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Broad substrate specificity of phosphotransbutyrylase from *Listeria monocytogenes*, a potential participant in an alternative pathway for provision of acyl CoA precursors for fatty acid biosynthesis

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Abstract

Listeria monocytogenes, the causative organism of the serious food-borne disease listeriosis, has a membrane abundant in branched-chain fatty acids (BCFAs). BCFAs are normally biosynthesized from branched-chain amino acids via the activity of branched chain α-keto acid dehydrogenase (Bkd), and disruption of this pathway results in reduced BCFA content in the membrane. Short branched-chain carboxylic acids (BCCAs) added as media supplements result in incorporation of BCFAs arising from the supplemented BCCAs in the membrane of *L. monocytogenes bkd* mutant MOR401. High concentrations of the supplements also effect similar changes in the membrane of the wild type organism with intact *bkd*. Such carboxylic acids clearly act as fatty acid precursors, and there must be an alternative pathway resulting in the formation of their CoA thioester derivatives. Candidates for this are the enzymes phosphotransbutyrylase (Ptb) and butyrate kinase (Buk), the products of the first two genes of the *bkd* operon. Ptb from *L. monocytogenes* exhibited broad substrate specificity, a strong preference for branched-chain substrates, a lack of activity with acetyl CoA and hexanoyl CoA, and strict chain length preference (C3–C5). Ptb catalysis involved ternary complex formation. Additionally, Ptb could utilize unnatural branched-chain substrates such as 2-ethylbutyryl CoA, albeit with lower efficiency, consistent with a potential involvement of this enzyme in the conversion of the carboxylic acid additives into CoA primers for BCFA biosynthesis.

1. Introduction

Listeria monocytogenes, the dangerous foodborne pathogen, grows actively at low temperatures and is a cause for concern due to the widespread use of refrigeration as a food preservation method [1]. *Listeria* outbreaks remain a continuing problem (http://www.cdc.gov/listeria/outbreaks). The economic costs of a 2008 *L. monocytogenes* outbreak

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in Canada linked to contaminated delicatessen meat from one processing plant, which resulted in 57 cases of listeriosis and 24 deaths, were estimated to be $242 million Canadian dollars [2].

A fluid membrane ensured by a high content of branched-chain fatty acids (BCFAs), which have low phase transition temperatures, supports the growth of *L. monocytogenes* at low temperatures [3–5]. Branched-chain amino acids, namely isoleucine, leucine and valine, through the activity of branched-chain amino transferase (Bcat) and branched-chain α-keto acid dehydrogenase complex (Bkd) result in the formation of the CoA derivatives of 2-methylbutyrate, isobutyrate and isovalerate respectively, which are then elongated by the wellcharacterized dissociated fatty acid biosynthesis (FAS II) system to form membrane BCFAs [6]. Mutants of *L. monocytogenes* (*cld-2*/MOR401) lacking functional Bkd have low membrane BCFA content and demonstrate diminished growth at refrigeration temperatures, reduced tolerance to adverse environmental conditions such as oxidative stress, and poor survival in macrophages [7,8]. Growth of *bkd* mutants in *Bacillus subtilis, L. monocytogenes* and *Staphylococcus aureus* could be rescued by the addition of branched-chain carboxylic acids (BCCAs) to the medium [9–11].

Extensive studies on medium supplementation by Kaneda [12] in *B. subtilis* and by Sen et al. [13] in *L. monocytogenes* show that addition of a specific BCCA results in an increase in membrane fatty acids biosynthesized from that particular BCCA. The carboxylic acids that are capable of causing an alteration of membrane fatty acid composition are not restricted to the normal products of BCAA metabolism but also include unnatural C6 BCCAs that have a similar structure such as 2-ethylbutyrate, 2-methylpentanoate, and straight-chain carboxylic acids (SCCAs) such as butyrate and propionate [12,13]. Incorporation of novel fatty acids into the membrane as a result of elongation of unnatural C6 BCCAs is indicative of the entry of the supplements into the FAS II pathway as their respective acyl CoA derivatives, since the only known source of membrane BCFAs is via biosynthesis [6]. Formation of acyl CoA derivatives despite the absence of functional Bkd confirms the presence of an alternate pathway which catalyzes the conversion of supplemented carboxylic acids into their respective acyl CoA end products [10,13]. Willecke and Pardee [11] first suggested the involvement of an alternate system in the conversion of BCCAs into precursors of fatty acids in a *bkd* mutant of *B. subtilis*. Integration of the products of such a wide range of substrates into the membrane implies that the pathway involved in their activation must be remarkably flexible in its substrate specificity [10,13]. This pathway has not been characterized in *L. monocytogenes* thus far despite ample evidence of its existence.

Two genes, namely phosphotransbutyrylase (*ptb*) and butyrate kinase (*buk*), have been identified upstream of the genes encoding the Bkd complex in the *bkd* operon of *L. monocytogenes* based on sequence similarity to the *bkd* operon in *B. subtilis* and *Enterococcus faecalis* [7,14–16]. *ptb* and *buk* are followed by the genes encoding the polypeptides of the Bkd complex in the *bkd* operon in *L. monocytogenes* [7]. The molecular organization of these two genes in *L. monocytogenes* is markedly different from *Clostridium acetobutylicum* in which *ptb* and *buk* form a separate operon expressed from a single promoter [17]. Buk (EC 2.7.2.7) is known to phosphorylate short chain carboxylic acids in the fermentative organism *C. acetobutylicum* and a marine spirochete MA-2 [18,19]. Ptb

(EC 2.3.1.19) from *C. acetobutylicum* is an enzyme catalyzing the reversible conversion of acyl phosphates to acyl CoAs [20] (Fig. 1). We propose that these two enzymes, given their functions and location in the *bkd* operon, reverse their physiological direction in the presence of exogenous carboxylic acids and catalyze the conversion of these substrates into their corresponding acyl CoA thioesters (Fig. 1).

Ptb has been well studied in *C. acetobutylicum,* an important producer of industrial solvents, and it plays a crucial role in the energy metabolism in the acidogenesis phase leading to the production of ATP by substrate phosphorylation [20–22]. Similar studies in *E. faecalis* demonstrate the involvement of Ptb in branched-chain amino acid catabolism resulting in energy production and secretion of catabolic end products such as 2-methylbutyrate and isovalerate [16]. These studies on Ptb were focused on its involvement in energy metabolism and have been limited in scope.

Ptb from *L. monocytogenes* is a putative 288 amino acid protein with 38.5% identity to the enzyme from *C. acetobutylicum* and 41% identity to Ptb from *E. faecalis*. In this work we describe the purification of Ptb from *L. monocytogenes* expressed in *E. coli* and investigation of its substrate preferences and enzyme kinetic constants (K_M and k_{cat}). The results indicate that Ptb demonstrates broad substrate specificity, which is consistent with our hypothesis of its role in the incorporation of exogenous carboxylic acids into the membrane fatty acids. SCCAs such as propionate and butyrate, which are used as food preservatives, additionally may contribute to the control of *L. monocytogenes* by resulting in the biosynthesis of straight-chain fatty acids that rigidify the membrane [10,13]. The substantial activity with these substrates suggests that Ptb may play a role in this process.

2. Materials and methods

2.1 Materials

Pentanoyl CoA was purchased from Crystalchem (Downers Grove, IL). All other materials used in this work including the chemicals utilized for synthesis of 2-methylbutyryl CoA and 2-ethylbutyryl CoA were purchased from Sigma Aldrich (St. Louis, MO).

2.2 Strains and plasmids

L. monocytogenes 10403S was grown in Brain Heart Infusion (BHI) broth (Becton Dickinson, Sparks, MD) and used for isolation of genomic DNA using a Masterpure genomic DNA purification kit according to the manufacturer's instructions (Epicenter, Madison, WI). Primers (forward 5' GGGGAGGTCGACAAATGACAAAAAGCAGATTTTTTTCA and reverse 5'GGGGAGCTCGAGTTTCTCAACTAGTCTTACAG) were designed with restriction sites for cloning and used for amplification of *ptb* from *L. monocytogenes* 10403S genomic DNA. The amplified gene (864 bp) was ligated into the expression vector pET28a (Novagen, Madison, WI) using T4 DNA ligase (Fermentas, Waltham, MA) to generate pET28a-*ptb* and transformed into competent *Escherichia coli* BL21 (DE3) cells. Kanamycin (50 μg/ml) was used as the selection agent for growth of pET28a and pET28a-*ptb* carrying *E. coli* cells in Luria broth (Becton Dickinson, Sparks, MD). Purification of DNA, ligation and

transformation were performed according to the manufacturer's instructions (Qiagen, Valencia, CA).

2.3 Purification of Ptb (EC 2.3.1.19)

An overnight culture of *E. coli* BL21 DE3 cells carrying pET28a-*ptb* was used to inoculate 500 ml Luria broth (2% inoculum) which was incubated at 37 °C until the OD_{600} reached 0.6. Overexpression of Ptb was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The cells were harvested by centrifugation at 4°C at 3,000 *g* and the pellets were stored at −80 °C. To purify Ptb, the cell pellet was resuspended in binding buffer (1.5 M sodium chloride-NaCl, 25 mM N-(2-Hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) [HEPES], 1 mM $MgCl_2$, 5 mM imidazole, 5% glycerol and 2 mM β-mercapto ethanol pH 7.5) and the cells were broken using a French press at 16,000 psi with two passes through the machine. Debris was removed by centrifugation at 20,000 *g* for 30 min and the cell-free extract was allowed to bind for one hour with nickel-chelated nitrilotriacetic acid (Ni^{2+}-NTA) resin (Thermo Scientific, Waltham, MA), which was pretreated with binding buffer. The resin was then packed into a column and washed with 5 column volumes of wash buffer (1.5 M NaCl, 25 mM HEPES, 1 mM $MgCl_2$, 10 mM imidazole, 5% glycerol and 2 mM β-mercaptoethanol, pH 7.5). The bound hexa histidine-tagged Ptb was then eluted with 4 ml of elution buffer (1.5 M NaCl, 25 mM HEPES, 1 mM $MgCl_2$, 250 mM imidazole, 25% glycerol and 2 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).

2.4 Synthesis of acyl CoA substrates

The acyl CoA substrates 2-methylbutyryl CoA and 2-ethylbutyryl CoA were prepared (Fig. 2) according to the protocol outlined by Stadtman [23]. Briefly, the mixed anhydride was prepared by the addition of 0.1 mol of the organic acid and anhydrous pyridine each to 10 ml icecold ethyl ether drop wise. This was followed by the addition of 0.1 mol of ethyl chloroformate drop wise to the mixture. The mixture was stirred for one hour in an ice bath. The insoluble pyridine hydrochloride that formed as a precipitate was removed by filtration and discarded. Next an aqueous solution of coenzyme A was prepared in 0.2 M KH_2CO_3 at pH 7.5. To this solution an equimolar amount of the mixed anhydride was added dropwise with constant stirring on ice for five minutes. The solution was tested for the presence of free CoA by the nitroprusside test as described by Toennies and Kolb [24]. Additional mixed anhydride was added until the nitroprusside test no longer revealed free CoA. The pH was then adjusted to 6.0 by the addition of 1 M HCl and the aqueous layer was extracted with an equal volume of ethyl ether three times to remove unreacted mixed anhydride. The remaining traces of ethyl ether were removed by bubbling nitrogen through the solution. The prepared acyl CoA substrate was quantitated by the hydroxylamine method [25].

2.5 Ptb assay

All kinetic studies were performed at room temperature (23–25 °C) in a reaction volume of 200 µl in microcuvettes (path length = 1 cm). Ptb was assayed in the acyl phosphate forming direction according to the method of Klotsch [26] as described by Weisenborn *et al.*, [20]. This assay was chosen for analysis of Ptb due to the commercial availability of acyl CoA

derivatives varying in the carbon skeleton and also due to the lack of available corresponding acyl phosphate derivatives. Ptb catalyzes the nucleophilic attack from inorganic phosphate on an existing acyl CoA derivative to produce the corresponding acyl phosphate derivative and concomitantly liberates coenzyme-A. The free sulfhydryl group on coenzyme-A was then quantified by reacting it with Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid), (DTNB)] [27]. Free thiols react with the disulfide bond in DTNB and liberate 5-thio-2-nitrobenzoic acid (TNB), which at neutral and alkaline pH ionizes to produce a yellow color which we measured spectrophotometrically (412 nm, Nanodrop 2000c UV-Vis spectrophotometer, Wilmington, DE). The production of TNB is stoichiometric with the amount of thiol present and thus was used calculate the initial velocity of Ptb production of each acyl phosphate derivative.

2.6 Steady state kinetic analysis

The assay for Ptb activity was standardized by using butyryl CoA as the substrate with a saturating concentration of inorganic phosphate (0.1 M) in the assay mixture. Enzyme-dependent rate of product formation in terms of the change in absorbance at 412 nm was studied and it was decided that a final concentration of Ptb in the assay of 5–10 nM would be used to obtain reaction velocities. Assay medium contained: 50 mM Tris (pH 7.5), 4 mM $MgCl_2$, 0.08 mM DTNB, 0.1 M KH_2PO_4 and varying amounts of acyl CoA (10 μM to 500 μM). Stock solutions of the acyl CoA substrates (10 mM) were prepared using 50 mM Tris-HCl (pH 4.0) and the concentrations were calculated based on the purity of the substrate as reported by the manufacturer. Tris buffer at low pH was chosen for preparing stock solutions in order to rapidly bring up the pH of the working solution prior to use. Each substrate was diluted to 1 mM concentration prior to use with 50 mM Tris-HCl (pH 7.5). Reactions were initiated by the addition of Ptb and absorbance (412 nm) was recorded at two second intervals for the duration of the reaction (60 sec). Initial rates were calculated from the linear portion of the reaction curve using Microsoft Excel and plotted against the substrate concentration, and apparent kinetic parameters were determined using KaleidaGraph software by fitting the data to the Michaelis-Menten equation, v = Vmax * [S] / (K_M +[S]) where Vmax is the maximum velocity and K_M is the substrate concentration that produces half maximal velocity and [S] is the substrate concentration (Kaleidagraph, Synergy software, Reading, PA). At least three replicates of each reaction were performed and the results were presented as the mean ± SEM. Coenzyme-A (25–125 μM) (Sigma-Aldrich, St. Louis, MO) was used to establish a standard curve. Assays were conducted in the absence of enzyme to determine background degradation of the substrate, if any, and also in the absence of the substrate to rule out interference of β-mercaptoethanol. The apparent K_M and k_{cat} values were determined for a variety of straight-chain and branched-chain acyl CoA compounds listed in Table 1. Ptb was assayed in the presence of excess isovaleryl CoA (200 μM) and varying inorganic phosphate concentrations (50 μM – 25 mM) in order to determine the kinetic constants with respect to inorganic phosphate.

Ptb assay in the acyl CoA forming direction was performed via colorimetric detection of released inorganic phosphate according to the method described by Helms *et al.* [28] based on the method of Brotherus and co-workers [29]. Briefly, the assay solution (200 μl) contained 100 mM Tris (pH 7.5), 500 μM CoA and varying concentrations of butyryl

phosphate (200 μM - 3 mM). The reaction was initiated by the addition of Ptb enzyme (5–10 nM final concentration). The reaction was incubated at 25 °C for 10 minutes and 50 μl of the assay solution was plunged into a microtiter plate well containing 80 μL of “ice-cold” stopping solution (0.5 M HCl, 0.129 g ascorbic acid and 0.2 ml of freshly prepared 10% ammonium molybdate). After incubation on ice for 10 minutes, 120 μl of ACG (Arsenite, Citrate, Glacial acetic acid) solution (150 mM sodium m-arsenite, 70 mM sodium citrate, 350 mM acetic acid) was added and incubated for 5 minutes at 37 °C. The resultant blue color was quantified via absorbance at 800 nm. Inorganic phosphate (100 – 500 μM) was utilized to build a standard curve. Data were plotted as a function of butyryl phosphate concentration and fitted to the Michaelis-Menten equation to determine kinetic constants.

3 Results

3.1 Purification of Ptb

Ptb was purified by its N-terminal His-tag through Ni^{2+}- NTA affinity purification as described in Methods. Loss of catalytic activity due to precipitation of purified Ptb was observed at concentrations ranging from 1–6 mg/ml in 50 mM Tris (pH 7.5) and in 25 mM HEPES (pH 7.5) buffers. Similar problems with stabilization of purified Ptb from *Clostridium beijerinckii* have been reported by Thompson and Chen [30], which were resolved by storage in high NaCl concentrations (1.3–3M). Ptb from *L. monocytogenes* could be stored for two weeks without detectable loss of activity by dilution of eluted protein to < 0.5 mg/ml in HEPES buffer with 1.5 M NaCl. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis confirmed the calculated molecular mass of Ptb (~34 kDa) and its purity (Fig. 3).

3.2 Kinetic analysis of Ptb

3.2.1 Straight chain acyl CoA substrates—It was of interest to understand the substrate specificity of Ptb in order to determine if it could fulfill the role of activation of different carboxylic acids. To this end Ptb was assayed systematically with acyl CoA substrates differing in carbon chain length by one carbon (C2–C6). The data were fitted to the Michaelis –Menten equation (Fig. 4A) and the kinetic constants presented in Table 1. As shown in Table 1, the K_M of butyrate was 79.9 μM and was the lowest of all the SCCAs tested. This K_M value was similar to those previously reported for Ptb from other organisms [20,31]. The shortest and longest substrates tested, acetyl CoA (C2) and hexanoyl CoA (C6), proved to be poor substrates for Ptb judging by their poor fit to the Michaelis-Menten equation and low catalytic efficiency (k_{cat} / K_M) suggesting that the carbon chain length was suboptimal for efficient binding to the active site. The overall catalytic efficiency and the turnover number (k_{cat}) for this series of substrates suggested that increase/decrease in chain length from the optimal size of C4 (butyryl CoA-8.43 μM^{-1} sec^{-1}) resulted in a decrease in catalytic efficiency with a sharp drop with an increase/decrease of two carbons (C2–0.3 μM^{-1} $sec^{-1}$and C6- 1.7 μM^{-1} sec^{-1}) (Table 1). From these data, we concluded that the ideal chain length of substrates for Ptb was C3–C5.

Ptb was also assayed in the acyl CoA forming direction using butyryl phosphate. The K_M for butyryl phosphate was 1.04 mM, which is higher than the previously reported values [20], with a corresponding catalytic efficiency (k_{cat} / K_M) of 1.05 (Fig. 4B).

3.2.2 Branched-chain acyl CoA substrates—In order to understand the substrate preferences of Ptb in detail we also examined its activity with a variety of branched-chain substrates. Our measurements were limited by the commercial availability of branched-chain substrates (isobutyryl CoA and isovaleryl CoA) and the substrates that we could prepare (2-methylbutyryl CoA and 2-ethylbutyryl CoA). Nonetheless, our studies utilized enough substrate structural diversity to deduce critical features of the Ptb active site (Fig. 4C). Presence of an alkyl side chain improved the binding affinity of the substrate to Ptb as evidenced by the K_M values (Table 1). Additionally, the K_M values of the different branched-chain substrates were similar suggesting that the presence of the alkyl side chain likely produces critical contacts within the active site resulting in improved binding. Perhaps the presence of the side chain restricted freedom of movement of the substrate in the active site, and thus resulted in increased turnover numbers (k_{cat}) of the branched-chain substrates in comparison to straight-chain substrates of the same chain length (e.g. butyryl-CoA-673.9 sec−1, isovaleryl-CoA 1043.4 sec^{-1}, 2-methylbutyryl-CoA-849.1 sec^{-1}) (Table 1). Furthermore, binding affinity did not appear to be influenced by the branch position nor by the length of the side chain as demonstrated by the similar K_M values for isovaleryl-CoA (β-position) and 2-methylbutyryl-CoA (α-position) and 2-ethylbutyryl-CoA (ethyl branch in the α-position) (Table 1). However, the increase in side chain length was found to be associated with lower k_{cat} values suggesting a decrease in product formation. Analysis of our experimental data (Table 1 & 2) indicated a strong preference for substrates with an alkyl side chain.

3.2.3. Inorganic phosphate—The kinetic constants of Ptb with respect to inorganic phosphate were determined at excess isobutyryl CoA, isovaleryl-CoA and propionyl CoA concentrations (200 μM) based on curve fitting to the Hill equation (Fig. 5A and 5B). Others have reported that high concentrations of inorganic phosphate can inhibit Ptb from some other bacteria [30]. It has also been reported that K_M values for phosphate can differ with various acyl-CoA substrates [30]. Consequently, we examined the phosphate parameters more closely in *L. monocytogenes* Ptb. We did not observe any inhibition by increasing concentrations of inorganic phosphate in our experiments.

When the specific activity of Ptb was plotted as a function of phosphate concentration (Fig. 5A. and 5B), product formation was minimal at low phosphate concentrations and an increase in activity was observed with concurrent increase in phosphate concentration (>1mM) until a maximum was obtained at about 15–20 mM of phosphate. The sigmoidal nature of the curve was readily apparent only at low concentrations of phosphate, thus confirming positive cooperativity, and curve fitting of the data to the Hill equation ($v = \mathrm{Vmax}\cdot [S]^n / K+[S]^n$, where n is the Hill coefficient) allowed the estimation of the parameters for binding of phosphate to Ptb (Fig. 5A. and 5B). The apparent K_M for phosphate when isobutyryl CoA was utilized as the cosubstrate was found to be 5.35 ± 0.55 mM with a Hill coefficient of 2.11± 0.37 and comparable parameters were obtained with isovaleryl CoA

(K_M - 2.78 mM) as the second substrate. However, the apparent K_M was found to be higher when the second substrate utilized was not a preferred substrate, as in the case of propionyl CoA (K_M =16.4 mM), a C3 compound which lacks a methyl branch unlike isobutyryl CoA and isovaleryl CoA.

3.3 Mechanism of action of Ptb

The initial rates of Ptb were further analyzed at varying concentrations of isovaleryl CoA in the presence of different fixed concentrations of inorganic phosphate (10, 25 and 100 mM). These data were used to determine the binding mechanism of Ptb which utilized the two substrates, acyl CoA and inorganic phosphate, with the formation of two products, CoA and acyl phosphate. Experiments were conducted to determine whether Ptb catalysis followed the pingpong model or required ternary complex formation. Data were normalized to the k_{cat} of isovaleryl CoA at 100 mM inorganic phosphate and fitted to a double reciprocal plot (Fig. 6). The double reciprocal fit of the data yielded lines intersecting at a single point on the Y-axis and was consistent with ternary complex formation rather than a ping-pong mechanism. The intersection of the lines at a single point on the Y-axis confirmed the mechanism to be that of rapid equilibrium ordered mechanism as outlined schematically in Fig. 7 [32]. Furthermore, Scheme 1 describing a simple ternary complex mechanism of action was mathematically simplified to the expression in equation 1 where x is the concentration of the varying substrate (isobutyryl CoA) and S is the concentration of the fixed substrate (inorganic phosphate). The initial rate data were fitted globally to equation 1 using the graphing software OriginPro (OriginLab, Northampton, MA) which confirmed the mechanism of action to involve ternary complex formation.

$$v = \frac{AxS}{(xS+B)} \quad \text{Equation 1}$$

4. Discussion

4.1 Substrate specificity of Ptb

The high membrane BCFA content of *L. monocytogenes* 10403S is ensured due to the strong affinity of its FabH enzyme towards the precursors of BCFAs [33]. Although the membrane fatty acid composition of the wild-type organism is affected only by high external concentrations of propionate, butyrate and certain unnatural BCCAs, the *bkd* mutant MOR401(*cld-2*) is more susceptible to manipulation by supplementation of the media by various carboxylic acids [10,13]. Sen *et al.* [13] showed that in addition to the natural products of amino acid catabolism, certain BCCAs with similar structural characteristics such as 2-ethylbutyrate and 2-methylpentanoate also cause significant alteration of the membrane fatty acid composition to reflect the incorporation of the fatty acids resulting from the elongation of the added BCCAs. These studies strongly indicate the presence of a bypass pathway forming CoA thioesters of the BCCAs and SCCAs for entry into the bacterial FAS II system. Ptb and Buk, whose activities resemble that of phosphotransacetylase (*pta*) and acetate kinase (*ack*) respectively, were concluded to be the most likely candidates due to their reported ability to utilize multiple substrates in other

organisms, reversibility of their catalytic activity, and their location in the *bkd* operon [18,20,34,35].

Our investigation into the kinetic characteristics of Ptb revealed its substantial activity with a wide range of acyl CoA substrates thus supporting our hypothesis. The substrates that were studied differed in their chain length and also in the presence and location of alkyl side chains. The broad substrate specificity of the enzyme is in agreement with the available data on Ptb from *C. acetobutylicum* and Ptb from *E. faecalis* [16,20]. Although Ptb has been studied in these organisms, the scope of these investigations has been limited to exploration of its activity with respect to the secreted products, such as butyrate in case of *C. acetobutylicum*, and the end products of BCAA catabolism in *E. faecalis,* with the inclusion of a few closely related compounds [16,20,22]. *L. monocytogenes* does not secrete these products under either aerobic or anaerobic growth conditions in defined medium according to a report by Romick et al. [36]. Additionally, a relationship between the activities of the *bkd* operon and membrane fatty acid composition could not be established in *E. faecalis* although 21% of the membrane fatty acids were BCFAs [16]. In contrast, Ptb from *L. monocytogenes* demonstrates considerable activity with the various substrates which have been previously demonstrated to influence the membrane fatty acid composition thus making it a likely candidate in the activation of exogenous carboxylic acids. Such a relationship between the activity of Ptb and the membrane composition has not been reported thus far.

4.2. Ptb exhibits chain length specificity

Although Ptb demonstrated broad substrate specificity, the activity is limited to substrates having a carbon chain length of C3–C5. Negligible activity with acetyl CoA as the substrate indicates that Ptb is distinct from Pta and that it is not involved in acetyl CoA metabolism. These data agree with previously published reports on Ptb from *C. acetobutylicum,* which likewise demonstrated no activity with acetyl CoA as the substrate, and the fact that addition of acetate into the medium does not cause any change in the membrane fatty acid composition [10,20,22]. Long chain fatty acids such as C14:0, C16:0 and C18:1 and addition of monolaurin and bile salts to the medium cause alteration of the membrane fatty acids to reflect the added substrates in *L. monocytogenes* [37–39]. While such fatty acids are incorporated into the membrane, their activation by Ptb is unlikely since a drastic drop in catalytic efficiency was exhibited by Ptb with hexanoyl CoA (C6) as its substrate when compared with its activity with pentanoyl CoA (C5) (Fig. 4A, Table 1 & 2). Recent reports by Parsons *et al.* [40,41] revealed a novel pathway involving the activation of long chain fatty acids by means of phosphorylation by a fatty acid kinase (Fak) thereby resulting in the production of fatty acyl phosphate. This pathway appears to be a more likely mechanism for activation of long chain fatty acids.

It is noteworthy that Ptb exhibited the highest affinity towards butyryl CoA among the straight chain substrates, and also exhibits significant product formation in the butyryl CoA forming direction (Fig. 4B). SCCAs, mainly acetate, propionate and butyrate, make up the majority of the byproducts of anaerobic metabolism by the mammalian gut commensal bacteria and *L. monocytogenes* is exposed to these carboxylic acids during infection [42].

Exposure to butyrate results in reduced expression of virulence genes in *L. monocytogenes* and *Salmonella enterica* Enteriditis thus indicating its role of a signal molecule [43,44]. The role of butyrate as a signal molecule is also apparent in Enterohemorrhagic *E. coli* (EHEC) in which it causes an upregulation of the virulence genes and increased motility [45]. Moreover, increased expression of solvent producing and stationary phase genes coinciding with a butyryl phosphate peak has been reported in *C. acetobutylicum*. Butyryl phosphate is hypothesized to behave as a small molecule phosphate donor similar to acetyl phosphate in *E. coli* in its role as a global gene regulator in *C. acetobutylicum* [46]. Additionally, high concentrations of butyrate result in growth inhibition due to incorporation of SCFAs in the membrane of *L. monocytogenes*, a property that could be used to aid in control of the organism [10,13]. Butyrate has been used as a feed additive to aid in the control *S. enterica* Enteriditis in turkeys and chicken [47,48].

4.3. Ptb demonstrates preference for branched-chain substrates

Ptb from *L. monocytogenes* preferred branched-chain CoA substrates, especially the natural degradation products of the branched-chain amino acids. The order of catalytic efficiency of Ptb in decreasing order was isobutyryl CoA > isovaleryl CoA > 2-methylbutyryl CoA > 2-ethylbutyryl CoA (Table 1). Such distinct preference of Ptb towards branched-chain substrates has not been established in any organism thus far. Ward et al. [16] showed that Ptb was involved in the catabolism of branched-chain amino acids by determining its activity with various substrates compared to that of butyrate. However, the kinetic constants were not determined for the other related acyl CoA substrates. FabH has been reported to prefer BCFA substrates in the order 2-methylbutyryl CoA > isovaleryl CoA > isobutyryl CoA at 30 °C in *L. monocytogenes* [33]. The substrate specificity of Ptb is unlike that of Bkd, the normal source of the intracellular acyl CoA pool, whose preference is α-keto methylvalerate > α-keto isovalerate > α-keto isocaproate and results in the formation of 2-methylbutyryl CoA, isobutyryl CoA and isovaleryl CoA respectively in *B. subtilis* [6]. This variation in the substrate specificities of the two enzymes may help explain the lack of restoration of the membrane fatty acid composition in the *bkd* mutant in *L. monocytogenes* (*cld-2*), to that of the wild type organism, despite the presence of equal amounts of the three BCCAs [7]. In other words, the significant branched-chain acyl CoA product formation by Ptb due to exogenous supplementation could outcompete the endogenous substrates for FabH and the substrate preference of Ptb could be reflected in the membrane fatty acid composition.

4.4. Ptb activity exhibits positive cooperativity with phosphate

Ptb from *L. monocytogenes* displays positive cooperativity with respect to phosphate in the absence of limiting conditions of acyl CoA substrate and is also not inhibited by increasing concentrations of phosphate. Wiesenborn et al. [20] investigated the binding of phosphate by Ptb from *C. acetobutylicum* and reported non-competitive inhibition at high concentrations of phosphate in the presence of excess butyryl CoA. Similar studies by Thompson and Chen [30] on Ptb from *C. beijerinckii* showed higher K_M values for phosphate when the cosubstrate was changed from butyryl CoA to a weaker substrate namely acetoacetyl CoA. Both the studies utilized a minimum concentration of 5 mM for phosphate for their kinetic investigations, which precluded a demonstration of positive cooperativity by the enzyme. We investigated Ptb from *L. monocytogenes* using a minimum concentration of 50 μM to a

maximum of 25 mM which allowed the observation of sigmoidal behavior by the enzyme, a confirmation of positive cooperativity. Although binding of phosphate molecules at more than one site was offered as an explanation for the nonlinearity of the double reciprocal plots by Wiesenborn et al. [20], our studies suggest that binding of the acyl CoA molecule likely influences the binding of phosphate within the active site. Thus binding of an optimal substrate, such as isobutyryl CoA, was accompanied by a lower K_M for phosphate, indicating higher affinity, whereas binding of a substrate that is suboptimal, such as propionyl CoA, resulted in a higher K_M for phosphate.

4.5. Ptb activity involves ternary complex formation

The mechanism of action of Ptb was confirmed to be that of rapid equilibrium ordered mechanism (Fig. 7) by the global fit of the initial rate data to the mathematical expression corresponding to the ternary complex formation or random Bi Bi binding scheme (Equation 1). This was similar to the mechanism determined for Ptb in *C. acetobutylicum* in which the data fit to a family of parabolic curves but was still not consistent with the fit for the ping pong mechanism. Formation of the ternary complex in phosphate acetyltransferase (*pta*) has been demonstrated in *Clostridium kluyveri* and also in the thermophilic archaeon *Methanosarcina thermophila* [34,49,50]. However, the double reciprocal plot of the data in *M. thermophila* shows lines intersecting to the left of the Y-axis and is unlike that of Ptb which shows the lines intersecting on a single point on the Y-axis [49]. Convergence of the lines to a point on the Y axis suggests the mechanism of action to be that of random Bi Bi and rapid equilibrium ordered mechanism wherein the enzyme forms a complex with both the substrates before the formation of products by chemical alteration [32]. This is the first report of the mechanism of action of Ptb from *L. monocytogenes*.

4.5. Conclusions

Analysis of Ptb from *L. monocytogenes* reveals that it is active with a large number of substrates and prefers substrates with a carbon chain length of C3-C5 and the presence of an alkyl side chain. Activity with natural SCCAs and BCCAs and also with unnatural substrates such as 2-ethyl butyrate, that are capable of altering the membrane fatty acid composition, strongly indicates that Ptb is the probable candidate for the alternate pathway for primer production for fatty acid biosynthesis. Butyrate is likely involved in regulation of gene expression since exposure to butyrate leads to decreased expression of virulence genes in *L. monocytogenes*. Additionally, high concentrations of butyrate also lead to diminished growth of *L. monocytogenes* suggesting a possible control strategy for the organism. The high activity of Ptb with butyryl CoA indicates that it could be involved in signal processing upon exposure of *L. monocytogenes* to butyrate.

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References

1. Walker SJ, Archer P, Banks JG. Growth of *Listeria monocytogenes* at refrigeration temperatures. J. Appl. Bacteriol. 1990; 68:157–162. [PubMed: 2108109]
2. Thomas MK, Vriezen R, Farber JM, Currie A, Schlech W, Fazil A. Economic cost of a *Listeria monocytogenes* outbreak in Canada, 2008. Foodborne Pathog. Dis. 2015; 12 fpd.2015.1965.
3. Annous BA, Becker LA, Bayles DO, Labeda DP, Wilkinson BJ. Critical role of anteiso-C15:0 fatty acid in the growth of *Listeria monocytogenes* at low temperatures. Appl. Environ. Microbiol. 1997; 63:3887–3894. [PubMed: 9327552]
4. Mastronicolis SK, Arvanitis N, Karaliota A, Magiatis P, Heropoulos G, Litos C, Moustaka H, Tsakirakis A, Paramera E, Papastavrou P. Coordinated regulation of coldinduced changes in fatty acids with cardiolipin and phosphatidylglycerol composition among phospholipid species for the food pathogen *Listeria monocytogenes*. Appl. Environ. Microbiol. 2008; 74:4543–4549. [PubMed: 18502923]
5. Zhu K, Ding X, Julotok M, Wilkinson BJ. Exogenous isoleucine and fatty acid shortening ensure the high content of anteiso-C15:0 fatty acid required for low-temperature growth of *Listeria monocytogenes*. Appl. Environ. Microbiol. 2005; 71:8002–8007. [PubMed: 16332779]
6. Oku H, Kaneda T. Biosynthesis of branched-chain fatty acids in *Bacillus subtilis*: A decarboxylase is essential for branched-chain fatty acid synthetase. J. Biol. Chem. 1988; 263:18386–18396. [PubMed: 3142877]
7. Zhu K, Bayles DO, Xiong A, Jayaswal RK, Wilkinson BJ. Precursor and temperature modulation of fatty acid composition and growth of *Listeria monocytogenes* cold-sensitive mutants with transposon-interrupted branched-chain alpha-keto acid dehydrogenase. Microbiology. 2005; 151:615–623. [PubMed: 15699210]
8. Sun Y, O'Riordan MXD. Branched-chain fatty acids promote *Listeria monocytogenes* intracellular infection and virulence. Infect. Immun. 2010; 78:4667–4673. [PubMed: 20823206]
9. Singh VK, Hattangady DS, Giotis ES, Singh AK, Chamberlain NR, Stuart MK, Wilkinson BJ. Insertional inactivation of branched-chain alpha-keto acid dehydrogenase in *Staphylococcus aureus* leads to decreased branched-chain membrane fatty acid content and increased susceptibility to certain stresses. Appl. Environ. Microbiol. 2008; 74:5882–5890. [PubMed: 18689519]
10. Julotok M, Singh AK, Gatto C, Wilkinson BJ. Influence of fatty acid precursors, including food preservatives, on the growth and fatty acid composition of *Listeria monocytogenes* at 37 and 10 °C. Appl. Environ. Microbiol. 2010; 76:1423–1432. [PubMed: 20048057]
11. Willecke K, Pardee AB. Fatty acid-requiring mutant of *Bacillus subtilis* defective in branched chain alpha-keto acid dehydrogenase. J. Biol. Chem. 1971; 246:5264–5272. [PubMed: 4999353]
12. Kaneda T. Incorporation of branched-chain C6 -fatty acid isomers into the related long-chain fatty acids by growing cells of *Bacillus subtilis*. Biochemistry. 1970; 10:340–347. [PubMed: 4992629]
13. Sen S, Sirobhushanam S, Hantak MP, Lawrence P, Thomas Brenna J, Gatto C, Wilkinson BJ. Short branched-chain C6 carboxylic acids result in increased growth, novel "unnatural" fatty acids and increased membrane fluidity in a *Listeria monocytogenes* branched-chain fatty acid-deficient mutant. Biochim. Biophys. Acta. 2015; 1851:1406–1415. [PubMed: 26225744]
14. Debarbouille M, Gardan R, Arnaud M, Rapoport G. Role of *bkdR*, a transcriptional activator of the *sigL*-dependent isoleucine and valine degradation pathway in *Bacillus subtilis*. J. Bacteriol. 1999; 181:2059–2066. [PubMed: 10094682]
15. Nelson KE, Fouts DE, Mongodin EF, Ravel J, DeBoy RT, Kolonay JF, Rasko DA, Angiuoli SV, Gill SR, Paulsen IT, Peterson J, White O, Nelson WC, Nierman W, Beanan MJ, Brinkac LM, Daugherty SC, Dodson RJ, Durkin AS, Madupu R, et al. Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species. Nucleic Acids Res. 2004; 32:2386–2395. [PubMed: 15115801]
16. Ward DE, Ross RP, van der Weijden CC, Snoep JL, Claiborne A. Catabolism of branched-chain alpha-keto acids in *Enterococcus faecalis*: the *bkd* gene cluster, enzymes, and metabolic route. J. Bacteriol. 1999; 181:5433–5442. [PubMed: 10464218]

17. Walter KA, Nair RV, Cary JW, Bennett GN, Papoutsakis ET. Sequence and arrangement of two genes of the butyrate-synthesis pathway of *Clostridium acetobutylicum* ATCC 824. Gene. 1993; 134:107–111. [PubMed: 8244020]
18. Hartmanis MGN. Butyrate kinase from *Clostridium acetobutylicum*. J. Biol. Chem. 1987; 262:617–621. [PubMed: 3027059]
19. Harwood CS, Canale-Parola E. Properties of acetate kinase isozymes and a branchedchain fatty acid kinase from a Spirochete. J. Bacteriol. 1982; 152:246–254. [PubMed: 6288660]
20. Wiesenborn DP, Rudolph FB, Papoutsakis ET. Phosphotransbutyrylase from *Clostridium acetobutylicum* ATCC 824 and its role in acidogenesis. Appl. Environ. Microbiol. 1989; 55:317–322. [PubMed: 2719475]
21. Cary JW, Petersen DJ, Papoutsakis ET, Bennett GN. Cloning and expression of *Clostridium acetobutylicum* phosphotransbutyrylase and butyrate kinase genes in *Escherichia coli*. J. Bacteriol. 1988; 170:4613–4618. [PubMed: 2844725]
22. Hartmanis MGN, Gatenbeck S. Intermediary metabolism in *Clostridium acetobutylicum* : Levels of enzymes involved in the formation of acetate and butyrate. Appl. Environ. Microbiol. 1984; 47:1277–1283. [PubMed: 16346566]
23. Stadtman ER. Preparation and assay of acyl coenzyme A and other thiol esters; use of hydroxylamine. Methods Enzymol. 1957; 3:931–941.
24. Toennies G, Kolb JJ. Techniques and reagents for paper chromatography. Anal. Chem. 1951; 23:823–826.
25. Lipmann F, Tuttle LC. A specific micromethod for the determination of acyl phosphates. J. Biol. Chem. 1945; 159:21–28.
26. Klotzsch HR. Phosphotransacetylase from *Clostridium kluyveri*. Methods Enzymol. 1969; 13:381–386.
27. Ellman GL. Tissue sulfhydryl groups. Arch. Biochem. Biophys. 1959; 82:70–77. [PubMed: 13650640]
28. Helms JB, Saunders LP, Meyer J, Costa CJ, Plowman E, Williford N, Corbitt M, Holden JP, Gatto C. Kinetic characterization of Na, K-ATPase inhibition by the acetaminophen metabolite. Open J. Mol. Integr. Physiol. 2015; 5:1–17.
29. Brotherus JR, Moller JV, Jorgensen PL. Soluble and active renal Na,K-ATPase with maximum protein molecular mass 170,000 + 9,000 daltons; Formation of larger units by secondary aggregation. Biochem. Biophys. Res. Commun. 1981; 100:146–154. [PubMed: 6266398]
30. Thompson DK, Chen JS. Purification and properties of an acetoacetyl coenzyme Areacting phosphotransbutyrylase from *Clostridium beijerinckii* ("*Clostridium butylicum*") NRRL B593. Appl. Environ. Microbiol. 1990; 56:607–613. [PubMed: 2317039]
31. Aboulnaga E-HH, Pinkenburg O, Schiffels J, El-Refai A, Buckel W, Selmer T. Effect of an oxygen-tolerant bifurcating butyryl coenzyme A dehydrogenase/electron-transferring flavoprotein complex from *Clostridium difficile* on butyrate production in *Escherichia coli*. J. Bacteriol. 2013; 195:3704–3713. [PubMed: 23772070]
32. Segel, IH. Enzyme kinetics: behavior and analysis of rapid equilibrium and steady-state enzyme systems. New York: John Wiley Sons; 1993.
33. Singh AK, Zhang Y, Zhu K, Subramanian C, Li Z, Jayaswal K, Gatto C, Rock CO, Wilkinson BJ. FabH selectivity for anteiso branched-chain fatty acid precursors in low temperature adaptation in *Listeria monocytogenes*. FEMS Microbiol. Lett. 2009; 301:1–8. [PubMed: 19811540]
34. Bergmeyer HU, Holz G, Klotzch H, Lang G. Phosphotransacetylase from *Clostridium kluyveri.* Culture of the bacterium, isolation, crystallization and properties of the enzyme. Biochem. Z. 1963; 338:114–21. [PubMed: 14087284]
35. Bock A, Glasemacher J, Schmidt R, Schönheit P, Glasemacher R. Purification and characterization of two extremely thermostable enzymes, phosphate acetyltransferase and acetate kinase , from the hyperthermophilic eubacterium *Thermotoga maritima*. J. Bacteriol. 1999; 181:1861–1867. [PubMed: 10074080]
36. Romick TL, Fleming HP, McFeeters RF. Aerobic and anaerobic metabolism of *Listeria monocytogenes* in defined glucose medium. Appl. Environ. Microbiol. 1996; 62:304–307. [PubMed: 8572709]

37. Juneja VK, Davidson PM. Influence of altered fatty acid composition on resistance of *Listeria monocytogenes* to antimicrobials. J. Food Prot. 1993; 56:302–305.
38. Tokarskyy O, Marshall DL. Mechanism of synergistic inhibition of *Listeria monocytogenes* growth by lactic acid, monolaurin, and nisin. Appl. Environ. Microbiol. 2008; 74:7126–7129. [PubMed: 18820062]
39. Gahan CGM, Hill C. *Listeria monocytogenes*: survival and adaptation in the gastrointestinal tract. Front. Cell. Infect. Microbiol. 2014; 4:9. [PubMed: 24551601]
40. Parsons JB, Broussard TC, Bose JL, Rosch JW, Jackson P, Subramanian C, Rock CO. Identification of a two-component fatty acid kinase responsible for host fatty acid incorporation by *Staphylococcus aureus*. Proc. Natl. Acad. Sci. 2014; 111:10532–10537. [PubMed: 25002480]
41. Parsons JB, Frank MW, Jackson P, Subramanian C, Rock CO. Incorporation of extracellular fatty acids by a fatty acid kinase-dependent pathway in *Staphylococcus aureus*. Mol. Microbiol. 2014; 92:234–245. [PubMed: 24673884]
42. Cummings JH, Pomare EW, Branch WJ, Naylor CP, Macfarlane GT. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut. 1987; 28:1221–1227. [PubMed: 3678950]
43. Sun Y, Wilkinson BJ, Standiford TJ, Akinbi HT, O'Riordan MXD. Fatty acids regulate stress resistance and virulence factor production for *Listeria monocytogenes*. J. Bacteriol. 2012; 194:5274–5284. [PubMed: 22843841]
44. Gantois I, Ducatelle R, Pasmans F, Haesebrouck F, Thompson A, Hinton JC, Van Immerseel F, Hautefort I. Butyrate specifically down-regulates *Salmonella* pathogenicity island 1 gene expression. Appl. Environ. Microbiol. 2006; 72:946–949. [PubMed: 16391141]
45. Tobe T, Nakanishi N, Sugimoto N. Activation of motility by sensing short-chain fatty acids via two steps in a flagellar gene regulatory cascade in enterohemorrhagic *Escherichia coli*. Infect. Immun. 2011; 79:1016–1024. [PubMed: 21149585]
46. Zhao Y, Tomas CA, Rudolph FB, Papoutsakis ET, Bennett GN. Intracellular butyryl phosphate and acetyl phosphate concentrations in *Clostridium acetobutylicum* and their implications for solvent formation. Appl. Environ. Microbiol. 2005; 71:530–537. [PubMed: 15640230]
47. Milbradt EL, Zamae JR, Araujo Junior JP, Mazza P, Padovani CR, Carvalho VR, Sanfelice C, Rodrigues DM, Okamoto AS, Andreatti Filho RL. Control of *Salmonella enteritidis* in turkeys using organic acids and competitive exclusion product. J. Appl. Microbiol. 2014; 117:554–563. [PubMed: 24797347]
48. Fernández-Rubio C, Ordóñez C, Abad-Gonzalez J, Garcia-Gallego A, Honrubia MP, Mallo JJ, Balaña-Fouce R, Fernandez-Rubio C, Ordonez C, Balana-Fouce R. Butyric acid-based feed additives help protect broiler chickens from *Salmonella enteritidis* infection. Poult. Sci. 2009; 88:943–948. [PubMed: 19359681]
49. Lawrence SH, Ferry JG. Steady-state kinetic analysis of phosphotransacetylase from *Methanosarcina thermophila*. 2006; 188:1155–1158.
50. Henkin J, Abeles RH. Evidence against an acyl-enzyme intermediate in the reaction catalyzed by *Clostridial* phosphotransacetylase. Biochemistry. 1976; 15:3472–3479. [PubMed: 952869]

Highlights

- Ptb from *L. monocytogenes* exhibited broad substrate specificity
- Ptb preferred substrates with a chain length of 3 to 5 carbons
- An alkyl side chain was required for higher catalytic efficiency
- Ptb could utilize unnatural substrates such as 2-ethyl butyryl CoA
- Ptb catalysis involved ternary complex formation

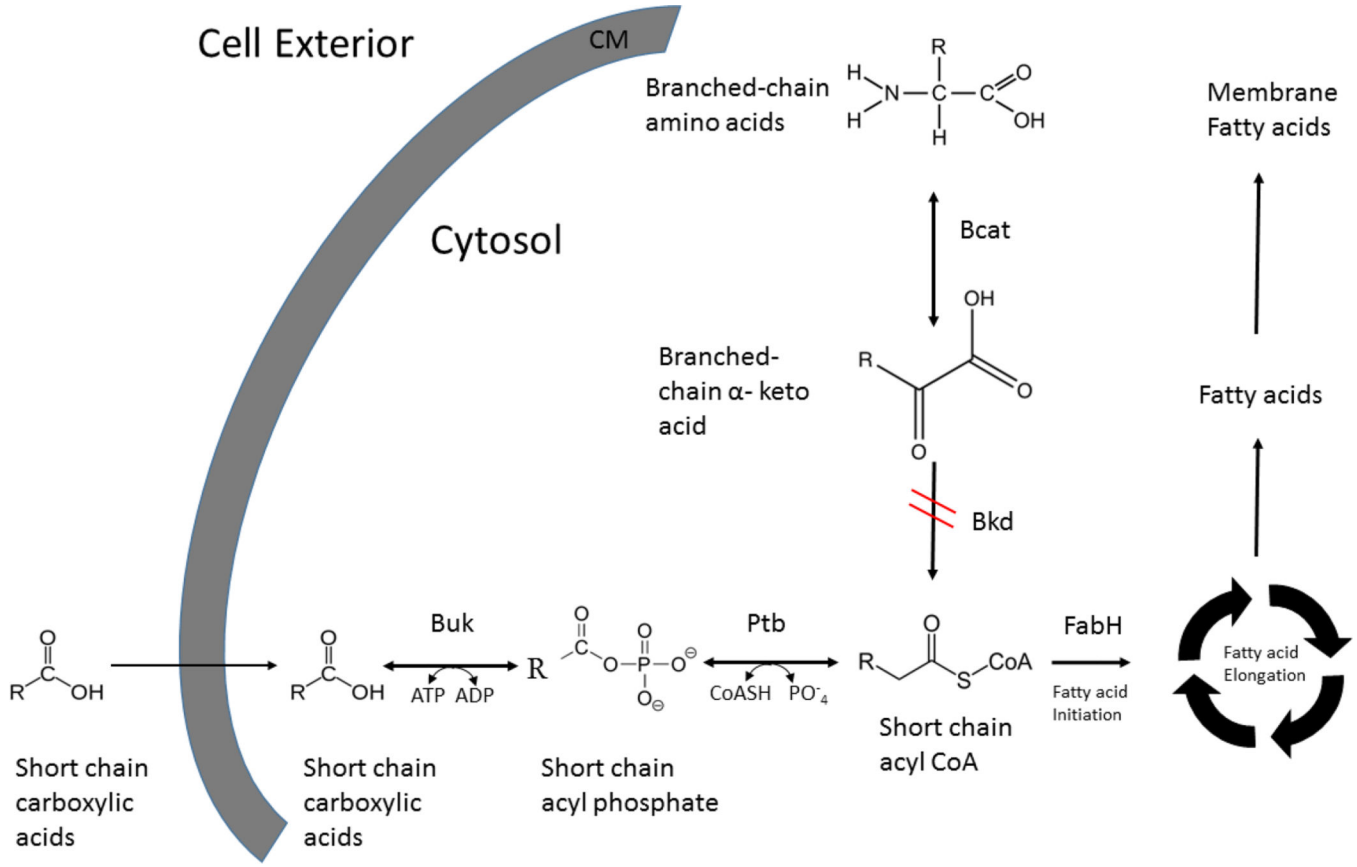


Fig. 1. Proposed alternative pathway for provision of acyl CoA precursors for fatty acid biosynthesis

Schematic showing the proposed sequential conversion of exogenous carboxylic acids into their acyl CoA derivatives by the activity of Buk and Ptb and their subsequent incorporation into membrane fatty acids. R-indicates SCCAs or BCCAs. Absence of functional Bkd allows greater incorporation of the products of the alternate pathway into the membrane fatty acids.

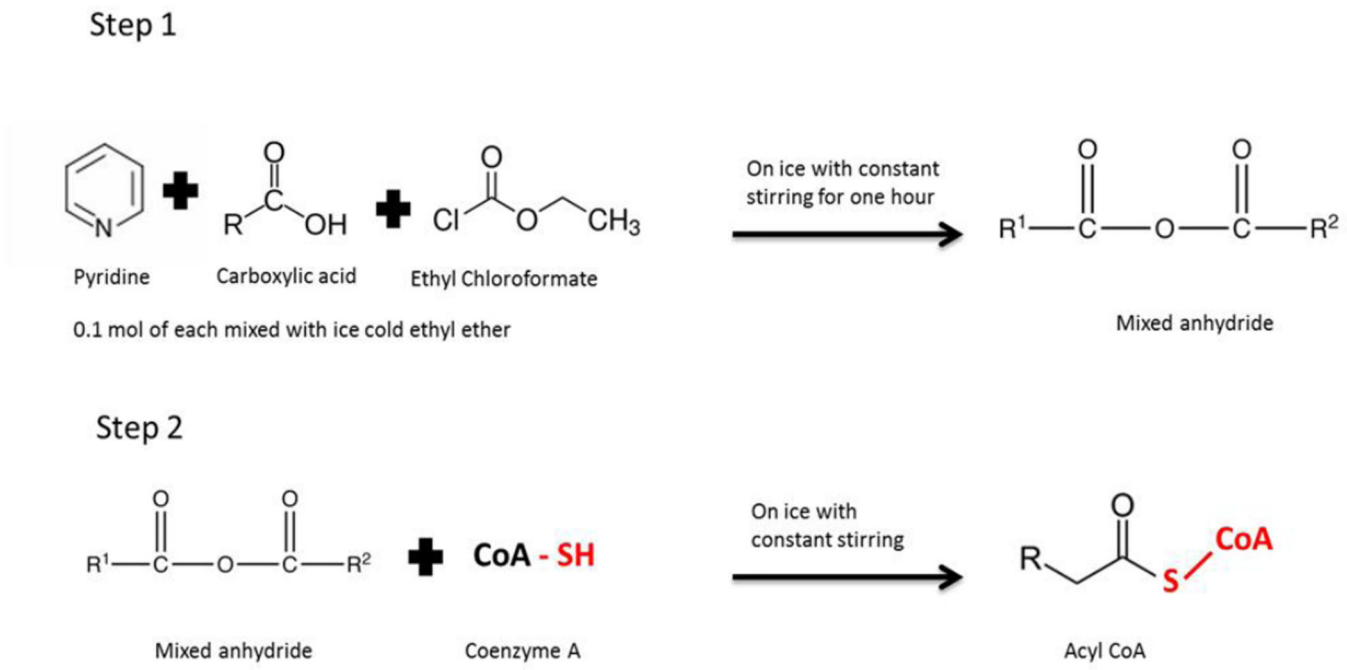


Fig. 2. Preparation of acyl CoA substrates

Step 1 shows the synthesis of mixed acyl anhydrides from the different carboxylic acids (R group-2-methylbutyrate and 2-ethylbutyrate). Step 2 shows the reaction of the mixed anhydride with an aqueous solution of CoA resulting in the formation of acyl CoA substrates according to the method outlined by Stadtman [23].

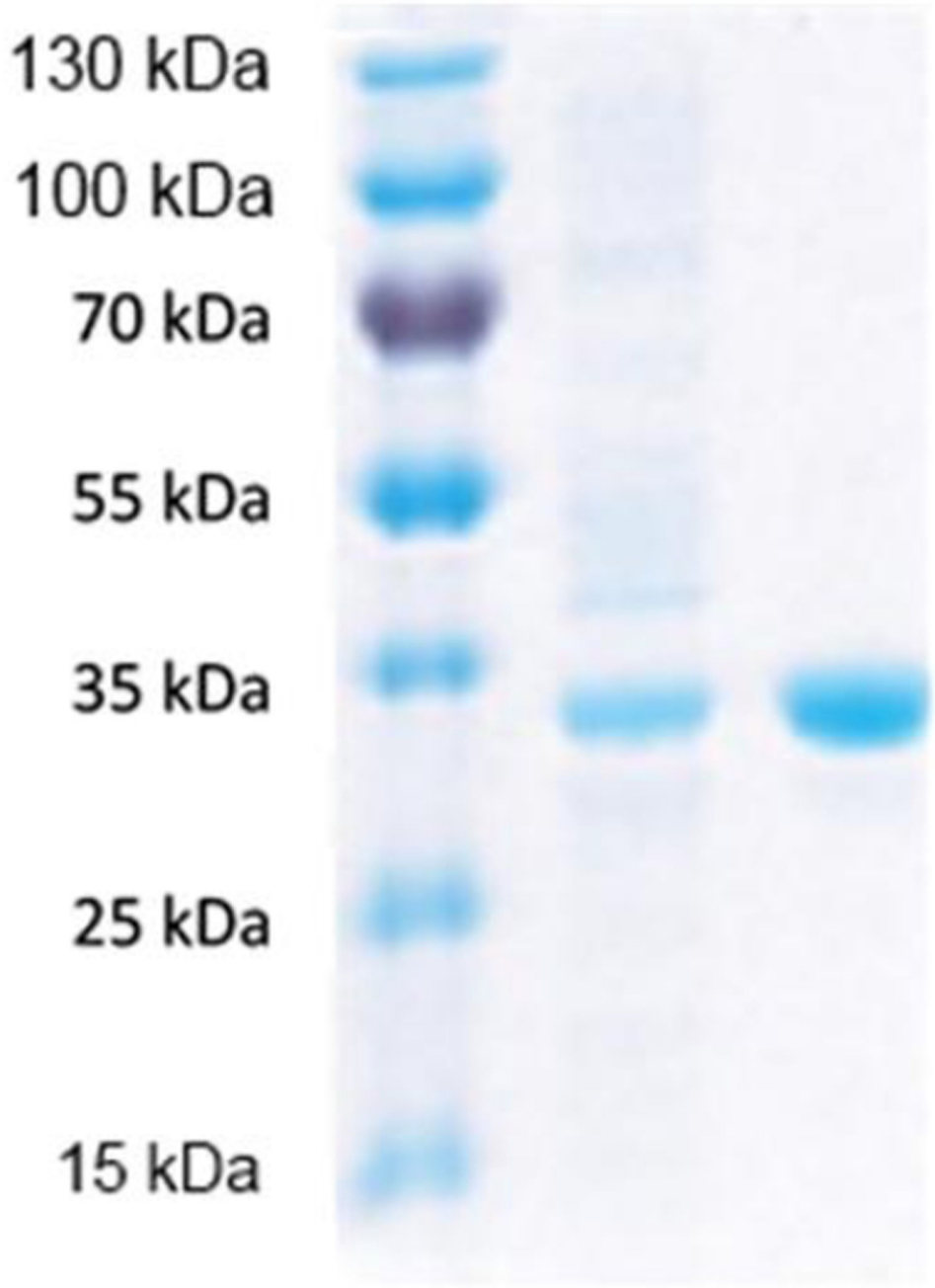
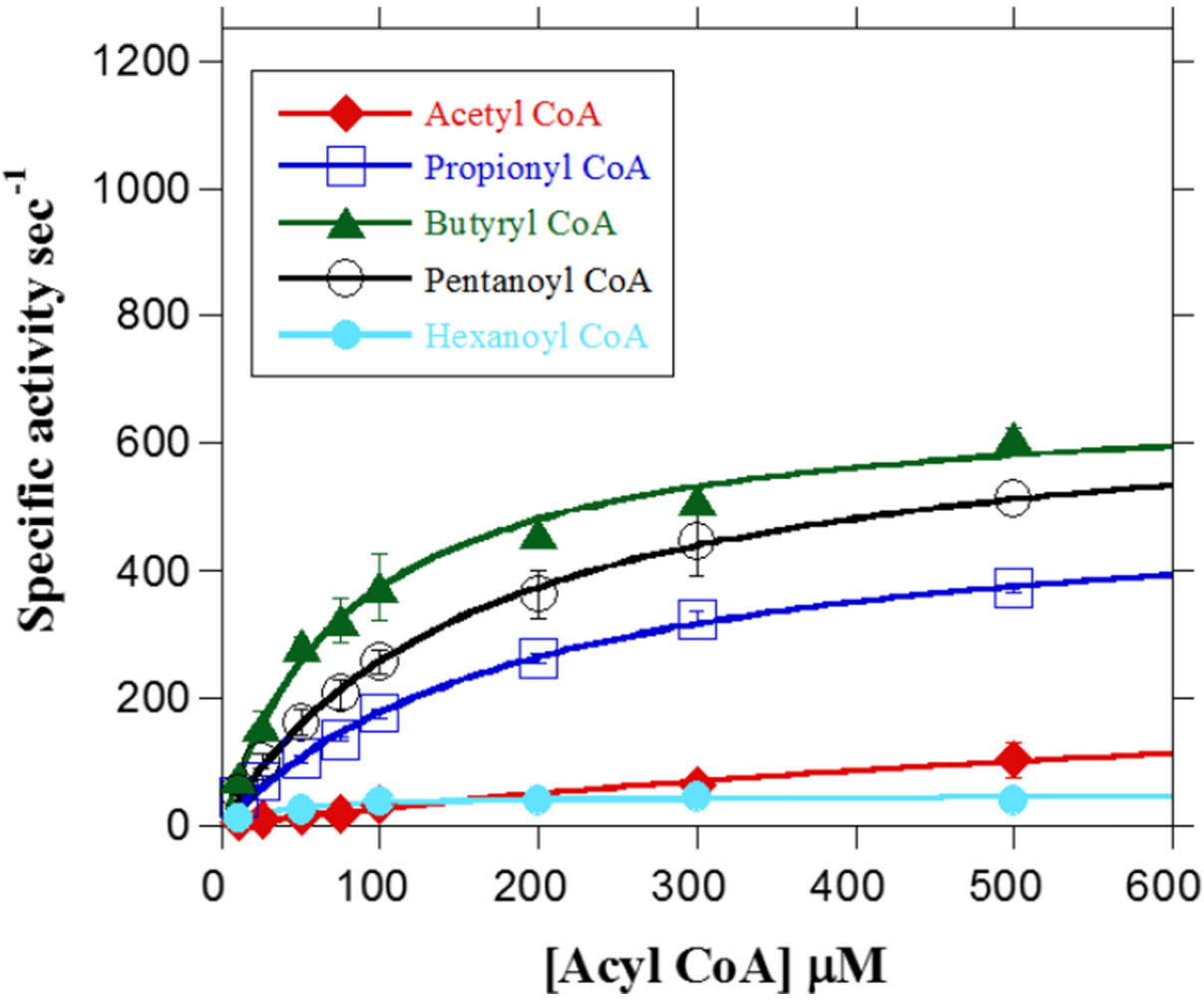


Fig. 3. SDS-PAGE analysis of purified preparation of Ptb

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 overexpression of Ptb, Lane 3 Recombinant Ptb after purification by Ni^{2+}- affinity chromatography.

A



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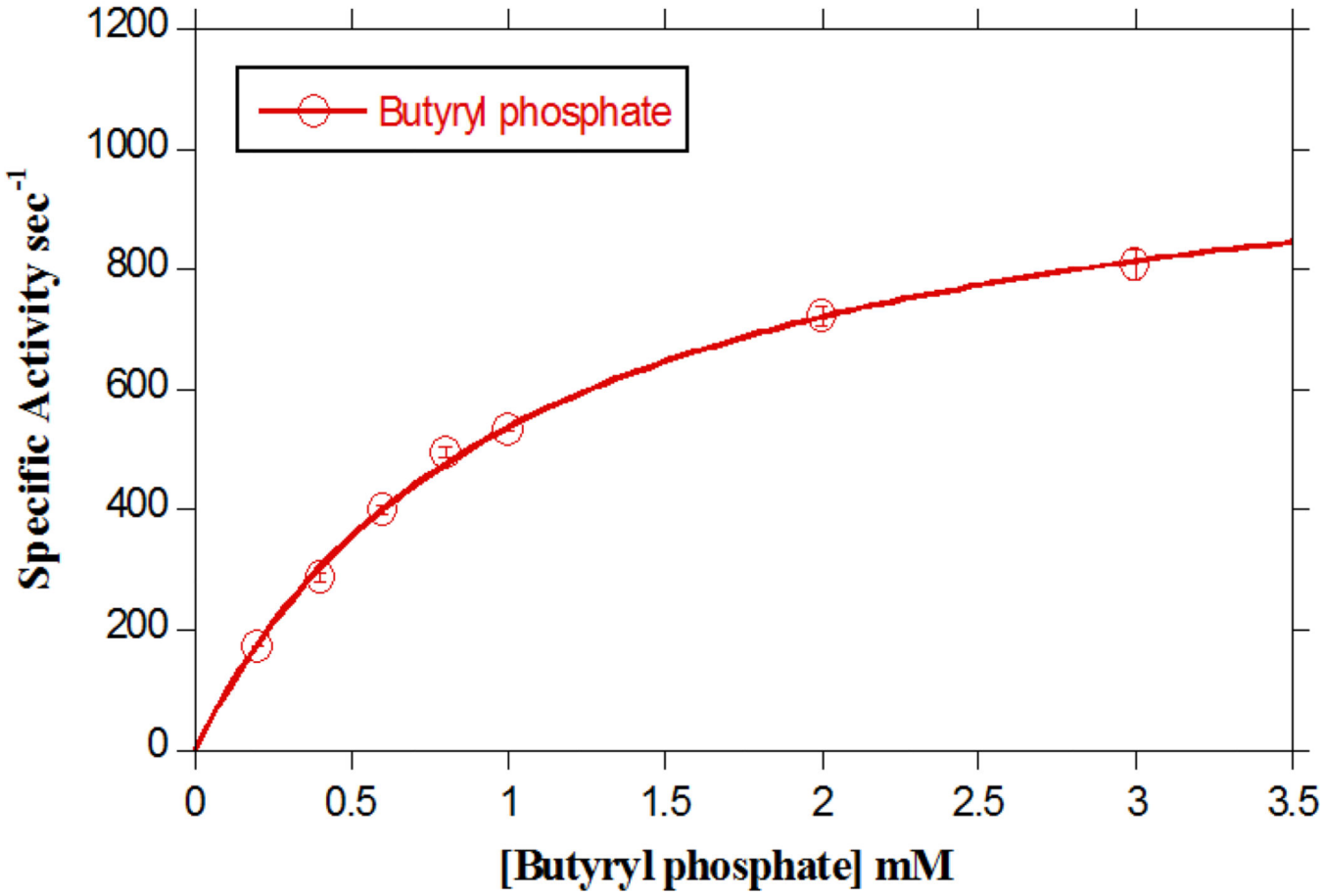

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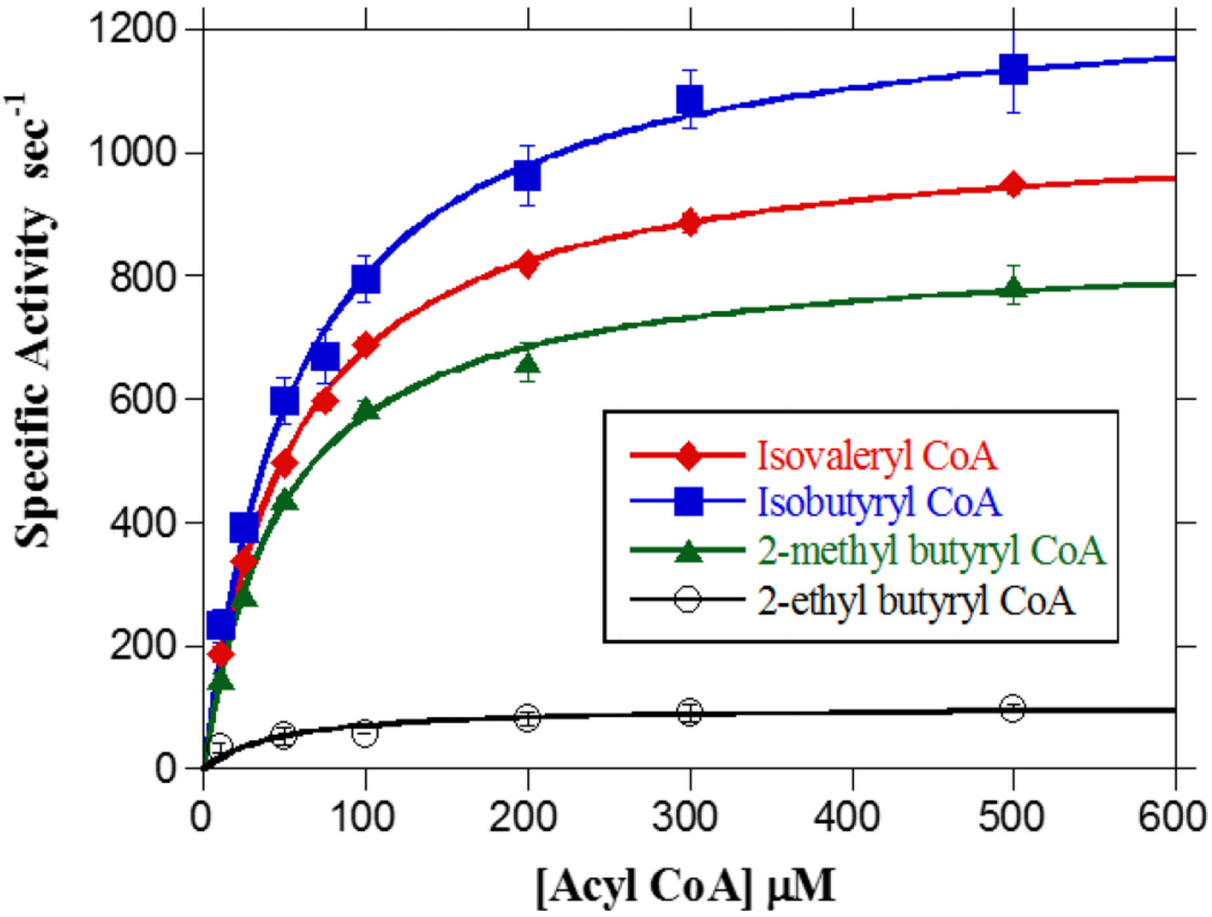


Fig. 4. Substrate preference of *Lm*Ptb

A. Concentration-dependence of straight-chain acyl CoA substrates on Ptb activity assayed in the in the acyl phosphate forming direction. Initial rates for Ptb utilization of the substrates acetyl CoA (closed diamonds), propionyl CoA (open squares), butyryl CoA (closed triangles) and pentanoyl CoA (open circles) and hexanoyl CoA (closed circles) were determined at 25 °C in the presence of 100 mM inorganic phosphate under standard assay conditions (see Materials and Methods). Data are plotted as a function of substrate concentration and K_M and k_{cat} were determined from fitting the data to the Michaelis-Menten equation using Kaleidagraph software. Data are means ± SEM of at least three experiments. **B**. Concentration-dependence of butyryl phosphate on Ptb activity assayed in the acyl CoA forming direction. Initial rates for Ptb utilization of butyryl phosphate (open

circles) were determined at 25 oC in the presence of 100 mM Tris and 500 μM CoA (see Materials and Methods). Data were plotted as a function of 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 ± SEM of three experiments. **C**. Concentration-dependence of branched-chain acyl CoA substrates on Ptb activity assayed in the in the acyl phosphate forming direction. Initial rates for Ptb utilization of the substrates isovaleryl CoA (closed diamonds), isobutyryl CoA (closed squares), 2-methyl butyryl CoA (closed triangles) and 2-ethyl butyryl CoA (open circles) were determined at 25 oC in the presence of 100 mM inorganic phosphate (see Materials and Methods). Data were plotted as a function of 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 ± SEM of at least three experiments.

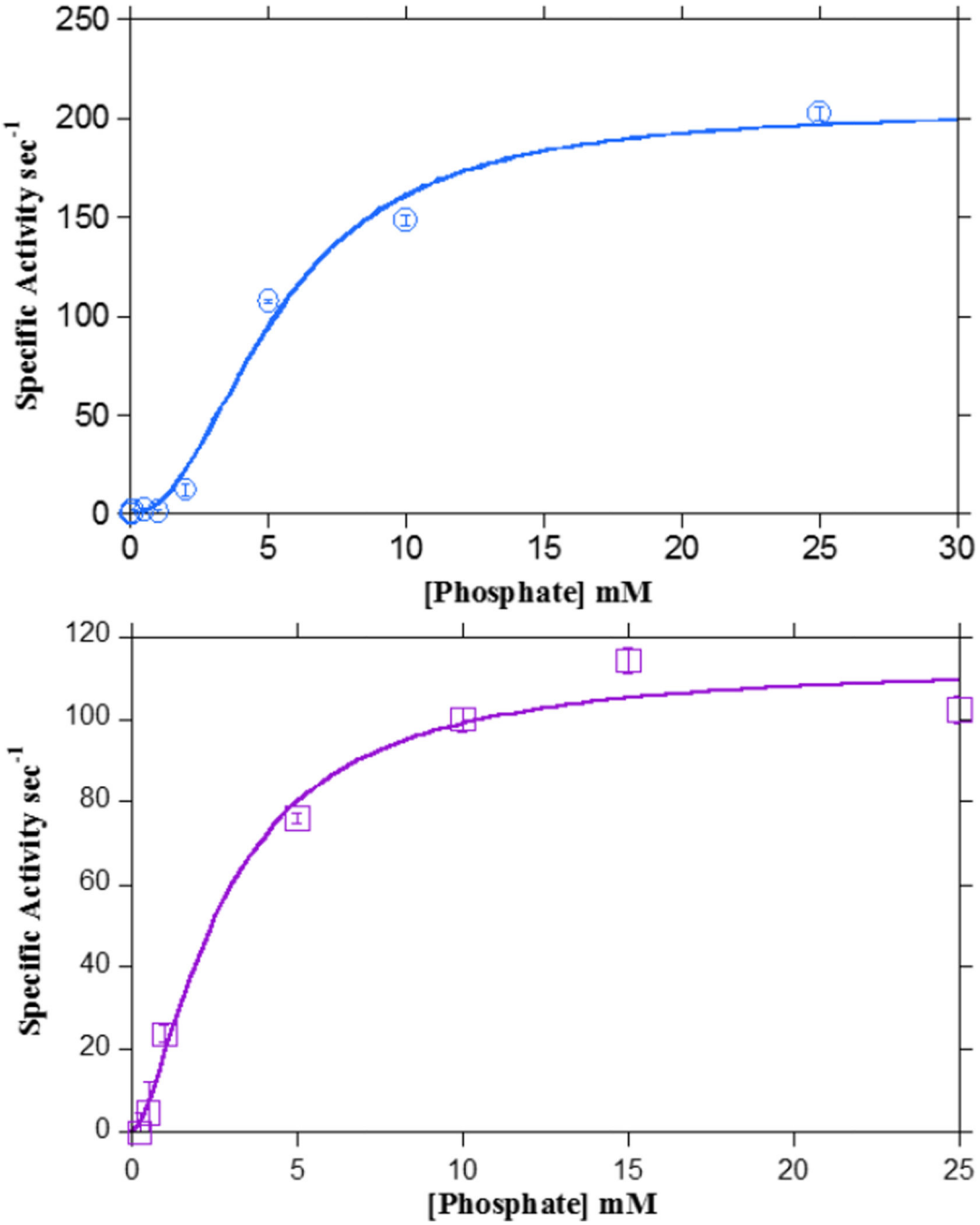


Fig. 5. Concentration dependence of phosphate utilization by *Lm*Ptb

Ptb activity was determined at 25oC in the presence of varying phosphate concentrations (10 μM – 25 mM) and excess **A)** isobutyryl CoA (200 μM) (open circles) or **B)** isovaleryl CoA (200 μM) (open squares). Data were fitted to the Hill equation using Kaleidagraph software providing values for K_M and k_{cat}. Means of data from at least three experiments ± SEM were plotted as reaction rate versus phosphate concentration.

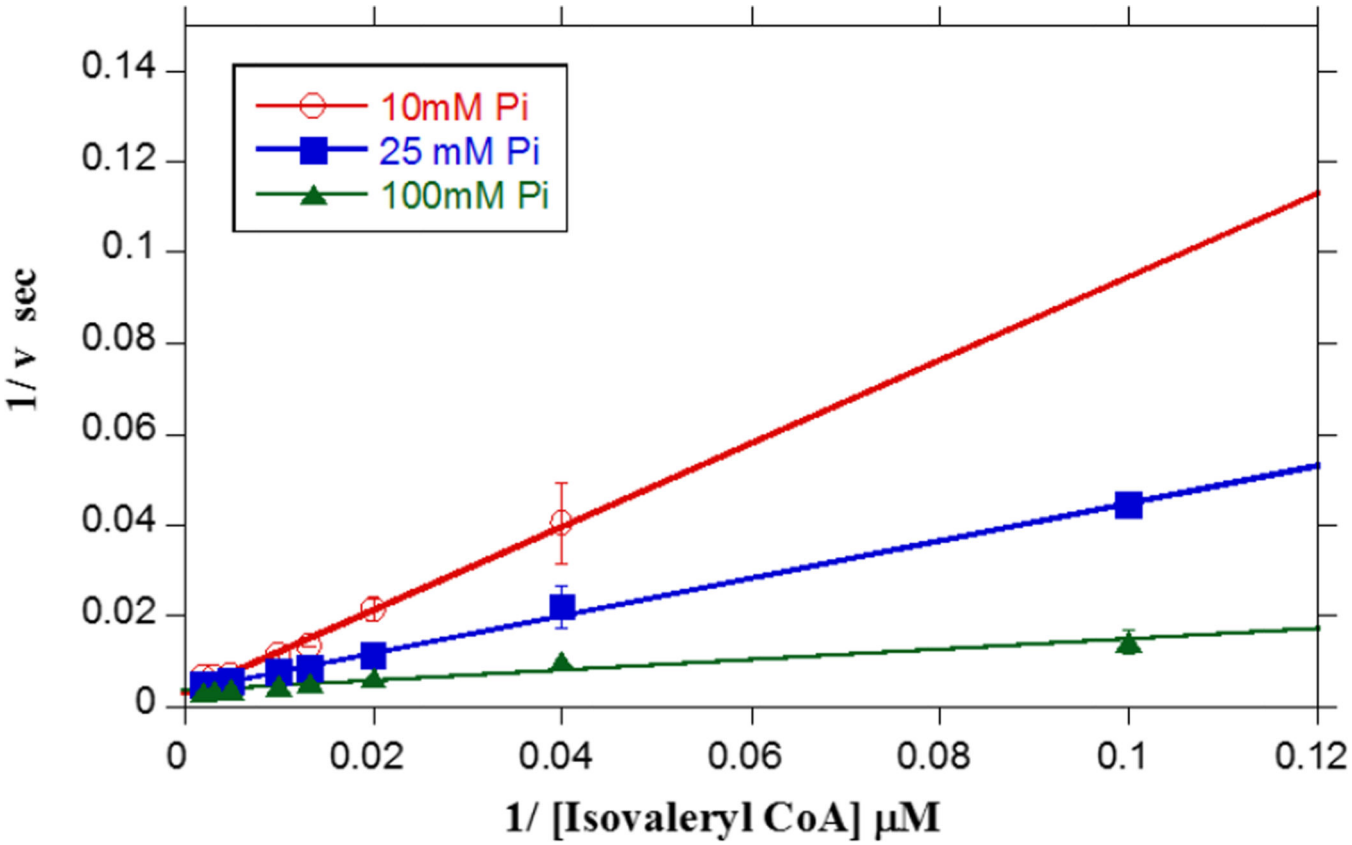


Fig. 6. Ptb activity follows a sequential kinetic model

The concentration dependence of isovaleryl-CoA on Ptb activity was determined in the presence of varying phosphate concentrations, i.e. 10 mM (open circles), 25 mM (closed squares), and 100 mM (closed triangles) under standard assay conditions at 25 °C. Double reciprocal (Lineweaver-Burk) plots 33 of means ± SEM from at least three experiments at each phosphate concentration were plotted. The three lines clearly intersect, which is indicative of a sequential kinetic mechanism (pingpong kinetics is characterized by parallel lines).

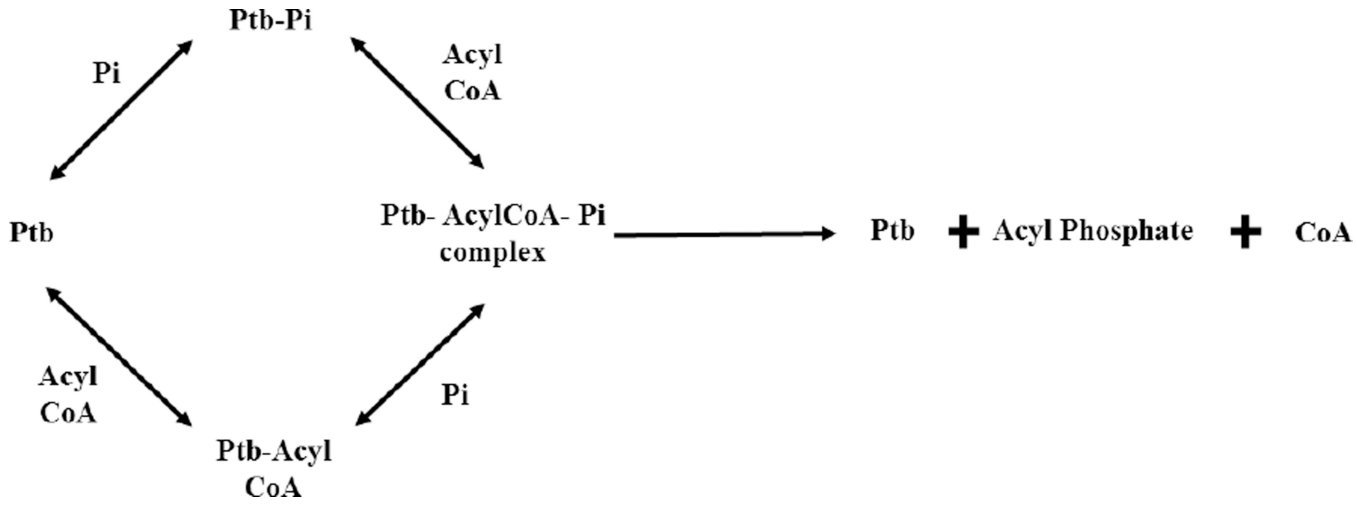
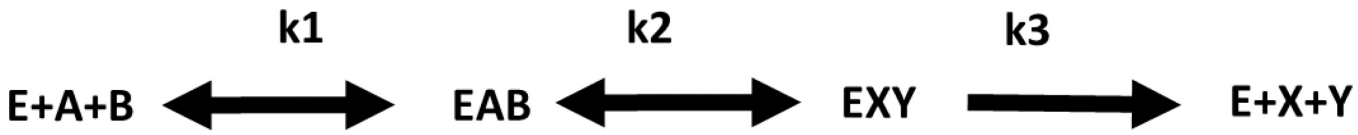


Fig. 7. Mechanism of action of Ptb

Schematic showing the proposed mechanism of action of Ptb involving the sequential binding of the substrates to Ptb to form the ternary complex followed by catalysis and subsequent release of products.



Scheme 1.

Table 1

Steady state kinetic analysis of Ptb from *L. monocytogenes*

Substrate	k_{cat} (sec^{-1})	K_M (μM)	k_{cat}/K_M ($\mu M^{-1}sec^{-1}$)
Straight-chain			
Acetyl CoA	302.4 ±103.9	992.1 ± 471.9	0.3
Propionyl CoA	518.8 ± 25.8	190.6 ± 20.9	2.72
Butyryl CoA	673.9 ± 25.2	79.9 ± 8.8	8.43
Pentanoyl CoA	676.1 ± 19	160.8 ± 10.5	4.2
Hexanoyl CoA	47 ± 2.6	31.5 ± 8.4	1.7
Branched-chain			
Iso butyryl CoA	1263.4 ± 31.7	57.9 ± 4.8	21.4
Isovaleryl CoA	1043.4 ± 12.2	53.2 ± 2.1	19.6
2-methylbutyryl CoA	849.1 ±18	48.1 ± 3.4	17.7
2-ethyl butyryl CoA	100.9 ± 11	43.8 ± 20.3	2.3

Initial velocities were measured at 25 °C in the presence of 100 mM phosphate 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 2

Relative activity of Ptb.

Substrate	Relative activity %
Isobutyryl CoA	100.0
Isovaleryl CoA	86.7
2-methyl butyryl CoA	73.8
Butyryl CoA	47.1
Pentanoyl CoA	32.6
Propionyl CoA	22.3
2-ethyl butyryl CoA	7.1
Hexanoyl CoA	4.8
Acetyl CoA	4.2

Relative activity was calculated from the experimental data obtained at 100 μM acyl CoA concentration and 100 mM phosphate measured at 25 °C under the same buffer conditions as stated in “Materials and Methods”. The activity of isobutyryl CoA (k_{cat}) was designated as 100% and the relative activity of the other substrates were calculated in comparison to isobutyryl CoA.