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YfmK is a novel N$^{\varepsilon}$-lysine acetyltransferase that directly acetylates the histone-like protein HBsu in *Bacillus subtilis*.

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Abstract

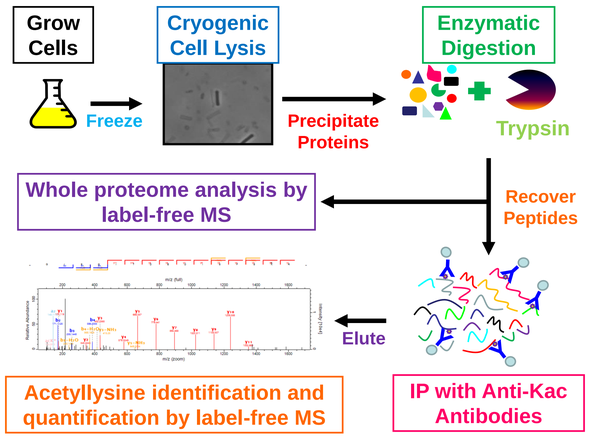
Recently, N$^{\varepsilon}$-lysine acetylation was realized to be a prevalent bacterial post-translational modification (PTM), contrary to the historical notion that this was a rare occurrence [1]. Acetylation can impact protein function in multiple ways, by modifying conformation, interactions, subcellular localization or activity. In bacteria, hundreds of proteins are known to be acetylated, including those involved essential processes such as DNA replication, nucleoid organization, translation, cell shape, central carbon metabolism, and even several virulence factors [1-6]. Despite the growing recognition that numerous proteins are acetylated, the biological significance of the vast majority of these modifications in *any bacteria* remains largely unknown. Previously, we characterized the *Bacillus subtilis* acetylome and found that the essential, histone-like protein HBsu contains seven novel acetylation sites *in vivo* [2]. HBsu is a bacterial nucleoid-associated protein, which is largely responsible for chromosome compaction and the coordination of DNA processes [7-10]. Despite the lack of sequence or structural homology, it is generally considered to be a functional homolog of eukaryotic histones.

We investigated whether acetylation is a regulatory component of the function of HBsu in nucleoid compaction. Using mutations that mimic the acetylated and unacetylated forms of the protein, we showed that the inability to acetylate key HBsu lysine residues results in a more compacted nucleoid. We further investigated the mechanism of HBsu acetylation. By screening deletions of the ~50 putative Gcn5-N-acetyltransferase (GNAT) domain encoding genes in *B. subtilis* for their effects on DNA compaction, five candidates were identified that may encode transacetylases acting on HBsu. Genetic bypass experiments demonstrated that two of these, YfmK and YdgE, can acetylate HBsu and their potential sites of action on HBsu were identified. Additionally, purified YfmK was able to directly acetylate HBsu *in vitro*, meaning that it is the second identified protein acetyltransferase in *B. subtilis.* We propose that at least one physiological function of the acetylation of HBsu at key lysine residues is to regulate nucleoid compaction, analogously to the role of histone acetylation in eukaryotes.

With the alarming rise in antibiotic resistance, the need to develop novel therapeutics is critical. Bacterial protein acetylation represents a world of untapped potential that may uncover new drug targets to replace or supplement our antiquated antibiotic arsenal. With proper study, the enzymes involved in regulation (i.e. acetylases and deacetylases) or the acetylated form of a key protein (i.e. virulence factors, essential genes, etc.) may provide valuable, druggable targets. The targeting of bacterial protein acetylation is a practical option, as targeting enzymes involved in acetylation regulation has been successful in treatment of certain cancers, latent viral and fungal infections [11-14].

Mass Spectrometry Workflow

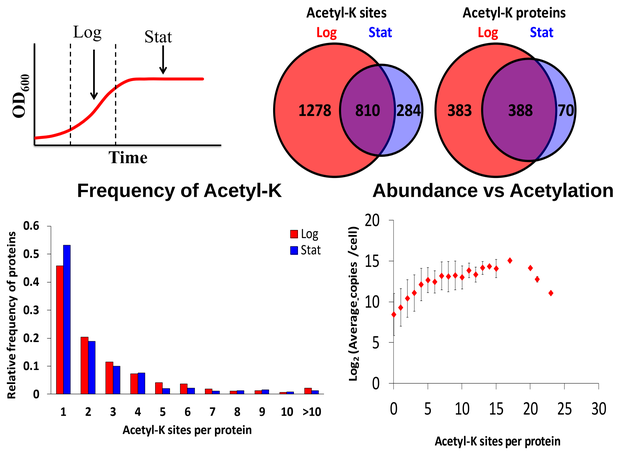
Proteome and acetyl-peptide capture analysis



Acetyllysine peptide capture was performed using a combination of commercially available anti-acetyllysine antibodies (Immune Chem, PTM Biolabs) conjugated to protein-A agarose beads [2]. Acetylated peptides were analyzed by nLC-CID and HCD MS/MS coupled directly to an LTQ-Orbitrap Velos mass spectrometer (ThermoFisher Scientific).

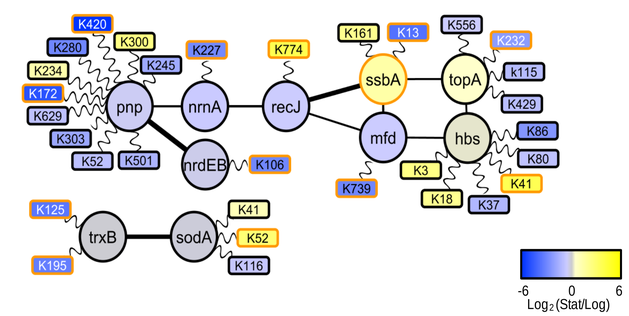
Results

Lysine acetylome in log versus stat phase



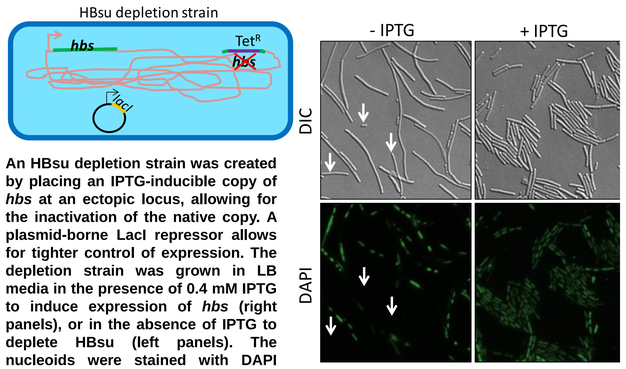
In total, 2372 unique acetylation sites on 841 proteins were identified. This accounts for ~20% of the proteome, similar to what was observed in human mitochondria [15].

Differential protein expression and acetylation networks



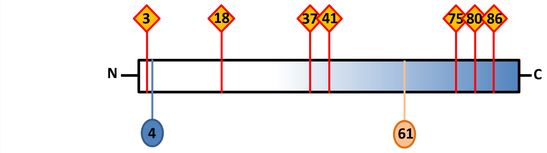
STRING relationships (solid edges) and identified acetylated lysine residues (wavy edges). Significant abundance changes are indicated by orange node outlines.

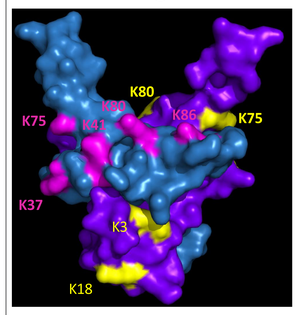
HBsu depletion leads to filamentation, nucleoid expansion and formation of anucleate cells



An HBsu depletion strain was created by placing an IPTG-inducible copy of *hbs* at an ectopic locus, allowing for the inactivation of the native copy. A plasmid-borne LacI repressor allows for tighter control of expression. The depletion strain was grown in LB media in the presence of 0.4 mM IPTG to induce expression of *hbs* (right panels), or in the absence of IPTG to deplete HBsu (left panels). The nucleoids were stained with DAPI (pseudo-colored green). White arrows denote anucleate cells.

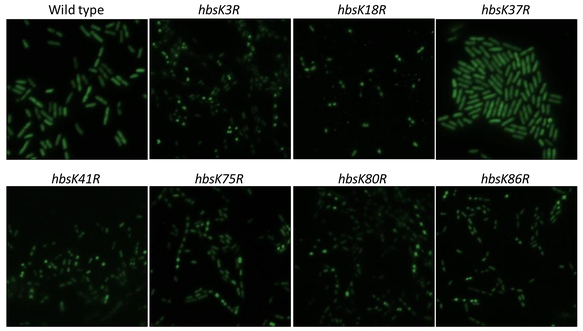
HBsu contains seven novel acetylation sites *in vivo*





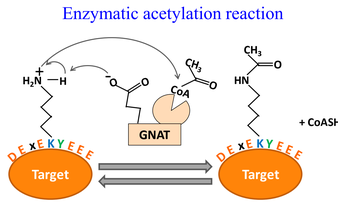
Top: Schematic displaying the 7 novel acetylation sites identified on HBsu (diamonds), and 2 identified phosphorylation sites (circles) [16-17]. All 14 acetyl mimic (K→Q) and unacetylated mimic (K→R) mutations were created at the *hbs* native locus. Left: A model of *B. subtilis* HBsu was created using PyMOL and the *B. stearothermophilus* structure as a template. The HBsu homodimer is displayed, with one monomer colored in cyan with the acetylated lysine sites labeled pink, and the other colored purple with the acetylated sites labeled yellow. The DNA molecule is threaded through the extended "arms" on top of the molecule, and may wrap down around it, which induces a severe bend in the DNA molecule [18].

Unacetylated mimic mutations (K →R) in 6/7 HBsu acetylation sites leads to compacted nucleoids

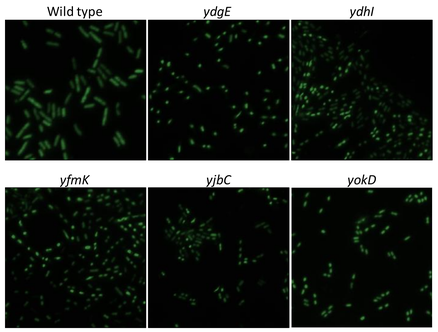


***Bacillus* cells were grown in minimal glucose media to exponential phase and their nucleoids were stained with DAPI (pseudo-colored green).**

An acetylase knockout candidate screen reveals five novel mutants that lead to nucleoid compaction.

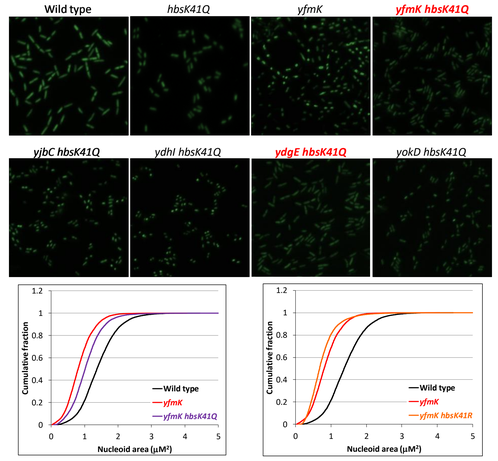


In bacteria, Gcn5-like N-acetyltransferases (GNAT) orthologs have been identified. These enzymes bind acetyl-CoA and use a conserved glutamate residue to catalyze the reaction. In *Bacillus*, there is one known GNAT protein (AcuA [27]), however, BLAST search reveals 50 potential GNATs.



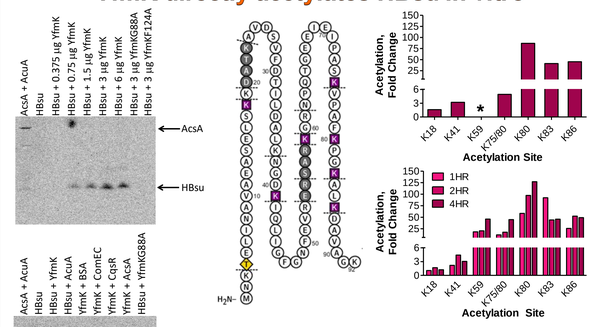
Knockout mutations of all 50 putative GNATs were analyzed for differences in nucleoid structure. *Bacillus* cells were grown in minimal glucose media to exponential phase and their nucleoids were stained with DAPI (pseudo-colored green). 5/50 mutants displayed a nucleoid compaction phenotype similar to that of the *hbs* R (unacetylated mimic) mutations.

YfmK and YdgE may directly acetylate HBsu *in vivo*



***Bacillus* cells were grown in minimal glucose media to exponential phase and their nucleoids were stained with DAPI (pseudo-colored green). Nucleoid areas of at least 4500 cells for each strain were determined. Cumulative distribution plots are displayed, where the 50th percentile represents the median of the population distribution. All distributions were significantly different from wild type (p-value < 0.0001), as determined using the Kolmogorov-Smirnov test [20]. YfmK also acetylates HBsu at K3 and K18 (not shown).**

YfmK directly acetylates HBsu *in vitro*



Left: *In vitro* acetylation assays were carried out in a reaction mixture [21], containing the indicated amount of YfmK incubated with 5 μg HBsu for 2 hours at 37°C in the presence of C^{14}-acetyl-CoA. A mixture of AcuA and AcsA was included as a positive control. YfmK was incubated with additional substrates, including BSA, a soluble domain of ComEC, AcsA and the *Vibrio cholera* protein CqsR. The catalytically inactive mutants, YfmKG88A and F124A were included for comparison.

Right: A) Wild-type HBsu protein sequence indicating acetylated (purple squares) and phosphorylated residues (yellow diamond) detected by tandem mass spectrometry. Predicted trypsin cleavage sites are indicated by dashed lines. Tryptic peptides that were not detected are indicated in grey. Relative quantification of HBsu lysine acetylation following incubation with YfmK compared to control. *, not quantified. Relative quantification of HBsu lysine acetylation following incubation with YfmK for 1, 2, and 4 hours compared to a time-matched control.

Conclusions

1. Acetylation is a prevalent, dynamic PTM in *B. subtilis,* and site selectivity does not solely depend on abundance or number of K residues (not shown).
2. The observed acetylation abundance changes between growth phases suggests potentially biologically relevant modification events.
3. *At least one* physiological function of acetylation of HBsu at key lysine residues is to regulate its function in controlling nucleoid compaction.
4. YfmK is the second lysine acetyltransferase to be identified in *B. subtilis.*

References & Acknowledgements

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