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Utilization of multiple substrates by butyrate kinase from *Listeria monocytogenes*

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Abstract

Listeria monocytogenes, the causative agent of listeriosis, can build up to dangerous levels in refrigerated foods potentially leading to expensive product recalls. An important aspect of the bacterium's growth at low temperatures is its ability to increase the branched-chain fatty acid anteiso C15:0 content of its membrane at lower growth temperatures, which imparts greater membrane fluidity. Mutants in the branched-chain α -keto dehydrogenase (*bkd*) complex are deficient in branched-chain fatty acids (BCFAs,) but these can be restored by feeding C4 and C5 branched-chain carboxylic acids (BCCAs). This suggests the presence of an alternate pathway for production of acyl CoA precursors for fatty acid biosynthesis. We hypothesize that the alternate pathway is composed of butyrate kinase (*buk*) and phosphotransbutyrylase (*ptb*) encoded in the *bkd* complex which produce acyl CoA products by their sequential action through the metabolism of carboxylic acids. We determined the steady state kinetics of recombinant His-tagged Buk using 11 different straight-chain and BCCA substrates in the acyl phosphate forming direction. Buk demonstrated highest catalytic efficiency with pentanoate as the substrate. Low product formation observed with acetate (C2) and hexanoate (C6) as the substrates indicates that Buk is not involved in either acetate metabolism or long chain carboxylic acid activation. We were also able to show that Buk catalysis occurs through a ternary complex intermediate. Additionally, Buk demonstrates a strong preference for BCCAs at low temperatures. These results indicate that Buk may be involved in the activation and assimilation of exogenous carboxylic acids for membrane fatty acid biosynthesis.

1. Introduction

Listeria monocytogenes, is the dangerous foodborne pathogen that causes listeriosis, a disease characterized by gastroenteritis, meningitis, spontaneous miscarriages and high mortality rate among infected individuals [1]. *L. monocytogenes* grows actively at temperatures as low as -0.1 °C, building up to dangerous levels, which is enabled by its highly fluid membrane [2]. The cellular membrane of *L. monocytogenes* is enriched with

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branched-chain fatty acids (BCFAs) which have greater cross sectional area, disrupt the close packing of membrane fatty acids and thus increase the fluidity of the membrane [3,4]. *L. monocytogenes* further increases the membrane content of anteiso C15:0, a survival strategy which supports its growth at low temperatures [2,5,6]. Expensive food product recalls due to outbreaks of *Listeria* underscore the growing need for better control strategies for this organism (<http://www.cdc.gov/listeria/outbreaks>).

BCFAs, which make up >90% of membrane fatty acid content of *L. monocytogenes*, are biosynthesized by the activity of branched-chain amino transferase (Bcat) and branched-chain α -keto acid dehydrogenase (Bkd) on branched chain amino acids (BCAAs), followed by elongation of the catabolic products by the bacterial fatty acid biosynthesis pathway (FAS II) [7]. Disruption of this pathway, as in the case of mutants of *L. monocytogenes* lacking functional Bkd (*cld-1*, *cld-2*/MOR401), results in reduced fitness and impairment of the homeoviscous adaptation strategies employed by the organism when exposed to low growth temperatures [4,5,8,9]. Such mutants are deficient in membrane BCFA content, unable to grow at low temperatures, and are significantly affected by unfavorable environmental conditions [4,5,8,10]. Addition of 2-methylbutyrate, the precursor of anteiso C15:0, restores the membrane BCFA content and thus rescues the growth of the mutant and leads to greater survival in macrophages and at low temperatures, as well as greater tolerance for acidity and alkalinity [4,9–12]. Restoration of membrane BCFA content by the addition of 2-methylbutyrate has also been observed in *bkd* mutants of *Staphylococcus aureus* and *Bacillus subtilis* [13,14].

Comprehensive investigation of media supplementation conducted by Kaneda [15] in *B. subtilis* and Sen *et al.* [16] in *L. monocytogenes* show that a wide range of carboxylic acids, which include straight chain carboxylic acids (SCCAs) such as propionate and butyrate, and branched-chain carboxylic acids (BCCAs, including 2-methylbutyrate and isobutyrate) are capable of alteration of membrane fatty acid composition. Additionally, supplementation of unnatural BCCAs such as 2-ethylbutyrate and 2-methylpentanoate result in the incorporation of novel fatty acids in the membrane arising from these compounds [11,15,16]. Since the only known source of BCFAs is via biosynthesis, it is presumed that these compounds are converted into their thioester derivatives by a pathway hitherto uncharacterized, and are utilized as primers by β -keto acyl ACP synthase III (FabH), the enzyme catalyzing the first condensation step in FAS II pathway [17]. The alternate pathway, first suggested by Willecke and Pardee in *B. subtilis* [14], must therefore exhibit broad substrate specificity to fulfill the requirements of activation of such a large range of substrates.

Homology of the *L. monocytogenes bkd* operon to the *bkd* operon in *Enterococcus faecalis* led to the identification of two genes butyrate kinase (*buk*) and phosphotransbutyrylase (*ptb*), present upstream of the *lpd* gene in the *bkd* operon [18–20]. Ptb is known to catalyze the reversible conversion of acyl CoA compounds into their corresponding acyl phosphate derivatives [21]. Ptb from *L. monocytogenes* demonstrates broad substrate specificity and shows a strong preference for branched-chain substrates [22]. Buk catalyzes the phosphorylation of a variety of carboxylic acids and is a BCCA kinase in *Spirochaeta isovalerica* MA-2 [23,24]. Although the Ptb-Buk pathway has been shown to be the source of butyrate secretion in the important industrial organism *Clostridium acetobutylicum*, we

hypothesize that presence of significant concentrations of exogenous carboxylic acids induces the formation of the acyl CoA products by the sequential activity of Buk and Ptb which are subsequently elongated by the FAS II pathway.

Buk is well characterized in the industrial fermentor, *C. acetobutylicum*, in which it is an important source of ATP during the acidogenesis stage of fermentation [24]. Other roles such as catabolism of branched-chain amino acids and ATP generation for survival have been attributed to Buk in *E. faecalis* and *S. isovaleric* [23,25]. We determined the substrate specificities of Buk by *in vitro* assay of the recombinant enzyme to investigate the potential role of Buk from *L. monocytogenes* in the phosphorylation of exogenous carboxylic acids for provision of precursors of fatty acid biosynthesis. Our results indicate that Buk is capable of conversion of a large number of carboxylic acids into their acyl phosphate derivatives.

2. Materials and methods

2.1 Materials

All the materials including antibiotics used as selection agents and acetate kinase were purchased from Sigma-Aldrich (St. Louis, MO).

2.2 Cloning and expression of Buk

Genomic DNA of *L. monocytogenes* 10403S, grown in Brain Heart Infusion (BHI) media (Becton Dickinson, Sparks, MD), was isolated using a Masterpure genomic DNA purification kit according to the manufacturer's instructions (Epicenter, Madison, WI). The *buk* gene was amplified using a *buk* forward primer with a *SacI* restriction site (bukF 5' ATGCGAGCTCATGTCTTTTGATGTTTT) and reverse primer with a *PstI* restriction site (bukR 5' ATGCCTGCAGTTAGTACTCTTTTCTT), which were designed based on the sequence of *L. monocytogenes* strain EGDe. The restriction sites were used for ligation of the *buk* gene into the expression vector pRSETa (Thermofisher, Waltham, MA) using T4 DNA ligase (Fermentas, Waltham, MA). Transformation of the plasmid pRSETa-*buk* into competent *E. coli* BL21 (DE3) cells was followed by confirmation of overexpression of the protein by Western blotting. DNA purification, ligation and transformation were performed according to the manufacturer's instructions (Qiagen, Valencia, CA). Ampicillin (50µg/ml) was used as the selection agent for growth of pRSETa and pRSETa-*buk* carrying *E. coli* cells in Luria broth (Becton Dickinson, Sparks, MD). Overnight culture of cells carrying the pRSETa-*buk* vector was diluted into 500 ml of Luria broth until the OD₆₀₀ reached 0.6. Addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 3 mM was used to induce the overexpression of Buk and the culture was incubated at 37 °C with shaking at 200 rpm for 3 hours. Cell pellets were obtained by centrifugation at 4°C at 3,000 *g* and were stored at -80 °C until use.

2.3 Purification of Buk

The cell pellet was resuspended in 25 ml binding buffer (200 mM NaCl, 25 mM N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) [HEPES], 1 mM MgCl₂, 5 mM imidazole, 5% glycerol and 5 mM β-mercaptoethanol pH 7.5). The cells were broken using a French press at 16,000 psi and two passes through the machine. Cell debris was removed

by centrifugation at 20,000 *g* for 30 min and the cell-free extract was allowed to bind by gravity flow with nickel-chelated nitrilotriacetic acid (Ni²⁺-NTA) resin (Thermo Scientific, Waltham, MA), which was pretreated with binding buffer. The resin was then washed with 5 column volumes of wash buffer (200 mM NaCl, 25 mM HEPES, 1 mM MgCl₂, 10 mM imidazole, 5% glycerol and 5 mM β-mercaptoethanol, pH 7.5). The bound His₆-tagged Buk was then eluted with 4 ml of elution buffer (200 mM NaCl, 25 mM HEPES, 1 mM MgCl₂, 250 mM imidazole, 25% glycerol and 5 mM β-mercaptoethanol, pH 7.5). The concentration of protein was determined by the Bradford assay using bovine serum albumin as the standard (Biorad, Hercules, CA). Addition of β-mercaptoethanol (4 mM) and glycerol (25%) was essential for the catalytic activity of Buk. Analysis of the preparation of Buk by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) confirmed the calculated molecular mass of Buk (~ 42 kDa) and the purity of the enzyme preparation (Fig. 1).

2.4 Standardization of Buk assay

Buk activity was measured in the acyl phosphate forming direction based on the assay developed by Lipmann and Tuttle [26] as described by Hartmanis [24] with minor modifications. Buk catalyzes the reversible transfer of the γ-phosphate of ATP to the carbonyl group of the carboxylic acid resulting in the formation of the high energy compound acyl phosphate as the product (Fig. 2. step 1). The acyl phosphate product was quantitated by the inclusion of neutralized hydroxylamine in the reaction mixture thereby resulting in the formation of its corresponding stable acyl hydroxamate (Fig. 2. Step 2). Acyl hydroxamate in the presence of acidic ferric chloride forms a purple ferric hydroxamic acid complex which was quantified spectrophotometrically at 540 nm. Lipmann and Tuttle [26] analyzed a variety of compounds and showed that color formation was specific for acyl anhydride compounds and color intensity did not vary with differences in the chain length of the compounds.

Commercial acetate kinase was utilized to standardize assay conditions. All assays of Buk activity were performed at 37 °C in a final volume of 200 μl. Buk-dependent formation of the corresponding acyl phosphate product was then studied in the presence of varying concentrations of butyrate and ATP. The assay medium contained 50 mM Tris-hydrochloride buffer (pH 7.5), 4 mM MnSO₄, 10 mM ATP, 400 mM neutralized hydroxylamine, 5 mM β-mercaptoethanol and varying amounts of carboxylic acid (100 μM to 300 mM). The reaction was initiated with the addition of purified Buk (1–5 μM final concentration) and arrested by the addition of 40 μl of 50% trichloroacetic acid (TCA). The precipitated protein was removed by centrifugation at 13,000 *g* for 2 minutes and the supernatant was transferred to a fresh tube. Color was developed by the addition of 400 μl of 1.25% ferric chloride solution in 1M hydrochloric acid and the intensity was measured using a Beckman DU-65 spectrophotometer at 540nm. Initial reaction rates were calculated as the amount of product formed per minute. Acetyl phosphate at varying concentrations (250 μM to 5 mM) was used to determine a standard curve. Assay medium without Buk was utilized as the blank.

2.5 Kinetic analysis of Buk

Initial velocities of Buk in the presence of varying concentrations of the carboxylic acid substrates were determined and plotted as a function of the substrate concentration. Non-linear curve fitting of the data to the Michaelis-Menten equation, $v = k_{cat} * [S] / (K_{0.5} + [S])$ where k_{cat} is the turnover number and $K_{0.5}$ is the substrate concentration that produces half maximal velocity and $[S]$ is the substrate concentration. However, inhibition of product formation was observed at higher concentrations of substrate in the case of hexanoate, 2-ethylbutyrate, 2-methylpentanoate and 3-methylpentanoate. Steady state kinetic parameters were computed for these substrates using KaleidaGraph software by fitting the data to the Michaelis-Menten equation incorporating substrate inhibition, $v = k_{cat} * [S] / (K_{0.5} + [S]) * (1 + [S]/[S_i])$ where all variables are the same with the additional factor S_i which is the substrate concentration that produces inhibition to half maximal velocity (Kaleidagraph, Synergy software, Reading, PA). Fits to this equation were used to determine the kinetic constants ($K_{0.5}$ and k_{cat}). At least three replicates of each assay were conducted and the results were presented as means \pm SEM. The apparent $K_{0.5}$ and k_{cat} values were determined for a variety of straight-chain and branched-chain carboxylic acid compounds listed in Table 1.

3 Results and Discussion

Butyrate kinase (EC 2.7.2.7) belongs to the ASKHA (acetate and sugar kinases / heat shock cognate / actin) superfamily of phosphotransferases and catalyzes the reversible phosphorylation of short-chain carboxylic acids in *C. acetobutylicum* [24]. The focus of this study was to investigate the substrate preferences of Buk from *L. monocytogenes* to determine if it was capable of phosphorylating the range of SCCAs and BCCAs which have induced alteration of the membrane fatty acid composition. To this end, Buk heterologously expressed in *E. coli* was purified by its N-terminal His-tag by affinity purification and assayed for activity by the hydroxamate method. Kinetic constants of Buk were determined in the presence of a saturating concentration of ATP (10 mM) and varying concentrations (0.1–300 mM) of various SCCAs and BCCAs.

3.1 Buk demonstrates broad substrate specificity

3.1.1 SCCAs—In order to understand the structural limits of substrates preferred by *LmBuk*, its activity was analyzed with several SCCAs with differing chain lengths (C2–C6). Among the SCCAs that were tested, Buk showed the highest catalytic efficiency ($5.5 \text{ mM}^{-1} \text{ min}^{-1}$) and affinity for pentanoate (C5) with an apparent $K_{0.5}$ of 13.1 mM. Butyrate (C4) also served as a good substrate with a corresponding $K_{0.5}$ value of 50.5 mM (Fig. 3A & Table 1). Buk activity with propionate (C3) showed that substantial product formation was also associated with lower affinity ($K_{0.5}=174.1 \text{ mM}$) compared to C4 and C5 SCCAs. The stronger preference for pentanoate demonstrated by *LmBuk* was similar to the branched-chain fatty acid kinase from *S. isovalerica* [23]. Additionally, the $K_{0.5}$ values exhibited by *LmBuk*, were relatively higher than reported values for *SiBuk* utilizing butyrate and propionate (Fig. 3A & Table 1) [23,24].

Acetate (C2) and hexanoate (C6) were poor *LmBuk* substrates (Fig. 3A & Table 1). However, elevated concentrations of acetate (>80 mM) did result in measurable product

formation (~10 % of the product with propionate as the substrate). Extremely low activity with acetate indicates that Buk is not involved in the secretion of acetate observed during aerobic and anaerobic growth in *L. monocytogenes* [27]. Furthermore, Buk is unlikely to be involved in phosphorylation of long chain fatty acids judging by the similarly low activity with hexanoate (C6) as the substrate. Thus, the modest integration of medium and long chain fatty acids reported earlier probably occurs via an alternate pathway [28].

These data indicate the broad substrate specificity and chain length preference (C3-C5) of *LmBuk*. The substantial activity in the phosphorylation direction (Fig. 3A and 3B; Table 1) supports our hypothesis that presence of sufficient concentrations of carboxylic acid precursors could drive the reaction in the acyl phosphate forming direction. Furthermore, *L. monocytogenes* Ptb has been shown to form butyryl-CoA from butyryl phosphate thus emphasizing the viability of the reversibility of this pathway [22]. The reversibility of the Ptb-Buk pathway has been utilized *in vitro* for the production of polyhydroxy alkanolic acids in *C. acetobutylicum* and *Bacillus megaterium* and the authors demonstrate the importance of broad substrate specificity of the Ptb-Buk pathway in this context [29,30].

It is also likely that the phosphorylated products of carboxylic acids serve a purpose other than formation of fatty acids. Butyrate is well known as an effector of gene expression. For example, butyrate causes an upregulation of virulence factor expression in enterohemorrhagic *E. coli* (EHEC) [31]. On the other hand, downregulation of virulence factor expression caused by exposure to butyrate has been observed in *Salmonella enterica* Enteritidis and *L. monocytogenes* [10,32]. Intracellular peak concentrations of butyryl phosphate, which likely behaves as a small molecule phosphate donor, is accompanied by altered expression of a large set of genes in *C. acetobutylicum* [33,34]. Thus, it is probable that Buk plays a significant role in signal transduction in *L. monocytogenes*, since the organism encounters high concentrations of short chain fatty acids in the mammalian gut during infection [35].

3.1.2 BCCAs—Buk from the marine spirochete MA-2 has been reported to be a true branched-chain fatty acid kinase [23]. We sought to determine if *LmBuk* exhibited similar behavior and kinetic constants were determined with various BCCAs differing in the size and branch position to this end. All BCCAs tested proved to be good substrates for *LmBuk*. Curiously, the presence of a methyl branch increased affinity of substrates which had a shorter chain length. For example, the $K_{0.5}$ values for propionate (174.1 mM) was ~20-fold higher than that of isobutyrate (C3 with a methyl branch at the 2nd position) (7.6 mM) revealing a substantial increase in affinity associated with the branched chain. A modest improvement in affinity was observed with the C4 substrates as demonstrated by a comparison of the $K_{0.5}$ for butyrate with that for isovalerate, 2-methylbutyrate and 2-ethylbutyrate (Fig. 3B & Table 1). However, the data derived from branched C5 substrates (2-methylpentanoate and 3-methylpentanoate) support lower binding affinity when compared with their straight chain counterparts (Fig. 3B & Table 1). Although the presence of the branch appears to increase affinity it also seems to cause a concomitant reduction in the turnover rate (Fig 3B, Table 1 & 2). Such a change might indicate that branched chain substrates have a reduced off-rate which would produce both of these observed enzymatic characteristics.

Although Buk activity is likely important in the incorporation of these compounds into the *L. monocytogenes* fatty acid pool, factors such as the substrate specificity of Ptb, FabH, and the other enzymes that participate in phospholipid biosynthesis probably play a larger role. Consistent with this is that although Buk showed the best catalytic efficiency with pentanoate, Ptb from *L. monocytogenes* prefers BCCA substrates, and does not utilize pentanoyl-CoA efficiently [22,36]. Additionally, synthesis of BCFAs has been reported to be dependent on the substrate specificity of FabH, which catalyzes the first committed step in fatty acid biosynthesis, thus these endogenous substrates likely outcompete the products of the Buk-Ptb pathway [37].

3.1.3 ATP—The ATP concentration dependence (10 μ M – 15mM) for *Lm*Buk was determined in the presence of 200 mM butyrate by fitting the data to the Michaelis-Menten equation as described in the “Materials and Methods”. The $K_{0.5ATP}$ for *Lm*Buk was 3.18 mM (Fig. 4), values which are in agreement with Buk from *C. acetobutylicum* and the marine spirochete *S. isovalerica* [23,24]. Additionally, presence of 2 mM ADP demonstrated competitive inhibition of the binding of ATP with an increase in the $K_{0.5ATP}$ to 6.09 mM (Fig. 4).

3.2 Buk utilized unnatural BCCAs as its substrates

*Lm*Buk also exhibited significant product formation with unnatural BCCAs such as 2-ethylbutyrate, 2-methylpentanoate, and 3-methylpentanoate (Fig. 3B & Table 1). Enzyme activity in the presence of these substrates was similar to hexanoate where activity begins to slow at higher concentrations. Thus, in these instances data were fitted to an equation that incorporated substrate inhibition (See Methods). Utilization of 2-ethylbutyrate showed the least product formation (k_{cat} 6.7 min⁻¹), which may be attributed to the longer side chain (ethyl) compared to 2-methylbutyrate (k_{cat} 11.3 min⁻¹). Additionally, while the presence of a methyl branch caused an increase in affinity, it also resulted in decreased product formation as evidenced by lower k_{cat} values (Fig. 3B & Table 1). To our knowledge, this is the first demonstration that Buk can utilize unnatural fatty acid precursors capable of altering the membrane fatty acid profile. *Lm*Ptb similarly can utilize unnatural BCCA derivatives as substrates indicating that these two enzymes could likely constitute a pathway to altering membrane composition [22]. Sen *et al.* [16] showed that novel fatty acids arising from these unnatural BCCAs imparted the biophysical characteristics necessary for survival of the organism at low temperatures. Our work here indicates that *L. monocytogenes* exploits the reversibility of the Ptb-Buk pathway in the presence of sufficient concentrations of unnatural substrates in its environment to supply these fatty acid precursors for elongation by the FAS II pathway. The requirement of higher concentrations in the case of the wild type organism indicates that the domination of Bkd activity in the production of endogenous substrates outcompetes the products from the bypass pathway [11].

3.3 Buk prefers BCCAs at low temperatures

L. monocytogenes *cld-2*MOR401 incorporates the products of numerous BCCAs in its membrane at low growth temperatures [8,16]. We determined whether Buk was capable of product formation with these substrates at low temperature as would be required if it contributes to increased membrane fluidity at these temperatures. Product formation at 10 °C

was observed with all BCCAs and SCCAs tested (Fig 3C & Table 2). Interestingly, substrate inhibition was not observed at low temperatures. We observed lower k_{cat} values for all of the substrates tested. However, the decrease was more pronounced for SCCAs compared to BCCAs (Fig 3C & Table 2), which may be due in part to an increase in the $K_{0.5}$ values for the SCCA substrates. This drastic reduction in Buk catalytic efficiency in the presence of SCCAs compared to BCCAs suggests that Buk demonstrates a strong preference for BCCAs at 10 °C (Fig 3C & Table 2). To our knowledge, this is the first report demonstrating a temperature-dependent switch in substrate preference by Buk from any organism. *LmFabH* also demonstrates a switch in its substrate preference at low temperatures to select for 2-methylbutyryl-CoA, the precursor of the low melting point fatty acid anteiso C15:0 enabling it to survive and grow at low temperatures [36]. The substrate specificities of *LmBuk* could thus be similar to other organisms which increase the membrane BCFAs in response to lower temperatures and can incorporate exogenous carboxylic acids. Perhaps these unique substrate specificities are indicative of evolutionary adaptations within *L. monocytogenes* providing a psychrotolerant selective advantage.

3.4 Buk catalysis occurs through a ternary complex intermediate

We sought to determine whether Buk catalysis occurs through a sequential or ping-pong mechanism. Enzyme activity was plotted as a function of butyrate concentration at different fixed concentrations of ATP (i.e. 5 mM, 7.5 mM and 10 mM). Double reciprocal plots (Fig. 5) showed that the lines converged to a point in the second quadrant. This is consistent with a reaction mechanism involving a sequential mechanism with the formation of a ternary complex. That is, *LmBuk* binds both butyrate and ATP forming a ternary complex, prior to catalysis and release of the products butyryl phosphate and ADP. Additionally, competitive binding of ADP (Fig. 4) in the active site is supportive of the sequential mechanism.

A similar mode of action was also suggested for the action of other phosphokinases such as acetate kinase (Ack) and glycerol kinase [38]. Although the apparent $K_{0.5}$ values and relative activity of Buk with various substrates were explored in *C. acetobutylicum* and the spirochete MA-2, the mechanism of action was not investigated in these organisms [23,24]. Cheek *et al.*, [39] classified and characterized the protein folding of kinases and showed the similarity of the structure of Ack and Buk. Buk is highly conserved among *Listeria* isolates and also demonstrates a significant identity with Buk from *C. acetobutylicum* and *Thermotoga maritima*. Additionally, substrate and nucleotide bound structures of *Salmonella typhimurium* propionate kinase showed that the carboxyl group of propionate is positioned at a distance of 0.5 nm from the γ -phosphate of ATP in the active site supporting a direct in-line transfer mechanism, i.e. a sequential mechanism [40].

3.5 Possible biological relevance of *LmBuk*

ptb and *buk* are immediately upstream of the *lpd* gene of the branched-chain α -keto acid dehydrogenase operon in *L. monocytogenes* [8]. The normal physiological function of Ptb and Buk may be in the catabolism of branched-chain α -keto acids to the corresponding free acids with formation of ATP as has been shown in *E. faecalis* [18], and in the synthesis of butyrate [41]. These two enzymes may function in the reverse direction in the formation of phosphate and CoA derivatives of SCCAs and BCCAs. There clearly is a pathway for the

formation of CoA derivatives for fatty acid biosynthesis from BCCAs not involving Bkd and branched chain amino acids. This is most readily seen in *L. monocytogenes bkd* mutants (*cld-1*, *cld-2* and MOR401) where BCCAs and SCCAs act as precursors for fatty acid biosynthesis [4,8,11,16]. However, even in wild type *L. monocytogenes* with intact *bkd* high concentrations of BCCAs such as 2-methyl butyrate, isobutyrate, and isovalerate act as precursors for anteiso odd, iso even and iso odd fatty acids respectively, and butyrate results in the biosynthesis of even straight-chain fatty acids [11]. Furthermore, the C6 BCCAs 2-ethylbutyrate and 2-methylpentanoate lead to the biosynthesis of 12-ethyltetradecanoate and 12-methylpentadecanoate respectively in wild type also. The substrate specificities of Ptb [22] and Buk are compatible with their function in production of CoA derivatives for fatty acid biosynthesis from carboxylic acids. Further verification of the roles of *ptb* and *buk* in fatty acid biosynthesis will require construction of mutants in these genes.

It is possible that sufficient concentrations of SCCAs and BCCAs in foods could impact the fatty acid composition of *L. monocytogenes*. Julotok *et al.* [11] showed that food preservatives such as acetate and lactate that are metabolized via acetyl CoA did not impact fatty acid composition, whereas propionate and butyrate at 25 mM and higher concentrations did. Both 2-ethylbutyrate and 2-methylpentanoate are used as food flavoring additives but at low levels, e.g., 40 ppm [42], and are therefore unlikely to impact fatty acid composition. Each food would be expected to have a unique concentration of BCCAs and SCCAs, but levels of these constituents are not normally reported as part of the United States Department of Agriculture Survey.

4 Conclusions

LmBuk exhibited substantial phosphorylation with a large number of SCCAs and natural and unnatural BCCA substrates indicating its broad substrate specificity. This suggests that Buk might play a role in provision of precursors for fatty acid biosynthesis under some conditions. Buk is distinct from acetate kinase and prefers substrates with a chain length of C3-C5 and is thus not involved in the activation of acetate or long chain fatty acids. Buk catalysis involves the formation of a ternary complex similar to other members of the phosphokinase protein family. Substantial product formation in the presence of butyrate (present in high concentrations in the mammalian gut) is consistent with a role for Buk in signal transduction during infection by *L. monocytogenes*.

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Highlights

- Buk from *L. monocytogenes* exhibited broad substrate specificity
- Buk preferred substrates with a chain length of 3 to 5 carbons
- An alkyl side chain improved binding among shorter chain substrates
- Buk could utilize unnatural substrates such as 2-ethylbutyrate, 2-methylpentanoate and 3-methylpentanoate
- Buk catalysis involved ternary complex formation

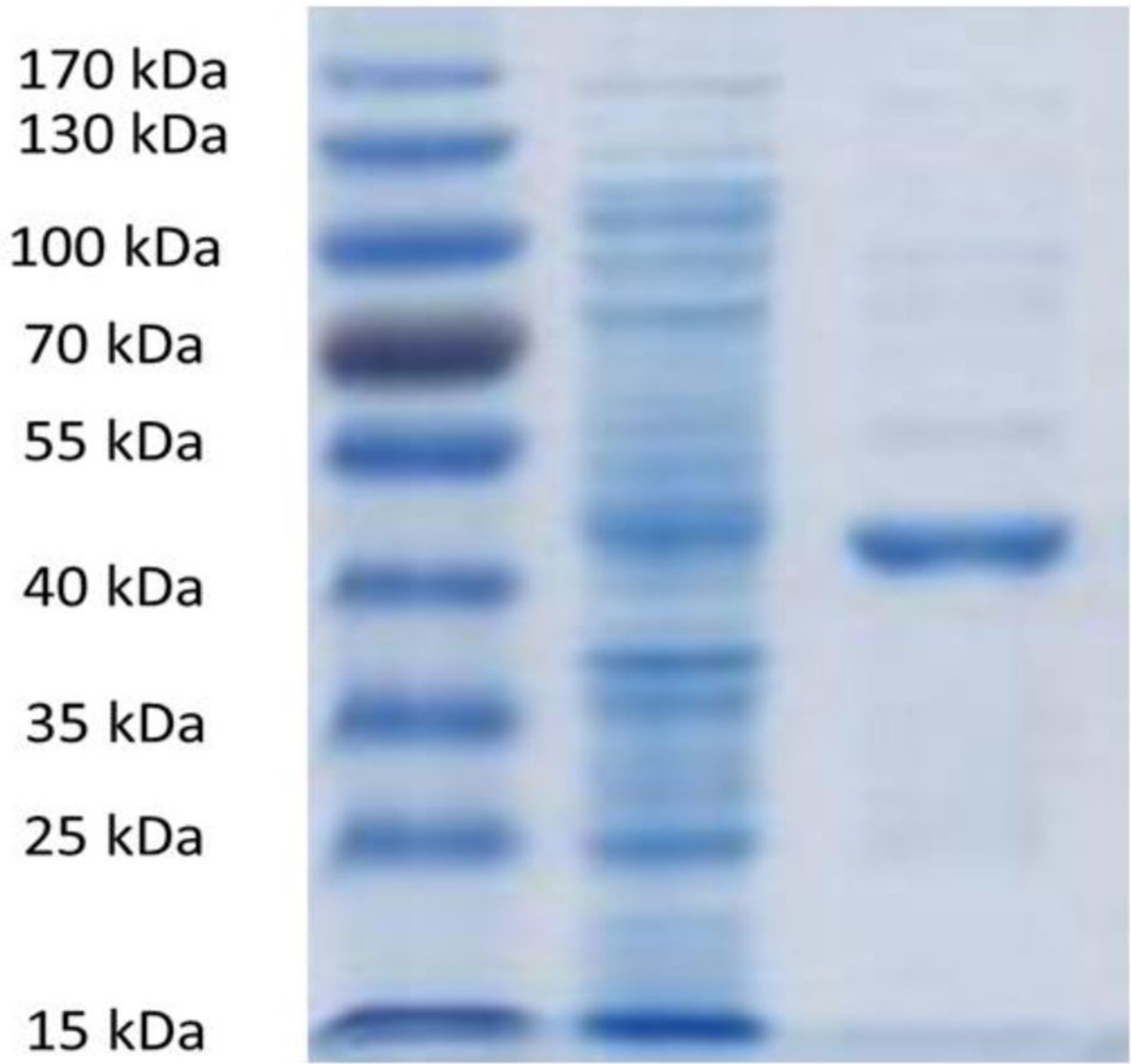


Fig 1. Analysis of purified *LmBuk* by SDS-PAGE

Samples were resolved on 10% (w/v) acrylamide gel and stained with Coomassie Blue.

Lane 1 protein molecular weight markers, Lane 2 Cell-free lysate showing expression of *LmBuk*, Lane 3 Recombinant *LmBuk* after purification by Ni²⁺-affinity chromatography.

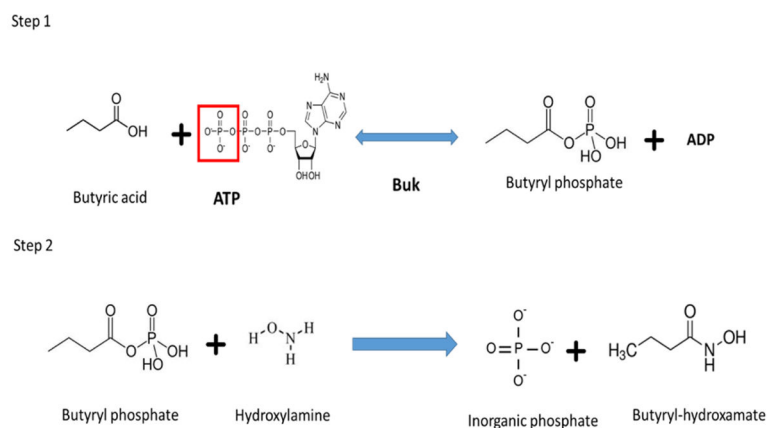


Fig 2. Reaction catalyzed by Buk

Step 1 shows the activity of Buk catalyzing the reversible transfer of the terminal phosphate moiety of ATP (highlighted in red) to the carbonyl group of butyric acid resulting in the formation of butyryl phosphate. Step 2 shows the irreversible conversion of the unstable butyryl phosphate to stable butyryl hydroxamate with the release of inorganic phosphate. Butyryl hydroxamate in the presence of acidic ferric chloride forms a purple complex which is quantified at 540 nm.

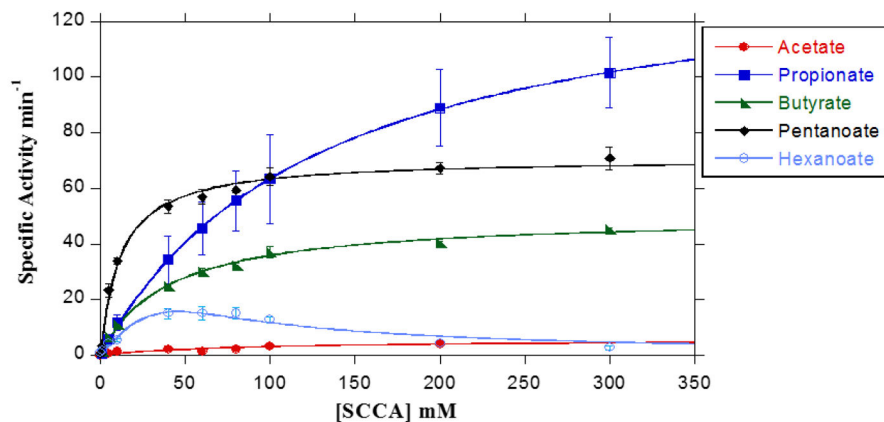


Fig. 3a

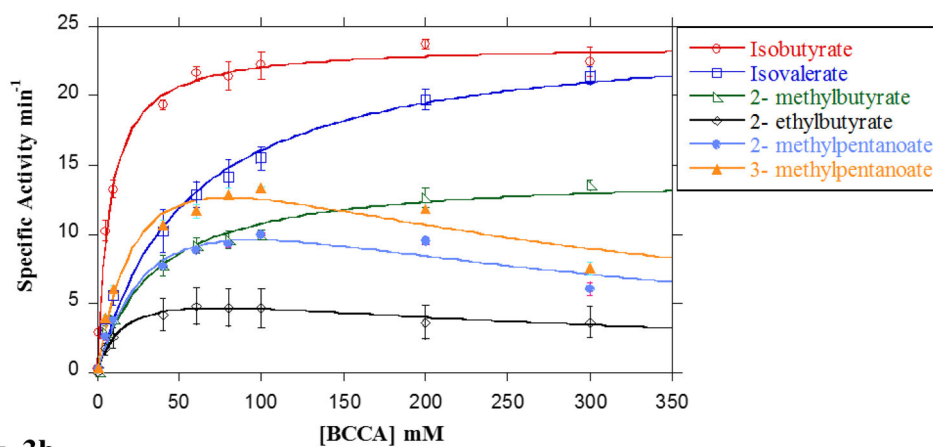


Fig. 3b

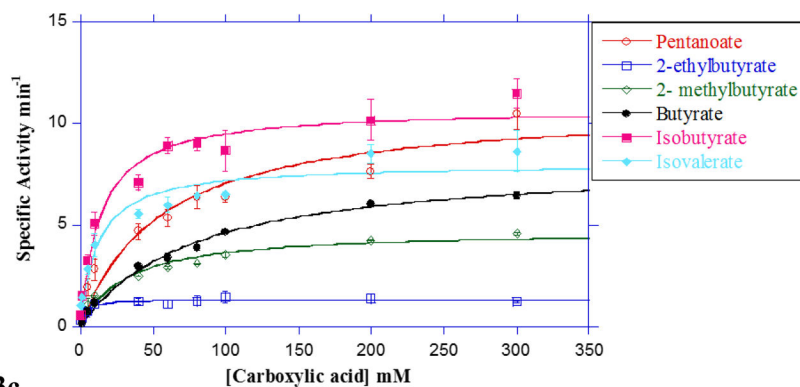


Fig. 3c

Fig 3. Substrate preference of *LmBuk*

A. Concentration-dependent activity of *LmBuk* in the presence of SCCA substrates at 37 °C. The observed initial rates of reaction for the substrates acetate (closed circles), propionate (closed squares), butyrate (closed triangles), pentanoate (closed diamonds) and hexanoate (open circles) catalyzed by *LmBuk* were determined at 37 °C in the presence of

10 mM ATP under standard assay conditions (see Materials and Methods). Data were plotted as a function of the substrate concentration and K_M and k_{cat} were determined from the fit of the data to the Michaelis-Menten equation using Kaleidagraph software. Data derived for hexanoate was fitted to the Michaelis-Menten equation to include substrate inhibition. Data are means \pm SEM of at least three experiments. **B. Concentration-dependent activity of *LmBuk* in the presence of BCCA substrates at 37 °C.** The observed initial rates of reaction for the isobutyrate (open circles), isovalerate (open squares), 2-methyl butyrate (open triangles) and 2-ethyl butyrate (open diamonds), 2-methyl pentanoate (closed circles) and 3-methyl pentanoate (closed triangles) catalyzed by *LmBuk* were determined at 37 °C in the presence of 10 mM ATP under standard assay conditions (see Materials and Methods). Data were plotted as a function of the substrate concentration and K_M and k_{cat} were determined from the fit of the data to the Michaelis-Menten equation using Kaleidagraph software. Data derived for 2-ethylbutyrate, 2-methylpentanoate and 3-methylpentanoate was fitted to the Michaelis-Menten equation to include substrate inhibition. Data are means \pm SEM of at least three experiments. **C. Concentration-dependent activity of *LmBuk* at 10 °C in the presence of SCCA and BCCA substrates.** The observed initial rates of reaction for the isobutyrate (closed squares), isovalerate (closed diamonds), 2-methyl butyrate (open diamonds) and 2-ethyl butyrate (open squares), pentanoate (open circles) and butyrate (closed circles) catalyzed by *LmBuk* were determined at 10 °C in the presence of 10 mM ATP as described under “Materials and Methods”. Data were plotted as a function of the substrate concentration and K_M and k_{cat} were determined from the fit of the data to the Michaelis-Menten equation using Kaleidagraph software. Data plotted are means \pm SEM of at least three experiments.

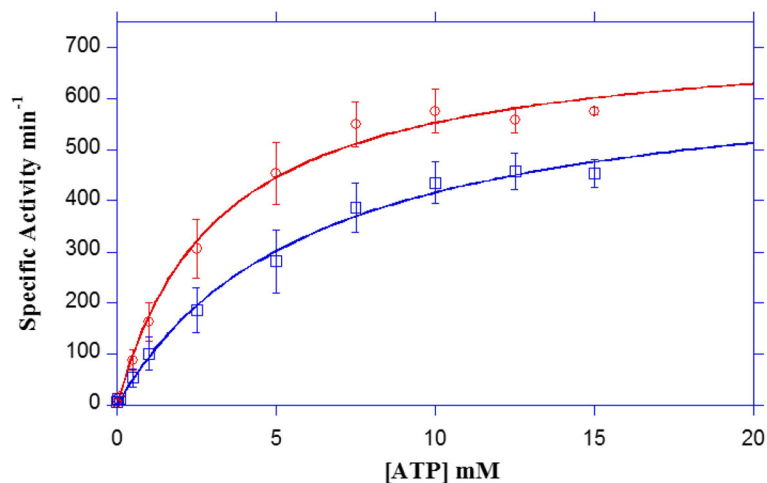


Fig 4. Concentration dependence of ATP utilization by *LmBuk* and competitive inhibition by ADP

LmBuk activity was determined under standard assay conditions in the presence of 200 mM butyrate and varying amounts of ATP (10 μ M–15 mM) and data (open circles) were plotted as a function of ATP concentration. Fit of the data to the Michaelis-Menten equation provided values for K_M and k_{cat} . ADP inhibition studies were performed using 2 mM ADP (open squares) in each reaction under the same experimental conditions as that of the ATP assay. ADP demonstrated competitive inhibition with respect to ATP. Data plotted are means \pm SEM of at least three experiments.

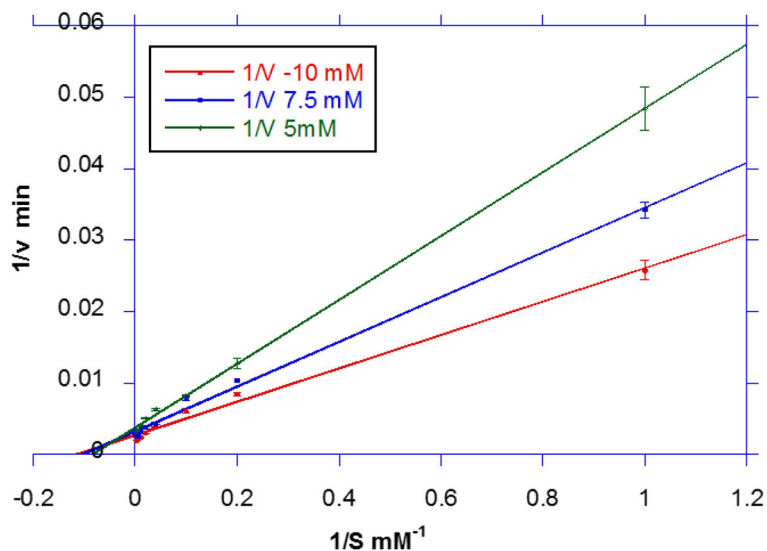


Fig 5. *LmBuk* catalysis occurs through a ternary complex intermediate

Concentration dependent activity of Buk in the presence varying concentrations of butyrate (0.1– 300 mM) at different fixed concentrations of ATP (5 mM (open diamonds), 7.5 mM (closed squares) and 10 mM (closed circles)) were determined under standard assay conditions at 37 °C. Means of reciprocals of specific activity from at least three experiments \pm SEM were plotted.

Table 1Steady state kinetic analysis of *LmBuk* at 37°C.

Substrate	k_{cat} (min ⁻¹)	K_M (mM)	k_{cat} / K_M (mM ⁻¹ min ⁻¹)
Straight-chain			
Propionate	155.6 ± 0	174.1 ± 68.9	0.89
Butyrate	50.2 ± 0.4	50.5 ± 5.8	0.99
Pentanoate	71.7 ± 2.2	13.1 ± 3.3	5.5
Hexanoate	1310.9 ± 474.9	1856 ± 676	0.7
Branched-chain			
Iso butyrate	23.7 ± 0.4	7.6 ± 1.3	3.1
Isovalerate	25 ± 0.1	57.1 ± 9.6	0.44
2-methylbutyrate	15.9 ± 1.5	36.2 ± 5.2	0.44
2-ethyl butyrate	6.7 ± 1.7	16.5 ± 0.9	0.4
2-methyl pentanoate	18.8 ± 2.4	44.2 ± 8.5	0.4
3-methyl pentanoate	21.2 ± 0.4	27.3 ± 0.8	0.8

Initial velocities were measured at 37 °C in the presence of 10 mM ATP at pH 7.5. The k_{cat} and K_M were calculated from the least squares fit of the experimental data from the different substrates to the Michaelis-Menten equation. The values indicated are the mean of experiments performed at least in triplicate ± SEM.

Table 2Steady state kinetic analysis of *LmBuk* at 10 °C.

Substrate	k_{cat} (min ⁻¹)	K_M (mM)	k_{cat} / K_M (mM ⁻¹ min ⁻¹)
Straight-chain			
Butyrate	8.2 ± 0.1	78.1 ± 3.8	0.1
Pentanoate	9.1 ± 0.4	42.5 ± 5.2	0.2
Branched-chain			
Isobutyrate	10.2 ± 0.3	9 ± 1.5	1.1
Isovalerate	7.6 ± 0.3	8.2 ± 1.4	0.9
2-methylbutyrate	4.3 ± 0.2	22 ± 4.3	0.2
2-ethyl butyrate	1.2 ± 0.1	4 ± 2.5	0.3

Initial velocities were measured at 10 °C in the presence of 10 mM ATP at pH 7.5. The k_{cat} and K_M were calculated from the least squares fit of the experimental data from the different substrates to the Michaelis-Menten equation. The values indicated are the mean of experiments performed at least in triplicate ± SEM.