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Research review paper

Bacterial protein acetylation and its role in cellular physiology and metabolic regulation

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ABSTRACT

Protein acetylation is an evolutionarily conserved posttranslational modification. It affects enzyme activity, metabolic flux distribution, and other critical physiological and biochemical processes by altering protein size and charge. Protein acetylation may thus be a promising tool for metabolic regulation to improve target production and conversion efficiency in fermentation. Here we review the role of protein acetylation in bacterial physiology and metabolism and describe applications of protein acetylation in fermentation engineering and strategies for regulating acetylation status. Although protein acetylation has become a hot topic, the regulatory mechanisms have not been fully characterized. We propose future research directions in protein acetylation.

1. Introduction

Cellular physiological processes from bacteria to higher animals are regulated at different levels to adapt to a changing environment and include transcriptional and posttranscriptional regulation of gene expression and allosteric regulation of enzyme activities. Post-translational modification (PTM) is a biochemical mechanism of covalent amino acid modification to regulate protein activity. Over the past 40 years of biotechnology development and related research, more than 200 types of PTM, such as phosphorylation, acetylation, ubiquitylation, and glycosylation, have been identified, as well as a large number of PTM sites (Minguez et al., 2012; Ren et al., 2017). Various PTMs can coexist in a given protein and synergistically affect its enzymatic activity.

Lysine is a small amphipathic molecule containing an additional positively charged amino group attached to its hydrophobic side chain. Protein acetylation on lysine residues is an evolutionarily conserved PTM closely related to cellular physiology and function. In the past 40 years, protein acetylation has been well researched in eukaryotes, especially in histone modification and gene transcription (Jenuwein, 2001; Norris et al., 2009). Clearly, lysine acetylation status is an important mediator of cellular replication, division, differentiation, and apoptosis. Abnormal modification states may trigger cancer, neurodegenerative, and cardiovascular diseases (McKinsey and Olson, 2004; Yang, 2004). The identification of acetylated mammalian mitochondrial proteins implies the possibility of modification occurring outside the nucleus and non-histone acetylated proteins from higher organisms to bacteria (Glozak et al., 2005; Gu and Roeder, 1997). Bacterial acetylation status has been studied using a combination of high-affinity immune separation and nano-HPLC/MS/MS (Yu et al., 2008). Since Allfrey and colleagues reported that histone acetylation can regulate gene expression in 1964, protein acetylation has been found not only to act as an important epigenetic regulator but also to regulate the cellular metabolism (Fig. 1). However, it remains unclear how protein acetylation affects global cellular metabolism. The complex regulatory mechanisms and physiological effects remain unclear, especially in bacteria.

Presently, protein acetylation has enzymatic and nonenzymatic mechanisms in bacteria (Verdin and Ott, 2013) (Fig. 2B). Enzymatic acetylation transfers an acetyl group from the donor acetyl coenzyme A (Acetyl-CoA) to target protein lysine residues via catalysis of a lysine

Abbreviations: AcP, Acetyl phosphate; Acetyl-CoA, Acetyl coenzyme A; KAT, Lysine acetyltransferase; KDAC, Lysine deacetylase; NAT, Nα-acetyltransferase; PTM, Posttranslational modification; TCA, Tricarboxylic acid; CCR, Carbon catabolite repression; ICDH, Isocitrate dehydrogenase; GapA, Phosphate dehydrogenase; AceA, Isocitrate lyase; AceK, Isocitrate dehydrogenase kinase/phosphatase; MDH, Malate dehydrogenase; CS, Citrate synthase; TopA, Topoisomerase I; *c*-di-GMP, Cyclic di-guanosine monophosphate; cAMP, Cyclic adenosine monophosphate; T3SS-1, Type III secretion system 1; SPI-1, pathogenicity island 1.

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acetyltransferase (KAT)(Starai and Escalante-Semerena, 2004a; Thao and Escalante-Semerena, 2011). The nonenzymatic mechanism involves the direct transfer of an acetyl group from acetyl phosphate (AcP) to protein lysine residues (Kuhn et al., 2014; Weinert et al., 2013). Lysine deacetylases (KDACs) can deacetylate some, but not all acetylated proteins produced by these enzymatic and nonenzymatic mechanisms (Abouelfetouh et al., 2014; Starai et al., 2002). Lysine acetylation regulates many biological processes, such as DNA replication, protein stability, enzymatic activity, cell metabolism, chemotaxis, and virulence.

Several good reviews have summarized the types and catalytic mechanisms of protein acetylation (Ali et al., 2018; Hentchel and Escalante-Semerena, 2015; Van Drisse and Escalante-Semerena, 2019; Verdin and Ott, 2014; Wolfe, 2015). This review mainly discusses the roles of protein acetylation in bacterial physiology and metabolism, the applications in fermentation engineering, and strategies for regulating cellular acetylation status. Finally, we forecast the future research of protein acetylation.

2. Protein acetylation and deacetylation in bacteria

There are several different types of amino acid acetylation. According to the position of amino acid acetylation, it can be divided into Nα-acetylation and Nε-acetylation. Nα-acetylation occurs at the amino terminus of any first amino acid of the substrate protein. Nε-acetylation involves the transfer of the acetyl group to the epsilon amine of lysine (Fig. 2A). Both Nα-acetylation and Nε-acetylation need a proper microenvironment for the acetyl donor and neutral amino group.

2.1. Nα-acetylation

Phillips first identified histone Nα-acetylation in 1963 (Phillips, 1963). Nα-acetylation is very common in eukaryotes, and approximately 80% of mammalian and 60% of yeast proteins exhibit this modification. In bacteria, some ribosomal proteins were found to be Nα-acetylated in posttranslation (Waller, 1963). Later, non-ribosomal proteins were also identified to be Nα-acetylated, such as chaperone SecB of *E. coli* (Smith et al., 1996), virulence factor ExsA of *Mycobacterium marinum* (Medie et al., 2014), deacetylase CobB long isoform ($CobB_L$) of *Salmonella enterica* (Parks and Escalante-Semerena, 2020). In eukaryotes, Nα-acetylation mainly occurs at the deformylated N-terminal methionine or the amino acid residue newly exposed after cleaving the N-terminal methionine during cotranslation (Fig. 2A).

Nα-acetylation is irreversible. Several Nα-acetyltransferases (NATs), including their substrates and subunits, have been identified in humans,

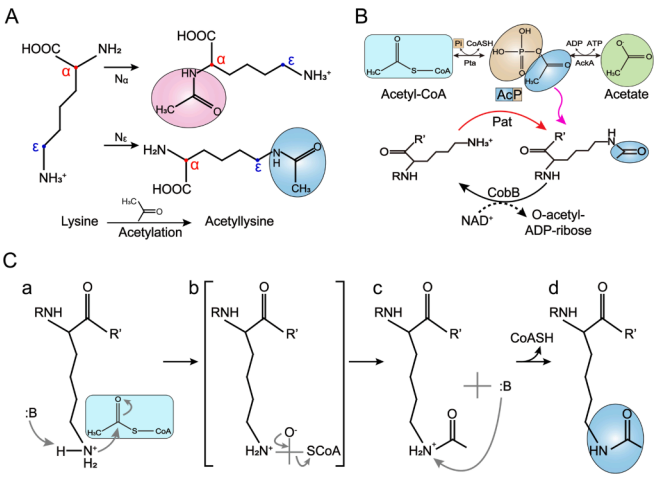

Fig. 2. Schematics of Nα- and Nε-acetylation. A: Classification of protein acetylation. Protein acetylation could be divided into two types according to different modification sites, acetylation of the N-terminal α-amino group (Nα-acetylation) (red) or acetylation of the ε-amino group of internal lysine residues (Nε-acetylation) (blue); B: Nε-lysine acetylation and deacetylation. Nε-lysine could be acetylated by acyltransferase (Pat), in which the acetyl group comes from acetyl-CoA (red), or through AcP-dependent non-enzymatic acetylation (pink). AcP is a high-energy compound composed of an acetyl and a phosphate group, produced from the phosphotransacetylase (Pta)-acetate kinase (AckA) pathway. Reversely, acetyllysine deacetylation is mediated by the NAD^+-dependent deacetylase CobB; C: Electron transfer process of Nε-acetylation from acetyl-CoA. (a) The Nε-lysine amino group is positively charged at neutral pH. The base (:B) abstracts a proton from the amino group. Then this lysine ε-amino group can act as a nucleophile to attack the electrophilic carbonyl carbon of acetyl-CoA. (b) The thiolate group is discharged and an intermediate is formed. (c) The base (:B) deprotonates the amino group to form the acetylated lysine (blue) and CoASH (d). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

yeast, and other eukaryotes (Polevoda et al., 2009). The conjugation of ubiquitin to proteins NH_2 groups is needed for protein breakdown. So, the physiological function of Nα-acetylation of NH_2-terminal was proposed to prevent protein degradation and promote protein stability (Hershko et al., 1984). However, in a subsequent study, Nα-acetylated proteins showed elevated protein degradation through generating some signals recognized by a certain ubiquitin ligase (Hwang et al., 2010). Since the Nα-acetyl modification is widespread in eukaryotic proteins,

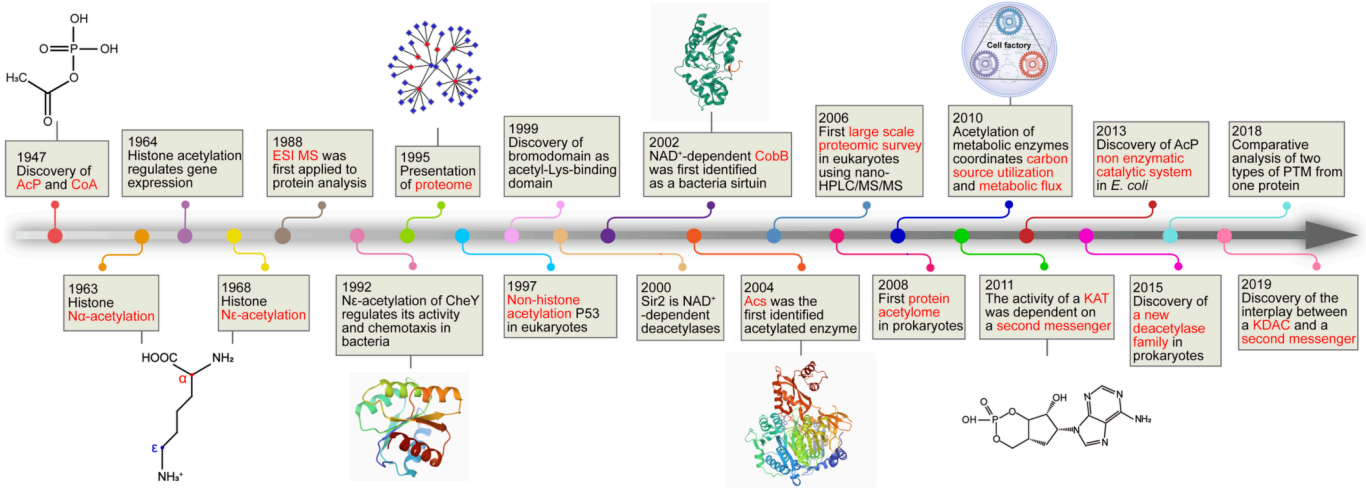

Fig. 1. Timeline of protein acetylation research. Abbreviations: AcP, acetyl phosphate; Sir2, silent information regulator 2; Acs, acetyl-CoA synthetase; KAT, lysine acetyltransferase; PTM, posttranslation modification; KDAC, lysine deacetylase.

other undiscovered mechanisms may exist to regulate protein stability in addition to Nα-acetylation effects.

2.2. Nε-acetylation

Allfrey et al. first reported the Nε-acetylation of f2a1 histone from calf thymus lymphocyte nuclei in 1968 (Gershey et al., 1968). In 1992, Nε-acetylated CheY was discovered in bacteria (Barak et al., 1992). CheY is an important protein of bacterial chemotaxis, and Nε-acetylation regulates its activity (Barak et al., 2006; Barak and Eisenbach, 2001). Unlike Nα-acetylation, Nε-acetylation only occurs at the lysine residue. Nε-acetylation increases protein size, alters the charge of the side chain, and affects protein stability, conformation, localization, and interactions with other macromolecules (Wolfe, 2015). Compelling evidence has revealed that Nε-acetylation is a prevalent PTM in eukaryotes and bacteria, affecting cellular physiology and metabolism (Norris et al., 2009; Verdin and Ott, 2014; Yu et al., 2008).

Enzymatic lysine acetylation and deacetylation are mediated by KAT and KDACs, respectively. Five KAT families have been discovered: (i) the Gcn5-related acetyltransferase (GNAT) family, (ii) the MYST family, (iii) the CBP/p300 coactivators, (iv) the TAFII 250 family, and (v) the SRC family (Marmorstein and Roth, 2001; Sterner and Berger, 2000). The GNATs family is identified in all three domains of life and encompasses more than 10,000 members (Ren et al., 2017; Vetting et al., 2005). However, the other four families are only present in eukaryotes (Marmorstein and Roth, 2001). In bacteria, almost all identified KATs belong to the GNAT family. KATs have many names such as YfiQ, Pat, PatZ and Pka in *E. coli* and *Salmonella*, and Pat is used in this review. The conventional mechanism of Nε-acetylation is that acetyltransferases promote the formation of a neutral amino group by deprotonation. Next, this neutral amino group attacks the carbonyl carbon of the donor acetyl-CoA, leading to lysine acetylation and free CoA release (Dyda et al., 2000) (Fig. 2C).

AcP-dependent acetylation is a nonenzymatic mechanism by which the acetyl group is directly transferred to the lysine ε-amino group (Kuhn et al., 2014; Weinert et al., 2013). AcP is an important high-energy molecule that serves as a global regulator by providing the phosphate and acetyl group (Kuhn et al., 2014; Weinert et al., 2013; Wolfe, 2010). Hundreds of genes involved in multiple bacterial phenotypes are regulated by AcP, including chemotaxis, protein degradation, flagella biosynthesis, and fermentative metabolism (Wolfe et al., 2010). Previous studies reported that acetylation level in a growth-arrested cell was closely related to the concentration of AcP and the Pta-AckA pathway in *E. coli* (Kuhn et al., 2014; Weinert et al., 2013). Additionally, acetylation levels correlated with AcP in a dose-dependent manner in vitro (Kuhn et al., 2014; Weinert et al., 2013). AcP-dependent nonenzymatic protein acetylation is the primary mechanism in *E. coli*, *Bacillus subtilis* and *Neisseria gonorrhoeae* (Kosono et al., 2015; Post et al., 2017; Schilling et al., 2015; Weinert et al., 2013).

2.3. Deacetylation of proteins

Presently, the identified KDACs are divided into two families and four classes: the zinc-dependent Rpd3/Hda1 family (classes I, II, and IV) and the NAD^+-dependent sirtuin family (class III) (Hentchel and Escalante-Semerena, 2015; Ren et al., 2017; Wolfe, 2015). Class III KDAC, commonly referred to as sirtuin, catalyzes lysine deacetylation with the help of NAD^+ (Sauve et al., 2006). By contrast, classes I, II, and IV catalyze this reaction through acyl group hydrolysis and do not need any cofactors (Hodawadekar and Marmorstein, 2007). Bacteria only encode one or two sirtuin homologs, far less than in eukaryotes (Hentchel and Escalante-Semerena, 2015). NAD^+ binds the catalytic site of sirtuin and initiates sirtuin-catalyzed deacetylation. In this reaction, NAD^+ functions as a cosubstrate and is cleaved to O-acyl-ADP-ribose. Next, an imidate intermediate is created after one-step ADP-ribosylation and a configuration inversion and finally completes the lysine deacetylation (Hentchel and Escalante-Semerena, 2015; Sauve, 2010). The NAD^+-dependent CobB in *S. enterica* was the first characterized and most deeply studied bacterial sirtuin (Van Drisse and Escalante-Semerena, 2019). Other bacterial species, such as *B. subtilis* and *E. coli*, also possess CobB homologs. Generally, lysine deacetylation catalyzed by CobB has no preference for AcP-dependent or enzymatic acetylation (Abouelfetouh et al., 2014). Only a fraction of acetylation in *Escherichia coli* has evidence of reversal so far (Abouelfetouh et al., 2014), and this fraction of acetylations are reversed by the deacetylase CobB. In recent years, several proteins were considered as putative deacetylases, such as YcgC in *E. coli* (Tu et al., 2015) and MSMEG 4620 in *Mycobacterium smegmatis* (Tan et al., 2015). Tu et al. reported that YcgC protein, a member of serine hydrolase family, catalyzed deacetylation of a transcriptional repressor RutR in vivo, involving Ser200 as the catalytic nucleophile instead of the Zn^{2+} or NAD^+ compared with other identified KDACs (Tu et al., 2015). However, this result could not be repeated by other researchers (Magdalena et al., 2018), and YcgC protein could not deacetylate isocitrate dehydrogenase (ICDH) in vivo or in vitro (Venkat et al., 2018a), turning out that YcgC is probably not a deacetylase. MSMEG_4620, a SIRT4 homolog, showed strong automatic ADP ribosylation activity and weak deacetylase activity in *M. smegmatis*. Notably, this SIRT4 homolog had important roles for the cell growth in environmental mycobacterial species (Tan et al., 2015).

2.4. Global analysis of lysine acetylation in bacteria

Before 2008, only a few bacterial acetylated proteins have been identified and characterized, and development of novel omic technologies has promoted discovery of bacterial acetylated proteins and elucidation of their physiological functions. The first bacteria acetylome was characterized in *E. coli* using high-affinity immunoseparation and nano-HPLC/MS/MS (Yu et al., 2008). This study identified 125 acetylated sites in 85 proteins involved in diverse physiological functions, including carbon metabolism, protein synthesis, and amino acid metabolism. The study also showed that acetylation was higher in the stationary phase than in the exponential phase (Yu et al., 2008). This research established a theoretical and technical basis for the protein acetylome and provided a framework for systematic analysis of the physiology role and regulatory mechanism of lysine acetylation in bacteria. In the following years, many groups conducted global screening and characterization of lysine acetylation in *E. coli* (Kuhn et al., 2014; Schilling et al., 2015; Wang et al., 2010; Weinert et al., 2013; Zhang et al., 2009). The number of acetylated sites and proteins expanded and important information of acetylome was identified, including the interaction network, localization, and gene ontology annotation. The acetylome of other bacterial species, such as *S. enterica*, *B. subtilis*, and *Mycobacterium tuberculosis*, have also been researched and characterized (Hentchel and Escalante-Semerena, 2015; Ren et al., 2017). All these results indicate that lysine acetylation is an evolutionarily conserved and abundant PTM from bacteria to mammals (Nakayasu et al., 2017). In the acetylome of *S. enterica*, approximately 50% of acetylated proteins participated in diverse metabolic pathways, and about 90% of the central metabolism enzymes were acetylated (Wang et al., 2010). However, how acetylation regulates cellular physiology and metabolism remains poorly understood.

3. Effects of protein acetylation on cellular physiology and metabolism

Lysine acetylation affects the protein function and cellular physiology (Fig. 3 and Table 1). Thus, more extensive and in-depth studies on protein acetylation will be helpful for better applications in cell physiology and metabolism regulation.

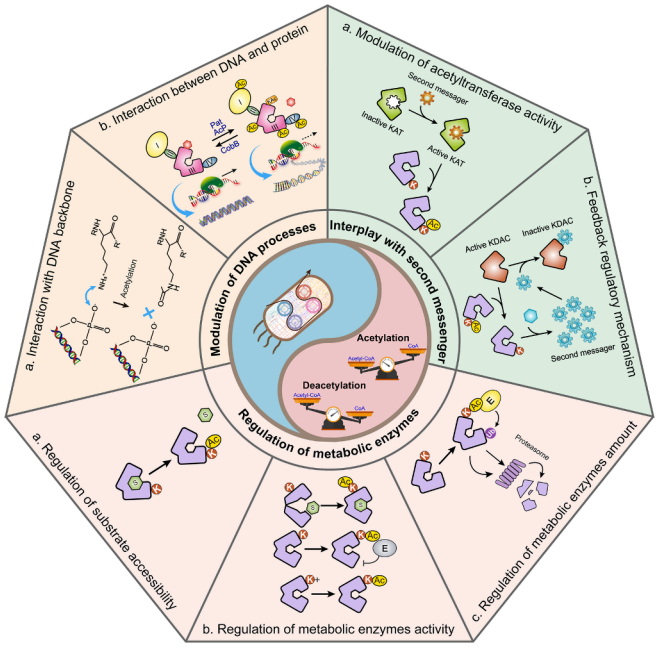

Fig. 3. The effects of acetylation on cellular physiology and metabolism. Regulation of metabolic enzymes: (a) Regulation of substrate accessibility through modifying the conserved lysine residues to hinder the entry of substrate. (b) Regulation of metabolic enzymes activity through causing allosteric changes, recruiting a negative regulator (E in gray) to inhibit metabolic enzymes, or neutralizing the positive charge of lysine residues near the active site. (c) Regulation of metabolic enzymes amount by promoting their degradation through proteasomal system. Modulation of DNA processes: (a) Causing a disruption in the interaction with DNA backbone. The negatively charged phosphate moieties of DNA backbone interacts with a positively charged deacetylated lysyl residues at cellular pH. Through acetylation, the charge on the side chain of lysyl residues is eliminated and no longer interact with the DNA. (b) Acetylation regulates the interaction between DNA and proteins. Acetylation affects the proteins binding to DNA and DNA supercoiling structure. Interplay with second messenger: (a) Acetyltransferase activity is activated by a second messenger; (b) Feedback regulatory between deacetylase and second messenger. The activity of deacetylase is inhibited by second messenger, thus affecting the deacetylation status of a protein, which involved in the synthesis of second messenger. Annotation: AC, acetylation; K, lysine residues; Metabolic enzymes and substrates (S) are colored in purple and light green, respectively. Active sites are indicated as the hexagonal shape inside the concave metabolic enzymes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.1. *Acetylation regulates the enzyme activity and metabolic flux distribution*

Lysine acetylation can increase or inhibit protein activity. The classic paradigm in bacteria is the acetylation regulation of acetyl-CoA synthetase (Acs). Acs catalyzes the irreversible conversion of acetate and acetyl-CoA, and it plays a critical role in acetate assimilation for producing metabolic intermediates and energy via glyoxylate shunt and tricarboxylic acid (TCA) cycle (Liu et al., 2015). It's worth noting that Jorge Escalante-Semerena's team did most of the work on CobB, Pat and their role in acetylating and regulating the activity of Acs and its homologs. Acs of *S. enterica* is the first enzyme reported to be controlled by *N*-lysine acetylation /deacetylation (Starai and Escalante-Semerena, 2004a). Acetylation of K609 by Pat inhibits Acs enzyme activity and deacetylation by CobB can restore it (Starai and Escalante-Semerena, 2004a; Starai et al., 2002). Similar acetylation-dependent regulatory modes of Acs were discovered in *E. coli* (Castaño-Cerezo et al., 2014), *B. subtilis* (Gardner et al., 2006), *M. smegmatis* (Xu et al., 2011) and *Rhodopseudomonas palustris* (Crosby et al., 2010). In vitro, Acs activity was increased 40-fold in the presence of deacetylases CobB and NAD^+ versus untreated controls, and Acs acetylation by Pat inhibited its activity (Castaño-Cerezo et al., 2014). A CobB mutant showed enhanced acetylation and a half reduction of acetyl-CoA synthetase activity (Castaño-Cerezo et al., 2014). Hence, Acs acetylation can be used to regulate the overflow metabolism, as discussed in section 4.2.

Flux distribution between some important metabolic branches, such as between glycolysis and gluconeogenesis, between TCA cycle and glyoxylate shunt, and between glycolysis and TCA cycle, are also affected by lysine acetylation. Glyceraldehyde phosphate dehydrogenase (GapA) catalyzes the reversible aldol condensation reaction of glyceraldehyde-3-phosphate and 1,3-bisphosphoglycerate in glycolysis and gluconeogenesis. In vitro, Pat treatment stimulates GapA activity in glycolysis and inhibit that in gluconeogenesis (Wang et al., 2010). Hence, modulating GapA activity by lysine acetylation can regulate the direction of the reversible reaction and redistribute the carbon flux between glycolysis and gluconeogenesis.

Isocitrate is an important branching point of carbon flux between TCA cycle and glyoxylate shunt. ICDH catalyzes the conversion from isocitrate to α-ketoglutarate, directing the carbon flow to TCA cycle. Isocitrate can also be split into succinate and glyoxylate by isocitrate lyase (AceA), directing the carbon flow to glyoxylate shunt. ICDH is the first bacterial enzyme identified to be regulated by reversible phosphorylation (Garnak and Reeves, 1979; LaPorte and Koshland, 1983). Isocitrate dehydrogenase kinase/phosphatase (AceK) can decrease ICDH activity by phosphorylation (Zheng and Jia, 2010). Meanwhile, activities of glyoxylate shunt enzymes AceA and AceK, and TCA cycle enzyme ICDH were also regulated by lysine acetylation, although effects of protein acetylation on activity of various enzymes are different. Acetylation led to a decrease in AceA specific activity but an increase in AceK activity (Wang et al., 2010), and the overall activity of ICDH was reduced by lysine acetylation (Venkat et al., 2018a). Hence, the metabolic flux distribution at the isocitrate branching point could be manipulated through the PTM regulations of AceA, AceK and ICDH. More specifically, reducing the acetylation of AceA protein, and/or increasing acetylation levels of AceK and ICDH could be efficient ways to channel carbon flow from TCA cycle to glyoxylate shunt. Glyoxylate shunt is a crucial pathway for acetate assimilation, furthermore it can avoid the two steps of carbon release in the TCA cycle and improve the economy of carbon atoms (Liu et al., 2017). Therefore, it is a powerful method for overcoming acetate overflow by the coregulation of acetylation and phosphorylation at the isocitrate branching point. On the basis of the crystal structure of ICDH, Venkat et al. chose eight lysine sites for acetylation and kinetic analyses. Their results demonstrated that acetylation was preferential to specific lysine sites (Venkat et al., 2018a). Acetylated ICDH lysine sites were located near S113, a key residue for reversible phosphorylation of ICDH (Doyle et al., 2001; Hurley et al., 1990; Venkat et al., 2018a). This phenomenon prompts the question of whether acetylation and phosphorylation exhibit crosstalk effects for enzyme activity.

Acetylation and phosphorylation modifications also coexist in malate dehydrogenase (MDH). MDH, catalyzing the conversion of malate and oxaloacetate, is a key enzyme in the TCA cycle. To determine whether the two modifications affect each other, researchers exploited a genetic code expansion strategy by codon selection and mutually orthogonal translation systems for simultaneously incorporating acetylation and phosphorylation into one protein (Venkat et al., 2018b). Next, they analyzed the effects of these two PTMs on the enzymatic activity of MDH in *E. coli*. They compared the enzyme activities of wild-type, acetyllysine (AcK) only containing, phosphoserine (SeP) only containing and AcK/Sep both containing MDHs. 140-AcK improved MDH enzyme activity by 3.5 folds (Venkat et al., 2018b; Venkat et al., 2017), while 280-SeP reduced the activity by approximately 70%. Surprisingly, the coexistence of 140-AcK and 280-SeP produced an enzyme activity similar to that of the wild type (Venkat et al., 2018b). These phenomena indicated that the two PTMs might independently regulate MDH enzyme activity. MDH acetylation occurred mainly via AcP-dependent chemical

Table 1
Effects of lysine acetylation on protein regulatory function.

Name	Description	Strain	Effect	Function	Reference
Enzyme activity					
Acs	acetyl-CoA synthetase	*Salmonella enterica Escherichia coli*	Acetylation decreases Acs activity	Acs acetylation regulates the acetate metabolism	Castaño-Cerezo et al. (2015); Starai et al. (2005)
GapA	glyceraldehyde phosphate dehydrogenase	*S. enterica*	Acetylation increases GapA activity in glycolysis and inhibit that in gluconeogenesis pathway	GapA acetylation regulates the carbon flux distribution between glycolysis and gluconeogenesis pathway	Wang et al. (2010)
AceA	isocitrate lyase	*S. enterica*	Acetylation decreases AceA activity	AceA acetylation regulates the activity of glyoxylate shunt and carbon flux distribution in isocitrate branch	Wang et al. (2010)
AceK	isocitrate dehydrogenase kinase/phosphatase	*S. enterica*	Acetylation increases AceK activity	AceK acetylation regulates the ICDH activity and carbon flux distribution in isocitrate branch	Wang et al. (2010)
ICDH	isocitrate dehydrogenase	*E. coli*	Acetylation decreases the overall ICDH activity	ICDH acetylation regulates the activity of TCA cycle and carbon flux distribution in isocitrate branch	Venkat et al., (2018)
MDH	malate dehydrogenase	*E. coli*	Acetylation increases the overall MDH activity	MDH acetylation regulates the activity of TCA cycle	Venkat et al. (2017)
CS	citrate synthase	*E. coli*	Acetylation decreases the overall CS activity	CS acetylation regulates the activity of TCA cycle	Venkat et al. (2019)
CheY	chemotaxis protein	*E. coli Salmonella typhimurium*	Acetylation increases CheY activity	CheY acetylation regulates the bacterial chemotaxis	Barak et al. (1992)
PatZ	acetyltransferase	*E. coli*	Autoacetylation promotes PatZ active octamer formation	PatZ autoacetylation regulates its stability and activity	(Puente et al. (2015)
AlaRS	alanyl-tRNA synthetase	*E. coli*	Acetylation decreases AlaRS activity	AlaRS acetylation regulates the protein synthesis	Kosono et al. (2018)
DNA-centered processes					
Hu	DNA-binding protein	*Mycobacterium tuberculosis*	Acetylation causes reduced DNA-binding ability of HU	Hu acetylation regulates the nucleoid structure and DNA-centered processes	Ghosh et al. (2016)
DosR	DNA-binding protein	*M. tuberculosis*	Acetylation causes reduced DNA-binding ability of DosR	Hu acetylation regulates the nucleoid structure and DNA-centered processes	Yang et al. (2018)
DnaA	chromosomal replication initiator protein	*E. coli*	Acetylation inhibits the binding of DnaA to oriC region and ATP	DnaA acetylation regulates the DNA replication initiation	Zhang et al. (2016)
TopA	topoisomerase I	*E. coli*	Acetylation reduces TopA activity and increases negative DNA supercoiling state.	TopA acetylation regulates the DNA supercoiling state	Zhou et al. (2017)
HilD	AraC/Xyls family transcriptional regulator	S. yphimurium	Acetylation causes reduced DNA-binding ability of HilD	HilD acetylation regulates the bacterial virulence	Sang et al. (2017)
PhoP	DNA-binding transcriptional dual regulator	*S. typhimurium*	Acetylation reduces the DNA-binding ability and phosphorylation level of PhoP	PhoP acetylation regulates the bacterial virulence and its transcriptional activity	Ren et al. (2019), (2016)
RcsB	DNA-binding transcriptional activator	*E. coli*	Acetylation inhibits RcsB activity	RcsB acetylation regulates the *rprA* transcription	Hu et al. (2013)
RpoA	RNA Polymerase subunit alpha	*E. coli*	Acetylation differentially affects RpoA activity	RpoA acetylation regulates *cpxP* transcription and stress responses	Lima et al. (2011, 2012)
LrP	leucine-responsive transcriptional regulator	*S.typhimurium*	Acetylation reduces the DNA-binding ability of Lrp	LrP acetylation regulates the fimbriae production	Ran et al. (2016)
CRP	transcription factor cAMP receptor protein	*E. coli*	Acetylation decreases the interaction with RNAP, and impairs CRP steady state	CRP acetylation regulates the transcription of many Class II promoters	Davis et al. (2017)
Acetylation and secondary messenger					
DgcZ	diguanylate cyclase	*E. coli*	Acetylation reduces the activity and stability of DgcZ	DgcZ acetylation regulates the production of *c*-di-GMP	(Xu et al., 2019)

catalysis, and the acetyltransferase YfiQ of *E. coli* did not work efficiently in vitro (Venkat et al., 2017).

Citrate synthase (CS), catalyzing the first reaction of TCA cycle, affects the metabolic flux distribution between glycolysis and TCA cycle. Venkat et al. also applied the genetic code expansion strategy to study the effects of lysine acetylation on CS enzyme activity (Venkat et al., 2019). CS was acetylated by AcP-dependent mechanism and deacetylated by CobB. The effects of lysine acetylation in specific sites varies on CS enzymatic activity. Acetylation impairs the overall CS activity and hinders the carbon flux into TCA cycle.

These studies provided direct biochemical evidence for lysine acetylation regulating enzyme activity in central metabolism. The change in enzyme activity affects the metabolic rate and eventually redirects metabolic flow. Thus, lysine acetylation has become a promising focus for enzyme engineering and metabolic regulation and has opened up a new engineering strategy for microbial synthesis.

3.2. Acetylation affects protein amount

The protein aggregation and inclusion body formation remain to be obstacles for the high-level production of recombinant proteins. Various factors affect the formation of inclusion bodies, such as molecular chaperones, inducer concentration, cultivation temperature, and production rate. Inclusion body formation is a dynamic and complex process that involves protein misfolding and rapid aggregation (Baneyx and Mujacic, 2004). To obtain biologically active proteins, inclusion bodies must be solubilized and renatured during purification. Inclusion body formation affects the stability, yield, biological activity, and purification cost of recombinant proteins (Baig et al., 2014; Baneyx and Mujacic, 2004; Marschall et al., 2017).

Lysine acetylation can change the charge, stability, size, and conformation of proteins, so researchers speculated that lysine acetylation may affect protein aggregation and inclusion body formation. To

test this hypothesis, a *pta-ackA* deletion mutant of *E. coli* was used to produce the aggregation-prone VP1GFP (Kuczyńska-Wiśnik et al., 2016). The mutation eliminated AcP synthesis and diminished protein acetylation through a nonenzymatic mechanism. Inclusion body formation was reduced in the *pta-ackA* mutant than that in the wild strain. In the CobB-knockout strain, inclusion body formation was enhanced because of the higher lysine acetylation status. These results suggested that protein acetylation promotes protein aggregates and the formation of inclusion bodies. In Δ*pta-ackA* mutant, slower synthesis rate of VP1GFP probably provided enough time for recombinant proteins to fold properly, resulting in lower aggregation and higher production. In Δ*cobB* strain, increased lysine acetylation can stabilize protein aggregate. However, it cannot exclude that protein aggregate is affected by some indirect factors resulted from other physiological changes caused by decreased AcP or CobB deacetylase loss.

Lysine acetylation also affects the protein half-life time and stability. HilD protein is a transcriptional regulator of the AraC/XylS family. In *S. enterica pat* knockout mutant, the half-life time of HilD was reduced 2.6 folds when compared with that in wild-type strain. The stability could be restored by complementing *pat* on the *pat* knockout mutant, suggesting lysine acetylation is responsible for maintaining the HliD stability (Yu et al., 2008). To further confirm this observation, the half-life times of HilD with the null mutant Pat (A811V) and wild-type Pat were compared. The results showed that the half-life time of HilD was 97 min in strain producing wild-type Pat protein, while it was reduced to 60 min with presence of Pat (A811V). Finally, they determined some HilD acetylation sites by mass spectrometry and identified lysine 279 was involved in the stability of HilD (Sang et al., 2017).

Thus, acetylation regulates protein amount by affecting the protein aggregation and half-life time. Combining with the effects of enzyme activity, protein acetylation regulates multiple processes on cellular physiology and metabolism.

3.3. Acetylation modulates the DNA-centered processes

DNA-centered processes, including DNA replication, transcription, repair, and recombination, are the foundation of life. Researchers have found that lysine acetylation modulates DNA-centered processes through two mechanisms. One is to regulate the DNA-binding protein to influence DNA structure, and the other is to regulate the enzyme activity involved in DNA-centered processes.

The effects of lysine acetylation on DNA-centered processes were first studied on histones in eukaryotes. Histones have lysine-rich domains that bind to the negatively charged DNA backbone (Sabari et al., 2017; Shen et al., 2015). The research on the protein acetylome in bacteria has made significant progress in recent years. Bacterial DNA-binding proteins that closely resemble histones have been identified. HU is a small 10 kDa DNA-binding protein that regulates DNA supercoiling with the help of topoisomerase (Rouvière-Yaniv et al., 1979; Van Drisse and Escalante-Semerena, 2019). So, far, only acetyltransferase Eis in *M. tuberculosis* has been identified for acetylating HU (Ghosh et al., 2016). In vitro, HU could be acetylated by Eis, changing its DNA binding capabilities (Ghosh et al., 2016). The C-terminal domain of HU contains 29 putative acetylated sites as determined by mass spectrometry, and the targets of Eis remain unknown. Additionally to HU, the *M. tuberculosis* histone-like protein DosR was confirmed to be acetylated by an acetyltransferase, RV0998 (Yang et al., 2018). Residue K182 of DosR showed a lower acetylation level under hypoxic conditions, which was assumed to increase DNA-binding capacity and facilitate gene expression to adapt to the hypoxic conditions during *M. tuberculosis* infection. A deep exploration of lysine acetylation in DNA-centered processes will contribute to understanding the bacterial pathogenesis in a new perspective.

DNA replication initiation catalyzed by DnaA is a key step in cell proliferation (Ozaki and Katayama, 2012). In this process, DnaA first binds to the AT-rich region of oriC, forming an initiation complex and facilitating the unwinding of the DNA duplex. Replication initiation is closely related to the cell cycle progression. When cells begin the transition from exponential to stationary phase, a series of cell morphology changes occur, such as the smaller cell shape, slower growth rate, reduced genome equivalents, and prolonged replication initiation cycle (Zhang et al., 2016; Zyskind and Smith, 1992). However, DnaA levels remain relatively constant throughout the growth (Ali Azam et al., 1999), suggesting that another mechanism may exist to regulate the replication initiation. Studies found that DnaA was acetylated by acetyltransferase Pat and nonenzymatic mechanism by AcP and was deacetylated by CobB (Zhang et al., 2016). DnaA acetylation status varied throughout growth and reached a peak at the stationary phase. Acetylated DnaA was inhibited for binding to oriC and ATP, reducing the frequency of DNA replication initiation (Zhang et al., 2016).

Topoisomerase I (TopA) regulates local and global DNA supercoiling. The DNA supercoiling state is primarily maintained by TopA and DNA gyrase. TopA mediates relaxation and DNA gyrase catalyzes supercoiling (Zechiedrich et al., 2000). Lysine acetylation modulates TopA enzyme activity (Zhou et al., 2017). TopA relaxation activity was reduced, and DNA negative supercoiling was increased in a CobB mutant and in vitro after the addition of AcP (Zhou et al., 2017). Hence, the physiological function of CobB is to protect TopA relaxation activity from excess lysine acetylation. These results presented a novel mechanism for regulating DNA-centered processes through the lysine acetylation of key enzymes and enriched our knowledge of systems that respond to environmental changes.

3.4. Acetylation and second messengers

Second messengers are information molecules produced in the cytoplasm, relaying the signals received from receptors on the cell surface. Besides their signal relay function, second messengers also enhance signal strength, transmit received information to effectors, and perform specific physiological functions. Few second messengers have been identified, but they regulate a wide range of biochemical and physiological processes by transmitting extracellular messages. The second messengers mainly include calcium ions (Ca^{2+}), cyclic nucleotides (e.g., cAMP and cGMP), inositol trisphosphate (IP3) and diacylglycerol (DAG).

Cyclic di-guanosine monophosphate (*c*-di-GMP) is a prevalent second messenger, playing important roles in bacterial transcription regulation and motility (Boehm et al., 2009; Xu et al., 2019). Diguanylate cyclase catalyzes the synthesis of *c*-di-GMP from GTP, and its degradation is catalyzed by phosphodiesterase. Recently, Xu et al. demonstrated the interplay between the second messenger *c*-di-GMP and deacetylase CobB (Xu et al., 2019). They discovered that the deacetylase activity of CobB was inhibited by *c*-di-GMP and further downregulated the cellular pool of acetyl-CoA by affecting the acetylation status of acetyl-CoA synthetase (Acs). Interestingly, DgcZ, involved in the synthesis of *c*-di-GMP, was the substrate of CobB. The deacetylation of DgcZ enhanced its activity and stability and thus improved *c*-di-GMP production.

Second messengers also affect the acetyltransferase activity (Xu et al., 2011). An *M. smegmatis* Pat is activated by the cyclic adenosine monophosphate (cAMP), indicating that cAMP signaling pathway regulates the MsPat-catalyzed lysine acetylation. MsPat is the first reported acetyltransferase activated by cAMP in mycobacteria, regulating the enzyme activity of central metabolism pathways. Since second messengers transmit varieties of extracellular signals, lysine acetylation can regulate cellular physiology and biochemistry processes by interplaying with second messengers.

3.5. Acetylation and bacterial virulence

The gram-negative bacterium *Salmonella* causes intestinal diseases in many animals. It is commonly used as a model for studying bacterial pathogenicity. Many studies have found that lysine acetylation plays an

essential role in bacterial virulence. The type III secretion system 1 (T3SS-1) encoded by pathogenicity island 1 (SPI-1) mediates invasion. HilD is a transcriptional regulator that induces the expression of SPI-1 (Ren et al., 2017). K297 acetylation of HilD can reduce its DNA binding capacity, inhibiting SPI-1 transcription and invasion, and SPI-1 can be expressed only with low level of acetylated HilD (Ren et al., 2017; Sang et al., 2017). As mentioned in section 3.2, acetylation of HilD K297 increased HilD stability (Sang et al., 2017). Hence, lysine acetylation can not only maintain its appropriate DNA-binding ability to ensure bacterial pathogenicity, but also keep a moderate protein level needed for cell growth due to the adverse effects of large amounts of HilD on *Salmonella* growth. It is an example that a single position of lysine acetylation regulates two different protein characteristics.

PhoP–PhoQ is a highly conserved bacterial two-component regulatory system that responds to many different signals and is closely related to bacterial virulence. PhoQ senses external signals and is activated via autophosphorylation. The phosphoryl group is sequentially transferred from PhoQ to PhoP, which activates its own expression and that of downstream-regulated genes. PhoP helps to maintain bacterial survival in the host and is considered the main contributor to virulence. PhoP in *Salmonella typhimurium* can be acetylated by Pat and an AcP-dependent nonenzymatic mechanism and is deacetylated by CobB (Ren et al., 2016). The conserved K201 in the DNA-binding domain is acetylated by Pat, reducing its DNA-binding ability. However, the phosphorylation necessary for PhoP activity was not counteracted by K201 acetylation (Ren et al., 2016). K201 acetylation also led to a dramatic attenuation of intestinal inflammation in a mouse infection model. These results suggest that acetylation of PhoP K201 has negative effects on pathogenesis.

In nonenzymatic acetylation of PhoP, the acetylation level of K102 was dependent on the concentration of AcP (Ren et al., 2019). Unlike enzymatic catalysis, K102 acetylation impaired PhoP phosphorylation and affected its transcriptional expression. K102 acetylation also reduced *Salmonella* virulence, similar to the acetylation by Pat. These findings revealed the importance of central metabolism on bacterial virulence through lysine acetylation. However, a deeper understanding of virulence-related proteins is necessary to uncover the underlying mechanisms of protein acetylation in bacterial virulence.

4. Applications of protein acetylation on metabolic engineering

Protein acetylation plays an important role in cellular physiology and metabolism and represents a potential tool for metabolic regulation to improve target production and conversion efficiency in fermentation, mainly through the regulation of overflow metabolism and global carbon flux redistribution.

4.1. Co-utilization of fermentable substrates

Carbon catabolite repression (CCR) in *E. coli* allows cells to consume glucose preferentially in a mixed carbon environment ((Brückner and Titgemeyer, 2002) Stülke and Hillen, 1999)(Fig. 4A). This mechanism usually causes a diauxic growth pattern and affects conversion efficiency in mixed carbon fermentation. Alleviating the CCR phenomenon and promoting the co-utilization of glucose and other substrates remains a crucial issue in fermentation. Acetate is considered a promising alternative carbon source because it is an inexpensive waste product of many industrial processes. For instance, acetate is a major unwanted byproduct from the pretreatment process of lignocellulosic biomass. Acetate is also produced from the anaerobic digestion of biomass (e.g., organic waste, sewage sludge, animal manure), and from syngas fermentations (Lim et al., 2018). Acetate has been used as a carbon source to produce some chemicals in *E. coli,* such as succinate (Li et al., 2016), phloroglucinol (Sun et al., 2020; Xu et al., 2017a), mevalonate (Xu et al., 2017b), 3-hydroxypropionate (Sun et al., 2020), isobutanol (Song et al., 2018) and itaconate (Noh et al., 2018). Acetate is assimilated into acetyl-CoA by high-affinity Acs node and low-affinity Pta-AckA node

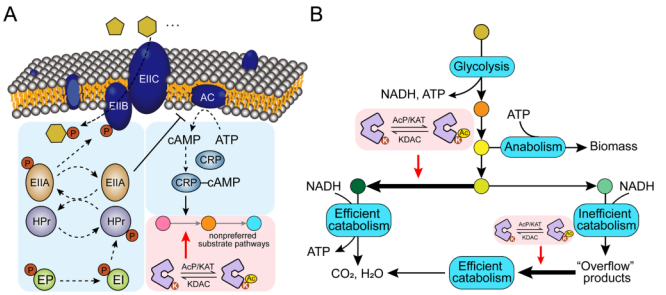

Fig. 4. Applications of protein acetylation on metabolic engineering. A: Protein acetylation promotes the co-utilization of fermentable substrates and alleviates the CCR effect. Glucose enters the cell using the phosphoenolpyruvate (PEP)-carbohydrate phosphotransferase system (PTS). PTS proteins enzyme I (EI) and phosphocarrier protein (HPr) participate in the phosphorylation cascade by transferring the phosphate group from PEP to EIIA. In the presence of preferred PTS sugars, the phosphate from EIIA is transferred to the hexose producing glucose-6-phosphate. In this case, the activity of adenylate cyclase (AC) decreased, which blocked the formation of cAMP-CRP complex and the functioning of nonpreferred substrate pathways. Protein acetylation can reactivate the metabolic enzymes involved in these pathways, which provides a new strategy for mixed carbon fermentation and CCR elimination. B: Protein acetylation regulates overflow metabolism. Protein acetylation promotes efficient catabolism or re-utilize "overflow" products, thereby affecting carbon flux redistribution. Annotation: EIIA, EIIB, EIIC, glucose specific enzyme II of PTS; P, phosphate group; CRP, cyclic AMP (cAMP) receptor protein; Red arrows represent promotion; Small circles labeled in different colors are indicated as substrates or intermediates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Thao and Escalante-Semerena, 2011). However, acetate cannot be co-utilized with glucose in *E. coli* K-12 strains because of the CCR.

As the most important enzyme for acetate assimilation, Acs is tightly controlled at the transcriptional and posttranslational levels (Tucker et al., 2014). Acs transcription is directly regulated by cAMP–CRP and the regulator FNR (Beatty et al., 2003), and is probably indirectly related to the DNA-binding transcriptional repressor IclR and many genes in acetate metabolism (Liu et al., 2015). Acs enzyme activity is regulated by lysine acetylation as described above. Leucine at position 641 is recognized by Pat, rendering acetylation of lysine at 609 and eliminating Acs activity. A Leu-641 Acs mutant was acetylation-insensitive (Starai et al., 2005), and an Acs (L641P) mutant was created in *E. coli* W and evaluated for the capacity to co-utilize glucose and acetate in batch and continuous culture (Novak et al., 2018). Acetate uptake was increased by 2.7-fold with glucose and a high concentration of acetate in batch fermentation. Additionally, *E. coli* W co-utilized glucose and acetate efficiently in an accelerostat (A-stat) with 0.20–0.70 h^{-1} dilution rates (Novak et al., 2018). This study provided a new candidate in metabolic engineering for CCR elimination and mixed carbon fermentation (Fig. 4A).

4.2. Overflow metabolism and regulation

Overflow metabolism is a common physiological phenomenon in the fermentation of industrial microorganisms when glucose is rapidly consumed in the presence of sufficient oxygen (Vemuri et al., 2006). Overflow metabolism is believed to be caused by the much higher activity of glycolysis versus the TCA cycle, leading to a significant accumulation of intermediates pyruvate and acetyl-CoA. For the urgent replenishment requirement of coenzyme A and energy, pyruvate and acetyl-CoA are converted to lactate and acetate (El-Mansi, 2004; Vemuri et al., 2006). Acetate excretion has adverse effects on gene expression, signal transduction, cell growth, and stress responses even at a low concentration of 0.5 g/L (Farmer and Liao, 1997). Acetate accumulation also affects carbon utilization efficiency. In previous reports, 10%–30% of carbon flux was distributed to acetate with glucose as the sole carbon

source during aerobic fermentation (Farmer and Liao, 1997). Many strategies have been described to alleviate overflow metabolism by fermentation control and genetic modifications, including maintaining as little residual glucose as possible in the fermentation system, reducing the glucose transport and consumption rate, activating the TCA cycle and glyoxylate shunt, and destroying the acetate biosynthetic pathways from pyruvate and/or acetyl-CoA (Eiteman and Altman, 2006; Liu et al., 2015).

Protein acetylation plays an important role in regulating overflow metabolism (Fig. 4B). Overflow metabolism was suppressed in glucose cultures, and cell growth rate was higher in acetate medium in an *E. coli* BL21 *pat* mutant strain. By contrast, when *cobB* was knocked out, the acetate concentration was increased in a glucose medium, and the cell growth rate was impaired in acetate cultures in *E. coli* strains K-12 and BL21 (Castaño-Cerezo et al., 2014). This phenomenon is partly due to the acetylation of key enzymes in central metabolism. By affecting enzyme activity, acetylation regulates the reaction rate of specific pathways and metabolic flux distribution, indirectly influencing overflow metabolism. For example, a key enzyme Acs in acetate metabolism, is regulated by acetylation, affecting the metabolic flux from acetyl-CoA to acetate and acetate secretion (Burckhardt et al., 2019; Castaño-Cerezo et al., 2014; Van Drisse and Escalante-Semerena, 2019(Starai and Escalante-Semerena, 2004b)). ICDH in the TCA cycle and AceA in glyoxylate shunt are also regulated by lysine acetylation. ICDH and AceA compete for the common substrate isocitrate, an important node in metabolic flux regulation. A previous study found that deletion of *cobB* led to a 34% reduction of carbon flux through the glyoxylate shunt (Castaño-Cerezo et al., 2014). The protein level of the glyoxylate shunt in the *cobB* mutant was approximately 50% of that observed in the wild-type strain, and the enzyme activity of AceA was significantly lower. The decrease in protein level and enzyme activity was highly consistent with the reduction in carbon flux through glyoxylate shunt. The glyoxylate shunt is the vital pathway for acetate assimilation, so acetylation can fine tune the glyoxylate shunt and affect overflow metabolism.

AcP-dependent nonenzymatic acetylation is the predominant form in *E. coli*. To describe the functional significance, Schilling et al. tracked the dynamics of Nε-lysine acetylation during the growth of wild-type *E. coli* K-12 and the CRP mutant growing in buffered tryptone broth (TB7, 1% (*w/v*) tryptone buffered at pH 7.0 with 100 mM potassium phosphate) with glucose or lactate carbon sources (Schilling et al., 2015). Each lysine in the studied protein showed considerable variations though overall acetylation extent and modification site increasing during cell growth process. The acetylation sites in some cases were both AcP- and glucose-dependent, and the latter acetylation was dominant in central metabolic pathways. cAMP-CRP is indispensable on glucose-dependent acetylation. When CRP was knockout, the global acetylation was correspondingly reduced. CRP-dependent acetylation also depends on AcP. Thus, the overall acetylation level was low in AcP synthesis-inhibited cells even CRP was overexpressed. In contrast, elevating the AcP level via the deletion of AcP degradation enzymes restored the dramatic acetylation loss caused by CRP deletion (Schilling et al., 2015). These results were consistent with a hypothesis that AcP synthesis was under the control of cAMP-CRP mediated transcription. In conclusion, AcP-dependent acetylation regulated by cAMP-CRP is believed to respond to overflow metabolism and mediate redistribution regulation of carbon flux in central metabolic pathways (Fig. 4B).

Compared with the traditional methods for regulating overflow metabolism, protein acetylation is a global regulation and shows metabolic fitness. For example, decreasing glucose transport by destroying the PTS system simultaneously affects the growth rate. Knockout of acetate synthetic pathway genes for alleviating overflow metabolism is also at the expense of cell growth. A common explanation is that Pta-AckA pathway is an ATP production source both in aerobic and anaerobic conditions (Ma et al., 2018; Mey et al., 2007). Besides, inactivation of the Pta-AckA pathway will make bacteria lose the ability to maintain redox homeostasis by diverting carbon away from the TCA cycle, and then impair fitness (Darrell et al., 2016; Won et al., 2021). To conclude, acetylation modification can be used as a fitness strategy to alleviate overflow metabolism and improve carbon utilization.

5. Regulation of protein acetylation status

Protein acetylation plays an important role in cellular physiology and is considered a promising strategy for metabolic regulation. The question is, how does one maintain or change protein acetylation status? We review some factors that affect protein acetylation status.

5.1. Regulation of AcP synthesis

The predominant acetylation in some bacteria is AcP-dependent nonenzymatic form. Since AcP is the primary acetyl donor and drains the KAT-dependent donor acetyl-CoA (Christensen et al., 2019), engineering pathways involved in AcP synthesis and acetate metabolism may be the first choice to change protein acetylation. The low affinity and reversible Pta-AckA pathway is the primary source for intermediate AcP (Christensen et al., 2019; Weinert et al., 2013). Pta catalyzes the reversible reaction between acetyl-CoA and AcP, whereas AckA catalyzes the interconversion of AcP and acetate. Higher AcP concentration and more lysine acetylation were observed in an *ackA* mutant than in the wild type in TB7 culture. By contrast, protein acetylation was decreased in a *pta-ackA* double mutant (Kuhn et al., 2014). However, when the *pta* mutant was grown in TB7 supplemented with acetate, the acetylation level increased, perhaps because of AcP synthesis from acetate via AckA catalysis (Kuhn et al., 2014).

As mentioned above, AcP-dependent acetylation can respond to overflow metabolism. Do the regulators involved in carbon flux control also influence protein acetylation? To explore this question, carbon regulators *arcA*, *cra*, *csrA*, *rcsB*, *crp*, and *cyaA* were deleted in *E. coli* BW25113 (Schilling et al., 2015). Obvious differences in acetylation patterns in these mutants were observed via western immunoblotting. The *crp* and *cyaA* mutants exhibited significantly lower global acetylation than did the wild-type strain (Schilling et al., 2015). To examine the relationship between CRP and AcP-dependent acetylation, Schilling et al. compared the acetylation status of *crp-ackA* double mutant to the *ackA* single mutant (Schilling et al., 2015). They demonstrated that AcP -dependent acetylation required CRP. Hence, genetic modifications of some carbon regulators can affect protein acetylation.

Genetic engineering of acetyltransferase and deacetylases is also a solution to be considered. Genetic modifications of these enzymes significantly changed protein acetylation, further affected the enzyme activity and metabolic flux distribution in vivo. Purified proteins incubated with acetyltransferase or deacetylase also change protein acetylation and enzyme activity. A large number of relevant results have been reviewed in section 3. In addition, other strategies, such as host selection, cell growth stage, carbon source and concentration have also been reported to affect protein acetylation. Thus, choosing an appropriate host and culture conditions also regulate protein acetylation and cell growth.

5.2. Host selection

E. coli is the most widely used host for laboratory and biotechnology applications. Phenotype, genome, and cellular physiology and metabolism have been compared in strains BL21 and K-12 (Liu et al., 2015). Differences in acetate metabolism between these strains are intriguing, and some researchers have attempted to unravel the mechanism underlying these differences. Noteworthily, acetate metabolism and protein acetylation are inextricably linked, prompting the question of whether the acetylation status of these strains also differs. Protein acetylation is more abundant in K-12 than in BL21, perhaps because of an elevated accumulation of AcP and increased acetate excretion (Weinert et al., 2013). Noronhae et al. compared the flux ratios through

glyoxylate shunt and TCA cycle between BL21 and K-12 strains by a comprehensive ^{13}C-isotopomer based model. They found that the flux through glyoxylate shunt was less in K-12, resulting in more acetate production, which can confirm the above assumption (Noronha et al., 2015). Other experiments were also conducted to elucidate the mechanism for differences in acetate metabolism and protein acetylation in BL21 and K-12 (Castaño-Cerezo et al., 2015). They compared the difference of protein acetylation between the wild-type BL21 and K-12 as well as *pat* and *cobB* mutant derivatives. Mutants of BL21 showed a more dramatic variation of protein acetylation than those of K-12 in glucose cultures. Similarly, the deletion of *cobB* led to a more than six-fold increase in acetate production in BL21 compared with a twofold increase in K-12. However, the protein acetylation of mutants in the K-12 background remained higher than the corresponding mutants in BL21. Thus, differences in protein acetylation due to the genetic background can influence acetate metabolism and growth rate.

Other studies sought to verify whether differences in gene expression lead to the differences in protein modification between BL21 and K-12. The expression of *acs*, *pat*, and *cobB* was measured in glucose and acetate media. The expression of *acs* and *pat* was higher in BL21 than in K-12 in glucose culture during the exponential phase growth. There was no obvious difference in *acs* expression, and *pat* expression in BL21 increased after 10 h growth in acetate culture. Unlike the expression pattern of *acs* and *pat* in glucose, *cobB* expression was very similar between the two strains. BL21 undergoes less of a CCR effect than the K-12 strain, leading to higher expression of *acs* and *pat* in BL21. BL21 can simultaneously utilize acetate and glucose, whereas K-12 only consumes acetate when the supply of glucose is exhausted (Castaño-Cerezo et al., 2015; Waegeman et al., 2012). These results proved the lower CCR effect and acetate production in BL21.

A regulatory scheme was proposed for lysine acetylation and acetate metabolism in BL21 and K-12 in the exponential growth phase in glucose culture (Castaño-Cerezo et al., 2015). In K-12, *acs* gene is not expressed because the transcription factor CRP is inactive due to the absence of cAMP, and acetate excretion depends only on the Pta-AckA pathway. In BL21, acetate is co-utilized with glucose because of the relaxed CCR, leading to higher cAMP concentration and contributing to *acs* expression. Thus, acetate metabolism was simultaneously regulated by Pta-AckA and Acs. Meanwhile, Acs enzyme activity was regulated by protein acetylation as discussed above. The lower acetylation status in BL21 leads to an increased Acs activity and a reduced acetate excretion.

Unlike the common strains BL21 and K-12, *E. coli* W has a special character of sucrose metabolism. Sucrose is produced from sugarcane, a most prevalent sustainable biomass. So, *E. coli* W clearly has great potential in industry applications. The genome of *E. coli* W was sequenced in 2011, and a sucrose catabolism (csc) regulon encoded by chromosome was identified (Sang et al., 2011). The *csc* regulon contains three functional genes of *cscB*, *cscA*, *cscK* and a repressor protein encoded gene *cscR*. When sucrose is absence, *csc* regulon is repressed by CscR, while it can be activated by cAMP-CRP in the absence of glucose due to the release of CCR (Deutscher et al., 2007). *E. coli* W shows a low acetate production, and Arifin et al. uncovers the possible reason using a systems biology approach combined with metabolite analysis (Arifin et al., 2014). They found that the flux in pentose phosphate pathway and glyoxylate shunt was reduced, and that in TCA cycle was increased. But the glyoxylate shunt was inactive when growing on sucrose, which was different from growing on other sugars. So, the low acetate accumulation may be result from the induction of acetate catabolic genes *acs* and *actP*, and is independent of the glyoxylate shunt. Although there were few reports on the comparison of acetylation status among the *E. coli* W, BL21 and K-12 strains, low acetate accumulation may lead to low acetylation level of *E. coli* W according to the existing theories.

The substantial differences in protein acetylation and acetate metabolism between *E. coli* strains make it critically important to select an appropriate host for microbial fermentation and metabolic engineering. Meanwhile, a deep exploration of the different regulatory mechanisms in these strains will provide more ideas for metabolic regulation.

5.3. Carbon source

Numerous studies have found that protein acetylation is affected by different carbon sources. The protein acetylome of *S. enterica* was observed with the presence of glucose and citrate, showing 15 enzymes with altered protein acetylation status based on carbon source, and all of them showed higher acetylation levels in glucose-containing medium (Wang et al., 2010). Furthermore, protein acetylation was enhanced by increased glucose concentrations (Venkat et al., 2017). Thus, cells could adapt to different carbon sources by altering protein acetylation and concomitantly regulating metabolic flux and cell growth.

Bacterial growth media usually contain a rich assortment of nutrients, including carbon, nitrogen, and inorganic salts. As the cofactor of many enzymes, magnesium is also frequently added. In recent years, Christensen et al. found an interesting phenomenon when they experimented with protein acetylation in *E. coli* in TB7 medium supplemented with glucose. At first, they observed that glucose was not fully consumed before the onset of the stationary phase. However, the glucose was depleted entirely in the exponential phase as magnesium was added to the medium (Christensen et al., 2017). Furthermore, cell size decreased and cell number and biomass increased, and similar results were observed in media containing other sugars or peptides (Christensen et al., 2017). Magnesium is indispensable for bacterial glucose consumption and cell growth, and is limited for cell growth in peptide-based media containing a sugar. As halting cell growth by whatever means leads to acetylation accumulation, magnesium supplementation not only promotes sugar consumption but also reduces protein acetylation. How does magnesium supplementation decrease protein acetylation? First, magnesium supplementation promotes more glucose to be converted into biomass instead of acetate, decreasing AcP-dependent acetylation. Second, the prolonged exponential phase by magnesium supplementation produces more nascent unmodified proteins, constantly diluting the overall acetylation level. Thus, magnesium supplementation will become a promising method to tune the acetylation level without using genetic manipulation, and will reduce the potential for accumulation of acetylation during recombinant protein production without impairing cell growth.

Noteworthily, no significant differences in protein acetylation were observed in cells cultured with the same concentration of glucose and xylose. However, more acetylation was obtained at higher concentrations of these two sugars (Schilling et al., 2019). This phenomenon suggested that it is more about the flux through glycolysis than identity of the carbon source. Hence, the acetylation level was not dependent on the specific carbon source per se but is the response to the levels of AcP. To conclude, selecting the appropriate carbon sources and optimizing the concentration and ratio of nutrients can also regulate protein acetylation and achieve a desirable result.

6. Conclusions and prospects

As a conserved global regulatory mechanism, protein acetylation plays an essential role in bacterial enzyme activity, cell cellular physiology, and metabolism. Genetic modifications and nonengineering methods can be used to regulate and change acetylation status. Lysine acetylation can be used for fermentation engineering to improve target production and conversion efficiency. However, there are many opportunities and challenges in the emerging field, for example, identification of novel acetyltransferase and deacetylases, interactions of acetylation and other PTMs. We must also understand what factors trigger protein acetylation, why the acetylation level and its effect on the activity of different enzymes vary even under the same conditions, and which lysine sites or protein types are more preferred by (de)acetylase. Functions of protein acetylation in many other bacteria still remain unclear, and more exploration is needed to define the regulatory roles

and mechanisms of protein acetylation.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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