of Buk’s role in utilizing BCFA precursors for membrane incorporation.

**Materials and Methods**

* **buk Mutant Construction**

Figure 2 outlines the creation of the cassette inserted into *L. monocytogenes* 10403S. To start, 1.25 kb regions directly upstream and downstream of *buk* (lmo1370) were amplified with Phusion DNA polymerase (Thermo Scientific). Primers used are described in Table 1. In the downstream section targeted for PCR, a small portion of *buk*
was included for the purpose of retaining a ribosome binding site, which helps to reduce polar effects of the knockout. Following purification (GeneJET PCR Purification Kit, Fermentas), the fragments were digested with BamHI and ligated together so that the downstream 1250 base pairs immediately follow the 1250 base pairs of the upstream portion (Rapid DNA Ligation Kit, Thermo Scientific). This was run on a 1% agarose gel and the correct band was excised and purified (Zymoclean Gel DNA Recovery Kit, Zymo Research). This construct was ligated to cloning vector pJET1.2/blunt (CloneJET PCR Cloning Kit, Thermo Scientific) and the resulting plasmid was transformed into TOP10 competent E. coli cells. After plasmid isolation (GeneJET Plasmid Miniprep Kit, Thermo Scientific), KpnI and SalI restriction enzymes were utilized to cut the upstream-downstream fragment from pJET. Following this, the construct was cloned into shuttle plasmid pKSV7 (Smith and Youngman, 2015) and once again transformed into TOP10 competent cells. pKSV7 isolation was carried out and digested with BamHI to match the similarly digested 802 base pair kanamycin resistance gene kanR from pCOM. The kanamycin gene was ligated into the digested pKSV7 and transformed into TOP10 competent E. coli cells. After confirmation of accurate construct creation, the vector was transformed by electroporation into L. monocytogenes 10403S cells, which were made competent by treatment with lysozyme and penicillin G (Park and Stewart, 1990). Plasmid integration and excision was accomplished with a protocol outlined specifically for use with thermosensitive vector pKSV7 (Jordan et al., 2014). Integration of pKSV7 was ensured through serial passage of the putative knockout strain at 42°C in the presence of chloramphenicol and kanamycin. Plasmid excision was accomplished with repeated
growth at 30ºC with kanamycin and testing for successful homologous recombination was confirmed with an absence of growth of the mutant on an agar plate containing chloramphenicol. Sequencing of the mutant was performed by the University of Illinois at Urbana-Champaign Core Sequencing Facility.

**Growth Studies**

The growth patterns of the *buk* mutant with and without supplementation of 1 mM 2-MB, 2-EB, 2-MP, and 100 mM butyrate were compared with WT strain 10403S and MOR401, a *bkd* mutant with a Tn917 transposon insertion (O’Riordan and Sun, 2012). Bacteria were grown overnight at 37°C with shaking at 200 rpm in BHI. Overnight cultures were diluted 1:50 into fresh BHI with a flask-to-medium ration of 5:1. Cultures were incubated at 37°C with shaking at 200 rpm and OD$_{600}$ was monitored until stationary phase.

**Membrane Fatty Acid Analysis**

Cultures were grown in BHI with or without supplementation and harvested at approximately an OD$_{600}$ of 0.6 (mid-log phase). The culture volume of 5 mL was centrifuged at 3000 x g for 15 minutes at 4°C and pellets were washed three times with cold distilled H$_2$O. Fatty acid profiles were determined by Microbial ID, Inc. (Newark, DE) where the fatty acids within the cells (wet weight 30 to 40 mg) were saponified, methylated, and extracted. The methyl esters were separated with an Agilent 5890 dual
tower gas chromatograph and determined using the MIDI microbial identification system (Sherlock 4.5 microbial identification system).

**Results**

After sequencing, the *buk* mutant was grown with supplementation of various precursors to confirm its inability to utilize BCFA primers (Figure 3). The *buk* mutant grown with and without supplementation of BCFA precursors displayed a long lag phase similar to that of MOR401. Decreased growth of the mutant in comparison to wild type could be due to the impact of butyrate kinase’s activity in other aspects of cell metabolism or stress brought on by the insertion of the kanamycin resistance gene. As expected, growth of MOR401 was restored in the presence of 2-MB (Sen *et al.*, 2015). The *buk* mutant reached a greater cell density than MOR401 by stationary phase, but was still below that of 2-MB-supplemented MOR401. Addition of 2-MB, 2-EB, and 2-MP to the media did not improve growth of the *buk* mutant, as is the case with MOR401, validating the postulation that Buk is necessary in priming precursors for incorporation into the membrane.

Fatty acid analysis was performed on supplemented cultures by Microbial ID Inc. to assess the *buk* mutant’s membrane fatty acid composition. As suggested by the growth curves of the *buk* mutant with the BCFA precursors, fatty acid composition was comparable with and without addition of primers (Table 2). Interestingly, 2-EB supplementation produced a detectable quantity of novel fatty acid, 12-ethyltetradecanoic acid, at 3.75%. This unnatural fatty acid was identified through its 2.73-minute retention
time; Sen et al. (2015) reported presence of 12-ethyltetradecanoic acid by the addition of 2-EB in MOR401 with a retention time of 2.75 minutes (Figure 1.4). Wild type 10403S supplementation with 1 mM 2-EB yields approximately 7% of 12-ethyltetradecanoic acid while the buck mutant yields just half of that value. The fatty acid composition of wild type grown with 2-MP shows the incorporation of 12-methylpentadecanoic acid while, in the buck mutant, none was detected. Overall, the observed results suggest that Buk is a factor in BCFA biosynthesis from exogenous short chain carboxylic acids, but redundant systems may also be involved as indicated by the incorporation of 2-EB.

**Discussion**

The pathways involved in maintenance of membrane fluidity by alteration of membrane fatty acid composition have been implicated in virulence (Sun et al., 2012), changes in membrane enzyme function, sensing for signal pathways involved in stress and gene expression, transport, and membrane permeability (Beney et al., 2001). In strain MOR401, resistance to stress and virulence were severely diminished (Sun et al., 2012). But in the event of BCFA precursors becoming available to the organism, regulation of virulence and stress pathways were once again restored. This study sought to confirm the involvement of one of the two suggested proteins, Buk, in priming exogenous BCFA precursors for elongation in *L. monocytogenes* by knocking out the buck gene.

Growth and fatty acid analysis of the mutant showed little effect of BCFA precursor supplementation. This leads us to believe that Buk is a critical role in the conversion of branched-chain carboxylic acids into BCFA precursors. Somewhat
expectedly, a small amount of 2-EB managed to become incorporated in Buk’s absence. It has been speculated that production of 12-ethyltetradecanoic acid may be a product of redundant systems in \textit{L. monocytogenes}. One possibility is the existence of a second butyrate kinase. The genome of \textit{C. acetobutylicum} strain ATCC 8244, another Gram-positive bacillus, contains two butyrate kinase coding sequences (CA_C1660 and CA_C3075). The small percentage of novel fatty acid may be due to a previously unknown second low-activity butyrate kinase in \textit{L. monocytogenes} 10403S.

Another proposal is that incorporation of 2-EB in the \textit{buk} mutant could have occurred via closely-related enzymes not previously associated with BCFA biosynthesis such as acetate kinase, or possibly a CoA transferase or CoA ligase, which have not been characterized yet in \textit{L. monocytogenes} (Horswill and Escalante-Semerena, 1999; Kumari \textit{et al.}, 1995).

Acetate kinase is an enzyme which takes part in excretion of acetate and ATP generation during anaerobic conditions via the phosphotransacetylase-acetate kinase pathway. Specifically, it catalyzes the reversible acetyl phosphate from acetate and ATP reaction (Wolfe, 2005). The Pta-AckA pathway has also been found to be involved with the DegU orphan response regulator, which directs motility, chemotaxis, biofilm formation, and ultimately virulence in \textit{L. monocytogenes} (Gueriri \textit{et al.}, 2008). The activity of acetate kinase is very similar to butyrate kinase. They perform the same reactions except acetate kinase is active with specific preference for acetate. Acetate and butyrate are very similar structurally due to butyrate possessing a carbon chain with two more carbon atoms than acetate. It is reasonable to suggest that acetate kinase could be
acting in place of Buk in the *buk* mutant and may be the reason for incorporation of small amounts of novel fatty acid precursor 2-EB.

Other possibilities include a CoA transferase or CoA ligase acting in place of the nonfunctional bypass pathway. *L. monocytogenes* encodes for several additional enzymes which perform similar functions as the proposed candidates of the bypass pathway of BCFA synthesis: propionate CoA-transferase (lmo2172), CoA-transferase (lmo2755), acetyl transferase (lmo0664), as well as fatty acid-CoA ligase (lmo0354) and acetate-CoA ligase (lmo2720). Substrates of these enzymes such as acetate, butyrate, and propionate can easily fit within the active sites of the listed enzymes. Use of bulkier C5 and C6 fatty acid precursors in our studies allowed us to target Ptb and Buk, which possess more flexible active sites and therefore are able to utilize a broad range of substrates (Sirobhushanam *et al.*, 2016; unpublished data). This is likely why there was a decreased amount of supplement incorporation; one or several of the aforementioned enzymes with more rigid active sites did not have the ability to fully utilize the supplemented BCFA precursors. We suggest that Buk and Ptb are the most probable candidates in the conversion of carboxylic acids into acyl CoA primers for BCFA synthesis.

It was thought that because of the likely occurrence of redundant systems in this organism, this study may have been best carried out by constructing a *ptb* knockout mutant considering the common phosphotransferase activity of Buk. However, after discovering many enzymes that perform very similar functions such as Pta reversibly
converting acetyl-CoA and inorganic phosphate into acetyl phosphate and CoA (Wolfe, 2005), a ptb mutant would have likely yielded similar results.

Redundant systems are very common in bacteria as well as higher organisms. A consequence of gene duplication events or horizontal gene transfer, redundant genes and their products are often brought about by selective pressures which induce stress (Gevers et al., 2004). When conditions again reach a normal state, duplicates can be lost except in the instance when one gene product performs their function more effectively than the other (Kochiwa et al., 2007). A comprehensive study on B. subtilis was performed to determine essential and nonessential genes by singly inactivating each of the 4,100 genes. Only 271 were found to be required for growth, which is just under 7% of the genome (Kobayashi et al., 2003). The vast majority of genes are perhaps just advantageous but not required for the survival of the organism, or have backup systems. buk was found to be nonessential in B. subtilis. Given the results of the present study where the knockout of buk yielded only slightly diminished growth and a normal membrane fatty acid profile, we also observed that Buk is nonessential in L. monocytogenes. Buk is active in butyrate production and is advantageous in the utilization of exogenous fatty acid precursors, but is not required. As is evident from the incorporation of 2-EB, there are probably several alternative enzymes which can act in place of our proposed bypass pathway.

Buk, as a factor in priming carboxylic acids for fatty acid biosynthesis, is also hypothesized to play a role in signal transduction when the organism encounters fatty acids in the gut during infection. Acetate, propionate, and butyrate are the three most common human intestinal fatty acids produced from bacterial fermentation of
carbohydrates (Scheppach, 1994). Buk displays relatively high phosphorylation activity with butyrate as well as pentanoate, isobutyrate, and isovalerate in *C. acetobutylicum* (Hartmanis, 1987). Also in *C. acetobutylicum*, high levels of phosphorylated gut carboxylic acids are associated with large changes in gene expression (Zhao et al., 2005). In light of these findings, it could be proposed that fatty acids found in the gut induce virulence factor activation in *L. monocytogenes* in part by the activity of PtB and Buk. *In vivo* studies on the *buk* mutant would elucidate the bypass pathway’s involvement in virulence.

Much is still not known about the implications of PtB and Buk’s roles beyond previous and present findings. For instance, prevalence of *ptb* and *buk* among different microorganisms is rather lacking, even in butyrate-secreting species. If this pathway were the only source of intestinal butyrate, many gut bacteria would have these genes. Instead, most microbes tend to utilize CoA transferase in the final steps of butyrate production (Louis et al., 2004). Since *L. monocytogenes* possesses genes encoding PtB, Buk, and CoA transferase, it is suggested that there is much more function to the PtB-Buk pathway than is presently known.
Figure 1.1 Gas chromatograph of MOR401 at 37°C.

Figure 1.2 Gas chromatograph of MOR401 supplemented with 2-MB at 37°C.
Figure 1.3 Gas chromatograph of MOR401 supplemented with 2-MP at 37°C.

Figure 1.4 Gas chromatograph of MOR401 supplemented with 2-EB at 37°C.
Table 1

*Primers used and corresponding restriction endonuclease sites*

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</tr>
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<td>upstream forward</td>
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</tr>
<tr>
<td>upstream reverse</td>
<td>AAAGGATCCTTGCTTTCTCCAAAAATTTAATTG (<em>BamHI</em>)</td>
</tr>
<tr>
<td>downstream forward</td>
<td>AAAGGATCCTATTGAGCAAACAAACTGGA (<em>BamHI</em>)</td>
</tr>
<tr>
<td>downstream reverse</td>
<td>AAAGTCGACTAATGAAGCCATCTGTATTCTCC (<em>SalI</em>)</td>
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Figure 2. Creation of the construct transformed into a *L. monocytogenes* 10403S background.
Figure 3. Influence of 2-methylbutyrate, 2-ethylbutyrate, and 2-methylpentanoate on the growth of wild type strain 10403S, MOR401, and the buk mutant at 37°C.
Table 2

*Fatty acid analysis of wild type 10403S, MOR401, and the buk mutant supplemented with various BCFA precursors at 37°C*

<table>
<thead>
<tr>
<th></th>
<th>WT 10403S</th>
<th>WT + 2-MB</th>
<th>WT + 2-EB</th>
<th>WT + 2-MP</th>
<th>WT + butyrate</th>
<th>MOR401 + 2-MB</th>
<th>MOR401 + 2-MB</th>
<th>buk mutant</th>
<th>buk + 2-MB</th>
<th>buk + 2-EB</th>
<th>buk + 2-MP</th>
<th>buk + butyrate</th>
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*Note.* ND - Not detectable (<1%)
REFERENCES


Gueriri, I., Bay, S., Dubrac, S., Cyncynatus, C., and Msadek, T. The Pta-Acka pathway controlling acetyl phosphate levels and the phosphorylation state of the DegU orphan response regulator both play a role in regulating *Listeria monocytogenes* motility and chemotaxis. Mol Microbiol 70, 1342-1357.


CHAPTER II

GROWTH OF A LISTERIA MONOCYTOGENES BKD-DEFICIENT MUTANT IN SERUM TO ANALYZE BRANCHED-CHAIN FATTY ACID PREFERENCE IN MEMBRANE INCORPORATION

Introduction

Listeria monocytogenes, a foodborne bacterial pathogen, is capable of growth at low temperature due to homeoviscous adaptation. This modulation of membrane fluidity in response to environmental conditions (temperature, pH, salt content, etc.) is performed by the incorporation of certain fatty acids (Annous et al., 1997; Giotis et al., 2007; Chihib et al., 2003). Addition of specific branched-chain fatty acids (BCFAs) is L. monocytogenes’ primary method of achieving greater membrane fluidity, which is unlike other microorganisms that prefer the incorporation of unsaturated fatty acids (Fulco, 1983). Unsaturated fatty acids are typically undetectable in L. monocytogenes while BCFAs make up greater than 90% of the membrane when grown in conventional laboratory medium (Zhu et al., 2005).

The fatty acid profile of L. monocytogenes grown in vivo has not yet been sufficiently studied. In vivo conditions can be replicated by growth of L. monocytogenes in fetal bovine serum (FBS). FBS contains a high amount of straight-chain unsaturated fatty acids (SCUFAs) such as oleic acid (C18:1Δ9) and arachidonic acid (C20:4), which are present at approximately 30% and 10% of fatty acids, respectively (Stoll and Spector,
Palmitic acid (C16:0), a straight-chain saturated fatty acid, also makes up a large proportion of fatty acids in FBS at 20%. Human serum is relatively similar in that it consists of high levels of palmitic acid (122 μM), oleic acid (122 μM), linoleic acid (84 μM, C18:2Δ9,12), and stearic acid (49 μM, C18:0) (Psychogios et al., 2011).

Previous studies have made it clear that *L. monocytogenes* does not prefer to induce changes in total unsaturated fatty acid content of the membrane in response to environmental stress (Li et al., 2002; Nichols et al., 2002). Previously, it was of interest to examine the potential for changes in unsaturated fatty acid content in two BCFA-deficient mutants *cld-1* and *cld-2*. Both contain a Tn917 transposon within the branched-chain α-keto acid dehydrogenase (Bkd) gene cluster, thus interrupting expression of functional Bkd and incorporation of BCFAs into the membrane. Annous et al. (1997) found no evidence for production of unsaturated fatty acids or activity of a desaturating system when *cld-1* and *cld-2* cultures were cold shocked. These studies were carried out in complex and defined medium.

MOR401 is a mutant with the *cld-2* transposon inserted into a clean wild type background; MOR401 shows reduced amounts of BCFAs and consequently, has a more rigid membrane (Sun et al., 2012; Sen et al., 2015). It is likely that MOR401 will also not favor unsaturated fatty acid incorporation into the membrane in standard medium. To what extent will this mutant lacking in membrane fluidity not utilize available, potential membrane-fluidizing SCUFAs? Growth in serum will assess MOR401’s urgency for increased membrane fluidity and inclination towards BCFA incorporation in the membrane. Additional stress by culturing at 10°C should escalate the mutant’s demand
for increased membrane fluidity. An absence or low levels of increased growth or incorporation of SCUFAs will confirm *L. monocytogenes*’ high preference for BCFAs. In addition, culturing of the mutant along with 2-methylbutyrate (2-MB) supplementation is also a test of preference; it is expected that the presence of anteiso C15:0 and anteiso C17:0 precursor 2-MB will override most of the uptake of SCUFAs into the membrane.

**Materials and Methods**

**Growth Studies**

In order to study the effects of serum on the growth and fatty acid composition of *L. monocytogenes*, wild type strain 10403S and MOR401 were grown in Brain Heart Infusion broth with 50% heat-inactivated FBS at 10°C. Heat inactivation of FBS, in order to destroy complement proteins, was done by incubation in a water bath at 56°C for 30 minutes along with mixing every 5 minutes to ensure even heat distribution. One flask of MOR401 culture in 50% FBS was supplemented with 1 mM 2-MB.

**Membrane Fatty Acid Analysis**

Cultures were grown in BHI with or without supplementation and harvested at approximately an OD<sub>600</sub> of 0.6 (mid-log phase). The culture volume of 5 mL was centrifuged at 3000 x g for 15 minutes at 4°C and pellets were washed three times with cold distilled H<sub>2</sub>O. Fatty acid profiles were determined by Microbial ID, Inc. (Newark, DE) where the fatty acids within the cells (wet weight 30 to 40 mg) were saponified, methylated, and extracted. The methyl esters were separated with an Agilent 5890 dual
tower gas chromatograph and determined using the MIDI microbial identification system (Sherlock 4.5 microbial identification system).

**Results**

To test the wild type strain and MOR401’s growth in the presence of high levels of SCUFAs and SCFAs, they were cultured at 10°C in BHI and 50% FBS. The large effect of MOR401’s growth in FBS and 2-MB tell us that SCUFAs are beneficial even to organisms without SCUFA production capability (Figure 1). MOR401 clearly benefitted from SCUFA incorporation; MOR401 grown in BHI failed to reach beyond an OD$_{600}$ of 0.2 whereas MOR401 cultured in serum neared 0.8. Additionally, MOR401 grown in both serum and 2-MB nearly reached an OD$_{600}$ of 1.4. Even though *L. monocytogenes* is not capable of producing SCUFAs, it is still able to benefit from them when available exogenously. And interestingly, the lag phase of MOR401 cultured in serum and 2-MB was shorter than serum-grown MOR401 without 2-MB. *L. monocytogenes*’ preference distinctly lies with BCFA precursor 2-MB over the SCFAs and SCUFAs found in serum.

In order to determine the membrane fatty acid content of MOR401 and wild type when grown in serum, fatty acid analysis was performed (Table 1). The results show that SCUFAs are incorporated in MOR401 to levels similar to that of *S. aureus* when grown in serum (Unpublished data). MOR401 takes up approximately 22% of SCUFAs, but in the addition of 2-MB, that amount decreases to levels resembling wild type SCUFA incorporation. The most prominent SCUFA incorporated into the membrane was oleic
acid (C18:1Δ9), which accounts for the majority of the membrane SCUFAs in each sample, followed by vaccenic acid (C18:1 Δ7) and palmitoleic acid (C16:1Δ7).

Wild type incorporates nearly 13% SCUFAs and 17% SCFAs at the expense of BCFAs, which only account for 69% of total membrane fatty acids when cultured in serum. The membrane of wild type grown in 100% BHI contains around 98% BCFAs and only 2% SCFAs (Sen et al., 2015). Even though SCUFAs and SCFAs were included in the membrane of serum-grown wild type strain to such an extent, wild type grown with and without serum showed nearly identical growth curves. SCUFAs are clearly as advantageous to the organism as BCFAs when available in the environment.

Discussion

Because SCUFAs yield similar amounts of membrane fluidity as BCFAs (Zhang et al., 2008), it is not surprising that L. monocytogenes utilizes these host-derived FAs. But then why do SCFAs get incorporated to such an extent to where they consist of 20% of membrane fatty acids when L. monocytogenes clearly prefers membrane-fluidizing fatty acids? Given the results of our experiment, it could be said that L. monocytogenes uses any accessible fatty acids and fatty acid precursors in its environment, even if some of them do not increase membrane fluidity and therefore, do not provide advantageous biophysical properties. This phenomenon may be due to several factors which include energy preservation and the activation of signaling pathways to alter gene expression in certain environments.
First, available fatty acid substrates are taken up and used in order to save the organism energy. *De novo* fatty acid biosynthesis is costly in terms of a cell’s use of energy and carbon. In fact, 95% of the energy used in phospholipid biosynthesis is required for fatty acid production (Parsons *et al.*, 2012). So, it is beneficial for the organism to take advantage of any available fatty acid substrate it encounters because that can lead to a large degree of overall energy savings.

Another reason for incorporation of non-advantageous fatty acids is that exogenous fatty acids added into the membrane could be involved in signaling mechanisms which lead to altered gene expression in certain environments. For example, when enterohemorrhagic *E. coli* (EHEC) is grown in the presence of common intestinal short chain fatty acids acetate, propionate, and butyrate, virulence factor expression is activated (Tobe *et al.*, 2011). Also, *Clostridium acetobutylicum* alters expression of many genes in response to fatty acid precursors such as those which involve flagella formation and stress (Zhao *et al.*, 2005). Gut fatty acids could serve as a trigger for the expression of genes which are advantageous during infection of a host. They could signal to the organism as to what sort of environment it currently resides in and whether virulence factors should be upregulated for proliferation and infection (Sun *et al.*, 2012).
Figure 1. Growth of wild type 10403S and MOR401 in 50% fetal bovine serum supplemented with 2-methylbutyrate at 10°C.
Table 1

Fatty acid profile of wild type 10403S and MOR401 grown in 50% fetal bovine serum and 1 mM 2-methylbutyrate at 10°C

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>MOR401 at 37°C</th>
<th>WT + FBS</th>
<th>MOR401 + FBS</th>
<th>MOR401 + 2-MB</th>
<th>MOR401 + FBS + 2-MB</th>
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<tbody>
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<td>BCFA</td>
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<td></td>
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Note. ND - Not detectable (<1%)
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CHAPTER III
THE EFFECTS OF NOVEL FATTY ACIDS DERIVED FROM SHORT BRANCHED-CHAIN C6 CARBOXYLIC ACIDS ON THE FUNCTIONAL MEMBRANE REQUIREMENTS OF A LISTERIA MONOCYTOGENES BCFA-DEFICIENT MUTANT

Introduction

Listeria monocytogenes is a foodborne pathogen implicated in listeriosis, a potentially fatal infection in pregnant women and individuals with compromised immune systems. One of this microorganism’s distinguishing and threatening features is its ability to grow at cold temperatures, which is due to the regulation of specific fatty acid incorporation into membrane phospholipids (Shabala et al., 2008; Giotis et al., 2007; Sun et al., 2012). Branched-chain fatty acids (BCFAs) typically make up greater than 90% of membrane fatty acids in most conditions while straight-chain fatty acids make up the remainder (Annous et al., 1997). BCFA anteiso C15:0 yields the greatest membrane fluidity, so this BCFA is incorporated to a higher degree when the organism is grown at low temperature (Edgcomb et al., 2000).

MOR401, a transposon mutant with an insertion in the branched-chain α-keto acid dehydrogenase operon, is deficient in BCFAs (Sun et al., 2010). However, supplementation of short branched-chain carboxylic acids such as 2-methylbutyrate (2-MB) restore the mutant’s growth, fatty acid composition, and membrane fluidity. It was
also determined that feeding MOR401 2-MB restored the mutant’s intracellular survival and growth, colocalization with actin, and production of listeriolysin. Later studies confirmed that supplementation decreased sensitivity to various antimicrobials and increased phagosomal survival (Sun et al., 2012). Growth with 2-MB was found to satisfy MOR401’s membrane functional requirements by the recovery of anteiso odd BCFA levels.

Recently, Sen et al. (2015) discovered that supplementation of growth media with several branched-chain C6 isomers, which are also BCFA precursors, such as 2-ethylbutyrate (2-EB) and 2-methylpentanoate (2-MP) led to production of various unnatural fatty acids in MOR401. When the mutant was supplemented with 2-MP, novel fatty acids were found to make up nearly 75% of membrane fatty acids. In addition, the precursors which produced these novel fatty acids restored BCFA membrane levels, growth at low temperature, and membrane fluidity.

It is unknown if addition of 2-EB and 2-MP, along with subsequent incorporation and creation of novel fatty acids, will also satisfy the previously studied functional requirements of the membrane, as 2-MB was capable of fulfilling. In order to determine whether these short branched-chain C6 carboxylic acids are also able to recover MOR401’s survival in the presence of antimicrobials, an assay was carried out as described by Sun et al. (2010).
Materials and Methods

Cultures were grown to an OD$_{600}$ of 0.6 where the cells were diluted $10^{-5}$ in 10 mM potassium phosphate buffer (pH 5.8) and aliquoted into a microtiter plate. The suspended cells were mixed with serially diluted solutions of polymyxin B, protamine sulfate, and mutanolysin and incubated for 1 hour at 37°C. Colonies after 0 hours and 1 hour were enumerated by plating on BHI agar. Percent survival was calculated with the following equation: $\%$ survival = ($\text{CFU}_{T=1 \text{ h}} / \text{CFU}_{T=0}$) x 100.

Results

To test how effectively the unnatural fatty acids in the membrane of 2-EB and 2-MP-supplemented MOR401 restore the mutant’s ability to survive under stress, MOR401 was grown with 2-EB and 2-MP followed by an hour-long incubation in the presence of polymyxin B (negative control), protamine, and mutanolysin. As shown in Figure 1, the assays testing incubation with polymyxin B and mutanolysin were not able to be replicated from Sun et al. (2010) despite numerous attempts to correct plating technique, buffer preparation, and antimicrobial dilution calculations. Yet, the assay analyzing MOR401 growth with protamine did yield the predicted results. When fed 2-EB and 2-MP, MOR401 displayed restored survival when grown with protamine. Wild type and MOR401 grown with 2-MB, 2-EB, and 2-MP were able to maintain viable cells when subjected to protamine.
Discussion

When BCFA-deficient MOR401 was fed 2-MB and cultured with various antimicrobials in Sun et al. (2012), membrane fluidity was restored and survivability of the mutant was drastically increased. More recently, it was discovered that unnatural BCFA precursors 2-EB and 2-MP also give rise to a more fluid membrane (Sen et al., 2015). This study sought to investigate the degree to which incorporation of 2-EB and 2-MP satisfy the functional requirements of the membrane.

However, problems were encountered. The assays testing MOR401 survival against polymyxin B and mutanolysin lead to unnaturally low colony counts of all strains tested, including wild type which is resistant to polymyxin B and mutanolysin under normal circumstances. Polymyxin B was supposed to have served as the negative control; it is a cationic antimicrobial peptide (CAMP) which only exhibits bactericidal activity against most Gram-negative bacteria. The peptide penetrates the organism’s outer membrane and increases its permeability by weakening interactions between lipopolysaccharides (Vaara et al., 1992). Mutanolysin from Streptomyces globisporus is similar to lysozyme, an enzyme present in macrophages, because it cleaves peptidoglycan through N-acetylmuramidase activity (Fliss et al., 1991). To bypass this, L. monocytogenes is supposed to be able to modify the targeted peptidoglycan by deacetylation of N-acetylglucosamine by PgdA (Boneca et al., 2007) and by O-acetylation due to OatA (Aubry et al., 2011).

Protamine is a CAMP whose antimicrobial activity involves electrostatic interactions between the peptide and bacterial envelope followed by disruption of the
membrane (Potter et al., 2004). This study has shown that incorporation of novel BCFAs into *L. monocytogenes*’ membrane largely contributes to protamine resistance.

Errors in calculating concentrations of mutanolysin or preparing the buffer could have contributed to the issues regarding the polymyxin B and mutanolysin assays. The acidic pH of the buffer was of concern at first, but this was found to be tested previously; acidic buffers with pH 5.0 and 7.0 were examined with wild type and MOR401 growth and little difference was observed (Sun et al., 2012). In fact, the acidity of the buffer causes cell wall peptidoglycan to become neutral or slightly positively charged, which causes CAMPs to be less attracted to peptidoglycan (Walkenhorst et al., 2013). Clearly, more work needs to be done in order to identify errors in experiment execution and eliminate issues involving buffer and antimicrobial preparation.
Figure 1. The survival of MOR401 grown in the presence of branched-chain fatty acid precursors against protamine sulfate, polymyxin B, and mutanolysin.
REFERENCES


