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Regulation of bacterial physiology by lysine acetylation of proteins

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Post-translational modification of proteins is a reversible mechanism of cellular adaptation to changing environmental conditions. In eukaryotes, the physiological relevance of N-ε-lysine protein acetylation is well demonstrated. In recent times, important roles in the regulation of metabolic processes in bacteria are being uncovered, adding complexity to cellular regulatory networks.

The aim of this mini-review is to sum up the current state-of-the-art in the regulation of bacterial physiology by protein acetylation. Current knowledge on the molecular biology aspects of known bacterial protein acetyltransferases and deacetylases will be summarized. Protein acetylation in *Escherichia coli*, *Salmonella enterica*, *Bacillus subtilis*, *Rhodopseudomonas palustris* and *Mycobacterium tuberculosis*, will be explained in the light of their physiological relevance. Progress in the elucidation of bacterial acetylomes and the emerging understanding of chemical acylation mechanisms will be discussed together with their regulatory and evolutionary implications. Fundamental molecular studies detailing this recently discovered regulatory mechanism pave the way for their prospective application for the construction of synthetic regulation networks.

Introduction – concept and historical overview

Post-translational modification (PTM) of proteins is an evolutionarily conserved strategy used by organisms for the efficient control of their biological activities, allowing them to exert rapid adaptive responses to environmental changes. Several types of PTMs exist in Nature. Among the more prominent are serine, threonine, and tyrosine phosphorylation; lysine ubiquitination, sumoylation, and neddylation; lysine acylation; lysine and arginine methylation; proline isomerization; and N- and O-glycosylation, which occurs on several residues, but mainly on serine, threonine and asparagine [1].

In the past years, protein acylation is receiving increasing attention because its involvement in several mitochondrial, nuclear and cytosolic processes [2–5]. Protein acylation can be defined as the transfer of an acyl group from a convenient biochemical donor molecule to an amino group on a protein. Many proteins can be acylated by activated acyl groups such as acyl-CoAs and acyl-phosphates. Thus, proteins can be acetylated, propionylated, butyrylated or succinylated [6,7]. Although some of these processes are catalyzed by specific transferases, they can also occur non-enzymatically. It is not clear yet whether these events are spontaneous or auto-catalytical. Among all these acylation modifications, protein acetylation is by far the best known.

Acetylation of lysine residues of proteins was first identified in histones more than 40 years ago. Recent reports have shown that a high number of metabolic enzymes are acetylated, which has led to suggesting an evolutionarily conserved mean of regulation of intermediary metabolism [8,9]. Protein acetylation and deacetylation is catalyzed by protein acetyltransferases and deacetylases, respectively, of which several families exist. In addition, several proteins acetylate in the presence of reactive

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acetyl derivatives, such as acetyl-phosphate, acetyl-CoA or acetyl-AMP [10–12].

Based on the chemical nature of the acetylated amino group, two types of protein acetylation can be considered, each one exhibiting specific characteristic features. The acetylation of the α-amino group of the N-terminal amino acid of proteins is very rare in bacteria [13] but frequent in eukaryotes (30–80% of proteins) and archaea (14–29% of proteins) [14]. On the other hand, the acetylation of proteins at the ε-amino group of internal lysine residues is a widely distributed PTM, frequent in all domains of life. This is a reversible post-translational process which has now been found on over 1500 eukaryotic proteins with diverse functions and locations [4,15,16]. Lysine acetylation exerts various effects on many of their critical functional properties, including interference with catalytic mechanisms, interaction with other proteins and DNA, stability, and sub-cellular distribution [2,4].

Until very recently, few bacterial proteins were known to be acetylated. However, the increasing power of high throughput proteomic techniques is starting to change this view [4,5]. Acetylation of microbial proteins has been excellently reviewed [14–16]. The aim of this mini-review is to sum up the current state of the art in the roles of protein acetylation in bacteria, with a special focus on the implications of chemical and enzymatic acetylation of proteins on cellular physiology.

Diversity of bacterial protein acetyltransferases and deacetylases

Protein acetyltransferases

N-acetyltransferases catalyze the transfer of an acetyl group from acetyl-CoA to a primary amine on a substrate. The acetylation of the ε-amino group of lysine residues of proteins is catalyzed by lysine acetyltransferases (often abbreviated to KATs or LATs), formerly known as histone acetyltransferases (HATs). Several KATs are known in eukaryotes, which are structurally diverse. All families contain a homologous acetyl-CoA binding core segment, which raises the possibility that they have evolved from a common ancestral protein [17].

Enzymes belonging to the Gcn5-related N-acetyltransferase (GNAT) superfamily (pfam00583) are conserved in all domains of life and are best described as enzymes that utilize acyl coenzyme A (CoA) as a donor for the acylation of the ε-amino group of lysine residues of proteins and small molecules. Many of the bacterial KATs described belong to this group. This is the case of *Salmonella enterica*: in 2004, the gene encoding the KAT *Se*Pat was identified in this organism. This protein is responsible for the acylation of acetyl-CoA and propionyl-CoA synthetases [18,19]. There is controversy on the acetylation of other metabolic proteins by *Se*Pat in *S. enterica*. Wang and col. described that the metabolic enzymes GapA, AceA and AceK are acetylated by *Se*Pat [9], but it has been claimed that these results cannot be reproduced [20].

*Se*Pat possesses two domains: a C-terminal domain homologous to GNAT acetyltransferases and a N-terminal domain with high homology to NDP-forming acetyl-CoA synthetase. However, it lacks acetyl-CoA forming activity because the replacement of a histidine residue which is critical for catalysis [19,21]. *Se*Pat shows sigmoidal kinetics and positive cooperativity for acetyl-CoA and a biphasic interaction with acetyl-CoA, which are unusual in GNATs. The structural basis for this behavior is the tetramerization of the protein in the presence of acetyl-CoA [21]. This biphasic acetyl-CoA binding relies on the acetyl-CoA synthetase-like domain, which allows it to respond to small variations in the acetyl-CoA concentration. This characteristic contributes to maintaining acetyl-CoA homeostasis during acetate uptake [21,22].

Other microorganisms contain KATs from the GNAT superfamily that lack the putative acetyl-CoA synthetase domain found in *Se*Pat (such as AcuA from *B. subtilis*) or have a regulatory domain absent in the *Se*Pat (such as *Mt*Pat from *Mycobacterium tuberculosis* which is responsive to cyclic nucleotides). In addition, many organisms that contain homologs to protein lysine deacetylases lack Pat homologs, an observation that suggests the existence of functional orthologs with distinct evolutionary lineage [19].

Protein deacetylases

Lysine deacetylases (KDACs) catalyze the cleavage of N-acetyl amide moieties. Eukaryotic KDACs are subdivided into four classes on the basis of phylogenetic criteria [23]. According to their reaction mechanism, two types of deacetylases can be discerned: simple hydrolases, which release the acetyl group as free acetate, and sirtuins, which are nicotinamide adenine dinucleotide (NAD^+)-dependent protein deacetylases. Sirtuins use NAD^+ as a co-substrate, which is cleaved in the catalytic cycle, generating nicotinamide and 2′-O-acetyl-ADP-ribose (OAADPr) as co-products. KDACs are conserved proteins with an ancient origin, and they are expected to participate in basic processes well conserved across organisms [23].

Sirtuins typically consist of a conserved catalytic core domain (~250 aa) and two optional and highly variable N- and C-terminal domain (50–300 aa) [23,24]. It is now evident that sirtuin protein sequences are broadly conserved within the three domains of bacteria, archaea and eukaryotes. Of special relevance is the involvement of sirtuins in the response to nutrients availability (calorie restriction response) and, therefore, in metabolic regulation [2].

Both types of KDACs are found in bacteria. In bacteria, the CobB protein in *S. enterica* is the best characterized sirtuin [25], although they have also been found in *E. coli* [26], *B. subtilis* [27], *Rhodopseudomonas palustris* [28], *Streptomyces coelicolor* [29] and other genera. Phylogenetic analysis reveals that bacterial sirtuins are related to the mitochondrial Sirt4 and Sirt5 from humans, while archaeal sirtuins are closer to eukaryotic Sirt6 and Sirt7. Eukaryotic Sirt1, Sirt2 and Sirt3 cluster separately, together with yeast sirtuins [24]. No recognizable hydrolytic (non-sirtuin) deacetylases have been found in many individual eubacterial species (e.g. *E. coli*) [23], although they have been described in *B. subtilis* [30] and *R. palustris* [28]. *M. smegmatis* possesses a predicted non-sirtuin deacetylase although its role has not been experimentally demonstrated [31].

Protein acetylation in bacteria: targeted pathways

Modulation of the activity of bacterial proteins by site-specific acetylation has long been known. One classical example is CheY, which regulates chemotaxis through the acetylation of two specific lysine residues which impairs binding to components of the flagellar motor switch complex and chemotaxis machinery [32,33]. Nevertheless, it was not until 2002, that the regulation of a bacterial enzyme by reversible acetylation/deacetylation was described for the first time [25]. Major advances in the identification of acetylated proteins have been done in *E. coli*, *S. enterica*, *B.*

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subtilis, *R. palustris* and *Mycobacterium* species. In this section, pathways targeted by protein acetylation in these microorganisms and their physiological roles will be summarized.

AMP-forming acyl-CoA synthetases

The members of the AMP-forming acyl-CoA synthetases family are the best characterized substrates of the protein acetylation system (Table 1). This large and diverse group of enzymes can activate several carboxylic acids into coenzyme A thioesters, including short-, medium- and long-chain fatty acids, dicarboxylic acids, aromatic acids and other carboxylated metabolites [28,34–36].

Acetyl-CoA synthetase (Acs) is a wide spread enzyme which activates acetate, allowing it to be scavenged and metabolized [34,37]. In 2002, Acs from *S. enterica* was the first bacterial protein whose activity was described to be regulated by reversible acetylation [25]. Currently, Acs orthologues in other bacterial species are also known to be regulated in the same way. Acetylation of Acs occurs at a conserved lysine residue which is necessary for catalysis, and inactivates the protein, blocking the rate limiting step for the metabolization of acetate [25].

*Acetylation of Acs in enterobacteria: **E. coli** and **S. enterica***

Acs from *S. enterica* is acetylated at a specific lysine residue in the vicinity of its active site, inactivating the enzyme. A careful analysis of the modified enzyme showed that formation of the reaction intermediary acetyl-AMP is blocked, while its transformation to acetyl-CoA proceeds normally [25]. Inactivation of Acs is reversed by deacetylation (Table 1). The KAT and the KDAC responsible for this reversible modification of Acs were identified [19,25]. The acetylation of Acs at high acetyl-CoA concentrations prevents its accumulation, while avoiding the depletion of free coenzyme A and metabolic energy [21,22]. In addition, we have recently shown that protein acetylation also responds to intracellular signals. Expression of the acetyltransferase *patZ* (formerly *yfiQ*) and the acetyl-CoA synthetase gene *acs* is up-regulated by cAMP in *E. coli* stationary phase glucose cultures (Fig. 1). In fact, the existence of concurrent up-regulation of *acs* gene expression and inhibition by acetylation and their dependence on cAMP and acetyl-CoA indicates that enzyme activities may be the result of finely tuned mechanisms, involving transcriptional, post-transcriptional and post-translational events [38].

*Acetylation of AcsA in the firmicute **Bacillus subtilis***

In *B. subtilis*, the metabolism of acetate and acetoin are highly related. The acetate activating enzyme (encoded by *acsA*) is specifically acetylated by the KAT from the GNAT family AcuA [30,39] and is deacetylated by the KDACs AcuC and SrtN [27] (Fig. 1). In this microorganism, deacetylation of AcsA is carried out by both AcuC and SrtN, despite their different catalytic mechanisms. In fact, growth on acetate as carbon source is as impaired in a double *acuC*/*srtN* knockout mutant as in the *acsA* mutant [27]. AcuA and AcuC are products of the *acuABC* operon (*ac*etoin *u*tilization), while SrtN is encoded by an independent gene. The *acuABC* operon and the *acsA* gene are adjacent in *B. subtilis* genome and these two transcriptional units are divergently co-transcribed [27].

The existence of two independent KDACs in *B. subtilis* and other bacteria such as *R. palustris* and *Mycobacterium sp.* suggests that these microorganisms may have evolved convergent strategies to deal with protein acetylation *in vivo*. It has been argued that the activity of NAD^+-dependent and independent KDACs is differently affected by small molecules such as nicotinamide nucleotides. Therefore these two strategies might be active under different growth conditions [27]. Therefore, although AcuC and SrtN activities can be viewed as redundant regarding deacetylation of AcsA, differences in their regulation by small molecules, and the anticipated differences in substrate specificities toward other acetylated proteins may explain why they co-exist in this bacterium. The existence of NAD^+-independent deacetylases has not been so far demonstrated in other microorganisms such as *E. coli*.

*Acetylation of acyl-CoA synthetases in **R. palustris***

R. palustris is a gram-negative purple non-sulfur α-proteobacterium which grows photoheterotrophically on aromatic compounds available in aquatic environments rich in lignin derived residues. When growing photosynthetically in the absence of oxygen, *R. palustris* converts aromatic compounds to acetyl-CoA, which is then used as carbon and energy source. At least 40 genes are annotated in *R. palustris* genome to encode putative AMP-forming acyl-CoA synthetases and many of them have the conserved lysine which is acetylated in Acs [4,34]. Two KATs (*Rp*Pat and *Rp*KatA) and two KDACs (*Rp*LdaA and *Rp*SrtN) have been identified in *R. palustris*. Detailed molecular analysis of protein acetylation by these KATs and KDACs demonstrated that their functions are not redundant but complementary. Crosby and col. identified 24 putative acetylated proteins, 14 of which were over-acetylated in a mutant devoid of both deacetylases. Nine of these proteins were AMP-forming acyl-CoA synthetases [20]. The activity of the aryl-CoA synthetases BadA (which activates benzoate) and HbaA (which activates 4-hydroxybenzoate) and the alicyclic acyl-CoA synthetase AliA (which activates cyclohexanecarboxylate) are regulated by N-ε-lysine acetylation [20,28]. The acetylation of these acyl-CoA synthetases avoids uncontrolled consumption of large amounts of ATP and free CoA, which might lead to growth arrest. In addition, these enzymes are reactivated by SrtN, which suggests that under high NAD^+/NADH ratio conditions, NAD^+ might signal a need to catabolize benzoate (and related compounds) to generate carbon and reducing power. In addition, it has been suggested that the transcriptional activator of the benzoate catabolism operon (BadR) may sense benzoyl-CoA rather than benzoate and, therefore, this regulation of benzoyl-CoA synthesis would co-ordinate post-translational and transcriptional regulation of aromatic compounds degradation [28].

Acetylation of AcsA in mycobacteria

Mycobacteria are of high biomedical interest because they are the causative agents of diseases such as tuberculosis and leprosy. Acetylation of proteins has been described recently in *M. tuberculosis*, which causes tuberculosis, and in the non-pathogenic *M. smegmatis*. Of special interest are the KATs from *M. tuberculosis* (*Mt*Pat) and *M. smegmatis* (*Ms*Pat), which possess a C-terminal GNAT domain fused to an N-terminal cyclic nucleotide binding domain. This is the only bacterial KAT allosterically regulated by cAMP binding [40]. Structural analyses revealed that cAMP binding to a cryptic site in the regulatory domain promotes an extensive conformational rearrangement that relieves autoinhibition of *Mt*Pat by a substrate-mimicking lid that covers the

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TABLE 1

Lysine acetylated bacterial proteins. Currently known functional effects of protein acetylation and deacetylation and the actual acetyltransferases and deacetylases involved in the post-translational modification are specified.

Species	Protein	Position[a]	Effect of acetylation/deacetylation	References
Escherichia coli	Acetyl-CoA synthetase (AMP forming)	K609	Acetylation of Acs impairs enzyme activity. Deletion of *cobB* deacetylase impairs growth on acetate as sole carbon source. The acetylation of Acs by PatZ and its deacetylation by CobB has been functionally demonstrated *in vivo*. CobB deacetylates Acs *in vitro*.	[26,38]
	CheY	K91 K109	Acetylation represses the binding of CheY to FliM, CheZ and CheA, components of the Flagellar Motor Switch Complex and chemotaxis machinery. Acetylation of CheY results in clockwise rotation of the flagella. CheY is chemically acetylated. CobB deacetylates CheY, regulating chemotaxis.	[49,51]
	Regulator of capsule synthesis (RcsB)	K180 K154	Acetylation of RcsB in K180 or K154 blocks DNA binding. Substitution of K180 by A, R or Q results in increased motility. Substitution of K154 by Q decreases transcription of the small RNA *rprA*. K180 is acetylated *in vitro* by *Se*Pat. K154 is chemically acetylated by acetyl-phosphate and deacetylated by CobB.	[47,65]
	RNase R	K544	Acetylation destabilizes the protein by increasing the strength of the binding of tmRNA-SmpB to the C-terminal region of RNase R in the exponential phase, leading to proteolytic degradation. Deacetylation in the stationary phase leads to the stabilization of the protein. RNase R is acetylated by PatZ (Pka, formerly YfiQ).	[53]
	N-hydroxyarylamine O-acetyltransferase (NhoA)	K214 K281	Acetylation of NhoA at K214 or K281 decreases N-hydroxyarylamine-O-acetyltransferase and aromatic amine N-acetyltransferase activity. Acetylation mechanism: unknown. Both lysines are deacetylated by CobB.	[66]
	α-subunit of RNAP	K291 K298	Acetylation of K298 contributes to *cpxP* promoter activity and K291 dampens this activation. These two lysine residues lie within the carboxy terminal domain (CTD) of α-subunit of RNAP. It is not clear how K291 or K298 are acetylated or deacetylated in *E. coli*.	[67]
Salmonella enterica	Acetyl-CoA synthetase (AMP forming) (Acs)	K609	Acetylation blocks the adenylating activity of the enzyme. Deletion of deacetylase impairs growth on acetate as sole carbon source. *Se*Pat and *Se*CobB control the acetylation of Acs.	[25]
	Propionyl-CoA synthetase (AMP forming) (Pcs)	K592	Acetylation blocks the adenylating activity of the enzyme. *Se*Pat and *Se*CobB control the propionylation of Acs.	[19]
	Glyceraldehyde-3-phosphate dehydrogenase (GapA)[b]	K321	Acetylation increases GapA enzymatic activity. *Se*Pat[b] and *Se*CobB control the acetylation of GapA.	[9]
	Isocitrate lyase (AceA)[b]	K34	AceA is inactivated by acetylation. *Se*Pat[b] and *Se*CobB control the acetylation of AceA.	[9]
	Isocitrate dehydrogenase phosphatase/kinase (AceK)[b]	K205	Acetylation activates the ICDH phosphatase activity. Deacetylation increases AceK's ability to inactivate ICDH. *Se*Pat[b] and *Se*CobB control the acetylation of AceK.	[9]
Bacillus subtilis	Acetyl-CoA synthetase (AMP forming) (Acs)	K549	Acetylation of K549 of *Bs*Acs blocks enzyme activity and impairs growth on acetate as the sole carbon source. *Bs*AcsA is acetylated by AcuA and deacetylated by AcuC (NAD^+-independent deacetylase) and SrtN (sirtuin type deacetylase).	[27,30]

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TABLE 1 (*Continued*)

Species	Protein	Position[a]	Effect of acetylation/deacetylation	References
R. palustris	Acetyl-CoA synthetase (Acs) Benzoyl-CoA synthetase (BadA) 4-hydroxybenzoyl-CoA synthetase (HbaA) Cyclohexanecarboxyl-CoA synthetase (AliA)	K512 (BadA)	Acetylation of these 4 acyl-CoA synthetases blocks enzyme activity and affects photoheterotrophic growth on benzoate. *Rp*Pat acetylates all 4 acyl-CoA synthetases. Two protein deacetylases are reported: SrtN (sirtuin-like, NAD^+-dependent) and LdaA (NAD^+ independent). BadA is activated by SrtN and LdaA. HbaA and AliA are activated by LdaA.	[28]
	9 AMP-forming acyl-CoA synthetases; glyceraldehyde-3-phosphate dehydrogenase; 4-oxalocrotonate tautomerase		Acetylation decreases activity of the enzymes by 70–97%. *Rp*Pat acetylates 9 acyl-CoA synthetases and *Rp*LdaA deacetylates 8 of them.	[20]
Streptomyces coelicolor	Acetyl-CoA synthetase (Acs)		Acetylation of *Sc*Acs blocks catalytic activity *in vitro*. *Sc*Acs is deacetylated by CobB1.	[29]
Streptomyces lividans	Acetoacetyl-CoA synthetase	K617	The enzyme is inactivated by the acetylation of K617. SlPat acetylates lysine 617.	[68]
Mycobacterium tuberculosis	Acetyl-CoA synthetase (Acs)	K617	Acs is inactivated by acetylation. *Mt*Pat acetylates *in vitro* Acs and this enzyme is deacetylated by the sirtuin deacetylase *Mt*SrtN	[42]
	Fatty acyl-CoA synthetase (FadD13)	K487	Mutation K487Q decreases activity. Lysine 487 is acetylated by *Mt*Pat in a cAMP dependent manner.	[69]
Mycobacterium smegmatis	Universal Stress Protein (USP)	K104	Unknown role. USP is acetylated by *Ms*PatA. Acetylation is regulated by cyclic AMP, which is an allosteric regulator of *Ms*PatA.	[40]
	Acetyl CoA synthetase (*Ms*Acs)	K589	The acetyl-CoA synthetase and propionyl-CoA synthetase activities of *Ms*Acs are inactivated by acylation (functional demonstration). *Ms*PatA and *Ms*SrtN control the acetylation of *Ms*Acs *in vivo*. Acetylation of *Ms*Acs is regulated by cyclic AMP, which is an allosteric regulator of *Ms*PatA.	[31]
	Propionyl CoA synthetase (Ms5404)	K586	Deletion of deacetylases alters growth in propionate minimal medium. Not demonstrated at the molecular level.	[31]

[a] Position of acetylated lysine in protein sequence.
[b] These results have been recently questioned in recent reports [20,44].

protein-substrate binding surface [41]. Cyclic nucleotides are universal second messengers which elicit diverse responses in bacteria. It has been reported that cAMP reprograms mycobacterial physiology, and its production by *M. tuberculosis* is critical for host-pathogen interaction and disease [40]. In fact, mycobacteria contain over a dozen genes identified as adenylate cyclases (ACs) [42], which activity increases under various physiological conditions. In particular, in *M. tuberculosis*, several of the ACs are specifically responsive to nitrogen and carbon limitation, pH, and bicarbonate [42]. The phagolysosome is an acidic, nutrient poor, and oxidatively hostile environment, and bacteria slow down their metabolism to survive [42]. When *M. tuberculosis* is phagocyted by macrophages, cAMP increases dramatically, and this cAMP is secreted to the phagolysosome, exerting responses in the host which are involved in pathogenesis [43].

Other pathways targeted by protein acetylation

Other metabolic enzymes

Important acetylation targets have been identified lately thanks to high throughput proteomics. This is the case of glyceraldehyde phosphate dehydrogenase (GapA), isocitrate lyase (AceA) and the isocitrate dehydrogenase (ICDH) kinase/phosphatase (AceK) in *S. enterica* [9]. However, it has been claimed that these results cannot be reproduced [20] and should be critically revised. Moreover, these authors suggested that Pat from *S. enterica* was capable of acetylating a wide range of substrates. A recent description of the molecular signature recognized by Pat from *R. palustris* demonstrates that this enzyme is quite specific and is very specialized in recognizing acetyl-CoA synthetases [44].

Non-metabolic targets: gene transcription, cell motility and chemotaxis

In addition to metabolic proteins, other interesting targets have been the subjects of classical molecular level studies in bacteria. In this section, we will focus on the regulation by acetylation of cell motility and chemotaxis, gene transcription and RNA stability.

Modulation of gene transcription: acetylation of RNA polymerase. In *E. coli*, the RNA polymerase (RNAP) is acetylated on almost 30 residues on different subunits (α, β and β′), and this acetylation pattern depends on the growth conditions [45]. Of special interest is the acetylation of the α-carboxy-terminal domain (CTD), which is involved in promoter recognition by interacting with a DNA region upstream the transcription initiation site known as the UP element and/or by interacting with certain TFs. In fact, this specific

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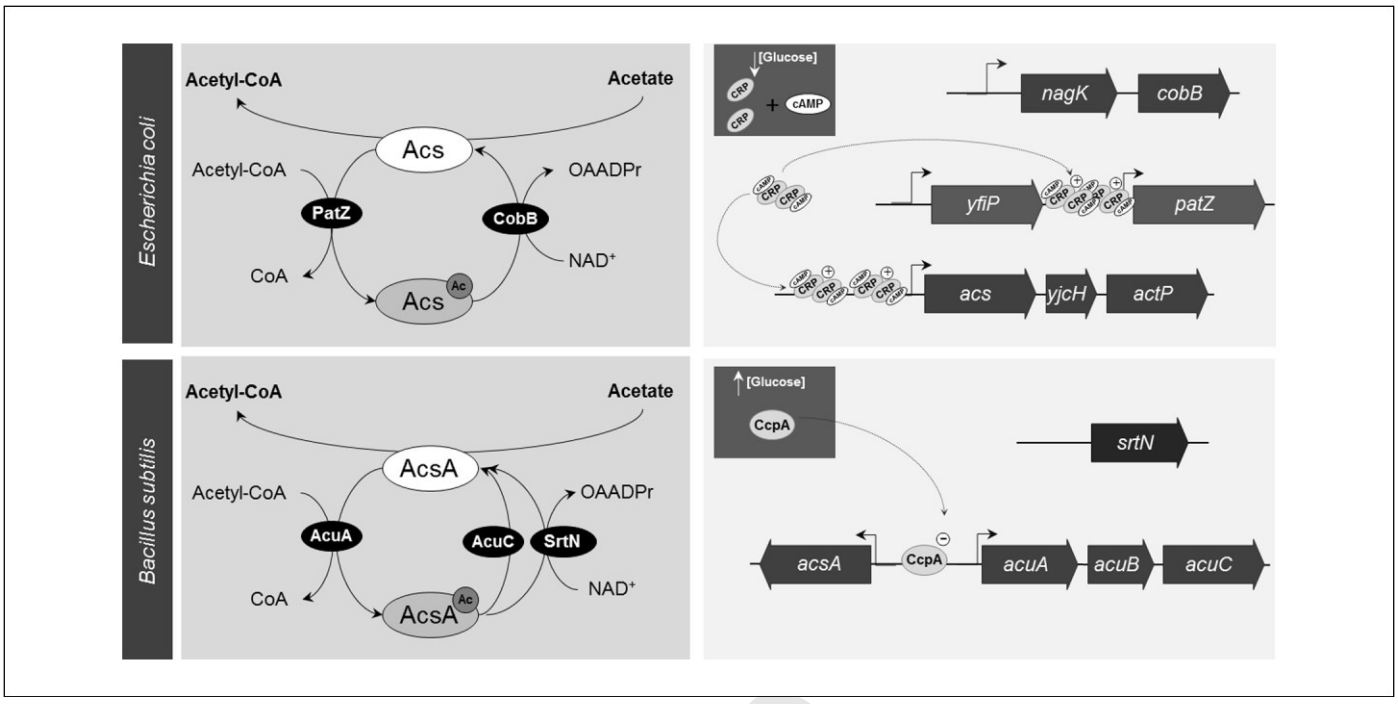

FIG. 1

Scheme of the regulation of (AMP-forming) acetyl-CoA synthetases by reversible acetylation/deacetylation in *E. coli* and *B. subtilis*. Protein acetyltransferases and deacetylases involved in protein acetylation (left panel). Transcriptional regulation of the genes involved in this post-translational modification (right panel). In *E. coli*, Acs is acetylated by PatZ and deacetylated by the sirtuin CobB. The expression of *patZ* and *acs* is up-regulated by cAMP-CRP, while the expression of *cobB* gene is constitutive [38]. In *B. subtilis*, Acs is acetylated by AcuA and deacetylated by AcuC (NAD^+-independent deacetylase) and SrtN (sirtuin) [27]. The *acsA* gene and the *acuABC* operon are divergently transcribed from the same promoter region and their transcription is regulated by the carbon catabolite regulator protein CcpA [84]. The regulation of *srtN* transcription has not been described.

domain is acetylated in response to glucose and other carbon sources. Moreover, acetylation is affected by PatZ (YfiQ) and CobB mutations, although there are no direct evidences that these proteins are responsible for these events. Interestingly, *cpxP* transcription responds to an acetyl-CoA to CoA imbalance that the cell perceives as a stress and may contribute to the regulation of protein acetylation and, probably, also to NAD^+ availability which would lead to competition of the deacetylase CobB with other enzymes for the free oxidized form of the nucleotide [45].

Modulation of cellular motility and chemotaxis: RcsB and CheY. The multicomponent RcsF/RcsC/RcsD/RcsA-RcsB phosphorelay system is involved in *E. coli* in the regulation of several genes related to the synthesis of the colanic acid capsule, cell division and motility, among others [46]. The DNA binding protein RcsB is the response regulator of this system. Acetylation or substitution by site-directed mutagenesis of a specific lysine residue of the protein (which lies within a helix-turn-helix motif and is essential for DNA binding) impedes *in vitro* binding to the RcsB box of a regulated promoter [47]. The active form of RcsB is phosphorylated [48] and, therefore, regulation involves two concurrent types of PTMs.

The CheY protein, which regulates chemotaxis, is also subjected to a similarly complex regulation by acetylation and phosphorylation. CheY is phosphorylated in response to environmental conditions and acetylated in response to altered metabolic state of cells. CheY is deacetylated by CobB [49]. These modifications shift the isoelectric point of the protein, avoiding the interaction with its partner proteins and regulating chemotaxis [49].

The concurrence of two independent PTMs regulating the same protein is intriguing. Both PTMs are linked and co-regulated. CheZ, dephosphorylates CheY and also increases its acetylation. These authors proposed that the physiological role of these mutual effects is at two levels: linking chemotaxis to the metabolic state of the cell, and serving as a tuning mechanism that compensates for cell-to-cell variations in the concentrations of regulatory proteins such as CheA and CheZ [50,51]. Therefore it seems possible that these two modes of regulation may have complementary roles.

The complementary regulation of protein activity by two PTMs (as described in CheY and RcsB) suggests the existence of a cross-talk between regulatory networks in *E. coli* [1]. Cross-talk between protein phosphorylation and lysine acetylation has also been recently evidenced in *Mycoplasma pneumoniae* [52]. In fact, both PTMs often co-occur within the same protein in this genome reduced bacterium and they are frequently observed at interaction interfaces and in multifunctional proteins. Deletion of protein kinases and phosphatases alters protein acetylation and, conversely, deletion of acetyltransferases affects protein phosphorylation. Altogether, this indicates that we have a very limited picture of the actual complexity of cellular regulatory networks.

Modulation of protein stability by acetylation: RNase R. RNase R is an exoribonuclease important for the degradation of structured RNAs in *E. coli*. This normally unstable protein increases its half-life in the stationary phase and certain stress conditions. Acetylation of RNase R by PatZ (YfiQ) in the exponential phase results in tighter binding of tmRNA-SmpB (the *trans*-translation machinery and its associated protein) and subsequent proteolytic degradation. In the

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stationary phase, RNase R is not acetylated, which results in longer protein half-life. This shift in the post-translational acetylation profile was explained on the basis of regulated expression of PatZ [53], which was not in agreement with previous reports [38], probably because different experimental conditions. It should be underlined that acetylation of RNase R only affects its stability and does not affect its catalytic activity [54].

Protein acetylomics in bacteria – state of the art

High throughput analysis of cellular acetylomes.

The number of proteins known to be post-translationally acetylated is constantly increasing because the improvement in mass spectrometry (MS)-based proteomics techniques [4,7,12,55]. These methods can be targeted for the identification of acetylated proteins by using antibodies to specifically immunoprecipitate acetylated peptides or increase the sensitivity of the technique by applying fractionation techniques to reduce the complexity of peptide mixtures subjected to mass spectrometric analysis [12,56]. In general, MS-based strategies allow the non-biased identification of a high number of targeted proteins/pathways. Other techniques, such as protein microarrays, have also been used for the identification of substrates of acetyltransferases, although *in vitro* specificities may not mirror what happens *in vivo* [47].

Several systems-wide studies of protein acetylation have been published to date, both in eukaryotic and prokaryotic organisms (Table 2). Overall, these works have demonstrated that acetylation in bacteria is more common and widespread than previously thought (maximum percentage of organisms ORFs found acetylated: 20% in rat, 24% in *E. coli*, 34% in *M. pneumoniae*) [12,52,57]. The percentage of acetylated ORFs is highly variable, from one organism to another, or even between different studies performed on the same organism. In fact, the output of these studies, that is the number or acetylation targets identified, is highly dependent on the techniques used previous to chromatographic analysis (e.g. immunoprecipitation with anti-acetyl-lysine antibodies, sample fractionation strategies, among others), the power and sensitivity of the MS/MS detection system used and even the data analysis software package used to inspect LC–MS/MS data.

A thorough validation of the targets identified by these high throughput analysis techniques is necessary to determine the impact of the acetylation on the functionality of the protein. In some of the works published, limited validation was done to confirm that the targets were actually acetylated or that these acetylation events had an effect on the protein targeted (Table 2).

It is quite remarkable that all these studies coincide in underlining that approximately 50% of the targeted proteins were related to metabolism. In principle this is because these proteins usually are highly abundant in the cells and, therefore, detection of acetylated peptides is more straightforward. However, it also supports that protein acetylation has a role in the regulation of metabolism in bacteria.

TABLE 2

Overview of LC–MS/MS-based studies of lysine acetylation in different organisms. Studies in eukaryotic systems (*A. thaliana*, *D. melanogaster*, *H. sapiens*, *R. norvegicus*, *S. cerevisiae* and *T. gondii*) are shown for comparison.

Organism	Number acetylated peptides/proteins	% Acetylated ORF	Validation targets[d]	Reference
Arabidopsis thaliana	91/71[a]	0.3	Y	[70]
	64/57[a]	0.2	Y	[71]
Bacillus subtilis	332/185[a]	4.4	N	[72]
Drosophila melanogaster	1981/1013[a,b,c]	7.3	Y	[73]
Erwinia amylovora	141/96 [a]	2.6	N	[74]
Escherichia coli	8284/1000[a,c,e]	24.1	N	[12]
	1070/349[a]	8.4	N	[75]
	138/91[a]	2.2	N	[55]
	125/85[a]	2.1	N	[76]
Geobacillus kaustophilus	253/114[a]	3.2	N	[77]
Homo sapiens	3600/1750[a,b,e]	8.7	N	[78]
Mycoplasma pneumoniae	719/221[a,e]	34.1	N	[52]
Rattus novergicus	15,474/4541[a,c,e]	19.6	Y	[57]
Plasmodium falciparum	421/230	4.3	Y	[79]
Rhodopseudomonas palustris	244/292	6.1	Y	[20]
Saccharomyces cerevisiae	2878/1059[a,b,e]	17.9	N	[80]
Salmonella enterica	235/191[a,e]	4.2	Y	[9]
Thermus thermophilus	197/128[a]	5.8	N	[81]
Toxoplasma gondii	411/274[a]	3.4	N	[82]
	571/386[a]	4.8	N	[83]

Data have been obtained using different techniques previous to LC–MS/MS:

[a] Immunoprecipitation (for enrichment in acetylated peptides).
[b] Isoelectric focusing fractionation (reduction of sample complexity).
[c] Micro-strong cation exchange fractionation (reduction of sample complexity).
[d] Validation of targets: Y (yes), N (no).
[e] Studies which quantified the acetylated peptides.

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Despite these efforts, the biological substrates and/or functions for a majority of bacterial KATs and KDACs and the particular effects of acetylation on the activity of most of the target enzymes remain largely unknown.

Chemical acetylation of proteins

The unexpectedly high abundance of protein acetylation has underlined the importance of chemical acetylation of proteins in eukaryotes and prokaryotes. First *in vitro* evidences of non-enzymatic acetylation of histones by high energy metabolites acetyl phosphate and acetyl-AMP were reported in the 70s [10]. Later on were reported physiological roles of chemical acetylation of proteins in bacteria. In *E. coli*, the chemotactic response regulator protein CheY was the first protein demonstrated to be chemically acetylated or autoacetylated [33,50,58,59]. Eisenbach and colleagues showed that CheY can be acetylated at different lysine residues with acetyl-CoA as acetyl donor. Even more, it was observed that the acetylation of CheY decreased in the absence of acetyl-CoA synthetase (Acs), indicating its involvement, probably by supplying the acetylating agent. In yeast, low levels of histones acetylation were observed after deletion of the *acs1* and *acs2* genes, altering gene transcription [60]. More recently, the slow kinetics of non-enzymatic acetylation of human histone H3 by acetyl-CoA have been shown [11]. Although the kinetics of chemical lysine acetylation have not been extensively studied, it seems reasonable that the rate of chemical acetylation may be protein and residue dependent. Moreover, chemical acylation of proteins is highly dependent on pH, probably because higher reactivity of lysyl groups at high pH values. In fact, the acetylation of proteins in the mitochondrial matrix by acetyl-CoA is stimulated by a pH increase [6].

Other acyl-CoAs can also act as sources of activated acyl groups. In fact, proteins can also be modified at lysines by succinylation, malonylation, propionylation or butyrylation. It has been observed that lysine succinylation can be enzyme independent. Succinylation overlaps lysine acetylation and an increase of succinyl-CoA levels in the cells can increase this post-translational modification in proteins [6,56].

In bacteria, acetylation of various proteins by acetyl-CoA has been described. More recently, the involvement of acetyl-phosphate in chemical acetylation of proteins in *E. coli* has been demonstrated. The authors of this study altered the pool of acetyl-phosphate deleting the enzymes involved in its production: acetate kinase (AckA) and phosphotransacetylase (Pta) [12]. Therefore, unbalanced synthesis and degradation of acetyl-phosphate may also alter the acetylation state of proteins in *E. coli*.

Altogether, these observations indicate that chemical acylation of proteins may be directly regulated by the levels of acyl-CoAs and acetyl phosphate and, therefore, depend on the metabolic state of the cells. Organisms have developed strategies to deal with the occurrence of chemical acylation of proteins. KDACs have evolved from ancient ancestors and exist in all domains of life [23]. Some sirtuins, such as CobB from *E. coli*, have broader substrate specificities, being capable of cleaving acyl-groups other than acetyl, such as propionyl- or succinyl [18,56,61]. In this evolution, NAD^+-dependent (sirtuins) and independent deacetylases appeared, probably because the need to deal with protein acylation under different physiological conditions.

To which extent protein acetylation is critical for the cell may be highly organism (and condition) dependent. Deacetylase deficient mutants have been constructed in several organisms. In *E. coli* and *S. enterica*, deletion of *cobB* affects growth on glucose very mildly, but much more importantly on acetate [22,38], a landscape where accumulation of acetyl-CoA and acetyl-phosphate is more likely. In *B. subtilis*, analysis of single and multiple mutants in the deacetylase genes showed that both genes are required for efficient growth on acetate [27]. In addition, these authors failed to construct a *pta acuC srtN* triple mutant, which suggests that this is a synthetic lethal mutant, probably because an over-acetylation of proteins because accumulated acetyl-phosphate. In fact, although an *acuA acuC srtN* mutant showed improved growth when compared to the *acuC srtN* strain, wild type growth was not recovered, suggesting either other KATs may be active in those conditions or that chemical acetylation occurred to a certain extent.

Protein acylation and synthetic biology

Fundamental Systems Molecular Biology studies are establishing the foundations of bacterial physiology regulation by protein acetylation. While functional rules are being uncovered, the potentialities of this novel level of regulation are also arising, creating a new playground for Synthetic Biology. The capability to interfere with natural protein acetylation networks and creating novel ones opens up the possibility to design *à le carte*, engineered cell factories with higher robustness for enhanced bioprocessing.

Novel strain engineering strategies are foreseen. Domains recognized in targeted lysine acetylated proteins can be engineered to alter the specificity of protein acetyltransferases and deacetylases. In this sense, the identification of the protein motifs which are recognized by protein acetyltransferases have already demonstrated to be useful [44,62]. On the other hand, protein deacetylases seem to be more active on unstructured protein domains, not recognizing specific motifs [12,63]. The implementation of tightly self-regulated networks is key for optimal performance of metabolically engineered microorganisms. These strategies have been successfully implemented, for instance, to sense and avoid the accumulation of toxic intermediates of over-expressed pathways [64]. Synthetic protein acetylation networks can be used to specifically regulate metabolic networks in response to physiologic signals.

Presently, the best characterized protein acylation targets are acyl-CoA synthetases. These proteins generally activate organic acids to be metabolically degraded or biotransformed, acting as bottlenecks for these pathways. However, the ever growing list of acylated proteins is not limited to enzymes, also affecting, for instance, transcriptional regulators, and provides novel paradigms of naturally regulated pathways (Table 1). A further understanding of the biological regulation of the levels of chemical acetylating agents (acyl-phosphates, acyl-CoAs, acyl-AMPs, among others) will be key in the following years. We foresee that the impact of the knowledge herein summarized will be highly relevant in the Synthetic Biology field.

Conclusions and outlook

Protein acetylation is a common modification affecting the functionality of targeted proteins. In bacteria, N-ε-lysine acetylation of proteins constitutes a widespread mechanism for the regulation of

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central metabolic pathways which has been previously overlooked. Proteins may be acetylated either chemically or enzymatically, and the reversibility of this process provides an efficient mean for rapid adaptation to changing environments. Although reversible regulation of AMP-forming acyl-CoA synthetases activity by acetylation is well characterized, this family of proteins is not the only target of this PTM. The list of modified proteins has been recently enriched with enzymes, transcription factors, components of the transcriptional machinery and other proteins. High throughput MS-based proteomics are uncovering the proteins which are acetylated *in vivo* and, more importantly, determining how protein acetylation status changes with environmental conditions. Although many of these modifications may have no effect, validation of proteomics results has demonstrated that, in many cases, protein acetylation affects protein activity, stability and protein-protein or protein-DNA interactions, therefore affecting cellular physiology.

Nowadays, molecular characterization of KATs and KDACs is still incomplete, although there are several evidences that their activity is modulated by intracellular levels of small molecules. To complete the picture, the role of acyl-CoAs, CoA, NAD^+, nicotinamide and OAADPr/ADPr in signaling and allosteric regulation of KATs and KDACs should be elucidated. The roles of NAD^+-dependent and independent KDACs in metabolism should be explored, to unravel *in vivo* regulation of their activity.

Current efforts in the analysis of acetylomes will allow completing the picture of cellular acetylated proteins, allowing us to understand how cells balance catalytic and chemical acetylation. Moreover, it will also decipher the cross-talk with the rest of overlapping post-translational regulation mechanisms.

This is an emerging field of research and, as such, several open questions remain to be answered. Novel insights into microbial physiology, biotechnological applications of microorganisms and host-pathogen interactions are anticipated as the list of regulated pathways expands. Even at this early stage, relevant implications in several aspects of Systems and Synthetic Biotechnology and benefits for basic and applied research are expected.

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