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Determinants within the C-Terminal Domain of *Streptomyces lividans* Acetyl-CoA Synthetase that Block Acetylation of Its Active Site Lysine *In Vitro* by the Protein Acetyltransferase (Pat) Enzyme

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**Abstract**

Reversible lysine acetylation (RLA) is a widespread regulatory mechanism that modulates the function of proteins involved in diverse cellular processes. A strong case has been made for RLA control exerted by homologues of the *Salmonella enterica* protein acetyltransferase (SePat) enzyme on the broadly distributed AMP-forming CoA ligase (a.k.a. acyl-CoA synthetases) family of metabolic enzymes, with acetyl-CoA synthetase (Acs) being the paradigm in the field. Here we investigate why the Acs homologue in *Streptomyces lividans* (SlAcs) is poorly acetylated *in vitro* by the *S. lividans* protein acetyltransferase (SpPat) enzyme. Chimeras of *S. enterica* Acs (SeAcs) and *S. lividans* Acs (SlAcs) constructed during the course of this work were acetylated by SpPat *in vitro*, retained most of their activity, and were under RLA control in a heterologous host. We identified SeAcs residues N- and C-terminal to the target lysine that when introduced into SlAcs, rendered the latter under RLA control. These results lend further support to the idea that Pat enzymes interact with extensive surfaces of their substrates. Finally, we suggest that acetylation of SlAcs depends on factors or conditions other than those present in our *in vitro* system. We also discuss possible explanations why SlAcs is not controlled by RLA as defined in other bacterial species.

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**Introduction**

Reversible lysine acetylation (RLA) is a post-translational modification that occurs in all domains of life [1] and affects diverse cellular processes and functions. Acetyltransferases transfer the acetyl moiety from acetyl-CoA to the ε-amino group of the target lysine. Lysine acetylation can affect enzyme activity [2], protein stability [3], protein-protein interactions, or DNA binding [4]. Yeast Gcn3 protein (yGcn3p)-related N-aceetyltransferases (a.k.a., GNATs), classified by amino acid sequence and structure [5], are the only class of acetyltransferases found in all domains of life [6]. GNATs were first identified for their role in modification of histones [7]. Crystal structures and biochemical analyses of the yGcn3p, the founding member of the GNAT family, with representative peptides from histones has provided valuable information about the substrate specificity and substrate recognition by GNATs [8,9].

Members of the GNAT family also acetylate metabolic enzymes. For example, in *Salmonella enterica*, the enzyme acetyl-CoA synthetase (SeAcs) is acetylated by the protein acetyltransferase (SePat), a two-domain acetyltransferase that contains a large domain of unknown function and a C-terminal GNAT domain [10]. SeAcs is a member of the AMP-forming CoA ligase family of enzymes that converts carboxylic acids to their CoA thioesters via an acyl-AMP intermediate [11]. Acetylation of the active site lysine of AMP-forming CoA ligases prevents the adenylylation of the carboxylic acid. In addition to Pat from *S. enterica*, GNATs are known to acetylate members of the of AMP-forming CoA ligase family (including Acs) in *Rhodopseudomonas palustris* [12,13], *Bacillus subtilis* [14], and *Mycobacterium smegmatis* [15]. The Acs homologue from *Streptomyces coelicolor* is acetylated *in vivo* [16], but the GNAT responsible for acetylation of *S. coelicolor* Acs is unknown.

Knowledge of the interactions of GNAT with their proteins substrates is limited. *R. palustris* encodes a single-domain GNAT (RpKatA) and a homologue of the SePat GNAT (RpPat). RpKatA and RpPat discriminate among members of the AMP-forming CoA ligase family produced by *R. palustris* [13]. In addition to the target lysine, RpPat recognizes a loop greater than 20 Å from the target lysine, suggesting that Pat enzymes interact with a large surface of the acceptor substrate [17]. As a proof of principle, the introduction of this recognition loop into *R. palustris* methylmalonyl-CoA mutase (RpMatB), an AMP-forming CoA ligase that is
not a substrate of RpPat, rendered RpMatB a target of acetylation by RpPat. Thus, synthetic chimeras of AMP-forming CoA ligases have yielded valuable information about how GNATs recognize protein substrates and have produced AMP-forming CoA ligases that are placed under the regulation of lysozyme acetylation. RpPat and SpPat enzymes acetylate their cognate Acs proteins. Although the GNAT responsible for the acetylation of Acs in S. coelicolor is unknown, the closely related actinomycete Streptomyces lividans encodes SpPatA, a two-domain homologue of SpPat and RpPat enzymes. Significantly, SpPatA does not efficiently acetylate the S. lividans Acs (SlAcs) in vitro [18], making this the first Acs enzyme that is not efficiently acetylated by a Pat acetyltransferase. In contrast, SpPatA efficiently acetylates SlAcs. Here we probe the amino acid sequences in SlAcs that rendered it a better substrate for SpPatA than SlAcs is. By replacing amino acids from SlAcs into the C-terminus of SlAcs, we constructed SlAcs–SeAcs chimera that were efficiently acetylated by SpPatA. One SlAcs–SeAcs chimera contained 41 amino acid differences from SlAcs. As a result of these changes, the SlAcs–SeAcs chimera was subject to regulation by SpPatA. We used a heterologous model system to demonstrate that the SlAcs–SeAcs chimera was subject to RLA regulation in vivo by SpPatA. In sum, we identified regions in SlAcs that were critical for recognition by SpPatA, and transferring of these residues into the poor substrate SlAcs resulted in SlAcs variant that was efficiently regulated by SpPatA.

Materials and Methods

Bacterial Strains and Growth Conditions

All strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Escherichia coli and Salmonella enterica strains were grown at 37°C in tryptic soy broth (LB, Difco) [19] or no-carbon essential (NCE) minimal medium [20] supplemented with sodium acetate (10 mM), MgSO4 (1 mM), and ampicillin (100 μg ml−1). When necessary, antibiotics were used at the following concentrations: ampicillin, 100 μg ml−1; tetracycline, 10 μg ml−1; chloramphenicol, 12.5 μg ml−1, kanamycin, 50 μg ml−1. L(+) arabinose was added at varying concentrations (5 or 200 μM) to induce the expression of S. enterica acs, S. lividans acs, and acs chimeras cloned into the expression vector pBAD30 [21]. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of IPTG (0–300 μM) to induce expression of S. lividans patA (EF660627) clones into the expression vector pSK-Km [22]. Growth experiments were performed at 37°C using a microtiter plate and a microtiter plate reader (Bio-Tek Instruments). All growth data are plotted as the mean of three data points.

Molecular Techniques

DNA manipulations were performed using standard techniques [23]. Restriction endonucleases were purchased from Fermentas. DNA was amplified using Pfu Ultra II Fusion DNA polymerase (Agilent) or Herculase II Fusion DNA polymerase (Agilent). Site-directed mutagenesis was performed using the QuikChange® Site Directed Mutagenesis kit (Agilent). Plasmids were isolated using the Wizard Plus SV Miniprep kit (Promega) and PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega). DNA sequencing was performed using BigDye® (ABI PRISM) protocols, and sequencing reactions were performed at the University of Georgia Genomics Facility.

Plasmids Used for Protein Overproduction

Chimeric proteins encoded by fusing different regions of S. lividans acs (EF660454) and S. enterica acs genes were generated by amplifying genomic DNA from S. lividans TK24 genomic DNA from S. enterica strain TR6583, respectively. Fusion plasmids encoding proteins in which the N-terminal domain of SlAcs was fused to the C-terminal domain of SeAcs were amplified by overlap-extension PCR [24], followed by standard cloning into plasmid pTEV5 [25]. Fusion plasmid encoding a protein in which an internal sequence of SlAcs was replaced by the corresponding sequence SlAcs were constructed as described below and in Table 2.

Plasmid pSlAcs14 (SlAcs 550–582 SeAcs) – the nucleotides encoding the first 582 residues of SlAcs fused to SeAcs were amplified from pSlAcs9, fused to the C-terminus of SlAcs, and cloned into pTEV5.

Plasmid pSlAcs15 (SlAcs 550–603 SeAcs) – the nucleotides encoding the first 603 residues of SlAcs fused to SeAcs were amplified from pSlAcs9, fused to the C-terminus of SlAcs, and cloned into pTEV5.

Plasmid pSlAcs23 (SlAcs 550–618 SeAcs) – the nucleotides encoding the first 618 residues of SlAcs fused to SeAcs were amplified from pSlAcs9, fused to the C-terminus of SlAcs, and cloned into pTEV5.

Plasmid pSlAcs17 (SlAcs 550–627 SeAcs) – the nucleotides encoding the first 627 residues of SlAcs fused to SeAcs were amplified from pSlAcs9, fused to the C-terminus of SlAcs, and cloned into pTEV5.

Plasmid pSlAcs18 (SlAcs 550–638 SeAcs) – the nucleotides encoding the first 638 residues of SlAcs fused to SeAcs were amplified from pSlAcs9 and cloned into pTEV5.

Plasmid pSlAcs19 (SlAcs 550–643 SeAcs) – the nucleotides encoding the first 643 residues of SlAcs fused to SeAcs were amplified from pSlAcs9 and cloned into pTEV5.

Plasmid pSlAcs26 (SlAcs 550–581 SeAcs, 591–627 SeAcs) – the nucleotides encoding the first 581 residues of SlAcs fused to SeAcs were amplified from pSlAcs9 with primers incorporating residues 582–590 from SlAcs, fused to the nucleotides encoding the 64 residues of SeAcs fused to SlAcs amplified from pSlAcs17, and cloned into pTEV5.

Plasmid pSlAcs27 (SlAcs 550–590 SeAcs, 598–627 SeAcs) – the nucleotides encoding the first 590 residues of SlAcs fused to SeAcs were amplified from pSlAcs9 with primers incorporating residues 591–597 from SlAcs, fused to the nucleotides encoding the 57 residues of SeAcs fused to SlAcs amplified from pSlAcs17, and cloned into pTEV5.

Plasmid pSlAcs28 (SlAcs 550–597 SeAcs, 603–627 SeAcs) – the nucleotides encoding the first 597 residues of SlAcs fused to SeAcs were amplified from pSlAcs9 with primers incorporating residues 598–602 from SlAcs, fused to the nucleotides encoding the 52 residues of SeAcs fused to SlAcs amplified from pSlAcs17, and cloned into pTEV5.

Plasmid pSlAcs29 (SlAcs 550–581 SeAcs, 603–627 SeAcs) – the nucleotides encoding the first 581 residues of SlAcs fused to SeAcs were amplified from pSlAcs9 with primers incorporating residues 582–602 from SlAcs, fused to the nucleotides encoding the 52 residues of SeAcs fused to SlAcs amplified from pSlAcs17, and cloned into pTEV5.

Plasmid pSlAcs44 (SlAcs 615–626 SeAcs) – the nucleotides encoding the first 614 residues of SlAcs were amplified from pSlAcs1, the nucleotides encoding the final 40 residues of SeAcs fused to SlAcs amplified from pSlAcs28, and cloned into pTEV5.

The C-terminal domain of SeAcs was amplified from strain TR6583. DNA fragments were cut with NheI and EcoRI and ligated into pTEV5 [25] cut with the same enzymes. The resulting plasmids directed the synthesis of SlAcs–SeAcs chimeras or SeAcs C-terminal domain (pACS38) each with an N-terminal H6 tag.
cleavable by recombinant tobacco etch virus (rTEV) protease prepared as described [26].

The C-terminal domain of SlAcs was amplified from S. lividans TK24 genomic DNA. The DNA fragments were cut with KpnI and HindIII and ligated into pKLD66 [25] cut with the same enzymes. The resulting plasmid pSlAcs7 directed synthesis of the SlAcs C-terminal domain with an N-terminal maltose-binding protein-His{sub}6-tag cleavable by rTEV protease as described above.

Construction of Untagged SlAcs Complementation Plasmid

The S. lividans acs was amplified from pSlAcs1 with the primers that included an optimized ribosome-binding site. The DNA fragment was cut with EcoRI and HindIII and ligated into pBAD30 [21], cut with the same enzymes. The resulting plasmid pSlAcs6 expresses SlAcs under the control of the P{sub}BAD promoter.

Construction of SeAcs, SlAcs, and SlAcs

Complementation vectors encoding H{sub}6-tagged SeAcs chimera C3. Genes encoding S. lividans SlAcs and the S. lividans/S. enterica SlAcs chimeras were amplified from pSlAcs1 and pSlAcs28, respectively, using primers that included an optimized ribosome-binding site and an N-terminal His{sub}6-tag. S. enterica acs was amplified from genomic DNA isolated from JE6583 using primers that included an optimized ribosome-binding site and an N-terminal His{sub}6-tag. The DNA fragments were cut with EcoRI and HindIII and ligated into pBAD30, cut with the same enzymes. The resulting plasmids pSlAcs47, pSlAcs48, and pACS59 produce SlAcs, S. lividans/S. enterica Acs chimera C3, and SlAcs{sup}WT, respectively, with His{sub}6-tags fused N-terminal with a Gly-Ser-Gly linker under the control of the P{sub}BAD promoter.

Purification of SlAcs-SeAcs chimeras, SlAcs C-terminal domain, and SlAcs C-terminal domain. Plasmids encoding tagged proteins were transformed with pRARE2 (EMD Millipore) into a Δpka derivative of E. coli strain C41(DE3) [27] (JE9314) to prevent acetylation prior to overproduction. The resulting strains were grown overnight and sub-cultured 1:100 (v/v) into two liters of LB containing ampicillin (100 μg ml{sup}-1) and chloramphenicol (12.5 μg ml{sup}-1). The cultures were grown shaking at 25°C to A{sub}600{sup}~0.7 and protein synthesis was induced with IPTG (0.25 mM). Upon induction, the cultures were grown overnight at 25°C. Cells were harvested at 6000 x g for 10 min at 4°C in an Avanti J-2 XPI centrifuge fitted with rotor JLA-8.1000 (Beckman Coulter). Cell pellets were re-suspended in 30 ml of cold His-bind
buffer (buffer A) 

\[
[\text{tris(hydroxymethyl)aminomethane-HCl (Tris-HCl)}} \text{ buffer (50 mM, pH 8), NaCl (500 mM), and imidazole (5 mM)} \text{ containing phenylmethanesulfonylfluoride (PMSF, 1 mM)}.
\]

Cells were placed on ice and lysed by sonication for 2 min (2-s pulse followed by 4 s of cooling) at level 7 in a model 550 sonic dismembrator (Fisher). The extract was cleared by centrifugation at 4°C for 30 min at 43,367 x g. His-SlAcs-SeAcs chimera was purified from clarified cell extract using a 1 ml settled bed volume of HisPur\textsuperscript{TM} Ni-NTA Resin (Pierce). Unbound proteins were eluted off the column by washing with buffer A. The resin was washed with 10 column volumes of buffer B [Tris-HCl buffer (50 mM, pH 8), NaCl (500 mM), and imidazole (15 mM)]. His-SlAcs-SeAcs chimera was eluted with 5 column volumes of buffer C [Tris-HCl buffer (50 mM, pH 8), NaCl (500 mM), and imidazole (250 mM)]. All fractions containing His-SlAcs-SeAcs chimera were combined. rTEV protease was added to His-SlAcs-SeAcs chimera and the SlAcs-SeAcs chimera/rTEV mixture was incubated at room temperature for 3 h. PMSF was added to the protein mixture and incubated 15 min at room temperature. The SlAcs-SeAcs chimera/rTEV mixture was dialyzed at 4°C against buffer D (Tris-HCl (50 mM, pH 8), NaCl (500 mM)) twice for 3 h and again against buffer D containing imidazole (5 mM) for 12 h. After cleavage and dialysis, protein mixtures were passed over 1 ml HisPur\textsuperscript{TM} Ni-NTA Resin (Pierce) using the buffers described above. Cleaved SlAcs-SeAcs chimera passed through the resin and eluted in the flow-through fractions. Purified SlAcs-SeAcs chimera was analyzed by SDS-PAGE. Fractions containing SlAcs-SeAcs chimera were pooled together. SlAcs-SeAcs chimera was stored in Tris-Cl buffer (50 mM, pH 8.0) containing NaCl (100 mM) and glycerol (20%, v/v). SlAcs concentration was determined by measuring absorbance at 280 nm. The molecular weights and molar extinction coefficients used to calculate His-SlAcs-SeAcs chimera concentrations are listed in Table 3. All enzymes were purified to >95% homogeneity.

### SeAcs Protein Purification

Plasmid pACS10 was transformed into a Δpho derivative of E. coli strain C41Δ(ΔE) (JE9314). The resulting strain was grown overnight and sub-cultured 1:100 (v/v) into two liters of LB containing ampicillin (100 µg ml\textsuperscript{-1}). The culture was grown

### Table 2. Plasmids used in this study.

| Plasmid   | Genotype                                                                 | Source or method             |
|-----------|--------------------------------------------------------------------------|------------------------------|
| pBAD30    | Promoter expression vector, bla<sup>+</sup>                              | [21]                         |
| pSlAcs6   | S. lividans acs<sup>+</sup> allele (EFD66247) in pBAD30, bla<sup>+</sup>  | Standard cloning             |
| pSlAcs47  | S. lividans acs<sup>+</sup> allele (EFD66247) with N-terminal His tag in pBAD30, bla<sup>+</sup> | Standard cloning             |
| pSlAcs1   | S. lividans acs<sup>+</sup> –S. enterica acs chimera allele with N-terminal His tag in pBAD30, bla<sup>+</sup> | Standard cloning             |
| pAC559    | S. enterica acs<sup>+</sup> allele with N-terminal His tag in pBAD30, bla<sup>+</sup> | Standard cloning             |
| pSRK-Km   | lac<sup>+</sup>-loc promoter-operator expression vector, kan<sup>+</sup>  | [22]                         |
| pSlPatA9  | S. lividans patA<sup>+</sup> allele (EFD66247) in pSRK-Km, bla<sup>+</sup> | [18]                         |
| pKLD66    | N-terminal, rTEV-cleavable MBP-His<sub>6</sub>-tag overexpression vector, bla<sup>+</sup> | [25]                         |
| pSlAcs7   | S. lividans acs<sup>+</sup> allele (EFD66247) C-terminal domain (DS19-D649) in pKLD66, bla<sup>+</sup> | Standard cloning             |
| pTEV5     | N-terminal, rTEV-cleavable His<sub>6</sub>-tag overexpression vector, bla<sup>+</sup> | [25]                         |
| pSlAcs1   | S. lividans acs<sup>+</sup> allele (EFD68454) in pTEV5, bla<sup>+</sup>   | [18]                         |
| pSlPatA1  | S. lividans patA<sup>+</sup> allele (EFD66247) in pTEV5, bla<sup>+</sup>  | [18]                         |
| pACS38    | S. enterica acs<sup>+</sup> C-terminal domain (DS18-S652) in pTEV5, bla<sup>+</sup> | Standard cloning             |
| pSlAcs8   | A1 chimera: SlAcs 520 SeAcs in pTEV5, bla<sup>+</sup>                   | Overlap-extension PCR        |
| pSlAcs9   | A2 chimera: SlAcs 550 SeAcs in pTEV5, bla<sup>+</sup>                   | Overlap-extension PCR        |
| pSlAcs12  | A3 chimera: SlAcs 560 SeAcs in pTEV5, bla<sup>+</sup>                   | Overlap-extension PCR        |
| pSlAcs22  | A4 chimera: SlAcs 566 SeAcs in pTEV5, bla<sup>+</sup>                   | Overlap-extension PCR        |
| pSlAcs10  | A5 chimera: SlAcs 582 SeAcs in pTEV5, bla<sup>+</sup>                   | Overlap-extension PCR        |
| pSlAcs11  | A6 chimera: SlAcs 617 SeAcs in pTEV5, bla<sup>+</sup>                   | Overlap-extension PCR        |
| pSlAcs14  | B1 chimera: SlAcs 550–582 SeAcs in pTEV5, bla<sup>+</sup> in pTEV5, bla<sup>+</sup> | Overlap-extension PCR        |
| pSlAcs15  | B2 chimera: SlAcs 550–603 SeAcs in pTEV5, bla<sup>+</sup> in pTEV5, bla<sup>+</sup> | Overlap-extension PCR        |
| pSlAcs23  | B3 chimera: SlAcs 550–618 SeAcs in pTEV5, bla<sup>+</sup> in pTEV5, bla<sup>+</sup> | Overlap-extension PCR        |
| pSlAcs17  | B4 chimera: SlAcs 550–627 SeAcs in pTEV5, bla<sup>+</sup> in pTEV5, bla<sup>+</sup> | Overlap-extension PCR        |
| pSlAcs18  | B5 chimera: SlAcs 550–638 SeAcs in pTEV5, bla<sup>+</sup> in pTEV5, bla<sup>+</sup> | Standard cloning             |
| pSlAcs19  | B6 chimera: SlAcs 550–643 SeAcs in pTEV5, bla<sup>+</sup> in pTEV5, bla<sup>+</sup> | Standard cloning             |
| pSlAcs26  | C1 chimera: SlAcs 550–581 SeAcs, 591–627 SeAcs in pTEV5, bla<sup>+</sup> | Overlap-extension PCR        |
| pSlAcs27  | C2 chimera: SlAcs 550–590 SeAcs, 598–627 SeAcs in pTEV5, bla<sup>+</sup> | Overlap-extension PCR        |
| pSlAcs28  | C3 chimera: SlAcs 550–597 SeAcs, 603–627 SeAcs in pTEV5, bla<sup>+</sup> | Overlap-extension PCR        |
| pSlAcs29  | C4 chimera: SlAcs 550–581 SeAcs, 603–627 SeAcs in pTEV5, bla<sup>+</sup> | Overlap-extension PCR        |
| pSlAcs44  | C5 chimera: SlAcs 615–626 SeAcs in pTEV5, bla<sup>+</sup>               | Overlap-extension PCR        |
| pSlAcs49  | K610A variant of C3 chimera in pTEV5, bla<sup>+</sup>                   | Site-directed mutagenesis    |

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shaking at 37°C to A600~0.7 and protein synthesis was induced with IPTG (0.25 mM). Upon induction, the cultures were grown overnight at 30°C. SeAc was purified and stored as described [2]. SlAcWT and SlPatWT were purified as described [18].

In vitro CoA Ligase Assays
Activity of SlAcWT, SeAcWT, and SlAcWT-SeAcWT chimera activities were measured using an NADH-consuming assay [12,28] with modifications. Reactions (100 μl total volume) contained 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES, 50 mM, pH 7.5), tris(carboxymethyl)phosphine (TCEP, 1 mM), ATP (2.5 mM) CoA (0.5 mM), MgCl2 (5 mM), KCl (1 mM), phosphonoarylpyruvate (3 mM), NADH (0.1 mM), pyruvate kinase (1 U), myokinase (5 U), lactate dehydrogenase (1.5 U) and acetate (0.2 mM). Reactions were started by the addition of AcCoA (5–100 pmol). The absorbance at 340 nm was monitored in a 96-well plate using the Spectramax Plus UV-visible spectrophotometer (Molecular Devices). Enzyme activities were determined to be in the linear range of the assay and were calculated as described [28].

In vitro Protein Acetylation Assay
Protein acetylation was observed using radiolabeled Ac-CoA as described [10,12,29]. Acetylation reactions contained 2-(6β-[3H]-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol (Bis-Tris-HCl) buffer (50 mM, pH 6.0), [1-14C]-Ac-CoA (20 mM), acetyl-CoA synthetase or acyl-CoA synthetase C-terminal domain (3 μM), glycerol (10%, v/v), and SlPatWT (1 μM). Reactions (20 μl total volume) were incubated for 60 min at 30°C. Samples (5 μl) were resolved using SDS-PAGE [30] and proteins were visualize by Coomassie Blue R staining [31]. Gels were dried and exposed 16 h to a multipurpose phosphor screen (Packard). Labeled proteins were visualized using a Typhoon Trio+ Imager (GE Healthcare) equipped with ImageQuant TL software (GE Healthcare). Acetylation was quantified as digital light units and is reported relative to SlAcWT acetylation.

The effect of acetylation on activity of SlAcWT, SeAcWT, and SlAcWT-SeAcWT chimera activity was determined as described [12] with modifications. SlAcWT, SeAcWT, or SlAcWT-SeAcWT (3 μM) was incubated with SlPatWT (1 μM) and 50 μM Ac-CoA for 90 min at 30°C using the buffer system described above. After 90 min, reactions were diluted into HEPES buffer (50 mM, pH 7.5 at 4°C). SlAcWT, SlAcWT, and SlAcWT-SeAcWT chimera activities were measured as described above.

In vitro Deacetylation Assays
Acetylated SlAcWT-SeAcWT chimera C3 was deacetylated with S. enterica CobB5 (S.CobB5) sirtuin deacetylase as described [29]. In vitro acetylated SlAcWT-SeAcWT chimera C3 (3 μM, radiolabeled) was incubated with S.CobB5 (3 μM) in deacetylation buffer containing HEPES buffer (50 mM, pH 7.0, NAD+ (1 mM) for 60 min at 37°C (10 μl reaction volume). Reaction mixture samples (5 μl) were resolved by SDS-PAGE, and subjected to phosphor imaging analysis to assess the acetylation state of SlAcWT-SeAcWT chimera C3 after incubation with S.CobB5.

H5-SlAc, H5-SeAc, or H5-Chimera C3 enzymes isolated from S. enterica were deacetylated with S.CobB5 as described above with modifications. H5-SlAc, H5-SeAc, or H5-Chimera C3 enzymes (1 mM) were incubated with S.CobB5 (1 μM) in deacetylation buffer containing HEPES buffer (50 mM, pH 7.0, NAD+ (1 mM) for 60 min at 37°C (25 μl reaction volume). Ac activity was measured using the CoA synthetase assay described above.

### Table 3. Molecular mass and molar extinction coefficients of proteins used in this study.

| Protein     | MM (Da) | ε (M⁻¹ cm⁻¹) |
|-------------|---------|--------------|
| SlAc       | 108369  | 57760        |
| SeAc       | 71045   | 135455       |
| A1 chimera | 71527   | 150925       |
| A2 chimera | 71541   | 150925       |
| A3 chimera | 71509   | 150800       |
| A4 chimera | 71432   | 149745       |
| A5 chimera | 71352   | 146455       |
| A6 chimera | 71115   | 135455       |
| B1 chimera | 71234   | 139925       |
| B2 chimera | 71471   | 150800       |
| B3 chimera | 71471   | 150925       |
| B4 chimera | 71350   | 150925       |
| B5 chimera | 71466   | 150925       |
| B6 chimera | 71751   | 150925       |
| C1 chimera | 71297   | 145425       |
| C2 chimera | 71627   | 150925       |
| C3 chimera | 71429   | 145425       |
| C4 chimera | 71293   | 139800       |
| C5 chimera | 71104   | 135330       |
| C6 chimera | 71429   | 145425       |
| SlPat      | 108369  | 57760        |

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Results

*S. lividans* Acetyl-CoA Synthetase (SlAcs) is Functional in vivo in a Heterologous System

The SeAcs homolog from *S. lividans* converts acetate to acetyl-CoA in *vivo* [10]. Alignment of the SeAcs and SlAcs amino acid sequences using BLAST revealed 52% sequence identity and 62% sequence similarity in amino acid sequence. To determine whether or not SlAcs functions in *vivo*, we expressed *S. lividans* acsA ectopically in a ΔacsA Δpta *S. enterica* strain (JE13238) demanding growth on low concentrations of acetate (10 mM). *S. enterica* uses two pathways for the conversion of acetate to acetyl-CoA via an acetyl-AMP intermediate. RLA controls SeAcs activity [2]. The protein acetyltransferase SlPat acetylates and inactivates SeAcs (discussed further below) [10], and SeAcs is deacetylated and reactivated by the sirtuin type deacetylase SeCobB [2,29]. In the second pathway, acetate kinase (Ack) and phosphotransacetylase (Pta) catalyze the conversion of acetate to acetyl-CoA via an acetyl-phosphate intermediate. AcS activity is used by the cell when the concentration of acetate in the environment is <10 mM, whilst Pta/Ack is the preferred pathway when *S. enterica* is growing on concentrations of acetate ≥25 mM. A *S. enterica* strain lacking the AcS and Ack/Pta pathways failed to grow on acetate (10 mM, Fig. 1B, squares). When SlAcs was produced ectopically, growth of an *S. enterica* Δacs Δpta strain was restored (Fig. 1B, circles), demonstrating that SlAcs was active and could substitute for SeAcs function in *vivo*.

**SIPatA Acetylates the C-terminal Domain of SeAcs, but not SlAcs**

AMP-forming CoA synthetases are two-domain enzymes that activate carboxylic acids to CoA thioesters in a two-step reaction. In the first half-reaction, an invariant lysine in the C-terminal domain (K609 of SeAcs) is buried in the active site cleft located between the N- and C-terminal domains [33]. Upon deacylation of the carboxylic acid substrate, the C-terminal domain undergoes a ∼140° domain rotation to allow for the thioesterification of the fatty acyl-AMP intermediate [34]. The catalytic lysine of AMP-forming CoA ligases is surface exposed when the enzyme is in the thioester-forming conformation [33], and this likely represents the conformation that is subject to acetylation by Pat.

Previously, we demonstrated that SlAcs was a poor substrate for the SIPatA enzyme in *vivo* [18]. That work identified SlAcs as the first example of an acetyl-CoA synthetase that was not recognized by the cognate Pat protein acetyltransferase in *vivo* [10,12]. However, SIPatA efficiently acetylated and inactivated the acetocetyl-CoA synthetase SlAacS from *S. lividans*, and the orthologous SeAcs enzyme [18], indicating that SIPatA was catalytically active, but somehow unable to acetylate SlAcs in *vivo*.

We considered the possibility that SlAcs favored the deacylation-conformation in *vivo*, which would likely render the target K610 inaccessible to SIPatA due to its location in the SlAcs active site. To differentiate the inaccessibility of SlAcs K610 from the inability of SIPatA to recognize SlAcs, we isolated the C-terminal domains of SeAcs (a good substrate of SIPatA) and SlAcs. In the absence of the N-terminal domain, the target lysine is no longer protected, thus it is accessible to the acetyltransferase.

Homogeneous C-terminal domains of SlAcs (residues D519–D649, 151 aa) and SeAcs (residues D518–S652, 135 aa) were incubated in the presence of SIPatA and radiolabeled [1-14C] acetyl-CoA. Differential migration of the C-terminal domains is likely due to differences in hydrophoby of [grand average of hydropathy (GRAVY)] scores [35] for SlAcs and SeAcs C-terminal domains are +0.023 and −0.160, respectively), which has been shown to affect gel mobility of protein in SDS-PAGE [36]. As shown in figure 2A, the C-terminal domain of SlAcs was acetylated, but the SlAcs C-terminal domain was not. These data showed that the N-terminal domain of SlAcs was not required for acetylation by SIPatA. Additionally, these results strongly suggested that inaccessibility of residue K610 was likely not the reason why SlAcs was poorly acetylated in *vivo*. We hypothesized that regions within the C-terminal domain of SlAcs enzyme prevented acetylation of SIPatA. As shown in figure 2B, the C-terminal domains of SlAcs and SeAcs share ∼50% sequence identity.

**Figure 1. SlAcs<sup>WT</sup> can substitute for SeAcs<sup>WT</sup> in S. enterica during growth on acetate.** A. *S. enterica* encodes a one-enzyme and a two-enzyme pathway for acetate activation. The one-enzyme pathway is comprised of acetyl-CoA synthetase (AcS), whose activity is modulated post-translationally by the protein acetyltransferase (Pat) and sirtuin deacetylase (CobB) enzymes. The two-enzyme pathway is comprised of acetate kinase (Ack) and phosphotransacetylase (Pta). B. Growth behavior of Δacs Δpta *S. enterica* strain JE13238 as a function of SlAcs<sup>WT</sup>. Experiments were performed on NCE minimal medium supplemented with acetate (10 mM), at 37°C using a microtiter plate and a plate reader (Bio-Tek Instruments). Synthesis of SlAcs<sup>WT</sup> was ectopically encoded (plasmid pSAcs6) and induced using L-(-)-arabinose (5 mM). Cloning vector (pBAD30) lacking *S. lividans* acsA was used as negative control. All S.D. <0.01 absorbance units. doi:10.1371/journal.pone.0099817.g001
Chimeras of SlAcs and SeAcs Reveal Regions in the SeAcs C-terminal Domain that are Critical for Acetylation by SlPatA

Based on regions of sequence conservation (Fig. 2B), we generated a set of precise fusions between the SlAcs and SeAcs that contained varying amounts of the SeAcs protein. A SlAcs chimera containing the SlAcs N-terminal domain fused to the SeAcs C-terminal (chimera A1) was strongly acetylated by SlPatA, confirming that the C-terminal domain of SlAcs was responsible for the poor acetylation of SlAcsWT (Figs. 3A, B).

We identified regions of the SeAcs C-terminal domain important for acetylation by SlPatA by constructing chimeras that contained decreasing amounts of the SeAcs C-terminal domain relative to chimera A1. To measure the efficiency of acetylation, each chimera was incubated with SlPatA and radiolabeled [1-14C] acetyl-CoA. SlPatA strongly acetylated chimeras containing at least the final 86 amino acids of SlAcs (chimeras A1, A2, A3, A4; Fig. 3). These chimeras contained at least 43 amino acids N-terminal to the acetylation site, a region previously reported to be important for acetylation of homologous AMP-forming CoA ligase enzymes by the R. palustris Pat homologue [17].

To narrow down the number of SeAcs residues required for acetylation of the SlAcs-SeAcs chimeras, we focused on SlAcs-SeAcs chimera A2, which had the fewest SeAcs-derived residues (Fig. 3B), and the highest level of acetylation (Fig. 3C).

We generated a second set of chimeras in which various stretches of SlAcs-derived residues were substituted into SlAcs-SeAcs chimera A2 (Fig. 3D). SlAcs-SeAcs chimeras B4, B5, and B6 that contained at least 45 residues of SlAcs (including the SlAcsK609 acetylation site) were strongly acetylated (Fig. 3C). Notably, the A10 loop of Acs, which contains the target lysine, is completely conserved between SlAcs and SeAcs (Fig. 2B). However, 17 amino acids C-terminal to the acetylation site of SlAcs were required for acetylation by SlPatA. This revealed a previously unrecognized region of the protein important for acetylation. Of this set of SlAcs-SeAcs chimeras, chimera B4 was the best substrate of SlPatA and contained the fewest SeAcs-derived amino acids.

To determine whether the 77 contiguous SeAcs-derived residues of chimera B4 were critical for acetylation, we identified regions of SlAcs and SeAcs with low amino acid sequence conservation and introduced those sets of SlAcs residues into chimera B4 (Fig. 3E). Of those tested, only SlAcs-SeAcs chimera C3 was acetylated with similar efficiency as SlAcs (Fig. 3F). Acetylation of each chimera was quantified relative to acetylation of SlAcs (Fig. 4A, gray bars).

Importantly, chimeras containing only the 60 SeAcs-derived residues N-terminal to K610 (chimera B2) or 11 SeAcs-derived residues C-terminal to K610 (chimera C5) were <30% acetylated relative to SeAcs. Thus, amino acid sequences N- and C-terminal to the target lysine were important for acetylation by SlPatA, and neither set of amino acids rendered SlAcs a strong acetylation target when introduced independently.

Assessment of the Enzymatic Activity of the Chimeras

Chimeras were tested for their AMP-forming acetyl-CoA ligase forming activity. Although the SlAcsWT and SeAcsWT C-terminal domains share a high degree of sequence conservation, not all chimeras were active (Fig. 4A, black bars). To identify active chimeras that were also targets of acetylation, the acetylation of each chimera was measured relative to SeAcs (Fig. 4A, gray bars). SlAcs-SeAcs chimera C3 (hereafter referred to as chimera C3) was identified as the single chimera with the fewest SlAcs residues that was active and efficiently acetylated by SlPatA. As shown in figure 4B, chimera C3 contained 41 amino acid differences from SlAcs. For comparison, we include the analogous sequence from Acs homologues known to acetylated by protein acetyltransferases in other bacteria. Notably, the wildtype SlAcs amino acid sequence replaced by SeAcs sequences shares some sequence homology with these Acs homologues.

Chimera C3 Activity is Modulated by Acetylation and Deacetylation

As shown in figure 5A, the catalytic residue K610 residue is the only residue of chimera C3 that was acetylated. To test whether the activity of chimera C3 was under the control of acetylation, the protein was incubated with SlPatA acetyltransferase in the presence and absence of the acetyl donor, acetyl-CoA. Upon acetylation, chimera C3 activity decreased >98%, similar to the regulation of SeAcs activity (Fig. 5B, gray bar). The SlAcs enzyme retains >75% activity upon incubation with SlPatA and Ac-CoA (Fig. 5B, gray bar). As mentioned above, acetylation of SeAcsWT is reversed by the NAD-dependent sirtuin deacetylase CobB in S. enterica, and deacetylation reactivates the SlAcsWT enzyme [2]. We
tested whether chimera C3 could be deacetylated by incubating acetylated chimera C3 with SCoB, the co-substrate NAD⁺, or both. When SCoB and NAD⁺ were present in the reaction mixture, chimera C3 was completely deacetylated (Fig. 5C), demonstrating that the reversibility of the acetylation process was not affected by the substitutions in chimera C3.

**SIACs-SeACs Chimera C3 is Acetylated in vivo in S. enterica**

To determine the efficiency of SPatA acetylation of chimera C3 in vivo, we used *S. enterica* acetate utilization (Fig. 1A) as a heterologous model to demonstrate the effects of SPatA acetylation on activity of the Acs homologues. In this system, His-tagged chimera C3, SIACs, and SeACs (H₆-chimera C3, H₆-SIACs, H₆-SeACs, respectively) were produced from plasmids in *S. enterica* acs pat cobB and *S. enterica* acs pat cobB strains JE9152 and JE9894, respectively. All the experiments were conducted in *S. enterica* pat strains to prevent acetylation by SPatA. We characterized the effect of SPatA acetylation on the H₆-Acs homologues by measuring growth of each strain harboring a plasmid with an inducible PatA allele or an empty cloning vector. Additionally, we isolated H₆-SIACsWT, H₆-SeACsWT, and H₆-chimera C3 from cells grown in the presence or absence of SPatA to quantify the effects of SPatA acetylation on each Acs protein. As shown in figure 6A, production of H₆-chimera C3, H₆-SIACs, or H₆-SeACs supported growth of *S. enterica* acs pat cobB strain (open symbols). This was the expected result, since the strain lacked Pat activity, thus the cell could not acetylate (i.e., inactivate) any of the Acs enzymes. We attributed the lag in the strain producing H₆-chimera C3 to the decreased activity of this chimera (Fig. 5B). When production of SPatA was induced in each strain (25 μM inducer), growth of *S. enterica* acs pat cobB strains producing H₆-SeACsWT or H₆-chimera C3 was significantly reduced, while growth of the *S. enterica* acs pat cobB strain producing H₆-SIACsWT was unaffected. Importantly, inhibition of an *S. enterica* acs cobB strain producing H₆-SeACsWT required high levels of SPatA induction (500 μM inducer, Fig. 6B). No growth inhibition occurred when SPatAWT was induced at low levels (≤5 μM inducer, Fig. 6C).

As expected, the presence of SCoBWT in a *S. enterica* acs pat cobB strain resulted in no significant growth defects upon SPatAWT induction in strains expressing H₆-SeACsWT or H₆-SeACsWT (Fig. 6D). However, we did note a slight inhibition of growth of a *S. enterica* acs pat cobB strain producing H₆-chimera C3. We surmised that such an effect was likely due to a decreased ability of SCoBWT to deacetylase and reactivate H₆-chimera C3 and restore growth. This idea was supported by the observation that increased induction of SPatAWT inhibited a *S. enterica* acs pat cobB strain producing H₆-chimera C3 (Fig. 6E), but not those producing H₆-SeACsWT or H₆-SeACsWT (Fig. 6F).

Since high levels of SPatA induction were required to inhibit growth of an *S. enterica* acs cobB strain producing H₆-SeACsWT, we expected that H₆-SeACsWT to be poorly acetylated by SPatAWT and thus more active in vivo. We also expected higher proportions of acetylated to non-acetylated H₆-SeACsWT and H₆-chimera C3 in vivo. To measure the effect of SPatAWT acetylation on the activity of H₆-SeACs of, H₆-SeACs of, and H₆-chimera C3, we grew *S. enterica* acs cobB strains expressing the corresponding Acs alleles while inducing SPatAWT at low levels (5 μM) to allow for growth and biomass accumulation for all strains (Fig. 6C). H₆-SeACsWT, H₆-SeACsWT, and H₆-chimera C3 enzymes were isolated from strains harboring plasmid-borne SPatAWT or empty vector.

As shown in figure 7, activity of the H₆-SeACsWT enzyme isolated from a strain producing SPatAWT was not significantly

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**Figure 3. Construction and acetylation of SIACs-SeACs chimeras.** A. A scheme of SIACsWT (white) and SeACsWT (grey) drawn to scale. Target lysine K610 for SIACsWT and K609 aligned and depicted by “K”. The N-terminal domains (520 residues) are shortened with a hatch in all remaining panels. B. Schematic representation of SIACs-SeACs chimeras A1–A6 in which the C-terminal portion of SIACsWT (white) was replaced with the corresponding amino acid sequence from SeACsWT (grey). All chimeras are drawn to scale for reference to the target lysine (denoted by “K”). Numbers denote the fusion points with respect to the SIACs protein sequence (i.e. either the first residue of SIACsWT replaced by SeACsWT sequence or the first residue of SIACsWT after the SeACsWT amino acid sequence). C. Acetylation of SIACs-SeACs chimeras A1–A6 and B1–B6 using SPatAWT and [1-14C]acetyl-CoA. D. Schematic or SIACs-SeACs chimeras B1–B6 in which internal portion of the C-terminal SIACs domain are replaced with the corresponding sequence from SeACsWT. E. Schematic of chimeras C1–C5. F. Acetylation of SIACs-SeACs chimeras C1–C6. doi:10.1371/journal.pone.0099817.g003
reduced compared to H6-SlAcsWT isolated from a strain with no SlPatWT. However, activities of the H6-SeAcsWT and H6-chimera C3 enzymes were significantly lower when isolated from strains expressing SlPatWT compared to those with no SlPatWT. Activities of the SeAcs and H6-chimera C3 were restored upon incubation with SeCobB deacetylase. These data suggested that SlPatWT more efficiently acetylated H6-SlAcs in a heterologous in vivo model.

Discussion

Herein we report the first Acs enzyme that is not a substrate of Pat homologues in vitro. This finding is important, since Acs is the paradigm for the analysis RLA in all metabolic systems reported thus far. Our results begin to shed some light onto why the SlAcs is not efficiently acetylated by the SlPatWT enzyme of S. lividans. By constructing chimeras of SlAcs that are acetylated by SlPatWT and retain biological activity we gained insights into structural, physiological and possibly evolutionary questions raised by this work.

Is Acs Activity under RLA Control in Streptomyces?

At present, the answer to this question is unclear. It is not known whether SlAcsWT is a bona fide substrate of SlPatWT in vivo in S. lividans. The literature adds to the challenge of determining whether or not in streptomycetes Acs is under RLA control. Work performed by others in Streptomyces coelicolor suggested that the Acs enzyme of this actinomycete may be under RLA control, because results of in vitro experiments showed that acetylated SeAcs was a substrate of a sirtuin deacetylase present in that bacterium [16]. The same authors also reported the isolation of acetylated SeAcs from S. coelicolor. Since the S. coelicolor genome contains a gene

Figure 4. SlAcs-SeAcs Chimera C3 is active and efficiently acetylated. A. Acetyl-CoA synthetase activity of each chimera and SlAcsWT relative to SeAcsWT (gray bars). Amount of acetylation in figure 3C and 3F was quantified and normalized to the total acetylation of SeAcs (black bars). SlAcs-SeAcs chimera C3, the most efficiently acetylated, active chimera with the fewest SeAcsWT-derived residues, is noted with a star. Values are reported as the mean ± S.D. of three experiments. B. Sequence alignment of SlAcsWT, SeAcsWT, chimera C3, Rhodopseudomonas palustris CGA009 Acs (RpAcs), and Mycobacterium smegmatis mc^155 Acs (MsAcs). Residues in chimera C3 that are derived from the SeAcsWT amino acid sequence are highlighted in black. SlAcs residues conserved in the MsAcs homologue are shown in bold typeface in the sequence of the latter. Black box indicates the target lysine.

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encoding a $\text{SIPat}$ homologue, they concluded that $\text{SAc}$ was under RLA control.

Our initial work with the $S. \text{livi}dans$ $\text{SIPat}^{\text{WT}}$ and $\text{SAc}^{\text{WT}}$ enzymes paints a complex picture for the regulation of $\text{SAc}^{\text{WT}}$ function in this organism, and by extrapolation, maybe in $S. \text{coelicolor}$. Because the specific activity of $\text{SAc}^{\text{WT}}$ is similar to that of $\text{SAc}^{\text{WT}}$ in vitro (Fig. 5B), we hypothesize that $\text{SAc}^{\text{WT}}$ activity is also tightly controlled by $S. \text{livi}dans$. To account for the inability of $\text{SIPat}^{\text{WT}}$ to acetylate $\text{SAc}^{\text{WT}}$, we propose that $\text{SIPat}^{\text{WT}}$ has evolved unique strategies for substrate recognition, or $\text{SIPat}^{\text{WT}}$ is not the primary modifier of $\text{SAc}^{\text{WT}}$. We discuss each possibility further below.

In vitro, $\text{SIPat}^{\text{WT}}$ does not Recognize $\text{SAc}^{\text{WT}}$

Pat homologues acylate $\text{Acs}$ in $R. \text{pala}stris$ and $S. \text{entero}tica$ [10,12]. Clearly, acetylation of $\text{SAc}^{\text{WT}}$ by $\text{SIPat}^{\text{WT}}$ does not occur efficiently in vitro or in a heterologous model system (Figs. 4B, 5A, 6A, 7) [18]. The following possibilities should be taken into consideration when thinking about the potential regulation of $\text{SAc}^{\text{WT}}$ by RLA. First, it is possible that $\text{SAc}^{\text{WT}}$ may have evolved to evade acetylation by $\text{SIPat}^{\text{WT}}$. Secondly, since $S. \text{livi}dans$ encodes $\sim 72$ predicted GNAT-type acetyltransferases (Pfam00583) it is possible that one of these GNATs, not $\text{SIPat}^{\text{WT}}$, acetylates $\text{SAc}^{\text{WT}}$. If a GNAT other than $\text{SIPat}$ acetylated $\text{SAc}$, it begs the questions of what selective pressure drove the conformational change $\text{SAc}$ to avoid recognition by $\text{SIPat}$, and what the physiological benefits of such a change are. And thirdly, the reversed domain organization of $\text{SIPat}$, relative to $Rp\text{Pat}$ and $S\text{Pat}$, may prevent recognition of $\text{SAc}^{\text{WT}}$ by $\text{SIPat}^{\text{WT}}$.

Substantial Changes in the C-terminal Domain of $\text{SAc}^{\text{WT}}$

Lead to its Recognition by $\text{SIPat}^{\text{WT}}$

Forty-one amino acid changes in the C-terminal domain of $\text{SAc}^{\text{WT}}$ were needed to allow $\text{SIPat}^{\text{WT}}$ to recognize and acetylate $\text{SAc}$ (Fig. 5). If we assumed that the domain organization of $\text{SIPat}^{\text{WT}}$ was not a factor in $\text{SAc}^{\text{WT}}$ recognition, such a large number of substitutions would suggest that the protein underwent dramatic evolutionary changes to prevent modification by $\text{SIPat}^{\text{WT}}$. Importantly, we note that some $\text{SAc}$ sequences that were replaced in the C3 chimera exhibit homology to $\text{Acs}$ homologues that are acetylated by GNAT enzymes in other bacteria (Fig. 4B). This suggests acetylation of $\text{Acs}$ and other AMP-forming acyl-CoA synthetases cannot be predicted by amino acid sequence [17]. Our results indicate, however, that $\text{SAc}$ recognition by $\text{SIPat}^{\text{WT}}$ is reversible by mutation, and that the resulting $\text{SAc}$ variant can be reversibly acetylated.

How do Changes in the C-terminal Domain of $\text{SAc}$ Affect its Acetylation and Activity?

Studies of $R. \text{pala}stris$ $\text{Pat}$ ($Rp\text{Pat}$) substrate specificity indicate that this enzyme recognizes a loop $\geq 20$ residues $N$-terminal to the target lysine in the substrate protein, suggesting that the $Rp\text{Pat}$ interacts with a relatively large surface of substrate proteins [17]. Here, we demonstrate that the identities of residues ranging from 8–52 amino acids $N$-terminal to the target lysine of $\text{SAc}^{\text{WT}}$ are critical for recognition of this substrate by $\text{SIPat}^{\text{WT}}$ in isolation. This indicates that $\text{SIPat}^{\text{WT}}$ recognizes several regions of the $\text{SAc}$ C-terminal domain including the target lysine, residues $N$-terminal to the target lysine, and residues C-terminal to the target lysine. It is possible that these regions of the $\text{SAc}$ C-terminal domain are necessary for direct interactions with the $\text{SIPat}$ protein. Alternatively, these regions may be necessary to position the target lysine for entry into the $\text{SIPat}$ active site. The crystal structure of $\text{SIPat}^{\text{WT}}$ substrate $\text{SAc}^{\text{WT}}$ is known (PDB 1PG3, 1PG4) [33]. Comparison of this structure with the structure of $\text{SIAc}$ (structure not known) may distinguish these possibilities. Efforts to obtain the crystal structure of $\text{SIAc}$ are ongoing.

Is $\text{SAc}^{\text{WT}}$ Regulated by One or More Protein Acetyltransferases?

As mentioned above, $\text{SAc}^{\text{WT}}$ may have evolved to evade acetylation specifically by $\text{SIPat}^{\text{WT}}$. However $\text{SAc}^{\text{WT}}$ may be acetylated in vitro by one of the additional $72$ predicted GNAT-type acetyltransferases (Pfam00583) encoded by the genome of this bacterium or by an enzyme independent pathway. The possibility that an alternative GNAT acylates $\text{SAc}^{\text{WT}}$ more efficiently than $\text{SIPat}^{\text{WT}}$ does is not unprecedented. It is known that the genome of $R. \text{pala}stris$ encodes a Pat homologue and a single-domain
Figure 6. Chimera C3 is regulated by SlPatA in vivo in S. enterica. Growth behavior of S. enterica in NCE minimal medium supplemented with acetate (10 mM). A. Growth of S. enterica Δacs pat ΔcobB producing H6-SeAcsWT (triangles), H6-SIAcswt (circles), or H6-Chimera C3 (squares) harboring either a plasmid expressing SlPatAWT (filled shapes) or an empty vector (open shapes). All media was supplemented with 25 μM IPTG. B. Growth of S. enterica Δacs pat ΔcobB (JE9152) producing H6-SIAcswt harboring a plasmid producing SlPatAWT induced with IPTG concentrations of 25 μM (open circles), 50 μM (light gray), 100 μM (medium gray), 250 μM (dark gray), or 500 μM (black). For reference, half-filled circles denote an equivalent strain producing H6-SIAcswt harboring an empty vector induced with 500 μM IPTG. C. Growth of S. enterica Δacs pat ΔcobB (JE9152) producing H6-SeAcsWT (triangles), H6-SIAcswt (circles), H6-Chimera C3 (squares), or empty vector (diamonds) harboring either a plasmid expressing SlPatAWT (filled shapes) or an empty vector (open shapes). All media was supplemented with 5 μM IPTG. D. Growth of S. enterica Δacs pat cobB+ (JE9894) producing H6-SeAcsWT (triangles), H6-SIAcswt (circles), or H6-Chimera C3 (squares) harboring either a plasmid expressing SlPatAWT (filled shapes) or an empty vector (open shapes). All media was supplemented with 25 μM IPTG. E. Growth of S. enterica Δacs pat cobB+ (JE9894) producing H6-SeAcs-ScobB chimera C3 harboring a plasmid producing SlPatAWT induced with IPTG concentrations of 10 μM (open triangle), 25 μM (light gray), 500 μM (medium gray), 100 μM (dark gray), or 250 μM (black). For reference, the inverted, filled triangles denote the growth of an equivalent strain producing H6-SeAcs-ScobB chimera C3 harboring an empty vector (no SlPatAWT) induced with 500 μM IPTG. F. S. enterica Δacs pat cobB+ strains (JE9894) producing H6-SeAcsWT (circles) or H6-SIAcswt (squares) are shown growing with high induction (250 μM IPTG) of empty vector control (open symbols) or a plasmids expressing SlPatA. All S.D. <0.015 absorbance units.

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Figure 7. Activities of Chimera C3 and SeAcswt are reduced in strains expressing SlPatAWT. H6-Chimera C3, H6-SIAcswt, and H6-SeAcs were produced in S. enterica Δacs pat ΔcobB strain JE9152 harboring either a plasmid producing SlPatAWT or an empty vector. Strains were grown in NCE minimal medium supplemented with acetate (10 mM). Acsc proteins were incubated in the presence or absence of SeCobB deacetylase and its co-substrate NAD+. Acsc activity was measured in an NADH-consumption assay. Values are reported as the mean ± S.D. of three activity measurements.

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GNAT protein acetyltransferase that share overlapping protein acetyltransferase substrates, and that both enzymes acetylate these substrates with different affinities [13]. Alternatively, SAcSWT may be acetylated directly and non-enzymatic by the reactive metabolite acetyl-phosphate. This phenomenon has been characterized in E. coli and has been shown to affect the activity of the target enzymes [37,38]. Therefore, the possibility of SPatWT not being the sole regulator of SAcSWT activity in S. lividans needs to be further investigated.

Does the Unique Domain Organization of SPatWT Affect Substrate Specificity?

Pat acetyltransferases are two-domain enzymes composed of a GNAT (acyltransferase) domain and a large domain whose function is likely regulatory. In SPatWT, the GNAT domain is located at the N-terminus of the protein [18]. In contrast, in R. palustris and S. enterica, the domain order is reversed (i.e., GNAT domain is at the C-terminus of protein). SPatWT also has a collagen-like Gly-Pro-Ser motif in the large domain [18]. S. enterica and R. palustris Pat homologues efficiently acetylate their cognate Acs enzymes in vitro [10,12]. The alternate domain organization of SPatWT may account for the poor acetylation of SAcSWT compared to SAcWT and SAcSWT in vitro [18]. If SPatWT has evolved strategies for recognition of protein substrates differently from SPat and RpPat, our in vitro assay may be missing a factor that promotes efficient SPatWT recognition of SAcSWT such as a small molecule, macromolecule (e.g., protein), or an as-yet-unknown intracellular condition. If this were the case, the amino acid changes introduced into SAcWT to generate the SAc-SacS chimera C3 obviate the need for additional factors or conditions for SPatWT recognition.

Author Contributions

Conceived and designed the experiments: ACT JCES. Performed the experiments: ACT. Analyzed the data: ACT JCES. Contributed to the writing of the manuscript: ACT JCES.

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