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REVIEW

Bacterial protein acetylation: new discoveries unanswered questions

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Abstract Nε-acetylation is emerging as an abundant post-translational modification of bacterial proteins. Two mechanisms have been identified: one is enzymatic, dependent on an acetyltransferase and acetyl-coenzyme A; the other is non-enzymatic and depends on the reactivity of acetyl phosphate. Some, but not most, of those acetylations are reversed by deacetylases. This review will briefly describe the current status of the field and raise questions that need answering.

Nε-acetylation is abundant in bacteria

Post-translational modifications control protein structure, stability and function. In eukaryotes, Nε-lysine acetylation is an abundant post-translational modification affecting thousands of proteins in diverse processes (Glozak and Seto 2007; Rardin et al. 2013; Verdin and Ott 2015; Yang and Seto 2008a). Compelling evidence exists that Nε-acetylation is also abundant in bacteria. Global surveys have revealed acetylation in diverse bacterial phyla (Baeza et al. 2014; Kim et al. 2013; Kosono et al. 2015; Kuhn et al. 2014; Lee et al. 2013; Wang et al. 2010; Weinert et al. 2013; Wu et al. 2013; Yu et al. 2008; Zhang et al. 2009,

2013). Recent studies have detected acetylation of 15–20 % of the *Escherichia coli* and *Bacillus subtilis* proteomes (Kosono et al. 2015; Kuhn et al. 2014; Weinert et al. 2013; Zhang et al. 2013). Here, I will provide a brief review and raise questions I consider to be most important.

Nε-acetylation can occur by two different mechanisms

The conventional mechanism for Nε-acetylation is enzymatic (Fig. 1i), relying on a lysine acetyltransferase (KAT) to catalyze donation of the acetyl group from acetyl-coenzyme A (acCoA) to the ε-amino group of a deprotonated lysine. This is the mechanism used by KATs to acetylate lysines in the unstructured N-termini of eukaryotic histones. KATs from diverse bacteria have been identified and some characterized [for reviews, see Bernal et al. (2014), Hentchel and Escalante-Semerena (2015), Hu et al. (2010), Jones and O'Connor (2011), Kim and Yang (2011), Soppa (2010) and Thao and Escalante-Semerena (2011b)].

In *E. coli,* researchers have identified a single KAT: a member of the GCN5-like acetyltransferase (GNAT) family called YfiQ. It was first identified and characterized in *Salmonella enterica* (Hentchel and Escalante-Semerena 2015; Starai and Escalante-Semerena 2004b; Thao and Escalante-Semerena 2011a), where it is called Protein AcetylTransferase or Pat. To avoid confusion, use of this acronym in *E. coli* should be discouraged, as it was previously assigned to Putrescine AminoTransferase. For a similar reason, an alternative acronym (Pka) should also be discouraged, as it is the widely used acronym for Protein Kinase A, which modifies eukaryotic proteins by phosphorylation.

It is possible that *E. coli* expresses KATs in addition to YfiQ, as several *E. coli* GNATs have not yet been

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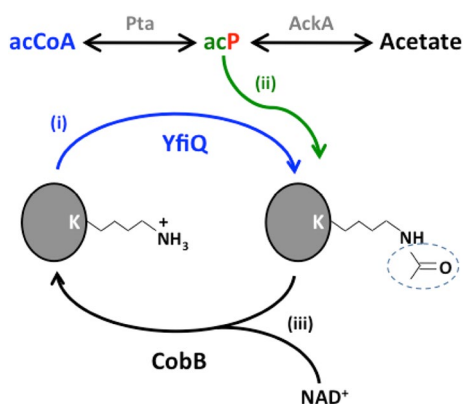
Fig. 1 Mechanisms for Nε-lysine acetylation and deacetylation. *i* Acetyltransferase (YfiQ)- and acCoA-dependent acetylation (*blue*). *ii* Non-enzymatic acP-dependent acetylation (*green*). *iii* The NAD⁺-dependent deacetylase CobB can remove acetyl groups from some acetyllysines. acP is the high-energy intermediate of the phospho-transacetylase (Pta)–acetate kinase (AckA) pathway

assigned a function. However, previous attempts to identify additional KATs have failed. New approaches should be applied.

Recent studies reveal a novel non-enzymatic mechanism, in which acetyl phosphate (acP) directly donates its acetyl group to the ε-amino group of a deprotonated lysine (Kuhn et al. 2014; Weinert et al. 2013). The result is the same as the enzymatic mechanism, acetylation of the Nε-amino group of a lysine (Fig. 1ii). In sheer numbers of sensitive lysines, acP-dependent acetylation dwarfs YfiQ-dependent acetylation (Kuhn et al. 2014; Weinert et al. 2013). A novel, label-free quantitative mass spectrometric (MS) method called Skyline 'MS1 Filtering' (Rardin et al. 2013; Schilling et al. 2012) was used to determine, with statistical significance, that 592 lysines from 292 proteins were sensitive to acP levels. In contrast, many fewer lysines (~6X) were sensitive to the KAT YfiQ (Kuhn et al. 2014). Using more conventional SILAC methods, others obtained similar results (Weinert et al. 2013). Thus, in terms of sheer numbers of acetylated lysines, the non-enzymatic, acP-dependent mechanism predominates in *E. coli*. Non-enzymatic, acP-dependent acetylation is not confined to *E. coli;* a similar story is unfolding in *B. subtilis* (Kosono et al. 2015).

The impact of acetylation is predicted to depend upon its stoichiometry. A highly regulated and common acetylation likely has more impact than a highly regulated but uncommon acetylation. To address this issue, John Denu and co-workers developed a method to calculate global acetyl-stoichiometry in cell lysates. They modified an approach previously developed by Brad Gibson's group to determine relative lysine reactivities in an isolated protein (Guo et al.

2008). Denu's team detected substantial acetyl-stoichiometry in *E. coli;* hundreds of quantified peptides had stoichiometries greater than 10 %, with 150 peptides acetylated at stoichiometries >20 % (Baeza et al. 2014). Since both acetylating mechanisms were operable in the tested cells, it is unclear whether the detected acetyllysines resulted from the enzymatic, acCoA-dependent mechanism or from the non-enzymatic, acP-dependent mechanism. Further analyses of stoichiometry should be pursued under a variety of physiologically relevant conditions. Such studies will help prioritize the study of acetyllysines predicted to have the largest impact.

The deacetylase CobB can reverse acetylations from either mechanism

Acetyllysines are quite stable; however, they can be enzymatically reversed by lysine deacetylases (KDACs) (Fig. 1iii). Two major families of KDACs have been identified: the zinc-dependent Rpd3/Hda1 family (Yang and Seto 2008b) and the NAD⁺-dependent sirtuin family (Blander and Guarente 2004). For each HDAC family, putative bacterial homologs have been identified [reviewed by Hildmann et al. (2007)], but only a few have been shown to serve as protein deacetylases [for a review, see Hentchel and Escalante-Semerena (2015)]. The best example of a bacterial sirtuin is CobB, first identified and characterized in *S. enterica* (Starai et al. 2002, 2003). In *E. coli,* the sirtuin CobB appears to be the sole (or predominant) KDAC (AbouElfetouh et al. 2015). It shows no preference for acetyl donors (AbouElfetouh et al. 2015); it can deacetylate both YfiQ-catalyzed acCoA-dependent acetyllysines (e.g., K609 of acetyl-CoA synthetase) and non-enzymatic acP-dependent acetyllysines (e.g., K154 of RcsB) (AbouElfetouh et al. 2015). Thus, it is not a member of a system designed specifically to reversibly acetylated a few key lysines, as suggested (Hentchel and Escalante-Semerena 2015), but instead appears to operate independently, at least in *E. coli*. How CobB recognizes its substrates remains to be determined; however, we know that specificity depends on accessibility and three-dimensional microenvironment of the target acetyllysine (AbouElfetouh et al. 2015). In contrast to dogma, most acetyllysines appear to be insensitive to CobB (Kuhn et al. 2014; Weinert et al. 2013). MS1 Filtering identified and quantified only 69 CobB-sensitive lysines. This contrasts with the 592 acP-dependent acetyllysines (Kuhn et al. 2014). Only 24 were sensitive to both acP and CobB (AbouElfetouh et al. 2015). Thus, the vast majority of acetyllysines are not reversed and a major unanswered question is how does the cell cope with acetylations that are not reversed?

acP-dependent acetylation is specific

Most lysines are not acetylated or only at very low undetectable levels. Some (e.g., K609 of Acs) are specifically acetylated by the enzymatic reaction (Hentchel and Escalante-Semerena 2015). Those that are acetylated in an acP-dependent manner exhibit different acetylation rates with different fold changes over time (Schilling et al. 2015). Most acetylated lysines reside in a three-dimensional environment conducive to non-enzymatic acetylation. This environment contains residues that bind the phosphoryl group; these residues are either positively charged or tend toward H-bond formation (Baeza et al. 2015; Kuhn et al. 2014). Also, the lysine must be deprotonated. In the enzymatic, acCoA-dependent mechanism, often a negatively charged residue on the enzyme surface acts as a general base catalyst to perform this function. In the non-enzymatic, acP-dependent mechanism, the catalyst appears to be a negatively charged residue adjacent to the target lysine (Kuhn et al. 2014). This model should be rigorously tested, but independent evidence supports it. A prime example is the cAMP receptor protein (CRP), the major carbon regulator in δ-proteobacteria, e.g., Enterobacteriaceae (Shimada et al. 2011) and Vibrionaceae (Colton and Stabb 2015). CRP has two adjacent lysines (K100 and K101) that exhibit strikingly different behaviors with respect to acetylation. AcP-dependent acetylation of K100 is almost always detected, whereas acetyl-K101 has never been detected (Baeza et al. 2014; Kuhn et al. 2014; Weinert et al. 2013; Zhang et al. 2013). The former includes all the aforementioned molecular features; the latter does not.

acP-dependent acetylation is a consequence of acetate fermentation

Enzyme-dependent acetylation is regulated at several levels. As mentioned previously, CobB deacetylates a subset of YfiQ-dependent acetyllysines in *S. enterica* and *E. coli* (AbouElfetouh et al. 2015; Garrity et al. 2007; Li et al. 2010; Starai et al. 2002; Zhao et al. 2004). In *S. enterica*, the myo-inositol catabolism repressor LolR activates transcription of *pat* and *cobB*, as well as that of *acs*, which encodes their substrate acetyl-CoA synthetase (Hentchel et al. 2015). Transcription of *pat*, but not *cobB*, decreases under acid stress. This might be due to reduced cAMP-CRP complex as acid stress also reduced *cya* and *crp* transcription (Ren et al. 2015) and *E. coli yfiQ* transcription depends on the cAMP-CRP complex (Castano-Cerezo et al. 2011). In *E. coli*, acP-dependent acetylation also requires cAMP-CRP (see below), but not YfiQ, and CobB can deacetylate a subset of acP-dependent acetyllysines (AbouElfetouh et al.

2015). Also, acP-dependent acetylation seems to result from overflow metabolism, which occurs if the CoA pool is overwhelmed, for example, when carbon exceeds nitrogen and/or oxygen. Cells solve this metabolic bottleneck by recycling CoA via mixed acid fermentation (Wolfe 2005). Species that perform mixed acid fermentation are found in the genera *Escherichia*, *Neisseria, Bacillus* and *Vibrio*, among others (Wolfe 2015). The major products of mixed acid fermentation are ATP and acetate. The intermediate of this pathway (Pta-AckA) is acP (Wolfe 2005). With millimolar levels of acP (Klein et al. 2007), many lysines become acetylated (Kuhn et al. 2014; Weinert et al. 2013). We propose a simple model: Overflow metabolism causes acP accumulation, and acP accumulation causes acetylation. This scenario is supported by a recent study that showed global acetylation increasing over time and correlating closely with glucose consumption and acetate excretion (Schilling et al. 2015). Attempts to further test this model are underway. If it is correct, then genetic or environmental manipulations that alter the timing of glucose consumption should simultaneously influence acetylation.

AcP-dependent acetylation is enriched in central metabolic pathways

If acetylation is a response to overflow metabolism, then acetylation might feed back to regulate central metabolism. If so, then central metabolic enzymes should be enriched for acetylation. Indeed, MS1 Filtering revealed that proteins with glucose-regulated acetyl sites are overwhelmingly involved in central metabolism, notably glycolysis, gluconeogenesis, the TCA cycle, the glyoxylate bypass, fatty acid biosynthesis and pyruvate metabolism, which links them all. Our group and others had previously shown that acetylation is enriched in central metabolic pathways (Kuhn et al. 2014; Wang et al. 2010; Weinert et al. 2013; Zhang et al. 2013), but this was the first demonstration that central metabolic enzymes become acetylated over time in response to glucose consumption. It also revealed that most glucose-induced acetylation was acP-dependent (Schilling et al. 2015). Moreover, this effect is not restricted to glucose, as similar results were obtained with other carbon sources (e.g., lactate) that result in acetate fermentation [(Schilling et al. 2015) and unpublished data].

cAMP-CRP regulates glucose-induced acP-dependent acetylation

Since glucose-regulated acetylation was enriched in central metabolism, we tested the hypothesis that some central metabolic regulator would regulate acetylation. Glucose

consumption and acetate excretion were delayed, and global acetylation was essentially eliminated in *cya* and *crp* mutants, but not in mutants lacking other central metabolic regulators, including *arcA, cra* and *csrA* (Schilling et al. 2015). Most CRP-dependent acetylation requires acP synthesis: acetylation was low in mutants that fail to synthesize acP, even when CRP was overexpressed. In contrast, acetylation was restored in *crp* mutants that accumulated acP. These results are consistent with a model in which acP is downstream of CRP. The mechanism by which cAMP-CRP-dependent transcription facilitates acP synthesis remains unknown, but it likely involves transcriptional control of carbon transport and flux through glycolytic pathways (Schilling et al. 2015).

The impact of acetylation on protein function and cellular physiology

We now know that protein acetylation occurs in diverse bacteria. It is abundant, stoichiometrically relevant, and regulated at multiple levels. As such, bacterial protein acetylation resembles eukaryotic protein acetylation, including non-enzymatic acetylation, which occurs at low levels in mitochondria (Lombard et al. 2015; Wagner and Payne 2013; Weinert et al. 2015). The inference is that acetylation in bacteria works much like it does in eukaryotes and that it exerts a similar impact on protein function and cellular physiology.

Most of what we know about the impact of Nε-acetylation of bacterial protein function comes from the study of a small subset of proteins. For acetyl-CoA synthetase (Acs), reversible acetylation serves as a simple on–off switch. A KAT (Pat in *S. enterica*, YfiQ in *E. coli*) inhibits Acs activity by acetylating a lysine (K609) located in Acs' active site (Gardner et al. 2006; Starai et al. 2002). The mechanism by which acetylation inhibits Acs activity is unknown; however, reactivation occurs upon deacetylation (Starai et al. 2002), catalyzed by the sirtuin CobB (Starai et al. 2002, 2003). For the response regulator CheY, acetylation of several lysines influences (1) reversible phosphorylation of an aspartyl residue, (2) rotational direction of the flagellar motor and (3) CheY's ability to form complexes with 3 protein targets (Barak and Eisenbach 2001; Barak et al. 1992, 2004, 2006; Fraiberg et al. 2014; Li et al. 2010; Liarzi et al. 2010; Yan et al. 2008). For RNase R, acetylation controls protein turnover: acetylation and thus neutralization of a lysine residue is proposed to break a salt bridge that sequesters the docking site for a chaperone that recruits proteases (Liang et al. 2011). For RcsB, it appears that acetylation of one lysine (K154) inhibits its function (Castano-Cerezo et al. 2014;

Hu et al. 2013), but the mechanism remains unknown. For the alpha subunit of RNA polymerase, acetylation of one lysine (K298) is proposed to enhance glucose-induced CpxR-dependent transcription of *cpxP* (Lima et al. 2011), whereas acetylation of a second lysine (K291) inhibits that transcription (Lima et al. 2012). Thus, acetylation of two different lysines on the same protein can differentially regulate its function.

As with protein function, the impact of acetylation on cellular physiology is poorly understood. Some patterns, however, have emerged. First, there is the obvious connection to central metabolism. The acetyl donors (acCoA and acP) are central metabolites as is the necessary substrate for deacetylation (NAD^+). The major carbon regulator in *E. coli* and other enteric bacteria CRP regulates both non-enzymatic acP-dependent acetylation and enzymatic acCoA-dependent acetylation and fermentation results in acP-dependent acetylation. Furthermore, reversible acetylation regulates Acs activity (Gardner et al. 2006; Starai et al. 2002; Starai and Escalante-Semerena 2004a, b). Because this activity involves both acCoA and ATP, acetylation modulates energy status with its attendant consequences (Chan et al. 2011). AcP-dependent and CobB-sensitive acetylation of K154 inhibits RcsB function (Castano-Cerezo et al. 2014; Hu et al. 2013), which responds to peptidoglycan stress (Cambre et al. 2015; Laubacher and Ades 2008), controls cell shape (Ranjit and Young 2013) and contributes to inherent antibiotic resistance (Laubacher and Ades 2008). RcsB also inhibits flagellar expression (Fredericks et al. 2006), activates extracellular polysaccharide biosynthesis (Fredericks et al. 2006), enhances the acid stress response (Castanie-Cornet et al. 2010) and controls transcription of a small RNA (*rprA*) that modulates the response to stationary phase stress (Hu et al. 2013; Majdalani et al. 2002). The link between acetylation and stress is supported by several other observations. Glucose-induced acetylation of alpha regulates transcription of *cpxP* (Lima et al. 2011, 2012), which encodes a chaperone involved the periplasmic unfolded protein response (Danese and Silhavy 1998). Furthermore, YfiQ/Pat and CobB have been associated with stresses associated with low pH (Ren et al. 2015), high temperature (Ma and Wood 2011) and reactive oxidative species (Ma and Wood 2011). Finally, acP has been associated with ATP-dependent proteolysis (Mizrahi et al. 2006) and both protein folding and aggregation (Mizrahi et al. 2009). ATP-dependent proteases are linked to carbon metabolism (Yang and Lan 2015). Most of these proteases and their chaperones are acetylated in an acP-dependent manner (Kuhn et al. 2014; Schilling et al. 2015). Since it is associated with stresses of multiple types, could acetylation be a response to stresses associated with rapid carbon flux through central metabolism?

Consequences for pharmaceutical production

Lysine acetylation has been detected following production in *E. coli* of recombinant therapeutic proteins, including the chemokine RANTES (d'Alayer et al. 2007), human basic fibroblast growth factor mutein (Suenaga et al. 1996), human carbonic anhydrase (Mahon et al. 2015), insulin lispro (Szewczak et al. 2015), interleukin-10 (Pflumm et al. 1997), interleukin-2 (Moya et al. 2002), interferon alpha (Takao et al. 1987), neurotropin-3 (Ross et al. 1996) and somatotropin (Violand et al. 1994). Nearly 30 % of currently approved recombinant therapeutic proteins are produced in *E. coli* (Huang et al. 2012). All post-translational modifications in a recombinant therapeutic protein could potentially influence its safety and efficacy and should be assessed for toxicity, immunogenicity and biological activity (Mahon et al. 2015). Thus, it is critical to identify the acetylating mechanism, understand its regulation and determine the effect that acetylation exerts on the function of these heterologously expressed proteins.

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