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Review Article

Propionylation of lysine, a new mechanism of short-chain fatty acids affecting bacterial virulence

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Abstract: Propionic acid (PA) is a major component of short-chain fatty acids produced by Bacteroidetes spp. Lysine propionylation is a novel type of protein regulatory posttranslational modification that is widespread in prokaryotes and eukaryotes, as well as in cellular processes, it affects DNA binding affinity, protein stability, and enzyme activity. In this review of published literature, we provide evidence that the level of propionyl modification is influenced by the concentration of PA and the PA metabolic intermediate (propionyl-CoA) and discuss the possibility of PA affecting enteropathogenic bacterial virulence. The understanding of propionyl modification is helpful to better understand the mechanism of PA-producing Bacteroidetes affecting the virulence of pathogenic intestinal bacteria. It may provide novel choices for the prevention and treatment of pathogenic intestinal bacteria.

Keywords: Propionic acid, posttranslational modification, lysine propylation, propionyl-CoA, bacterial virulence

Introduction

Propionic acid (PA) is a major component of short-chain fatty acids (SCFAs). PA is produced by anaerobic bacteria (especially members of Bacteroides) that ferment sugars in the gut [1]. SCFAs are the main metabolite of anaerobic fermentation in the mammalian gut and are mainly composed of acetate, propionate, and butyrate in the human gut (molar ratio 60:20:20). Concentrations range from 7-20 mM in the ileum to 60 mM-150 mM in the large intestine, depending on the host diet and microbial community composition [2]. SCFAs are effectively absorbed by the intestinal mucosa, providing energy to intestinal cells, maintaining the acidic intestinal environment, promoting integrity of the epithelial barrier, and reducing the production of pro-inflammatory molecules [3, 4]. In addition, SCFAs regulate the replication, colonization, and expression of virulence genes of intestinal pathogens (including Klebsiella, enterohemorrhagic Escherichia coli, and Salmonella) *in vitro* and in *vivo* [5-7].

Posttranslational modification (PTM) is an important molecular regulatory mechanism in all kingdoms of organisms [8-10]. Acylation of N_{ε}-lysine residues is an important PTM in both Eukaryotes and Prokaryotes [11-13], which widely affects DNA binding affinity, protein-protein interactions, protein stability, enzyme activity, or protein localization [14-17]. With the development of posttranslational modified proteomics based on high throughput and sensitive mass spectrometry, many new PTMs such as short-chain lysine acylation have been identified. SCFA can be converted into homologous acyl-coenzyme A (CoAs) and then used directly as a cofactor in the acylation of certain proteins such as propionylation [18, 19], malonylation [20], butyrylation [18], 2-hydroxyisobutyrylation [21, 22], β-hydroxybutyrylation [23], crotonylation [24], succinylation [25-27], and glutarylation [28, 29]. These findings give us a better understanding of PTM.

More than 60,000 acetylated and approximately 20,000 propionylated and succinylated gut microbial peptides were recently identified from human microbiome samples [26]. These findings indicate that acylation modification is common in intestinal microflora. At present, the most studied lysine acylation is acetylation.

Protein acetylation participates in bacterial chemotaxis, metabolism, DNA replication, and other cellular processes. However, there is growing evidence that propionate in intestinal SCFAs and its intermediate, propionyl-CoA, are closely related to propionylation. Propionylation may also be involved in affecting the virulence of bacteria. In this review, we summarize the advances in knowledge of propionylation of bacterial proteins and its relationship to propionate and propionyl-CoA. We also discuss the potential of propionate as a propionylation-mediated virulence factor of intestinal pathogenic bacteria.

Overview of propionylation

Lysine is one of the 15 amino acid residues encoded by modified ribosomes [30]. The electron-rich and nucleophilic properties of the lysine side chain make it suitable for covalent PTM reactions with different substrates. Acetylation is the earliest and most comprehensively studied of all lysine acylation, which are mechanisms of acetylation that include regulation of transcriptional regulators of bacterial virulence, and roles of acetylation in biofilm formation and antibiotic resistance that have been previously reviewed in detail [31-34]. In bacteria, acetyl-metabolizing intermediates, such as acetylphosphates and acetyl-CoA, can perform enzymatic lysine acetylation by deenzymatic acetate metabolizing enzymes or by providing acetyl donors. Up to 40% of proteins can be acetylated due to both enzymatic and nonenzymatic acetylation mechanisms [35-38]. Acetylation demonstrated higher levels in microorganisms compared to other PTMs that regulate metabolic processes, such as phosphorylation [9]. So, microbes evolved an elegant mechanism for regulating cellular metabolism through acetylation [39]. In 2007, Zhao et al. first described data that found that propionyl-CoA and acetyl-CoA are the same high-energy molecule, with a structure close to that of acetyl-CoA. Here the novel post-translational modification involving propionylation of lysine residues were first reported [18].

Propionylation involves the addition of propionyl (CH3-CH2-CO-) supplied by propionyl-CoA to the epsilon amino group of the target lysine residue catalyzed by propionyl transferase, which changes the charge state of lysine from +1 to 0. Propionylation of lysine was first discovered in histone proteins [18]. Subsequently, propionylation of non-histone substrates in mammals was demonstrated [40]. Propionylation is a dynamic process in which propionyl is bound to the substrate protein by some p300/CBP acetyltransferases. In prokaryotes, *Salmonella enterica* and *Bacillus subtilis* Gcn-5-associated N-acetyltransferases Pat and AcuA can catalyze the propionylation of lysine at site 592 of propionyl-CoA synthetase PrpE from S. enterica [41]. The corresponding class of sirtuin deacetyltransferase of *S. enterica* CobB catalyzes the removal of propionyl modifications from PrpE under NAD (+) conditions. In bacteria, acetylphosphate (ACP) produced by acetyl-CoA can also chemically acetylate lysine residues [38, 42, 43] without enzyme catalysis. Similarly, high concentrations of propionyl-CoA induce non-enzymatic propionylation *in vitro*. However, non-enzymatic propionylation has less effect on protein activity (< 20%) than enzymatic propionylation involving acyltransferase AcuA [44]. This also reflects the differences in the mechanism and effects of propionylation via two different pathways [45] (**Figure 1**). Interestingly, the authors also found that human SIRT2 (hSirT2), SIRT3 (hSirT3) and bacteria *T. maritima* Sir2 (Sir2Tm) proteins were able to remove propionyl modifications within one hour, but human (hSirT1), human SIRT4 (hSirT4), and mouse SIRT1 (mSirT1) showed no depropionylase activity [41]. This study provided important guidance for subsequent propionation-related studies. By combining high-affinity anti-propionyl-lysine pan-antibodies with high-precision mass spectrometry (MS) analysis, propionylation of proteins has been established as being common in bacteria and fungi at different growth stages, with differences in propionylation levels in different species.

The consortium of propionylated proteins is often called the propionylome. For example, 121 propionyl sites were identified in 80 proteins at the intermediate exponential stage and 323 propionyl sites were identified in 163 proteins at the late stable stage in a proteomic study of *Thermus thermophilus* HB8, the number of sites and the degree of propionylation showed significant differences independent of protein abundance [46]. The proteome of *T. rubrum* was analyzed by propionylation in the

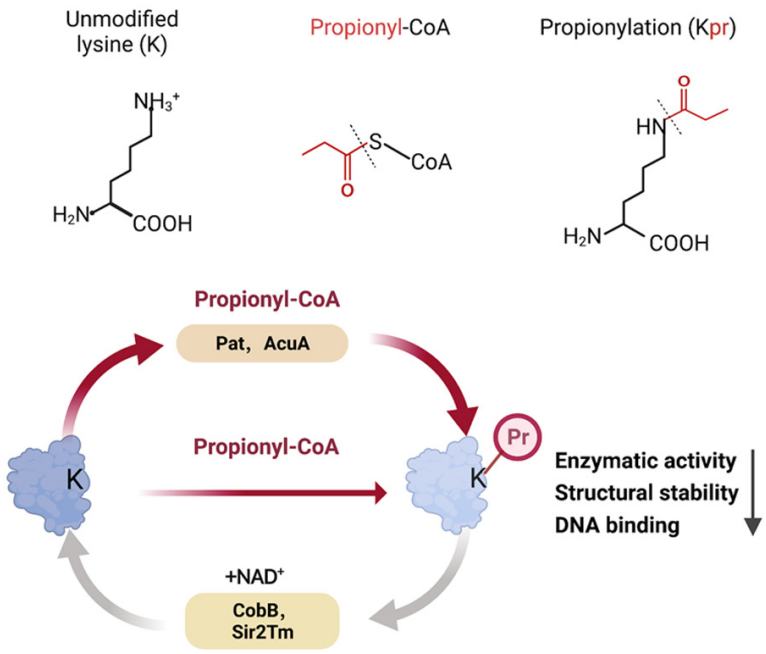

Figure 1. Mechanisms of propionylation in *Salmonella enterica*. Propionylation can be catalyzed by lysine acyltransferase using propionyl-CoA as the propionyl donor, or non-enzymatically by propionyl-CoA. Some, but not all, propionylation can be reversed by lysine deacylase. Pat, AcuA: acylaed transferases; CobB, Sir2Tm: deacylated transferases; K, Lysine.

Table 1. Characterization of propionylation of lysine residues of proteins

Species	Kpr proteins	Kpr sites	Percentage of total proteins (%)	Reference
T. rubrum	115	157	1.1	[47]
T. thermophilus	183	361	8	[46]
G. kaustophilus	55	83	1.6	[81]
E. coli	603	1467	9.5	[49]
Synechocystis	68	111	1.9	[48]
A. hydrophila	59	98	1.4	[82]
M. smegmatis	18	19	0.3	[83]

Kpr: propionylation.

conidia and mycelium stages. Seventy propionyl sites of 54 proteins were identified in conidia and 96 propionyl sites of 70 proteins were identified in mycelium. The propionyl sites of each protein were slightly more in mycelium than in conidia [47]. A protein propionylation test based on the results of propionylation of PCC 6803 (*Synechocystis*) under various stress conditions during the growth process suggested that propionylation was related to the stress response of *Synechocystis* [48]. In *E. coli*, propionylated proteomics analysis identified 956 propionylation (Kpr) sites in LB culture and 126 Kpr sites in M9 medium supplemented with 0.8% glucose. Following the addition of glucose to LB medium (0.8%), 166 Kpr sites were identified, indicating extensive substrate diversity [49] (**Table 1**).

Propionate and propionyl-CoA can affect propionylation

Enzymatic acylation modification cannot occur in prokaryotes without the acylating transferases Pat [41] and AcuA [41], and the CobB deacylating transferase [41, 49]. In addition, propionyl-CoA is needed as a propionyl source. Propionyl-CoA is the substrate of the propionylation reaction, and its concentration can directly affect the level of the reaction [47]. Propionyl-CoA is an intermediate product of PA metabolism and can be directly produced from PA by propionyl-CoA synthase (PrpE) and acetyl-CoA synthase (ACS) [50, 51]. In the methyl citrate cycle, PA is first activated by PrpE to yield propionyl-CoA. Propionyl-CoA then condenses with oxalacetic acid, as catalyzed by methyl citrate synthase (PrpC), to form methyl 2-citrate. Methyl citrate dehydratase/hydratase (PrpD) then dehydrates methyl 2-citrate to produce methyl aconite. The latter is then cleaved by methyl isocitrate lyase (PrpB) to produce pyruvic acid and succinic acid. Interestingly, ACS, PrpE, PrpC, PrpD, and PrpB can be propionylated in the presence of PA [49]. The activities of PrpE and ACS are simultaneously controlled by acetylation and propionylation (**Figure 2**).

Increasing evidence indicates that PA and its intermediate, propionyl-CoA, are closely related to propionylation. Proteomics studies in *E. coli* have demonstrated the marked increases in

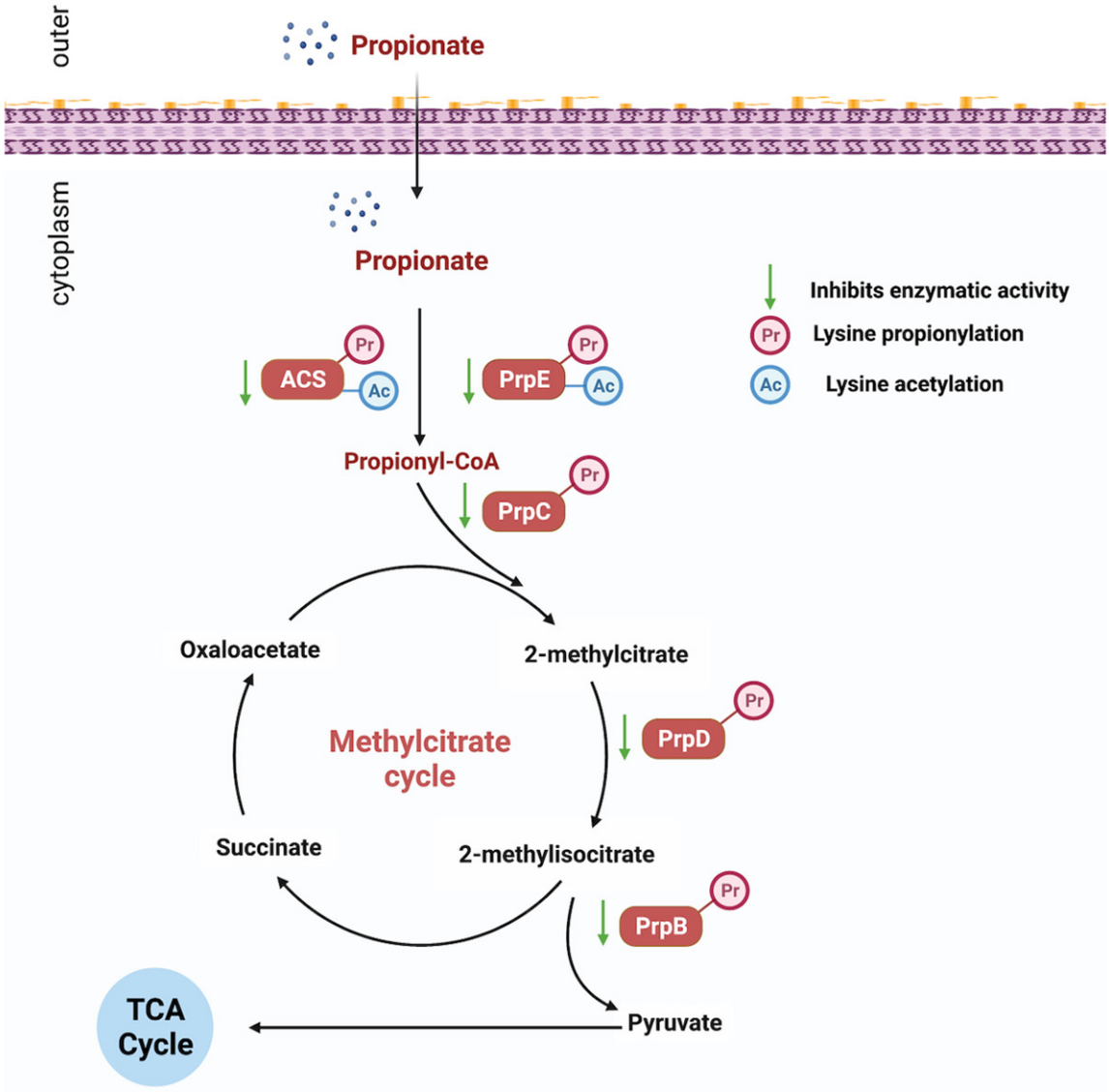

Figure 2. Simplified model of propionate catabolism in *E. coli*. Two enzymes involved in propionate metabolism (ACS and PrpE), as well as enzymes involved in the methylcitrate cycle (PrpC, PrpD, and PrpB), are propionylated in response to propionate treatment. TCA: tricarboxylic acid cycle; ACS: acetyl-CoA synthase; PrpE: propionyl-CoA synthase; PrpC: methyl citrate synthase; PrpD: Methyl citrate dehydratase/hydratase; PrpB: methyl isocitrate lyase.

the global propionylation level of lysine and the number of propionylation sites (increase of 713) [49] after propionate treatment. Among 69 proteins from PCC 6803 (*Synechocystis*), 111 lysine propionyl sites were identified [48]. A total of 157 propionyl sites were identified in 115 *T. rubrum* proteins. The level of propionyl modification was closely related to the concentration of propionyl-CoA and sodium propionate [47]. Similarly, the excess supply of propionyl-CoA during erythromycin production in *Saccharpolyspora erythraea* results in excess propionylation that affects erythromycin production [44, 52]. The link between propionylated modification and propionate was revisited in a transcriptional activity study of phosphate regulator PhoP in *S. erythraea*. The authors reported that the addition of propionate increased the level of propionylated modification in PhoP cells, resulting in a loss of the response to phosphate [19].

There are related reports in eukaryotic cells. For example, propionate-induced acetylation and propionylation in SCFAs reportedly up-regulated MICA/B expression on colon cancer

cells [53]. In another study, cells were similar to propionate as a model to study the increased propionylation of proteins [54]. These studies demonstrated that the propionylation of lysine occurs in a variety of proteins and affects a variety of biological functions. In particular, the propionylation of lysine contributes to the overall metabolic regulation and cellular stress response in mice [55]. This may be one of the mechanisms regulating cell metabolism and response to stress conditions in bacteria and mammals [46], and has important associations with propionate.

Effects of PA on bacterial virulence

PA is an abundant carbon source in nature and can be used as the only carbon source by many aerobic bacteria and some anaerobic bacteria. In contrast, the addition of propionate to growth media inhibits the growth of most microorganisms, even in the presence of other carbon sources, due to the accumulation of toxic metabolic intermediate propionate-CoA. Reflecting this, high concentrations of PA are widely used as an antimicrobial agent in food and agriculture. However, despite its widespread use, how propionate affects microbial growth remains unclear [56, 57]. *S. enterica* serovar Typhimurium exposed to propionate reduces the expression of *Salmonella* pathogenic island 1 (SPI-1). The propionyl-CoA metabolic intermediate can modify and decrease the stability of HilD, affect the expression of invasion-related genes, and ultimately inhibit the invasion of *Salmonella* [58]. In addition, preincubation of *S. enteritidis* with propionate and butyrate can decrease epithelial cell invasion [59]. Infection rates of salmonella were reduced in a pig model of infection by adding a mixture of organic acids, including propionate, to their food [60]. Recent findings have demonstrated that propionate can directly affect bacterial virulence. Propionate produced by *Bacteroides fragilis* in the gut directly inhibited the growth of pathogens *in vitro* by disrupting intracellular pH homeostasis, to mediate the relationship between colonization resistance of *S.* Typhimurium [61]. Therefore, supplementing the diet with SCFAs, especially propionate, may be a promising intervention strategy to reduce *Salmonella* infection. Propionate produced by the host through the intestinal microflora may prevent local inflammation and tissue damage by controlling the host's response to symbiosis [62].

PA is generally considered safe for human and animal use. However, a study that examined on the adherence of invasive *E. coli* (AIEC) demonstrated that PA positively regulated the virulence of strain LF82. Increased exposure to PA and increased intestinal concentrations in mice resulted in a more than 20-fold increase in persistence and increased the most prominent phenotypic features of AIEC, such as intestinal epithelial adhesion, invasion, and biofilm formation [63]. Another study reported that intestinal PA resulted in increased ethanolamine metabolism by AIEC. To overcome the toxic byproducts associated with the use of ethanolamine, AIEC synthesized and then excreted bacterial microcompartments (MCPs). In addition, the use of a concentration of ethanolamine comparable to that in the human gut in the external environment of macrophages stimulated rapid proliferation of AIEC [64]. A recent report also described that *S.* Typhimurium can overcome propionate inhibition by using propionate as a carbon source for anaerobic respiration. Nitrate-dependent propionate metabolism by *S.* Typhimurium provided an inflammation-dependent colonization advantage [65]. Therefore, in addition to promoting colonization resistance, propionate from microbial populations may also contribute to colonization by intestinal pathogens during infection.

PA can affect bacterial virulence via propionylation

Acetylation has recently been widely reported to affect toxicity in a variety of ways, including intracellular survival [66, 67], regulation of transcription factors [68-70], biofilm formation [71, 72], interaction with the host [73, 74], and antibiotic resistance [75, 76]. In the genus *Salmonella*, propionate is involved in posttranslational modification of HilD via propionyl-CoA. Propionyl-CoA reduces the stability of HilD and affects the expression of invasion-related genes [58]. The stability and DNA binding ability of HilD are related to reversible acetylation [77, 78]. However, these studies focused on the absence of Pat in the acyltransferase and ignoring both Pat and AcuA can act as propionyltransferases (AcuA has 5.5-times higher propionylating activity than Pat; [41]). Thus, it is likely that the post-translational modification

Table 2. The effect of propionylation on protein function

Species	Kpr protein	Functional Kpr site	Function	Consequence of propionylation	Reference
S. enterica	PrpE	K592	Activate propionate to propionyl-CoA	inhibits enzymatic activity	[41]
M. smegmatis	FadD35	K519	synthesizes CoA esters from short to long-chain fatty acids	inhibits enzymatic activity	[83]
Synechocystis	FbpI	K156, K336	photosynthetic carbon fixation and gluconeogenesis	inhibits enzymatic activity	[48]
	PsaD	K132	associated with photosynthesis	decreased structural stability of PsaD	[48]
A. hydrophila	MDH	K168	Involved in glucose metabolism	inhibits enzymatic activity	[82]
S. erythraea	SACE_0337 SACE_4729 SACE_3848	Multiple	Synthesis of propionyl-CoA	inhibits enzymatic activity	[44]
	mmsA2	K94	synthesis of propionyl-CoA	inhibits enzymatic activity	[52]
	PhoP	K198, K203	two-component system	inhibits DNA binding	[19]
E. coli	ACS	K609	rate-limiting enzyme in propionate metabolism	inhibits enzymatic activity	[49]

Kpr: propionylation; PrpE: propionyl-CoA synthase; FbpI: bifunctional enzyme fructose-1,6/sedoheptulose-1,7-bisphosphatase; PsaD: subunit II of photosystem I; MDH: malate dehydrogenase; ACS: acetyl-coenzyme A synthetase.

related to HilD is also closely related to propionylation. PhoP has been shown in *S.* Typhimurium. In the presence of acetyl-CoA, the K201 and K88 sites were acetylated by acetyltransferase Pat and ACP respectively, which affected PhoP activity, and significantly decreased the ability of *S.* Typhimurium survive in macrophages [79, 80]. In *Saccharopolyspora erythraea*, a propionate level of 20 mM enhances propionyl modification of PhoP and affects nucleic acid binding [19]. Similarly, in the presence of propionate, binding of PhoP to bind to nucleic acids was restored when the sites of PhoP K198 and K203 were mutated simultaneously to arginine to mimic non-propionylated modifications [19]. The environment of pathogenic intestinal bacteria naturally contains PA. It has been demonstrated that PA treatment can significantly enhance propionylation-related modification. Given the similarities between propionylation and acetylation, and the fact that both can be modified by acyltransferase, Pat, and CobB deacetyltransferase, the binding of nucleic acids by modified protein usually decreases. It is conceivable that propionate-enhanced propionyl-related modification can modulate the pathogenicity of intestinal bacteria.

Bacterial propionylated proteins are involved in metabolism

Propionylated proteins are involved in various metabolic processes and play an important role in metabolic regulation (**Table 2**).

As mentioned above, propionylation is a ubiquitous protein modification involved in many cellular physiological processes. These include carbon source utilization, gluconeogenesis, two-component systems, and others.

Research strategies of propionylation

Propionylation is generally studied by combining proteomics with *in vivo* and *in vitro* techniques. In the past, mass spectrometry-based proteomics was used to identify and verify propionyl sites. With the refinement of the technology, stable isotope labeling can be used for quantitative propionyl group analysis of specific proteins and their sites. In this approach, proteins are deranged and digested with trypsin. Peptides were labeled by light or heavy stable isotope dimethyl reagents, Solutions of formaldehyde (CH_2O, light-labeled) and deuterated formaldehyde (CD_2O, heavy-labeled) are added to the peptide mixture of different groups [49]. Propionyl lysine peptides can be enriched by affinity chromatography with pan-anti-propionyl lysine antibody bound to agarose beads and analyzed by nanoscale high-performance liquid chromatography coupled to tandem mass spectrometry. Compared with the previously used mass spectrometry propionyl group analysis method, the refined method more directly compares the differences in levels of propionyl between the control and experimental groups. Western blot, immunohistochemistry, and enzyme-linked immunosorbent assays based on propionylated antibody can be used to

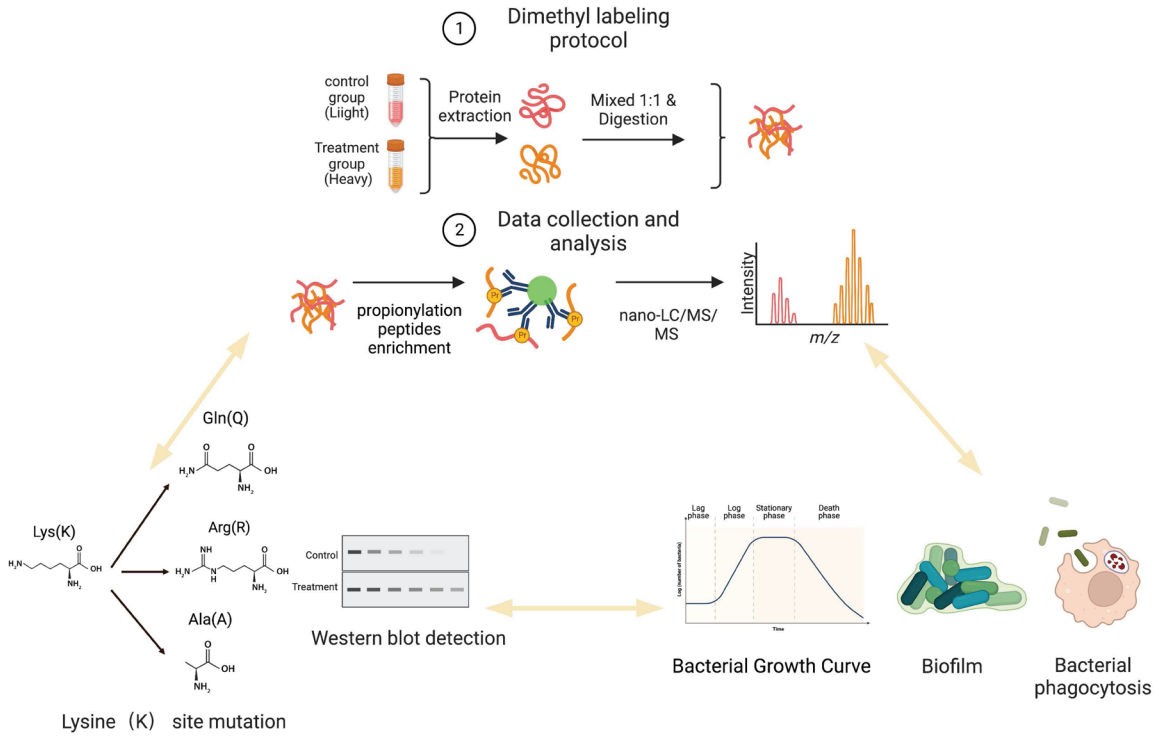

Figure 3. Strategies of propionylation research. Identification and quantification of lysine propionylation substrates can be achieved using quantitative proteomic analysis and the stable isotope dimethyl labeling method. Strains are cultured for protein extraction. After digestion, peptides are labeled by light or heavy stable isotope dimethyl reagents. Antibody to propionyl lysine is used for modified peptide enrichment. Samples are analyzed by high-resolution mass spectrometry. In combination with phenotypic and molecular experimental data, the function of propionylated proteins is verified.

verify the difference of protein modification in different samples, such as control and experimental groups. In molecular biology examinations, the propionylation state can be simulated by site-directed mutagenesis of propionylation sites *in vitro*, using glutamine (Q) instead of neutralizing positive charge to simulate propionylation. The substitution of arginine (R) or alanine (A) with a simulated unpropionated state. (R) can maintain a positive charge while avoiding propionylation, instead, (A) only removes the charge [19, 49]. These results can be validated *in vivo* and *in vitro* by a combination of phenotypic tests. In addition, the determination of the concentration of propionyl-CoA and analyses of propionyl transferase and depropionyl transferase mutants can also help to determine the mechanism of propionylation and the corresponding effects (**Figure 3**).

Challenges in propionylation research

Propionylation is a ubiquitous protein modification involved in many cellular physiological processes, such as carbon source utilization, gluconeogenesis, two-component systems, and others. This modification can inhibit the activity of many kinds of enzymes. However, whether propionylation can directly affect the virulence of bacteria in a manner like acetylation is unclear. This is worth exploring since the ubiquity and diversity of propionylation also means that it has more potential. In this context, the foregoing findings are helpful to provide a theoretical basis for studies of the pathogenicity and drug resistance of bacteria.

Conclusions

PA produced by the intestinal resident anaerobic Bacteroides spp. and propionyl-CoA, an intermediate metabolite of bacterial metabolism PA, affects a novel protein post-translational modification (PTM)-lysine propionylation (Kpr) in a concentration-dependent manner. This mechanism has a wide variety of substrates and can be dynamically regulated under different nutrient conditions. It can affect

the metabolism of bacteria by regulating DNA binding affinity, changing protein stability and enzyme activity, and even affect the expression of virulence factors of enteric pathogenic bacteria. The study of propionylation of lysine provides a new opportunity to understand the mechanism by which PA-producing Bacteroides affects the virulence of enteric pathogens.

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Disclosure of conflict of interest

None.

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